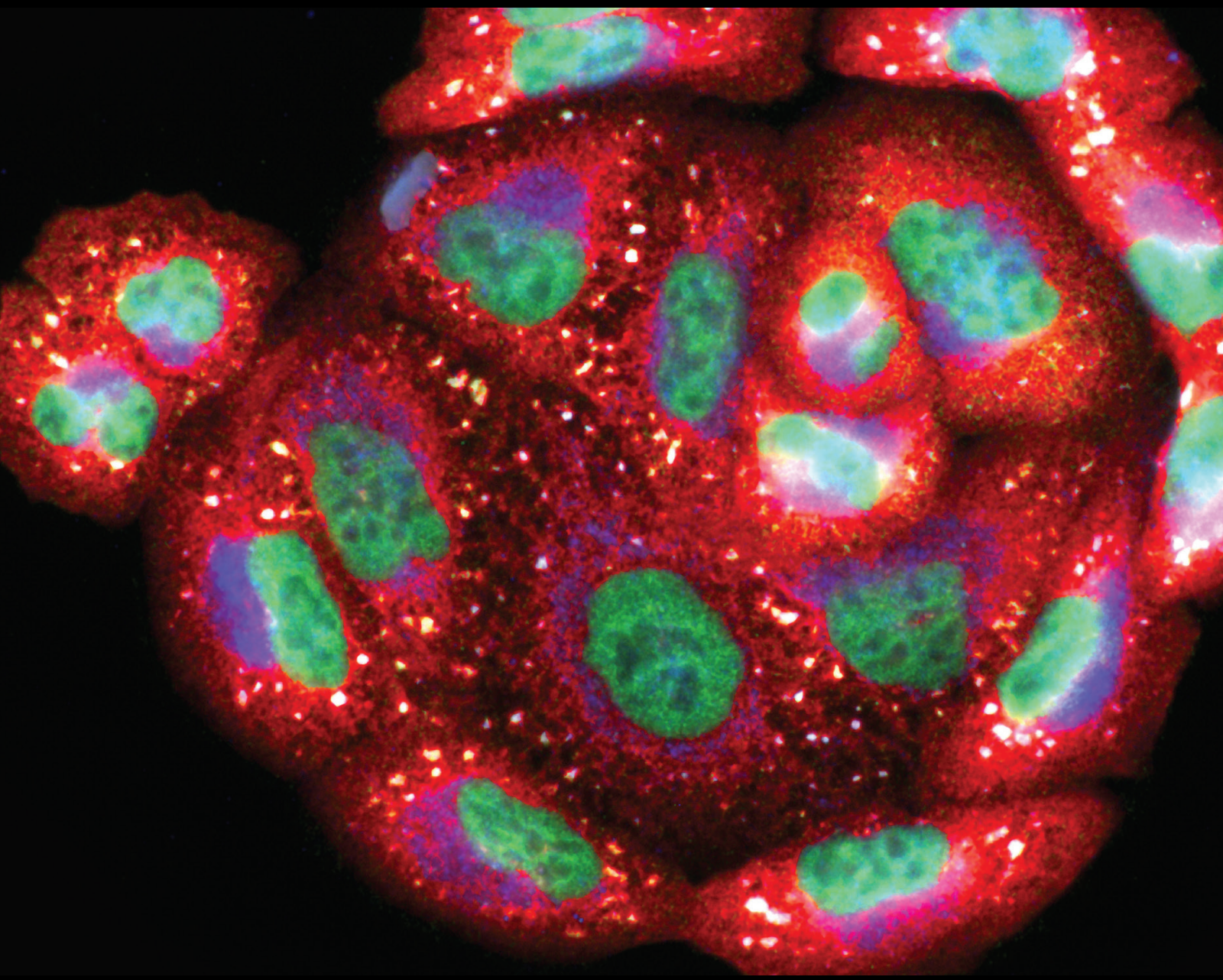
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Age-Related Chronic Disease: From Bench to Bedside

Lead Guest Editor: Fiammetta Monacelli
Guest Editors: Michele Cea, Nicola Amodio, and Chirag Acharya



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Erratum

Erratum to "Antioxidant and Anti-Inflammatory Properties of Anthocyanins Extracted from Oryza sativa L. in Primary Dermal Fibroblasts"

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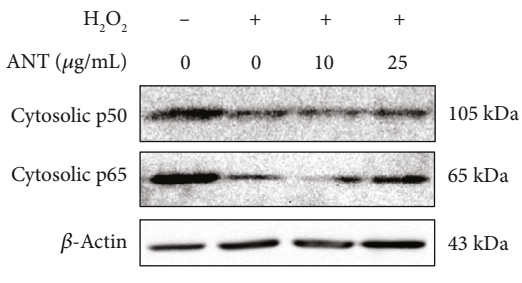
In the article titled "Antioxidant and Anti-Inflammatory Properties of Anthocyanins Extracted from Oryza sativa L. in Primary Dermal Fibroblasts" [1], the Western Blots in Figure 8(a) were duplicated in Figure 8(c) during the production process. The correct Figure 8(c) is shown below.



(c)

Figure 8

References

[1] P. Palungwachira, S. Tancharoen, C. Phruksaniyom et al., "Antioxidant and anti-inflammatory properties of anthocyanins extracted from *Oryza sativa* L. in primary dermal fibroblasts," *Oxidative Medicine and Cellular Longevity*, vol. 2019, Article ID 2089817, 18 pages, 2019.

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Review Article

Central Role of Oxidative Stress in Age-Related Macular Degeneration: Evidence from a Review of the Molecular Mechanisms and Animal Models

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Guest Editor: Fiammetta Monacelli

Age-related macular degeneration (AMD) is a common cause of visual impairment in the elderly. There are very limited therapeutic options for AMD with the predominant therapies targeting vascular endothelial growth factor (VEGF) in the retina of patients afflicted with wet AMD. Hence, it is important to remind readers, especially those interested in AMD, about current studies that may help to develop novel therapies for other stages of AMD. This study, therefore, provides a comprehensive review of studies on human specimens as well as rodent models of the disease, to identify and analyze the molecular mechanisms behind AMD development and progression. The evaluation of this information highlights the central role that oxidative damage in the retina plays in contributing to major pathways, including inflammation and angiogenesis, found in the AMD phenotype. Following on the debate of oxidative stress as the earliest injury in the AMD pathogenesis, we demonstrated how the targeting of oxidative stress-associated pathways, such as autophagy and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, might be the futuristic direction to explore in the search of an effective treatment for AMD, as the dysregulation of these mechanisms is crucial to oxidative injury in the retina. In addition, animal models of AMD have been discussed in great detail, with their strengths and pitfalls included, to assist inform in the selection of suitable models for investigating any of the molecular mechanisms.

1. Introduction

Age-related macular degeneration (AMD) is a neurodegenerative disease that affects the central retina of an aging eye, resulting in a progressive loss of vision and a common cause of visual impairment and disability in the aging population [1]. The global burden of AMD is estimated at 8.7% and dry AMD accounts for approximately 90% of the total number of people with this vision-threatening condition [2]. Currently, anti-vascular endothelial growth factor (anti-VEGF) therapy is approved only for the treatment of the wet form of AMD and involves the inhibition of VEGF from binding to VEGF receptors in the retina. The search for an effective treatment for dry AMD is still ongoing and depends on the understanding of the sequence of molecular mechanisms that are involved in the pathogenesis of this eye disease.

Studies in human populations and donor eyes from AMD patients have provided significant insight into the understanding of the pathogenesis of AMD. Evidence indicates that AMD is a multifactorial disease, having both genetic and environmental risk factors [3]. The risk of AMD is greater in persons with a family history of the disease than those without [4]. Observational studies have identified major environmental risk factors such as cigarette smoking, obesity, nutritional factors, and alcoholism [3]. However, investigation of the pathogenesis of AMD is limited by the inability to study the molecular mechanisms involved as they might have happened long before the diagnosis of the condition. Also, it is challenging to study this condition because of the complex nature of AMD which may arise from interactions among those risk factors involved. Hence, the use of animal models of retinal degeneration under

controlled conditions in studying AMD provides crucial insight into the disease. In addition, the inducement of retinal degeneration in animals takes a relatively shorter time and provides prompt information than studying AMD in humans. As a result, studies on animal models have played a pivotal role in the preclinical evaluations of interventions, such as anti-VEGF treatments in neovascular AMD, before trials P of such treatments in human [5].

Experimental models of AMD have been established in many species including drosophila, mice, rats, guinea pigs, and monkeys. While the primate models may be preferable due to their similarities in retina structure and drusen formation and composition with humans [6], the longer time required for inducement and challenge in breeding them make the murine models much preferred for studying AMD because of lower cost, faster disease progression, and ease of genetic engineering. However, no existing animal model yet fully recapitulates the retinal changes found in human AMD. Notwithstanding, the rodent (murine) models show retinal changes including subretinal deposits, thickening of the Bruch's membrane (BrM), loss of retinal pigment epithelium (RPE) and photoreceptors, and choroidal neovascularization (CNV), which are the characteristics of AMD [7].

The objective of this review was to evaluate evidence in support of the involvement of oxidative stress, inflammation, dysregulated lipid metabolism, and dysregulated angiogenesis in the pathogenesis of AMD, relying on the information from human studies and existing animal models of AMD, to help illustrate the roles of these mechanisms. The strengths and pitfalls of each animal model were reviewed to assist inform in the selection of suitable models for investigating any of the molecular mechanisms. We demonstrated the primary role that oxidative stress may play in triggering each of the mechanisms and illustrated why the targeting of mechanisms including autophagy, Nrf2, and lipid metabolism in the retina might be the futuristic research direction to explore in the search of treatment for a AMD.

1.1. Overview of AMD Pathobiology. The histopathology of the AMD retina reveals that this ocular disease is characterized by localized destruction of the retinal layers at the macular region. Retinal changes observed in the AMD eyes are varied and include (1) loss of the RPE and photoreceptor layer, (2) accumulation of lipids and protein deposits beneath the RPE or in the BrM, and (3) choriocapillaris dropout, CNV, and disciform scarring [8, 9]. In addition to these is inflammation response through the recruitment of macrophages and microglia, and complement activation [10, 11].

Although controversial, most studies have proposed the RPE as the primary site of injury in AMD [8, 12]. The RPE *in situ* performs several functions to maintain retinal homeostasis, some of which include (1) regulating the transport of nutrients and metabolites, (2) absorption of light, (3) recycling of the retinal visual pigment for the continuity of phototransduction, and (4) phagocytosis of shed photoreceptor outer segments. Experimental data support that a malfunction of the RPE leads to retinal degeneration in animal models [13]. One of the early molecular event believed to be associated with RPE malfunction in AMD is the age-related accumulation of lipofuscin, a remnant from poorly degraded phagocytosed photoreceptor outer segments [14, 15]. Lipofuscin in the RPE may contribute to oxidative damage through the generation of free radicals, as well as inhibition of phagocytotic degradation of damaged biomolecules and organelles [16–19].

Adjacent to the RPE pathological changes in AMD eyes are extracellular deposits, which include the basal lamina deposit, basal linear deposits, and drusen. Drusen and other basal deposits in the BrM are important risk factors in the development of AMD [20]. Generally, two processes are believed to contribute in the formation of the subretinal lipid/protein deposits: (1) inefficient RPE metabolism, inefficient degradation of substrate, and/or damaged RPE cells give rise to debris, and (2) local chronic inflammation due to complement activation as activated microglia are recruited to the site of debris [6, 11, 21, 22]. Apart from the immunogenic properties, these lipid deposits become easily oxidized contributing to oxidative stress [23, 24]. In addition, the BrM serves as a semipermeable membrane facilitating the diffusion of nutrients and metabolites between the outer retina and the choriocapillaris; hence, accumulation of deposits within the BrM impairs the transportation of molecules and lead to damage of the RPE and photoreceptor layers [24, 25]. While pathological changes in the RPE may be widely accepted to be the earliest damage in AMD, it was argued by some researchers that the pathophysiology in AMD may differ between the dry AMD and wet AMD [9, 26]. Mcleod and coworkers reported that in dry AMD, regions of complete RPE atrophy in the AMD retina preceded the adjacent areas of loss in the choriocapillaris, indicating the RPE as the primary site of insult. In contrast, they observed that in wet AMD or choroidal neovascularization (CNV), choriocapillaris loss preceded RPE atrophy, implicating the choriocapillaris as the focus of injury in wet AMD, which in turn could induce hypoxia in the adjacent RPE, upregulating vascular endothelial growth factor (VEGF), and promoting CNV [9].

2. Molecular Mechanisms and Models of AMD

Evidence suggests that AMD is a complex disease having multiple risk factors and molecular mechanisms. The studies on experimental models of AMD suggest that these molecular mechanisms involved in AMD could be categorized broadly into (1) oxidative stress-mediated [27, 28], (2) dysregulated antioxidant mechanisms, (3) inflammation [29, 30], (4) dysregulated lipid metabolism [31–33], and (5) dysregulated angiogenesis [34, 35]. This review focuses on recent developments that explain each of these mechanisms in AMD and in particular describes the various murine models employed in these studies.

3. Oxidative Stress and AMD

Oxidative stress appears to be central in the development of AMD due to its relationship with other molecular

mechanisms found in AMD. Generally, oxidative stress is characterized by increased levels of reactive oxygen species (ROS) resulting in the damage or modification of cellular proteins, lipids, and DNA, thereby impairing their physiological roles [36]. Several physiologic conditions favor the generation of ROS and oxidative stress in the retina, which includes higher oxygen metabolism, higher polyunsaturated fatty acid content (PFA), and presence of photosensitive molecules (rhodopsin and lipofuscin) and retinal illumination [28].

A huge body of literature supports the involvement of oxidative stress in AMD. Blood serum from AMD subjects showed increased levels of oxidative stress indicated by increased levels of malondialdehyde (MDA), protein carbonyls, and 8-hydroxy-2-deoxyguanosine (8-OHdG) compared to normal non-AMD cohorts [37], thus suggesting that systemic oxidative stress is related to AMD [37]. Concurrently, studies have shown increased oxidative stress in the retina from donors' eyes with AMD [38–41]. Cultured RPE cells from AMD donors' eyes revealed higher ROS production and malfunctioning of the mitochondria [41]. Oxidative damage to mitochondrial and nuclear DNAs was observed in the RPE of AMD subjects [39, 42]. Donor eyes with AMD showed a higher level of the carboxyethylpyrrole (CEP) in the BrM compared to normal eyes [43]. CEP is a lipid peroxidation product formed from docosahexaenoic acid (DHA) under oxidative stress [44]. Since the photoreceptor outer segments are largely composed of DHA [45], the relative increase of CEP in the BrM from donor's eyes with AMD could suggest increased vulnerability to oxidative damage and/or greater exposure to oxidative stress compared to the non-AMD retina. Also, an accumulation of damaged proteins and impairment of autophagy, which is a proteolytic mechanism of efficient antioxidant capability, were observed in AMD [46]. In addition, the finding of cigarette smoking as a major risk factor of AMD in most epidemiological studies emphasized the crucial role of oxidative stress in the development of this retinal disease [47, 48]. The relationship between cigarette smoking and AMD has been demonstrated experimentally in vitro and in wild-type mice and was shown to be directly linked to the oxidants in cigarette smoke [49, 50]. The cigarette smoke extract (CSE) was observed to increase lipid peroxidation by 8-fold in RPE [51]. Investigating the neuroprotection of antioxidants in the development of AMD may be helpful to further entrench the significant role of oxidative stress in AMD as existing literature is inconclusive [52, 53]. Next, we provide a comprehensive review of the animal models to provide insight into the involvement of oxidative stress in the development of AMD.

3.1. Cigarette Smoke Model. The exposure of mice to cigarette smoke is an important way to investigate the role of oxidative stress in AMD because cigarette smoking is the most significant modifiable risk factor in AMD [47, 54]. Cigarette smoke contains several potent oxidants, including hydroquinone (HQ), nicotine, and cadmium [55, 56]. This animal model has helped to understand possible molecular events that might lead to AMD. HQ upon entry into the circulation through the lungs diffuses into cells and affects the mitochondria, resulting in the overproduction of superoxide anion and damage to mitochondrial membranes [49]. Leakage of superoxide into the cytoplasm generates ROS, which mediates protein oxidation and lipid peroxidation. In addition, complement activation was found in the cigarette-smoke-mediated retinal degeneration [51, 57].

Espinosa-Heidmann and colleagues demonstrated that HQ from cigarette smoke could cause subretinal deposits, a hallmark of AMD [49]. The protocol for inducement normally involves repeated daily exposure of adult mice to cigarette smoke pumped into sealed chambers for a certain time period of the day. Exposed mice showed elevated serum HQ levels and oxidative stress accompanied by retinal changes including BrM deposits and thickening, inflammation, and choroidal neovascularization [49, 50, 58]. This rodent model shows that oxidative stress could induce other molecular mechanisms to generate the AMD phenotype. The severity of the retinal degeneration was shown to depend on the length of exposure. In a study where mice were exposed to cigarette smoke for 2 hours daily for 4.5 months, there was less damage to the RPE compared to the result from another study which used a 5 hr daily exposure for 6 months [49, 58]. The extended exposure time to cigarette smoke led to apoptosis of the RPE [58].

3.2. Light-Induced Model. The damaging effect of light on the retina has been studied and reported to affect mainly the outer retina and RPE [59–61]. Intense retinal illumination has been associated with a reduction in the thickness of the outer nuclear layer and accumulation of deposits within the RPE [62]. Most studies of retinal damage by light have used the BALB/c mice or SD rats, known for their genetic susceptibility to light. Recently, a novel protocol by which retinal degeneration could be induced in the commonly available C57BL/6 J mice was described [63]. The wide use of the light-induced retinal degeneration model for studying AMD is because it offers advantages such as synchronized photoreceptor death occurring with light exposure and is easy to induce within a short time [64]. Also, this model offers the possibility to vary the severity through manipulation of light intensity and duration, and more importantly because light is a natural risk factor involved in many retinal diseases [62].

One of the mechanisms accounting for the retinal damage is due to the interaction between light and photosensitive molecules such as rhodopsin and lipofuscin [59, 65]. The activation of rhodopsin coupled with another cascade of events in the phototransduction process has been associated with photoreceptor cell death [64, 65]. It is likely that such a rhodopsin-mediated mechanism is related to oxidative stress because antioxidant interventions have been found to preserve photoreceptors [66–68]. In addition, light causes the formation of lipid peroxidation from the DHA content of the rods' outer segment membranes [69, 70]. The first light-induced model of retinal degeneration was demonstrated by Noell et al. in albino rats [71]. Since then, several researchers have used similar protocols that differ with respect to the presence of genetic susceptibility of the animal, intensity, wavelength, and duration of light exposure [72–74]. Shorter wavelengths closer to

the blue region of the electromagnetic spectrum have been found to have the highest risk for retinal degeneration compared to the longer wavelengths [59]. The susceptibility to retinal damage by the blue light is due to the increased generation of ROS by the photoactivated pigments rhodopsin and lipofuscin in response to this specific range of the electromagnetic spectrum [75, 76].

3.3. Carboxyethylpyrrole Immunization Model. The carboxyethylpyrrole (CEP) model elucidates the mechanism by which oxidative damage to cellular molecules including lipids and proteins could result in inflammation and retinal degeneration. CEP-modified protein adduct is generated by oxidative damage to DHA with subsequent reaction with the lysine moiety of adjacent proteins. CEP protein adducts found in AMD are immunogenic, inducing autoantibody production and inflammation in the retina [43, 77]. The immunization of mice with CEP-modified mouse serum albumin (CEP-MSA) induced antibodies against CEP and led to inflammatory responses such as the deposit of complement component-3 in BrM and macrophage infiltration [78]. Retinal changes observed after 12-14 months of single immunization included loss of RPE, drusen formation, and thickening of the BrM. These retinal degenerative changes could be induced within a shorter duration (i.e., 2-3 months) in mice through repeated immunization. This model may be useful in investigating mechanisms by which oxidative stress may mediate inflammation in AMD. Based on the outcome of the cigarette smoke, light damage, and CEP immunization in mouse's retina, the pathways by which oxidative stress may lead to AMD have been described (Figure 1).

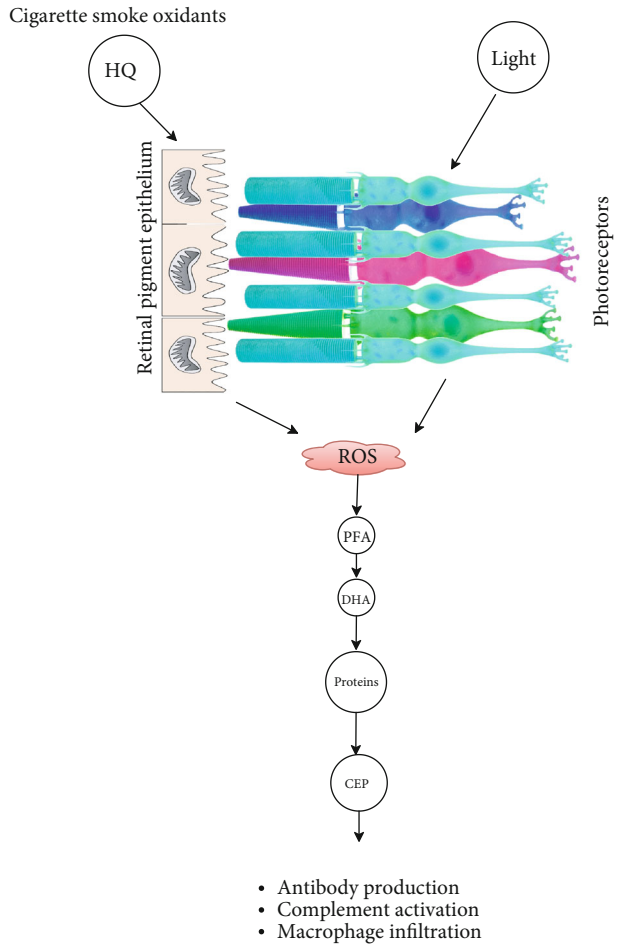

Figure 1: Molecular mechanism in oxidative stress-induced retinal degeneration in mice. ROS levels increase in the retina following (1) exposure to the cigarette smoke prooxidant, hydroquinone (HQ), causing mitochondrial damage in the RPE, and (2) retinal illumination resulting in the photoactivation of rhodopsin. A chain of reactions results in the formation of the carboxyethylpyrrole (CEP) from the docosahexaenoic acid (DHA), a polyunsaturated fatty acid content (PFA) constituent in the retina. CEP is immunogenic, leading to an inflammatory response.

4. Dysregulated Antioxidant Mechanisms and AMD

Antioxidants and other antioxidant-related mechanisms play important roles in reducing oxidative stress. Antioxidants are intracellular molecules that scavenge reactive oxygen species (ROS) and enzymes which degrade superoxides and hydroperoxides, protecting against oxidative stress [79]. Vitamins (A, C, and E) and carotenoids (lutein and zeaxanthin) are potent and effective antioxidants essential in retinal function. Carotenoids, in particular, zeaxanthin, are found in the central retina constituting the macular pigment shown to be protective against light-induced oxidative damage through absorption of the near-blue wavelength of light [80]. Vitamin E (α-tocopherol) may protect the retina from oxidative damage by acting as a scavenger of lipid peroxyl radicals [81]. Studies have shown that increased antioxidants in diet or serum could be protective against AMD progression. According to a longitudinal clinical study, dietary intake of antioxidant/zinc was found to reduce the risk of early AMD in a highly susceptible population due to genetic polymorphisms of complement factor H (CFH) Y402H and LOC387715 A69S [82]. Although some studies found no protective effect of antioxidants against early AMD [83], there is little controversy over its protective role in late AMD [84]. Also, experimental studies in monkeys have found that the consumption of antioxidant-deficient diet (vitamins A and E, and B carotene deficiencies) was associated with photoreceptor degeneration and lipofuscin accumulation in the RPE [85, 86].

In addition to the antioxidants, autophagy and nuclear factor erythroid 2-related factor 2 (Nrf2) and their associated antioxidant enzymes have been found to be highly beneficial for retinal survival under both normal and adverse conditions [87, 88]. Autophagy is a cellular recycling mechanism possessing efficient antioxidant properties and protective against neurodegenerative diseases [87]. An age-related upregulation of autophagy occurs in the retina of non-AMD donors and mice, indicated by an increase in the number of the autophagosome, autophagy-related proteins, and autophagy flux [46]. In contrast, donor eyes with AMD showed a decline in autophagy, suggesting it may be involved in the disease [46, 89]. One master regulator of the cellular antioxidant mechanism is Nrf2, a transcription factor that

regulates the production of antioxidant enzymes against oxidative stress. Under quiescent conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytosol, inactive, and predestined for degradation by the ubiquitin-proteasome pathway [90]. However, Nrf2 dissociates from Keap1 under oxidative stress resulting in its upregulation and translocation into the nucleus. This leads to the upregulation of several antioxidant genes and enzymes including heme oxygenase 1 (HO-1), NAD(P)H-quinone oxidoreductase (NQO1), glutathione S-transferase (GST), superoxide dismutase (SOD), ferritin, and glutathione reductase. SOD is a ubiquitous family of enzymes present in all oxygen-metabolizing cells. It constitutes the first line of defense against superoxide radical (O_2^-) and other ROS [91]. The O_2^- radical is a highly potent oxidative agent because each free radical rapidly gains three electrons to rebalance itself, unlike other ROS. Also, O_2^- could generate other ROS, particularly hydrogen peroxide and hydroxyl radicals.

In the retina, Cu-Zn (SOD1), Mn-SOD (SOD2), and Fe-SOD (SOD3) are found in the cytosol, mitochondrial matrix, and tissue extracellular space, respectively [92]. Mouse models of retinal degeneration induced by compromising the antioxidant mechanisms provide a platform to understand the role of oxidative stress in AMD.

4.1. Autophagy Deficiency Models. The protective role of autophagy against AMD has been demonstrated *in vivo* through the impairment of autophagy to observe any consequential changes in the retina [89, 93]. Yao and colleagues found that the deletion of the RPE gene encoding RB1CC1 (inducible coiled-coil 1) inhibited autophagy in mouse's RPE and accompanied by age-dependent retinal changes including RPE atrophy, microglial infiltration, sub-RPE deposits, and CNV. Subsequently, photoreceptors degenerated and loss of retinal functions occurred [89]. Similar results were observed in mouse's retina following the deletion of RPE-specific Atg5 and Atg7 [93]. Mice aged 8 months old developed retinal signs of early AMD such as abnormal RPE thickness and photoreceptor degeneration [93]. This occurrence of oxidative damage in the retina following the inhibition of autophagy is an indication that autophagy could be an important antioxidant mechanism in the retina. In addition, impairment of autophagy is shown to induce inflammation through the recruitment of inflammasome-activated macrophages [94]. Liu and coworkers showed that impairing autophagy in the eyes of mice via intravitreal injection of wortmannin, an autophagy inhibitor that irreversibly inhibits class III PI3-Kinase12, transiently reduced autophagy activity for one week and led to photoreceptor and RPE death by apoptosis [94]. These findings support the involvement of autophagy in the maintenance of normal homeostasis in the aging retina, and its impairment may play a role in aging retinal degeneration.

4.2. Nrf2 Deficiency Model. Retinal degenerative changes, the presence of hard and soft drusen and RPE atrophy, are observed in adult Nrf2 knockout mice by fundus examination [95]. Retinal function assessment revealed a decline in the a-wave and b-wave amplitudes in electroretinograms (ERGs). Microscopic examination of their retinas showed thickening of the BrM and sub-RPE deposits comprising complement components C3d and vitronectin, which are indicators of complement pathway activation. In some eyes, CNV lesions and loss of photoreceptors were also observed. The age-dependent retinal damage occurring in Nrf2-deficient mice elucidates the importance of dysregulated antioxidant mechanisms and oxidative stress in the development of AMD. According to Zhao and coworkers, there was additional downregulation of autophagy in the Nrf2-deficient mice, which they described as an increase in autophagosome and autolysosome and accumulation of oxidatively damaged protein aggregates and organelles [95]. However, the impact of Nrf2 deficiency on autophagy inhibition may require further investigation to be established since increased autophagosome and autolysosome could as well be indications of upregulated autophagy when the stage of binding to these to the lysosomes is uncompromised [96, 97]. Even though this Nrf2 deficiency model may be helpful in addressing some questions in AMD, its use is limited by the fact that the role of Nrf2 in AMD remains unclear. It is yet to be demonstrated whether Nrf2 is differentially expressed in AMD donor eyes compared to normal healthy eyes. However, recent experimental data in mice have also supported the association between Nrf2 deficiency and AMD, as it was found that the Nrf2 mRNA expression under oxidative stress was impaired in the RPE of aged mice compared to younger mice, thus making this association probable in humans [98].

4.3. SOD Deficiency Model. The retinal changes occurring in SOD1- and SOD2 knockout mice emphasize a major role of oxidative stress in the pathogenesis of AMD. *Sod1*$^{-/-}$ aged 10 months or older developed retinal drusen, thickened BrM, and CNV. In addition, degenerative changes were found in the RPE and photoreceptors of some mice [99]. Sandbach and colleagues demonstrated that the deficiency in SOD2 expression was associated with an increased mitochondrial ROS production [100]. SOD2 deficiency is lethal, and SOD2 knockout mice die within one week from systemic abnormalities related to oxidative damage [101, 102]. Hence, to study retinal changes arising due to SOD2 deficiency, these mice were treated with the SOD2 mimetic, manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP), which extended their lifespan to 3 weeks. MnTBAP-treated SOD2 knockout mice showed thinning of the inner retinal layer and photoreceptor layer compared to wild type. No change was observed in the RPE and BrM. Perhaps, either the short lifespan of SOD2 knockout mice does not allow sufficient time for additional age-related retinal abnormalities to develop or the MnTBAP treatment for the SOD2 knockout mice have protective properties.

Also, other researchers investigated retinal damage occurring in SOD2 deficiency using a gene therapy approach to overcome the problem of lethality with SOD2 knockout mice [103]. The adeno-associated virus (AAV) expressing a ribozyme gene (Rz432) was administered by subretinal injection into the eyes of adult C57BL/6 mice to target the RPE and knockdown SOD2 expression in adult mice [104].

Eyes treated with Rz432 had reduced SOD2 proteins and increased oxidative damage. At 4 months posttreatment, retinal changes typical of human AMD such as the loss of a- and b-waves amplitudes of ERG, accumulation of oxidized proteins in RPE and degeneration, thickening of BrM, apoptotic photoreceptor death, and increased deposit of A2E, the lipofuscin fluorophore, were observed in most treated eyes [103]. However, the role of SOD in human AMD remains uncertain since an earlier genetic study reporting an association between SOD polymorphism and AMD [105] has recently been challenged [106]. Also, results from a previous study investigating SOD enzyme levels in RPE from donor's eyes with or without macular degeneration found no significant correlations between SOD and aging or macular degeneration [107], making this mechanism only speculative.

4.4. α-Tocopherol Deficiency Model. α-Tocopherol (vitamin E) is a potent fat-soluble antioxidant known for its role as a scavenger of lipid peroxyl radicals. The physiological role of α-tocopherol in the body is evident from systemic conditions such as neurological dysfunction, myopathies, and diminished erythrocyte lifespan associated with its deficiency. Following absorption, α-tocopherol is transported to parenchymal cells of the liver for storage [108]. The serum concentration of α-tocopherol is regulated by α-tocopherol transfer protein (α-TTP) which is involved in its transport from the liver to other body organs [109]. Mutations in the gene encoding α-TTP are linked to ataxia with isolated vitamin E deficiency [109, 110].

Since the retina has a rich lipid content, it was thought that α-tocopherol could have an impact. Therefore, to investigate the protective effect of vitamin E on oxidative stress, α-TTP knockout mice were generated and fed vitamin E-deficient diet [111]. There was increased lipid peroxidation and degeneration of neurons, and α-TTP-deficient mice showed changes in retinal function indicated by attenuation in a- and b-waves in ERG at 12 months, as well as loss of outer and inner segments of photoreceptors by 20 months. This outcome shows that α-tocopherol is a potent antioxidant in the retina protective against oxidative stress-related retinal degeneration. However, long-term clinical trials investigating the neuroprotection of vitamin E supplement intake on the development or progression of AMD have consistently found no significant clinical effect [112, 113]. The fact that vitamin E deficiency in humans is rare and often found in isolated cases of abnormal dietary fat absorption or metabolism, instead of a diet low in vitamin E [114, 115], may explain why the intake of it as a supplement may not be of additional benefit in persons with AMD having normal metabolism.

5. Inflammation and AMD

Numerous reports have discussed the roles of inflammation in AMD pathogenesis extensively [30, 116]. Analysis of drusen from donated eyes with AMD revealed the presence of complement components C3 and C5 and membrane-attack complex (MAC), suggesting the activation of the complement pathways. In addition, negative regulators of the complement pathways, including vitronectin and clusterin, were also found in drusen [22, 29, 43, 117]. Activation of the complement system causes proinflammatory responses such as the production of MAC, leading to cell lysis and release of chemokines to mediate recruitment of inflammatory cells including microglia and macrophages [29, 118]. The role of inflammation in the pathogenesis of AMD is solidified by genetic studies showing that the Y402H polymorphism of the complement factor H (CFH), a soluble glycoprotein regulating complement activation, is found in more than half of AMD cases and that the presence of this polymorphism is associated with a higher risk of this disease [119–122]. Other indicators of inflammation in AMD are the presence of chemokines and the accumulation of immune cells such as macrophages and microglia in the retina of AMD subjects [123, 124]. Also, the ability to create animal models of retinal degeneration through manipulation of the immune response further highlights the importance of inflammation in AMD. Various animal models supporting inflammation in AMD are discussed in this review.

5.1. Chemokine Models. Chemokines and their G-protein-coupled receptors contribute significantly to inflammation in AMD. The role of chemokines in AMD is evident from the increased infiltration of activated macrophages and microglia in the milieu of drusen and atrophic lesions [117, 123]. The enhanced recruitment of these immune cells is due to the differential expressions of chemokines and its receptors in AMD [125, 126]. Chemokines are grouped into four families depending on their conserved cysteine residues: CXC, CX3C, CC, and C [127, 128]. CCL2/CCR2 and CX3CL1/CX3CR1, which are ligand/receptor pairs, have been implicated in macrophages and microglia recruitment, respectively, in AMD [129].

5.1.1. $Ccl2^{-/-}$ or $Ccr2^{-/-}$ Mouse Model. This model presumed that the recruitment of macrophages into the retina was protective. Ambati et al. showed that knocking out *Ccl2*/*Ccr2*, a chemokine receptor expressed by macrophages and its binding molecule, respectively, in mice led to the inhibition of macrophage recruitment and retinal degeneration. Signs of retinal degeneration found in the adult (16 months and older) mouse retina were drusen, lipofuscin, thickening of the BrM and geographic atrophy, or CNV [130]. These findings, however, have been challenged by others who demonstrated that $Ccl2^{-/-}$ or $Ccr2^{-/-}$ mice showed no change in the thickness of either the BrM or RPE, as well as the photoreceptors [131, 132]. Intriguingly, the same controversy on the involvement of *CCR2*/*CCL2* in AMD is found in studies using human participants too. A case-control study of the association between *CCL2*/*CCR2* polymorphisms and AMD showed no significant association between these genes and AMD [133]. Furthermore, quantitative PCR reactions evaluating the expression of these genes in laser-dissected RPE from 13 AMD donor eyes and 13 control eyes found no significant difference in the expression of these genes between normal subjects and those with AMD. Controversially, another case-control study with relatively fewer subjects subsequently reported a significant difference in the genotype

and allele frequency for *CCL2*/*CCR2* between AMD and normal controls and concluded that individuals possessing both single nucleotide polymorphisms (SNPs) were at a higher risk of developing AMD [134]. More recently, the demonstration that different functional macrophage subtypes may exist (either protective or injurious) may help to resolve the controversy [135]. Mice given an intravitreal injection of M2 macrophages, a subtype accumulating in wet AMD, displayed exacerbated CNV lesions while mice injected with M1 macrophages displayed ameliorated CNV lesions [135]. The exact role of macrophages and their receptor-ligand pairs (CCR2/CCL2) in AMD require further investigation.

5.1.2. Cx3cr1$^{-/-}$ Mouse Model. Contrary to the proposal that infiltration of macrophages into the retina is protective, the *Cx3cr1*$^{-/-}$ mouse model suggests an accumulation of microglia, central nervous system (CNS)-resident macrophages, in the retina is harmful [136]. An in vitro study showed that microglial cells induced the death of photoreceptor cells [137]. Microglia are the first and main form of active immune defense in the CNS [138]. Combadière and colleagues demonstrated that *Cx3cr1*$^{-/-}$ mice had impaired microglial egress from the retina, resulting in its accumulation [136]. The *Cx3cr1*$^{-/-}$ mice developed sub-RPE deposits, photoreceptor degeneration, and CNV. Recently, a meta-analysis of findings from five long-term studies suggested no association between common *CX3CR1* variants and AMD [139]. Again, no agreement has been reached on the role of CX3CR1 in human population studies.

5.1.3. CCL2$^{-/-}$/CX3CR1$^{-/-}$ Double Knockout Model. The *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ double knockout murine model was employed to overcome the shortcomings of the longer average time taken for either *Ccl2*$^{-/-}$ or *Cx3cr1*$^{-/-}$ single knockout mice to express AMD phenotype. The researchers reported success in the creation of a murine model that took between 4 and 6 weeks to exhibit visible drusen-like lesions, as well as histological signs of AMD including thickening of BrM, localized hypopigmentation, and degeneration of RPE, and photoreceptor atrophy [140, 141]. CNV was also observed in some of the mice. In addition, there was an increased deposit of lipofuscin granules and its component N-retinylidene-N-retinylethanolamine (A2E) in the retina of the mice. Also, signs of active inflammation were found in the double knockout mouse model, including complement C3, macrophages, and activated microglia [142]. However, the reproducibility of this murine AMD model is doubtful as works by others have challenged that there could be some other genetic mutation in the breeding pair of mice used [129]. Assuming that the outcomes reported in the different chemokine models were valid, then inflammation may be crucial in the development of CNV and AMD.

5.2. Complement Activation Models. Even though several reports pointed to activation of the complement pathways in the AMD retina [22, 117], the discovery that polymorphisms in the gene for CFH were associated with greater susceptibility to AMD demonstrates the contribution of this mechanism to the pathogenesis of this disease [119–122]. One of the reasons for the susceptibility of the Y402H polymorphism of *CHF* in AMD is that the impaired binding of CFH to the BrM results in unregulated complement activation and chronic local inflammation [143]. CFH regulates the complement system by inhibition of the alternative pathway through direct inactivation of C3b or binding to C3b, ultimately inhibiting the synthesis of C3 convertase [144]. Depletion of C3 convertase is necessary otherwise it could lead to MAC formation causing lysis of RPE. CFH reaches the retina mainly by circulation, although some amount is also synthesized by the RPE [145, 146]. Apart from the CFH gene, polymorphisms in *C3* have also been found to be associated with increased susceptibility to AMD [147, 148]. Meanwhile, polymorphisms in complement factor B and complement components 2 (C2) have been found to be protective against AMD [149, 150].

5.2.1. Models of Complement Factor H Deficiency. The role of CFH in inflammation and AMD is supported by the retinal degenerative changes observed in the *Cfh*$^{-/-}$ mice and *Cfh*$^{+/-}$ mice. Older surviving *Cfh*-/- mice developed characteristic AMD signs including visual functions and the accumulation of subretinal deposits [151, 152]. Similarly, adult *Cfh*+/- mice fed on a high cholesterol diet developed similar functional and structural changes [152]. These models elucidated the pathophysiological roles of CFH in the retina. The increased sub-RPE deposits in the *Cfh*$^{+/-}$ and *Cfh*$^{-/-}$ mice were due to the competition between CFH and lipoprotein for binding to the BrM. *Cfh*$^{+/-}$ and *Cfh*$^{-/-}$ mice, having a deficiency in CFH expression, had lipoprotein accumulation in the BrM forming sub-RPE deposits. They further demonstrated that the *Cfh*$^{+/-}$ mice showed dysregulated activation of complement in the BrM causing inflammation. However, a systemic difference exists between *Cfh*$^{-/-}$ mice and *CFH* polymorphism. CFH knockout mice have decreased plasma C3 concentration, unlike *CFH* polymorphisms which do not present with changes in C3 levels [151].

5.2.2. Humanized CFH Mice. This transgenic murine model was developed to evaluate the importance of Y402H polymorphism in AMD. Obviously, a mouse model of AMD based on the Y402H variants, the commonest genetic risk factor found in AMD, would help elucidate the pathological mechanisms of this human disease. To create the Y402H *CFH* transgenic mouse, zygotes from wild-type mice were injected with plasmids containing Y402H variants of human *CFH* to substitute the mouse *Cfh* [153]. Matured transgenic mice aged 12 to 14 months showed drusen-like deposits in the central retina. There was an accumulation of macrophage and microglia, basal laminar deposits, and lipofuscin granules in the retina of these mice. In addition, there were signs of complement activation and inflammation in the retina. The inflammation may be brought about by the reduction in the affinity of CFH to bind to MDA, a lipid peroxidation product arising from oxidative stress in the retina. As such, the free MDA molecules could bind and activate macrophages resulting in inflammation [154]. It is possible that the Y402H polymorphisms lessen the efficiency of CFH to deal with oxidative stress making the aging retina vulnerable to AMD. However, there are conflicting results on the

suitability of the humanized *CFH* mice as a model of AMD. Ding et al. generated humanized CFH mice by crossing transgenic mice having a full-length human *CFH* bacterial artificial chromosomes with $Cfh^{-/-}$ mice [144]. Normal retinal morphology and function were preserved in those humanized *CFH* mice. In fact, even in humans, not all Y402H variants develop AMD. How Y402H polymorphism contributes to AMD remains to be examined.

5.2.3. C3-Overexpressing Mice. The retina from AMD eyes has increased complement expression and activation compared to normal eyes [117]. Therefore, to study whether increased complement activation underlies AMD, adult wild-type mice were administered subretinal injections of murine *C3*-carrying recombinant adenovirus [155]. C3 is a common converging point for the complement pathways, and its breakdown into C3a and C3b initiates the final process leading to the formation of MAC. Scotopic electroretinography showed functional deficits in these exogenous C3-overexpressing mice within 2 weeks. Histology and immunohistochemistry revealed pathological signs including RPE atrophy, loss of photoreceptor outer segments, reactive gliosis, and retinal detachment. The deposition of MAC was observed in the outer segments of photoreceptors. While this model corroborates the role of the complement activation in AMD, its challenge is dealing with the involvement of adenoviruses themselves in the retinal pathological changes. Other than the surgical skill required, it could be a model of choice for investigating therapeutic interventions targeting the complement pathway due to the comparatively shorter duration required for creating this model.

6. Dysregulated Lipid Metabolism and AMD

AMD is characterized by the accumulation of sub-RPE deposits including drusen, basal linear, and basal laminar deposits, which are largely composed of lipid [32, 156]. Another evidence supporting dysfunctional lipid metabolism in AMD is based on the association between aging and the accumulation of lipoproteins in the BrM. Lipoprotein accumulation could lead to the formation of a lipid wall, impairing the exchange of nutrients between choriocapillaris and RPE across the BrM and compromising retinal functions [156]. Interestingly, the location of this lipid wall is the same as the sub-RPE deposits found in AMD; possibly, it might be the precursor of these deposits. Lipoproteins found in the retina are either produced locally by the RPE or come from circulation [157]. Lipoproteins transport cholesterol across the BrM to/from the RPE and photoreceptors. Apolipoprotein is the protein constituent found in lipoproteins. Other evidence implicating dysfunctional lipid metabolism in AMD comes from the association between apolipoproteins and AMD [158]. APOE and APOB of low-density lipoproteins (LDL) facilitate lipid metabolism through binding to specific receptors on the liver and other cells. *APOE4* polymorphism is protective against AMD whereas *APOE2* polymorphism is associated with the increased risk of AMD [158, 159]. The protective role of APOE4 has been linked to its increased receptor-binding affinity as compared with APOE2. Thus, APOE4 may facilitate greater lipid metabolism due to its increased binding affinity to the liver, the primary site of lipid metabolism. Others have also reported that APOE4 is associated with higher macular pigment optical density, which also might confer protection against AMD [160]. Also, an association between atherosclerosis and AMD has been reported [161]. Based on the fact that increased serum cholesterol-lipoprotein is a hallmark of atherosclerosis, then by extension, it implicates increased cholesterol in AMD [162]. A direct association between AMD and increased serum cholesterol has been supported by a population-based study showing that higher serum HDL concentration in aged persons doubled their risk of developing AMD [163]. Also, the contribution of dysfunctional lipid metabolism to AMD is upheld by the finding that an intervention modulating lipid metabolism was effective in managing AMD [164]. In this follow-up study of 23 subjects diagnosed with AMD having large soft drusen, a high dose of atorvastatin treatment resulted in a regression of the drusen and vision gain in 10 patients. No subject progressed to advanced neovascular AMD. In addition, the induction of retinal degeneration in animals by the manipulation of genotypes responsible for lipid metabolism further supports the involvement of this mechanism in AMD.

6.1. Humanized Apolipoprotein and $Apoe^{-/-}$ Mouse Models. Researchers have demonstrated an association between dysfunctional lipid metabolism and AMD in mice through the expression of variant apolipoproteins. It was shown that adult humanized transgenic mice expressing one of the three human APOE isoforms (APOE2, APOE3, OR APOE4) and fed high-cholesterol-containing diets for 8 weeks developed age-related retinal degenerative changes [165]. However, the results sharply contrast the findings from human genome studies which show that APOE4 is protective against AMD [158, 166]. Also, other models such as $Apoe^{-/-}$ mice, humanized *APO*E3-Leiden* mice, and humanized *APOB100* mice support a relationship between hypercholesterolemia and AMD [33, 167, 168]. All these adult transgenic mice when fed high-fat diets showed hyperlipidemia accompanied by thickening of the BrM and subretinal deposit resembling basal linear deposits which were all age-related except for the $Apoe^{-/-}$ mice [33]. In humans, the APO*E3-Leiden, a defective human APOE-3 variant, was associated with hyperlipoproteinemia (i.e., inability to break down cholesterol and triglycerides) and early onset atherosclerosis [169, 170]. Apolipoprotein B100 (APOB100) is another type of low-density lipoprotein (LDL) involved in cholesterol transport. It is one of the components in sub-RPE deposits in AMD [168]. Several researchers have studied the role of APOB100 in AMD, and most have directly linked the development of retinal degeneration to increased cholesterol accumulation in the retina [171–173]. However, Espinosa-Heidmann and colleagues have contested that there were no obvious increased cholesterol deposits in the BrM and suggested that it is solely due to hyperlipidemia in the APOB100 mice [172]. They showed that the retinal degeneration occurred much faster in younger humanized ApoB100 mice fed a high-fat diet following exposure to blue-green light, suggesting the

importance of lipid peroxidation. Furthermore, it was shown that prophylactic treatment of APOB100 mice with subcutaneous injection of the antioxidant tocopherol prevented retinal degeneration in the APOB100 mice. These together reveal that a higher risk of lipid peroxidation in the retina may be a plausible mechanism by which hyperlipidemia may be associated with retinal damage.

6.2. Cd36$^{-/-}$ Mice. The *Cd36*$^{-/-}$ mouse model shows the significance of phagocytosis and breakdown of photoreceptor outer segment (POS) by the RPE as a mechanism for maintaining normal retinal homeostasis. CD36, also referred to as fatty acid translocase (FAT), is a membrane glycoprotein used by cells for recognition and binding to specific oxidized low-density lipoproteins, oxidized phospholipids, and long-chain fatty acids, for transport into cells [174, 175]. In the retina, CD36 is abundantly expressed by the RPE and involved in the recognition and phagocytosing of oxidized POS [176, 177]. *Cd36* knockout mice, therefore, have an accumulation of oxidized LDL, thickening of BrM and photoreceptor death [177]. Hence, this model, like the apolipoprotein and *Apoe*$^{-/-}$ mice, supports increased lipid peroxidation in AMD.

6.3. Ldl Receptor$^{-/-}$ *and Vldl Receptor*$^{-/-}$ *Mice.* Findings from the *Ldl* receptor$^{-/-}$ and *Vldl* receptor$^{-/-}$ mice enhance our understanding of the association between AMD and dysfunctional lipid metabolism by showing that the lack of LDL/VLDL in mice resulted in changes in the vascular endothelial growth factor (VEGF) expression and retinal neovascularization [178, 179]. The LDL receptors bind to APOB- and APOE-containing lipoproteins to facilitate lipid metabolism [180, 181]. These receptors are abundantly expressed by the liver, to aid in the uptake of cholesterol [182]. Similar to the variant humanized apolipoprotein mice and Apoe$^{-/-}$ mice, mice lacking LDL receptors had increased plasma cholesterol due to impaired cholesterol metabolism [183]. Thickening of the BrM was observed in the *Ldl* receptor$^{-/-}$ mice fed a high-fat diet. In addition, these mice expressed increased levels of VEGF in the outer retinal layers [179]. The VLDL receptors are also involved in the binding and uptake of ApoE-containing lipoproteins. VLDL receptors are expressed in the retinal vascular endothelium and RPE of mice [178]. Interestingly, mice lacking VLDL receptors showed normal serum cholesterol levels but developed retinal degeneration characterized by neovascularization at 2 weeks postnatal, followed by photoreceptor degeneration, RPE hyperplasia, and subretinal fibrosis at the end stage [178, 184]. The results of these mouse models support LDL/VLDL receptors as negative regulators of retinal neovascularization, and interventions targeting them might prove beneficial in the management of wet AMD. The Wnt pathway has been proposed as the mechanism by which VLDL receptors modulate VEGF expression and neovascularization [185].

6.4. LDL Injection Model in Rats. This model supports a relationship between hyperlipidemia and AMD by showing that repeated intravenous injections of LDL for 7 days resulted in the accumulation of apolipoprotein B100 in BrM, as well as early AMD-like retinal changes including thickening of BrM, the death of photoreceptors, and inflammation in rat retina [186].

7. Dysregulated Angiogenesis and AMD

Angiogenesis describes the formation of new blood vessels from existing blood vessels by either splitting or sprouting [187]. It is essential in development, reproduction, and repair. Dysregulated angiogenesis underlies several human diseases [188]. Progression of AMD may be associated with the development of new choroidal blood vessels into the central retina, an indication of dysregulated angiogenesis [189]. This wet form of AMD characterized by CNV is a major cause of blindness in the elderly. Results from large population-based studies suggest that some populations may be more prone to wet AMD [190]. The formation of new capillaries involves a cascade of events beginning with the degradation of the underlying basement membrane of the existing blood vessel, by the proteolytic activity of the plasminogen activator system and matrix metalloproteinases [191]. This is followed by chemotactic migration and proliferation of endothelial cells into the extracellular matrix stroma, formation of lumen, and maturation of the endothelium [187, 192].

Most evidence implicates VEGF as the proangiogenic factor underlying CNV [34, 35, 193]. These are as follows: (1) vitreous VEGF levels were found to be significantly higher in patients with AMD and CNV compared to healthy controls [194], and (2) clinical trials involving the administration of the anti-VEGF agents, including ranibizumab, bevacizumab, aflibercept, and pegaptanib, markedly suppressed neovascularization and vascular permeability in humans and sustained gain of vision in many AMD patients [195]. However, since VEGF is synthesized *in situ* by the RPE under both normal physiological conditions, it is argued that an equally potent inhibitory regulator must be involved in maintaining homeostasis in the normal retina [196–198]. One antiangiogenic regulator synthesized by RPE is pigment epithelium-derived factor (PEDF). All the regulators are tightly controlled under normal physiological conditions. Conditions such as hypoxia, ischemia, and inflammation promote neovascularization by tilting the balance in favor of increased VEGF expression [199, 200].

In mammals, VEGF binds to 3 VEGF receptors which differ in their affinity and function (VEGFR-1, VEGFR-2, and VEGF-3). The binding of VEGF to VEGFR-2 induces vascular permeability and angiogenesis in vivo [201]. Binding of VEGF to VEGF-R1 may not produce an effect in itself, but VEGFR-1 negatively regulates activation of VEGFR-2 by acting as a decoy receptor that competitively binds to VEGF, thereby modulating the amount of VEGF that binds to VEGFR-2 [202, 203]. VEGFR-3, previously thought to be limited to angiogenesis in the embryo, has been found to be expressed on quiescent vascular endothelial cells in capillaries and is imputed for the induction of human gliomas and colon carcinomas [204, 205]. Further details on

the affinity of VEGF receptors to the subtypes of VEGF are comprehensively reviewed by others [206].

7.1. Laser-Induced CNV Model. CNV was accidentally discovered as a complication of argon laser photocoagulation treatment of the eye. It was later demonstrated experimentally in monkeys that laser trauma resulting in rupture of the BrM could induce CNV [207]. A CNV-inducement protocol has been described for mice to allow repeatability of outcome [208]. Inflammation occurs following the rupture of BrM by laser trauma which may instigate increased VEGF expression in the retina [209, 210]. Also, Berglin and coworkers demonstrated the involvement of matrix metalloproteinase-2 (MMP-2) in laser-included CNV [211]. They compared the size of CNV lesions created by laser trauma between mice lacking MMP-2 and normal wild-type mice and showed that laser exposure altered the MMP-2 gene and protein expressions resulting in bigger CNV lesions in wild-type mice than the MMP-2 knockout mice. The laser-induced CNV model has helped in the understanding of CNV and led to the development of effective therapy for CNV in human AMD. However, its usefulness is limited due to the substantial damage to the neural retina and BrM by laser treatment.

7.2. Light-Induced CNV. Light-induced CNV is another useful model supporting oxidative stress as an upstream mechanism to angiogenesis [212]. Oxidative stress enhances the expression of VEGF and PEDF in RPE, believed to protect against oxidative damage [213, 214]. Albert and colleagues found that repeated exposure of albino rats to intense light for 12 hrs daily for 1 month resulted in an increased level of retinal lipid peroxidation product and retinal changes, including RPE and photoreceptor degeneration, sub-RPE deposits, and CNV [212]. Six months of intense light exposure resulted in complete loss of the outer nuclear layer and the appearance of vast areas of CNV in rats [212]. The progressive retinal changes seen in this light-induced model resemble human AMD and may be appropriate for investigating the pathophysiology of AMD.

7.3. VEGF Overexpression Models. These models have been used to investigate the role of VEGF overexpression in CNV through either injection of adenoviral vectors expressing VEGF into the RPE or subretinal injection of microbeads containing RPE, the primary source of VEGF in the retina [215–217]. Spilsbury and coworkers showed that subretinal injection of an adenoviral vector expressing rat VEGF resulted in overexpression of the exogenous VEGF and was accompanied by CNV in the rat eye 2 weeks after injection [216]. At about 3 months there was a loss of RPE and photoreceptors. Similar results were observed by Baffi and coworkers, who used a subretinal injection of an adenoviral vector expressing the human *VEGF* gene in rats [217]. Early retinal changes observed at 4 weeks of postinjection included subretinal exudates and CNV. In addition, there was a shortening of photoreceptor outer segments and reduction of the outer nuclear layer at overlying areas of neovascularization.

Oshima and coworkers, however, argued that increased expression of VEGF alone in vivo could not cause CNV based on the finding that transgenic mice with increased expression of VEGF in RPE had normal retina and choroid [218]. It was further demonstrated that when there was a subretinal injection of a gutless adenoviral vector expressing Ang2, CNV consistently occurred. According to them, VEGF or angiopoietin 2 (Ang2) could not reach the choriocapillaris to induce neovascularization because of the tight junctions between RPE cells. There breaking of the barrier occurs during subretinal injections of the adenoviral vector or microbeads. Also, the adenoviral vectors themselves could induce inflammation [219]. As a result, Wang and colleagues proposed the subretinal injection of an adeno-associated viral vector (AAV) encoding human VEGF in rats [220]. Unlike adenovirus, AAV causes little or no inflammatory response and, hence, not likely to contribute to CNV [221]. While these models provided understanding into the association between increased retinal VEGF and AMD, the compromise of the retinal-blood barrier resulting from the subretinal injection could trigger an inflammatory response and promote the growth of new blood vessels [222].

7.4. Matrigel Injection Model. Matrigel is a basement membrane extract that can be used for investigating the roles of different angiogenic substances in CNV [223]. It is liquid at 4°C but solidifies to form a plug following injection into tissues [224]. Matrigel may be composed of structural proteins and growth factors of choice depending on the requirement of an experiment. Implanted Matrigel can also be easily removed from tissue for quantification of angiogenesis by immunohistochemistry or histology. Shen and colleagues demonstrated that the subretinal injection of Matrigel induced CNV and other signs of retinal degeneration in the eyes of mice [225]. Histological examination conducted at week 4 postinjection revealed solid Matrigel located beneath the neuroretina and above BrM. At 12 weeks, RPE and photoreceptor degeneration and various degrees of CNV were also observed. In addition, macrophage infiltration and mild inflammation were seen in some CNV lesions. However, the reported success rate for inducing CNV in mice was low ranging from 30 to 55%.

8. Conclusion and Perspective

Rodent models of retinal degeneration provide valuable evidence for the mechanisms involved in AMD and help elucidate how these mechanisms may be interrelated with each other (Figure 2). Oxidative stress appears, however, to be the common link to all the molecular mechanisms. The role of oxidative stress is implicated in the inducement of inflammation in the Y402H polymorphism, which is considered the most important genetic risk of AMD. In the Y402H polymorphism, there is a marked reduction in the ability of the complement regulator CFH to bind to MDA, a lipid peroxidation product [154], leading to uncontrolled MDA-induced inflammation. This is because the binding of MDA to resident macrophages results in the release of cytokines by the macrophages [154], and CFH serves as a decoy to MDA from binding to the macrophages. Thus, the higher risk of AMD associated with Y402H polymorphism is related to an

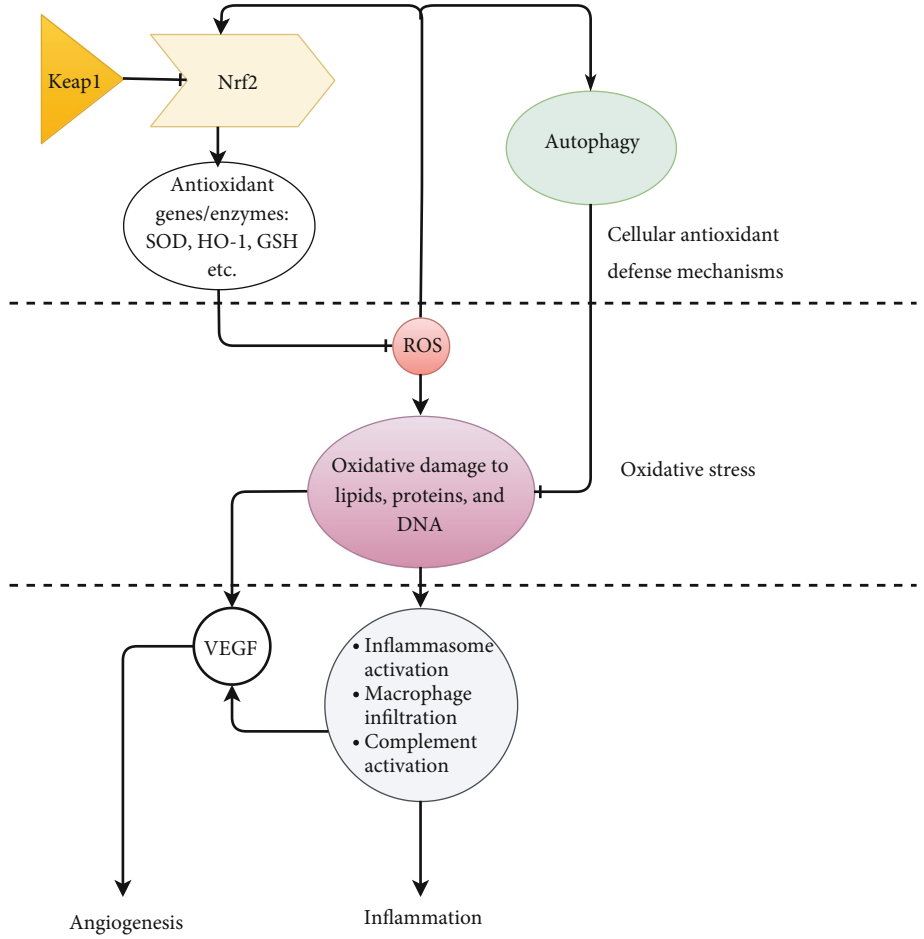

Figure 2: The interrelationships between the molecular mechanisms involved in AMD show the potential therapeutic role of autophagy and Nrf2 activation in the disease. Oxidative damage to lipids, proteins, and DNA is seemingly the primary insult leading to age-related macular degeneration. The accumulation of lipids due to an inhibition of lipid metabolism promotes oxidative stress by increasing lipid peroxidation in the retina. Oxidative stress could initiate inflammation through the activation of the inflammasome, complement, and macrophages. Oxidative stress may also upregulate VEGF expression in the retina and induce choroidal neovascularization. The antioxidant mechanisms, including autophagy and Nrf2, which are upregulated under oxidative stress counteract further oxidative damage and maintain retinal homeostasis.

increased vulnerability to oxidative stress in the retina. The role of hyperlipidemia in AMD development also appears to be through increased oxidative stress. Analyzing data from rodent models generated to express the human apolipoproteins shows that these mice showed signs of hyperlipidemia, oxidative stress, and retinal degeneration. The hyperlipidemia (due to impaired lipid metabolism) causes a block of retinal blood vessels and thickening of BrM, leading to ischemia and hypoxia, and the induction of oxidative stress. Last but not the least, photooxidative damage in mouse's eyes has been shown to induce CNV. It is explained that increased oxidative stress promotes increased VEGF production by the RPE cells, as a response to counteract the harm from oxidative stress in the eye. The unregulated VEGF production, however, stimulates the development of new blood vessels. Therefore, targeting VEGF or inflammation (the current focus for AMD treatment), although is proven to be an effective approach, addresses the late stage of AMD. The autophagy and Nrf2 mechanisms are cellular defense mechanisms found to support survival under oxidative stress. The relationship between oxidative stress and autophagy or Nrf2 is bidirectional; increased oxidative stress activates autophagy or Nrf2, and these antioxidant defense mechanisms, in turn, could inhibit oxidative damage in the retina. Since impaired autophagy, Nrf2, or dyslipidemia are factors that could promote oxidative stress in the retina, studies to target these pathways as alternative therapeutic options for AMD are warranted.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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References

[1] C. F. Chou, M. Frances Cotch, S. Vitale et al., "Age-related eye diseases and visual impairment among U.S. adults," *American Journal of Preventive Medicine*, vol. 45, no. 1, pp. 29–35, 2013.

[2] W. L. Wong, X. Su, X. Li et al., "Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis," *The Lancet Global Health*, vol. 2, no. 2, pp. e106–e116, 2014.

[3] L. Sobrin and J. M. Seddon, "Nature and nurture- genes and environment- predict onset and progression of macular degeneration," *Progress in Retinal and Eye Research*, vol. 40, pp. 1–15, 2014.

[4] H. Shahid, J. C. Khan, V. Cipriani et al., "Age-related macular degeneration: the importance of family history as a risk factor," *British Journal of Ophthalmology*, vol. 96, no. 3, pp. 427–431, 2012.

[5] C. H. Meyer and F. G. Holz, "Preclinical aspects of anti-VEGF agents for the treatment of wet AMD: ranibizumab and bevacizumab," *Eye*, vol. 25, no. 6, pp. 661–672, 2011.

[6] S. Umeda, M. T. Suzuki, H. Okamoto et al., "Molecular composition of drusen and possible involvement of anti-retinal autoimmunity in two different forms of macular degeneration in cynomolgus monkey (*Macaca fascicularis*)," *The FASEB Journal*, vol. 19, no. 12, pp. 1683–1685, 2005.

[7] A. C. Bird, N. M. Bressler, S. B. Bressler et al., "An international classification and grading system for age-related maculopathy and age-related macular degeneration," *Survey of Ophthalmology*, vol. 39, no. 5, pp. 367–374, 1995.

[8] M. A. Zarbin, "Current concepts in the pathogenesis of age-related macular degeneration," *Archives of Ophthalmology*, vol. 122, no. 4, pp. 598–614, 2004.

[9] D. S. McLeod, R. Grebe, I. Bhutto, C. Merges, T. Baba, and G. A. Lutty, "Relationship between RPE and choriocapillaris in age-related macular degeneration," *Investigative Opthalmology & Visual Science*, vol. 50, no. 10, pp. 4982–4991, 2009.

[10] N. Gupta, K. E. Brown, and A. H. Milam, "Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration," *Experimental Eye Research*, vol. 76, no. 4, pp. 463–471, 2003.

[11] R. Natoli, N. Fernando, H. Jiao et al., "Retinal macrophages synthesize C3 and activate complement in AMD and in models of focal retinal degeneration," *Investigative Ophthalmology & Visual Science*, vol. 58, no. 7, pp. 2977–2990, 2017.

[12] P. S. Mettu, A. R. Wielgus, S. S. Ong, and S. W. Cousins, "Retinal pigment epithelium response to oxidant injury in the pathogenesis of early age-related macular degeneration," *Molecular Aspects of Medicine*, vol. 33, no. 4, pp. 376–398, 2012.

[13] O. Strauss, "The retinal pigment epithelium in visual function," *Physiological Reviews*, vol. 85, no. 3, pp. 845–881, 2005.

[14] J. R. Sparrow and M. Boulton, "RPE lipofuscin and its role in retinal pathobiology," *Experimental Eye Research*, vol. 80, no. 5, pp. 595–606, 2005.

[15] J. Z. Nowak, "Age-related macular degeneration: pathogenesis and therapy," *Pharmacol Reports*, vol. 58, pp. 353–363, 2006.

[16] R. D. Glickman, A. E. Dontsov, M. A. Ostrovsky, N. Kumar, M. Vendal, and M. A. Gonzales, "Photo-oxidative stress in the eye: role of retinal pigment epithelial pigments," in *1999 International Conference on Biomedical Optics*, pp. 428–438, Wuhan, China, 1999.

[17] S. C. Finnemann, L. W. Leung, and E. Rodriguez-Boulan, "The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 6, pp. 3842–3847, 2002.

[18] A. Höhn and T. Grune, "Lipofuscin: formation, effects and role of macroautophagy," *Redox Biology*, vol. 1, no. 1, pp. 140–144, 2013.

[19] M. M. Olchawa, J. A. Furso, G. M. Szewczyk, and T. J. Sarna, "Lipofuscin-mediated photic stress inhibits phagocytic activity of ARPE-19 cells; effect of donors' age and antioxidants," *Free Radical Research*, vol. 51, no. 9-10, pp. 799–811, 2017.

[20] C. A. Curcio and C. L. Millican, "Basal linear deposit and large drusen are specific for early age-related maculopathy," *Archives of Ophthalmology*, vol. 117, no. 3, pp. 329–339, 1999.

[21] L. V. Johnson, W. P. Leitner, M. K. Staples, and D. H. Anderson, "Complement activation and inflammatory processes in drusen formation and age related macular degeneration," *Experimental Eye Research*, vol. 73, no. 6, pp. 887–896, 2001.

[22] D. H. Anderson, R. F. Mullins, G. S. Hageman, and L. V. Johnson, "A role for local inflammation in the formation of drusen in the aging eye," *American Journal of Ophthalmology*, vol. 134, no. 3, pp. 411–431, 2002.

[23] G. S. Hageman, P. J. Luthert, N. H. Victor Chong, L. V. Johnson, D. H. Anderson, and R. F. Mullins, "An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration," *Progress in Retinal and Eye Research*, vol. 20, no. 6, pp. 705–732, 2001.

[24] J. C. Booij, D. C. Baas, J. Beisekeeva, T. G. M. F. Gorgels, and A. A. B. Bergen, "The dynamic nature of Bruch's membrane," *Progress in Retinal and Eye Research*, vol. 29, no. 1, pp. 1–18, 2010.

[25] R. Guymer, P. Luthert, and A. Bird, "Changes in Bruch's membrane and related structures with age," *Progress in Retinal and Eye Research*, vol. 18, no. 1, pp. 59–90, 1999.

[26] A. C. Bird, R. L. Phillips, and G. S. Hageman, "Geographic atrophy: a histopathological assessment," *JAMA Ophthalmology*, vol. 132, no. 3, pp. 338–345, 2014.

[27] S. G. Jarrett and M. E. Boulton, "Consequences of oxidative stress in age-related macular degeneration," *Molecular Aspects of Medicine*, vol. 33, no. 4, pp. 399–417, 2012.

[28] S. Beatty, H.-H. Koh, M. Phil, D. Henson, and M. Boulton, "The role of oxidative stress in the pathogenesis of age-related macular degeneration," *Survey of Ophthalmology*, vol. 45, no. 2, pp. 115–134, 2000.

[29] L. A. Donoso, D. Kim, A. Frost, A. Callahan, and G. Hageman, "The role of inflammation in the pathogenesis of age-related macular degeneration," *Survey of Ophthalmology*, vol. 51, no. 2, pp. 137–152, 2006.

[30] A. Kauppinen, J. J. Paterno, J. Blasiak, A. Salminen, and K. Kaarniranta, "Inflammation and its role in age-related macular degeneration," *Cellular and Molecular Life Sciences*, vol. 73, no. 9, pp. 1765–1786, 2016.

[31] K. B. Ebrahimi and J. T. Handa, "Lipids, lipoproteins, and age-related macular degeneration," *Journal of Lipids*, vol. 2011, Article ID 802059, 14 pages, 2011.

[32] A. U. Kishan, B. S. Modjtahedi, E. N. Martins, S. P. Modjtahedi, and L. S. Morse, "Lipids and age-related macular degeneration," *Survey of Ophthalmology*, vol. 56, no. 3, pp. 195–213, 2011.

[33] S. Dithmar, C. A. Curcio, N. A. Le, S. Brown, and H. E. Grossniklaus, "Ultrastructural changes in Bruch's membrane of apolipoprotein E-deficient mice," *Investigative Ophthalmology & Visual Science*, vol. 41, no. 8, pp. 2035–2042, 2000.

[34] A. Witmer, "Vascular endothelial growth factors and angiogenesis in eye disease," *Progress in Retinal and Eye Research*, vol. 22, no. 1, pp. 1–29, 2003.

[35] J. S. Penn, A. Madan, R. B. Caldwell, M. Bartoli, R. W. Caldwell, and M. E. Hartnett, "Vascular endothelial growth factor in eye disease," *Progress in Retinal and Eye Research*, vol. 27, no. 4, pp. 331–371, 2008.

[36] R. Kohen and A. Nyska, "Invited Review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification," *Toxicologic Pathology*, vol. 30, no. 6, pp. 620–650, 2002.

[37] Y. Totan, R. Yağcı, Y. Bardak et al., "Oxidative macromolecular damage in age-related macular degeneration," *Current Eye Research*, vol. 34, no. 12, pp. 1089–1093, 2009.

[38] C. M. Ethen, C. Reilly, X. Feng, T. W. Olsen, and D. A. Ferrington, "Age-related macular degeneration and retinal protein modification by 4-hydroxy-2-nonenal," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 8, pp. 3469–3479, 2007.

[39] M. R. Terluk, R. J. Kapphahn, L. M. Soukup et al., "Investigating mitochondria as a target for treating age-related macular degeneration," *The Journal of Neuroscience*, vol. 35, no. 18, pp. 7304–7311, 2015.

[40] K. Renganathan, Q. Ebrahem, A. Vasanji et al., "Carboxyethylpyrrole adducts, age-related macular degeneration and neovascularization," in *Recent Advances in Retinal Degeneration*, vol. 613 of Advances in Experimental Medicine and Biology, , pp. 261–267, Springer, 2008.

[41] N. Golestaneh, Y. Chu, Y.-Y. Xiao, G. L. Stoleru, and A. C. Theos, "Dysfunctional autophagy in RPE, a contributing factor in age-related macular degeneration," *Cell Death & Disease*, vol. 8, no. 1, article e2537, 2018.

[42] J. Blasiak, S. Glowacki, A. Kauppinen, and K. Kaarniranta, "Mitochondrial and nuclear DNA damage and repair in age-related macular degeneration," *International Journal of Molecular Sciences*, vol. 14, no. 2, pp. 2996–3010, 2013.

[43] J. W. Crabb, M. Miyagi, X. Gu et al., "Drusen proteome analysis: an approach to the etiology of age-related macular degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 23, pp. 14682–14687, 2002.

[44] L. Lu, X. Gu, L. Hong et al., "Synthesis and structural characterization of carboxyethylpyrrole-modified proteins: mediators of age-related macular degeneration," *Bioorganic & Medicinal Chemistry*, vol. 17, no. 21, pp. 7548–7561, 2009.

[45] B. G. Jeffrey, H. S. Weisinger, M. Neuringer, and D. C. Mitchell, "The role of docosahexaenoic acid in retinal function," *Lipids*, vol. 36, no. 9, pp. 859–871, 2001.

[46] S. K. Mitter, C. Song, X. Qi et al., "Dysregulated autophagy in the RPE is associated with increased susceptibility to oxidative stress and AMD," *Autophagy*, vol. 10, no. 11, pp. 1989–2005, 2014.

[47] S. Velilla, J. J. García-Medina, A. García-Layana et al., "Smoking and age-related macular degeneration: review and update," *Journal of Ophthalmology*, vol. 2013, Article ID 895147, 11 pages, 2013.

[48] J. Thornton, R. Edwards, P. Mitchell, R. A. Harrison, I. Buchan, and S. P. Kelly, "Smoking and age-related macular degeneration: a review of association," *Eye*, vol. 19, no. 9, pp. 935–944, 2005.

[49] D. G. Espinosa-Heidmann, I. J. Suner, P. Catanuto, E. P. Hernandez, M. E. Marin-Castano, and S. W. Cousins, "Cigarette smoke–related oxidants and the development of sub-RPE deposits in an experimental animal model of dry AMD," *Investigative Ophthalmology & Visual Science*, vol. 47, no. 2, pp. 729–737, 2006.

[50] A. L. Wang, T. J. Lukas, M. Yuan, N. du, J. T. Handa, and A. H. Neufeld, "Changes in retinal pigment epithelium related to cigarette smoke: possible relevance to smoking as a risk factor for age-related macular degeneration," *PLoS One*, vol. 4, no. 4, article e5304, 2009.

[51] K. Kunchithapautham, C. Atkinson, and B. Rohrer, "Smoke exposure causes endoplasmic reticulum stress and lipid accumulation in retinal pigment epithelium through oxidative stress and complement activation," *Journal of Biological Chemistry*, vol. 289, no. 21, pp. 14534–14546, 2014.

[52] J. R. Evans and K. S. Henshaw, "Antioxidant vitamin and mineral supplements for preventing age-related macular degeneration," *Cochrane Database of Systematic Reviews*, article CD000253, 2008.

[53] H. P. Y. Sin, D. T. L. Liu, and D. S. C. Lam, "Lifestyle modification, nutritional and vitamins supplements for age-related macular degeneration," *Acta Ophthalmologica*, vol. 91, no. 1, pp. 6–11, 2013.

[54] R. A. Armstrong and M. Mousavi, "Overview of risk factors for age-related macular degeneration (AMD)," *Journal of Stem Cells*, vol. 10, no. 3, pp. 171–191, 2015.

[55] P. Leanderson and C. Tagesson, "Cigarette smoke-induced DNA-damage: role of hydroquinone and catechol in the formation of the oxidative DNA-adduct, 8-hydroxydeoxyguanosine," *Chemico-Biological Interactions*, vol. 75, no. 1, pp. 71–81, 1990.

[56] T. Horinouchi, T. Higashi, Y. Mazaki, and S. Miwa, "Carbonyl compounds in the gas phase of cigarette mainstream smoke and their pharmacological properties," *Biological & Pharmaceutical Bulletin*, vol. 39, no. 6, pp. 909–914, 2016.

[57] A. Woodell and B. Rohrer, "A mechanistic review of cigarette smoke and age-related macular degeneration," in *Retinal Degenerative Diseases*, vol. 801 of Advances in Experimental Medicine and Biology, , pp. 301–307, Springer, 2014.

[58] M. Fujihara, N. Nagai, T. E. Sussan, S. Biswal, and J. T. Handa, "Chronic cigarette smoke causes oxidative damage and apoptosis to retinal pigmented epithelial cells in mice," *PLoS One*, vol. 3, no. 9, article e3119, 2008.

[59] J. Wu, S. Seregard, and P. V. Algvere, "Photochemical damage of the retina," *Survey of Ophthalmology*, vol. 51, no. 5, pp. 461–481, 2006.

[60] J. Lanum, "The damaging effects of light on the retina. Empirical findings, theoretical and practical implications," *Survey of Ophthalmology*, vol. 22, no. 4, pp. 221–249, 1978.

[61] J. J. Hunter, J. I. W. Morgan, W. H. Merigan, D. H. Sliney, J. R. Sparrow, and D. R. Williams, "The susceptibility of the retina to photochemical damage from visible light," *Progress in Retinal and Eye Research*, vol. 31, no. 1, pp. 28–42, 2012.

[62] D. T. Organisciak and D. K. Vaughan, "Retinal light damage: mechanisms and protection," *Progress in Retinal and Eye Research*, vol. 29, no. 2, pp. 113–134, 2010.

[63] X. Zhong, B. Aredo, Y. Ding, K. Zhang, C. X. Zhao, and R. L. Ufret-Vincenty, "Fundus camera-delivered light-induced retinal degeneration in mice with the *RPE65* Leu450Met variant is associated with oxidative stress and apoptosis," *Investigative Ophthalmology & Visual Science*, vol. 57, no. 13, pp. 5558–5567, 2016.

[64] A. Wenzel, C. Grimm, M. Samardzija, and C. E. Remé, "Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration," *Progress in Retinal and Eye Research*, vol. 24, no. 2, pp. 275–306, 2005.

[65] M. Rózanowska and T. Sarna, "Light-induced damage to the retina: role of rhodopsin chromophore revisited," *Photochemistry and Photobiology*, vol. 81, no. 6, pp. 1305–1330, 2005.

[66] H. Tomita, Y. Kotake, and R. E. Anderson, "Mechanism of protection from light-induced retinal degeneration by the synthetic antioxidant phenyl-*N*-*tert*-butylnitrone," *Investigative Ophthalmology & Visual Science*, vol. 46, no. 2, pp. 427–434, 2005.

[67] Y. Fang, T. Su, X. Qiu et al., "Protective effect of alpha-mangostin against oxidative stress induced-retinal cell death," *Scientific Reports*, vol. 6, no. 1, article 21018, 2016.

[68] M. B. Rozanowska, "Light-induced damage to the retina: current understanding of the mechanisms and unresolved questions: a symposium-in-print," *Photochemistry and Photobiology*, vol. 88, no. 6, pp. 1303–1308, 2012.

[69] A. Catalá, "An overview of lipid peroxidation with emphasis in outer segments of photoreceptors and the chemiluminescence assay," *The International Journal of Biochemistry & Cell Biology*, vol. 38, no. 9, pp. 1482–1495, 2006.

[70] H. Pasantes-Morales and C. Cruz, "Taurine and hypotaurine inhibit light-induced lipid peroxidation and protect rod outer segment structure," *Brain Research*, vol. 330, no. 1, pp. 154–157, 1985.

[71] W. K. Noell, V. S. Walker, B. S. Kang, and S. Berman, "Retinal damage by light in rats," *Investigative Ophthalmology*, vol. 5, no. 5, pp. 450–473, 1966.

[72] C. Grimm and C. E. Remé, "Light damage as a model of retinal degeneration," in *Retinal Degeneration*, vol. 935 of Methods in Molecular Biology, , pp. 87–97, Humana Press, 2012.

[73] F. Hafezi, C. Grimm, B. C. Simmen, A. Wenzel, and C. E. Remé, "Molecular ophthalmology: an update on animal models for retinal degenerations and dystrophies," *British Journal of Ophthalmology*, vol. 84, no. 8, pp. 922–927, 2000.

[74] G. H. Kim, H. I. Kim, S.-S. Paik, S. W. Jung, S. Kang, and I.-B. Kim, "Functional and morphological evaluation of blue light-emitting diode-induced retinal degeneration in mice," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 254, no. 4, pp. 705–716, 2016.

[75] M. Boulton, A. Dontsov, J. Jarvis-Evans, M. Ostrovsky, and D. Svistunenko, "Lipofuscin is a photoinducible free radical generator," *Journal of Photochemistry and Photobiology B: Biology*, vol. 19, no. 3, pp. 201–204, 1993.

[76] G. C. Demontis, B. Longoni, and P. L. Marchiafava, "Molecular steps involved in light-induced oxidative damage to retinal rods," *Investigative Ophthalmology & Visual Science*, vol. 43, pp. 2421–2427, 2002.

[77] X. Gu, S. G. Meer, M. Miyagi et al., "Carboxyethylpyrrole protein adducts and autoantibodies, biomarkers for age-related macular degeneration," *Journal of Biological Chemistry*, vol. 278, no. 43, pp. 42027–42035, 2003.

[78] J. G. Hollyfield, V. L. Bonilha, M. E. Rayborn et al., "Oxidative damage–induced inflammation initiates age-related macular degeneration," *Nature Medicine*, vol. 14, no. 2, pp. 194–198, 2008.

[79] S. Hosaka, M. Obuki, J. Nakajima, and M. Suzuki, "Comparative study of antioxidants as quenchers or scavengers of reactive oxygen species based on quenching of MCLA-dependent chemiluminescence," *Luminescence*, vol. 20, no. 6, pp. 419–427, 2005.

[80] E. Loskutova, J. Nolan, A. Howard, and S. Beatty, "Macular pigment and its contribution to vision," *Nutrients*, vol. 5, no. 6, pp. 1962–1969, 2013.

[81] E. Niki, "Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence," *Free Radical Biology & Medicine*, vol. 66, pp. 3–12, 2014.

[82] L. Ho, R. van Leeuwen, J. C. M. Witteman et al., "Reducing the genetic risk of age-related macular degeneration with dietary antioxidants, zinc, and ω-3 fatty acids: the Rotterdam study," *Archives of Ophthalmology*, vol. 129, no. 6, pp. 758–766, 2011.

[83] E. W.-T. Chong, T. Y. Wong, A. J. Kreis, J. A. Simpson, and R. H. Guymer, "Dietary antioxidants and primary prevention of age related macular degeneration: systematic review and meta-analysis," *BMJ*, vol. 335, no. 7623, p. 755, 2007.

[84] T. Michikawa, S. Ishida, Y. Nishiwaki et al., "Serum antioxidants and age-related macular degeneration among older Japanese," *Asia Pacific Journal of Clinical Nutrition*, vol. 18, pp. 1–7, 2009.

[85] F. Steele and J. E. O'Tousa, "Rhodopsin activation causes retinal degeneration in drosophila *rdgC* mutant," *Neuron*, vol. 4, no. 6, pp. 883–890, 1990.

[86] M. R. Malinow, L. Feeney-Burns, L. H. Peterson, M. L. Klein, and M. Neuringer, "Diet-related macular anomalies in monkeys," *Investigative Ophthalmology & Visual Science*, vol. 19, no. 8, pp. 857–863, 1980.

[87] S. Giordano, V. Darley-Usmar, and J. Zhang, "Autophagy as an essential cellular antioxidant pathway in neurodegenerative disease," *Redox Biology*, vol. 2, pp. 82–90, 2014.

[88] H. E. de Vries, M. Witte, D. Hondius et al., "Nrf2-induced antioxidant protection: A promising target to counteract ROS-mediated damage in neurodegenerative disease?," *Free Radical Biology & Medicine*, vol. 45, no. 10, pp. 1375–1383, 2008.

[89] J. Yao, L. Jia, N. Khan et al., "Deletion of autophagy inducer *RB1CC1* results in degeneration of the retinal pigment epithelium," *Autophagy*, vol. 11, no. 6, pp. 939–953, 2015.

[90] N. Wakabayashi, K. Itoh, J. Wakabayashi et al., "*Keap1*-null mutation leads to postnatal lethality due to constitutive Nrf2 activation," *Nature Genetics*, vol. 35, no. 3, pp. 238–245, 2003.

[91] J. M. Matés, C. Pérez-Gómez, and I. Núñez de Castro, "Antioxidant enzymes and human diseases," *Clinical Biochemistry*, vol. 32, no. 8, pp. 595–603, 1999.

[92] A. Behndig, B. Svensson, S. L. Marklund, and K. Karlsson, "Superoxide dismutase isoenzymes in the human eye," *Investigative Ophthalmology and Visual Science*, vol. 39, no. 3, pp. 471–475, 1998.

[93] Y. Zhang, S. D. Cross, J. B. Stanton, A. D. Marmorstein, Y. Z. Le, and L. Y. Marmorstein, "Early AMD-like defects in the RPE and retinal degeneration in aged mice with RPE-specific deletion of *Atg5* or *Atg7*," *Molecular Vision*, vol. 23, pp. 228–241, 2017.

[94] J. Liu, D. A. Copland, S. Theodoropoulou et al., "Impairing autophagy in retinal pigment epithelium leads to inflammasome activation and enhanced macrophage-mediated angiogenesis," *Scientific Reports*, vol. 6, no. 1, article 20639, 2016.

[95] Z. Zhao, Y. Chen, J. Wang et al., "Age-related retinopathy in NRF2-deficient mice," *PLoS One*, vol. 6, no. 4, article e19456, 2011.

[96] M. Komatsu, H. Kurokawa, S. Waguri et al., "The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1," *Nature Cell Biology*, vol. 12, no. 3, pp. 213–223, 2010.

[97] A. Jain, T. E. Rusten, N. Katheder et al., "P62/sequestosome-1, autophagy-related gene 8, and autophagy in *Drosophila* are regulated by nuclear factor erythroid 2-related factor 2 (NRF2), independent of transcription factor TFEB," *Journal of Biological Chemistry*, vol. 290, no. 24, pp. 14945–14962, 2015.

[98] M. M. Sachdeva, M. Cano, and J. T. Handa, "Nrf2 signaling is impaired in the aging RPE given an oxidative insult," *Experimental Eye Research*, vol. 119, pp. 111–114, 2014.

[99] Y. Imamura, S. Noda, K. Hashizume et al., "Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 30, pp. 11282–11287, 2006.

[100] J. M. Sandbach, P. E. Coscun, H. E. Grossniklaus, J. E. Kokoszka, N. J. Newman, and D. C. Wallace, "Ocular pathology in mitochondrial superoxide dismutase (Sod2)-deficient mice," *Investigative Ophthalmology & Visual Science*, vol. 42, no. 10, pp. 2173–2178, 2001.

[101] Y. Li, T. T. Huang, E. J. Carlson et al., "Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase," *Nature Genetics*, vol. 11, no. 4, pp. 376–381, 1995.

[102] S. Melov, P. Coskun, M. Patel et al., "Mitochondrial disease in superoxide dismutase 2 mutant mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 3, pp. 846–851, 1999.

[103] V. Justilien, J. J. Pang, K. Renganathan et al., "SOD2 knockdown mouse model of early AMD," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 10, pp. 4407–4420, 2007.

[104] X. Qi, A. S. Lewin, W. W. Hauswirth, and J. Guy, "Optic neuropathy induced by reductions in mitochondrial superoxide dismutase," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 3, pp. 1088–1096, 2003.

[105] K. Kimura, Y. Isashiki, S. Sonoda, T. Kakiuchi-Matsumoto, and N. Ohba, "Genetic association of manganese superoxide dismutase with exudative age-related macular degeneration," *American Journal of Ophthalmology*, vol. 130, no. 6, pp. 769–773, 2000.

[106] M. Kan, F. Liu, X. Weng et al., "Association study of newly identified age-related macular degeneration susceptible loci *SOD2*, *MBP*, and *C8orf42* in Han Chinese population," *Diagnostic Pathology*, vol. 9, no. 1, p. 73, 2014.

[107] M. R. Liles, D. A. Newsome, and P. D. Oliver, "Antioxidant enzymes in the aging human retinal pigment epithelium," *Archives of Ophthalmology*, vol. 109, no. 9, pp. 1285–1288, 1991.

[108] S. K. Jensen, J. V. Nørgaard, and C. Lauridsen, "Bioavailability of α-tocopherol stereoisomers in rats depends on dietary doses of *all-rac-* or RRR-α-tocopheryl acetate," *British Journal of Nutrition*, vol. 95, no. 3, pp. 477–487, 2006.

[109] N. Kono, U. Ohto, T. Hiramatsu et al., "Impaired α-TTP-PIPs Interaction Underlies Familial Vitamin E deficiency," *Science*, vol. 340, no. 6136, pp. 1106–1110, 2013.

[110] K. Ouahchi, M. Arita, H. Kayden et al., "Ataxia with isolated vitamin E deficiency is caused by mutations in the α–tocopherol transfer protein," *Nature Genetics*, vol. 9, no. 2, pp. 141–145, 1995.

[111] T. Yokota, K. Igarashi, T. Uchihara et al., "Delayed-onset ataxia in mice lacking α-tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 26, pp. 15185–15190, 2001.

[112] H. R. Taylor, G. Tikellis, L. D. Robman, M. C. CA, and M. N. JJ, "Vitamin E supplementation and macular degeneration randomised controlled trial," *BMJ*, vol. 134, no. 6, p. 940, 2002.

[113] W. G. Christen, R. J. Glynn, E. Y. Chew, and J. E. Buring, "Vitamin E and age-related macular degeneration in a randomized trial of women," *Ophthalmology*, vol. 117, no. 6, pp. 1163–1168, 2010.

[114] D. Manor and S. Morley, "The α-tocopherol transfer protein," *Vitamins and Hormones*, vol. 76, pp. 45–65, 2007.

[115] D. P. R. Muller, J. K. Lloyd, and O. H. Wolff, "Vitamin E and neurological function," *The Lancet*, vol. 321, no. 8318, pp. 225–228, 1983.

[116] J. Ambati, J. P. Atkinson, and B. D. Gelfand, "Immunology of age-related macular degeneration," *Nature Reviews Immunology*, vol. 13, no. 6, pp. 438–451, 2013.

[117] M. P. Kawa, A. Machalinska, D. Roginska, and B. Machalinski, "Complement system in pathogenesis of AMD: dual player in degeneration and protection of retinal tissue," *Journal of Immunology Research*, vol. 2014, Article ID 483960, 12 pages, 2014.

[118] M. Gemenetzi and A. J. Lotery, "Complement pathway biomarkers and age-related macular degeneration," *Eye*, vol. 30, no. 1, pp. 1–14, 2016.

[119] J. L. Haines, M. A. Hauser, S. Schmidt et al., "Complement factor H variant increases the risk of age-related macular degeneration," *Science*, vol. 308, no. 5720, pp. 419–421, 2005.

[120] J.-M. Lin, Y.-Y. Tsai, L. Wan et al., "Complement factor H variant increases the risk for early age-related macular degeneration," *Retina*, vol. 28, no. 10, pp. 1416–1420, 2008.

[121] A. O. Edwards, Ritter R 3rd, K. J. Abel, A. Manning, C. Panhuysen, and L. A. Farrer, "Complement factor H polymorphism and age-related macular degeneration," *Science*, vol. 308, no. 5720, pp. 421–424, 2005.

[122] R. J. Klein, C. Zeiss, E. Y. Chew et al., "Complement factor H polymorphism in age-related macular degeneration," *Science*, vol. 308, no. 5720, pp. 385–389, 2005.

[123] M. C. Killingsworth, J. P. Sarks, and S. H. Sarks, "Macrophages related to Bruch's membrane in age-

related macular degeneration," *Eye*, vol. 4, no. 4, pp. 613–621, 1990.

[124] J. M. Skeie and R. F. Mullins, "Macrophages in neovascular age-related macular degeneration: friends or foes?," *Eye*, vol. 23, no. 4, pp. 747–755, 2009.

[125] M. K. Falk, A. Singh, C. Faber, M. H. Nissen, T. Hviid, and T. L. Sørensen, "CX3CL1/CX3CR1 and CCL2/CCR2 chemokine/chemokine receptor complex in patients with AMD," *PLoS One*, vol. 9, no. 12, article e112473, 2014.

[126] M. Grunin, T. Burstyn-Cohen, S. Hagbi-Levi, A. Peled, and I. Chowers, "Chemokine receptor expression in peripheral blood monocytes from patients with neovascular age-related macular degeneration," *Investigative Opthalmology & Visual Science*, vol. 53, no. 9, p. 5292, 2012.

[127] K. J. Laing and C. J. Secombes, "Chemokines," *Developmental and Comparative Immunology*, vol. 28, no. 5, pp. 443–460, 2004.

[128] S. A. Lira and G. C. Furtado, "The biology of chemokines and their receptors," *Immunologic Research*, vol. 54, no. 1-3, pp. 111–120, 2012.

[129] W. Raoul, C. Auvynet, S. Camelo et al., "CCL2/CCR2 and CX3CL1/CX3CR1 chemokine axes and their possible involvement in age-related macular degeneration," *Journal of Neuroinflammation*, vol. 7, no. 1, p. 87, 2010.

[130] J. Ambati, A. Anand, S. Fernandez et al., "An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice," *Nature Medicine*, vol. 9, no. 11, pp. 1390–1397, 2003.

[131] U. F. O. Luhmann, S. Robbie, P. M. G. Munro et al., "The drusenlike phenotype in aging *Ccl2*-knockout mice is caused by an accelerated accumulation of swollen autofluorescent subretinal macrophages," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 12, pp. 5934–5943, 2009.

[132] U. F. O. Luhmann, L. S. Carvalho, S. J. Robbie et al., "*Ccl2*, *Cx3cr1* and *Ccl2*/*Cx3cr1* chemokine deficiencies are not sufficient to cause age-related retinal degeneration," *Experimental Eye Research*, vol. 107, pp. 80–87, 2013.

[133] D. D. G. Despriet, A. A. B. Bergen, J. E. Merriam et al., "Comprehensive analysis of the candidate Genes *CCL2*, *CCR2*, and *TLR4* in age-related macular degeneration," *Investigative Ophthalmology & Visual Science*, vol. 49, no. 1, pp. 364–371, 2008.

[134] A. Anand, N. K. Sharma, A. Gupta et al., "Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration," *PLoS One*, vol. 7, no. 11, article e49905, 2012.

[135] S. Zandi, S. Nakao, K. H. Chun et al., "ROCK-isoform-specific polarization of macrophages associated with age-related macular degeneration," *Cell Reports*, vol. 10, no. 7, pp. 1173–1186, 2015.

[136] C. Combadière, C. Feumi, W. Raoul et al., "CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration," *The Journal of Clinical Investigation*, vol. 117, no. 10, pp. 2920–2928, 2007.

[137] R. S. Roque, A. A. Rosales, L. Jingjing, N. Agarwal, and M. R. al-Ubaidi, "Retina-derived microglial cells induce photoreceptor cell death in vitro," *Brain Research*, vol. 836, no. 1-2, pp. 110–119, 1999.

[138] J. K. Olson and S. D. Miller, "Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs," *The Journal of Immunology*, vol. 173, no. 6, pp. 3916–3924, 2004.

[139] D. A. Schaumberg, L. Rose, M. M. DeAngelis, R. D. Semba, G. S. Hageman, and D. I. Chasman, "Prospective study of common variants in *CX3CR1* and risk of macular Degeneration," *JAMA Ophthalmology*, vol. 132, no. 1, pp. 84–95, 2014.

[140] J. Tuo, C. M. Bojanowski, M. Zhou et al., "Murine *Ccl2*/*Cx3cr1* deficiency results in retinal lesions mimicking human age-related macular degeneration," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 8, pp. 3827–3836, 2007.

[141] C. C. Chan, R. J. Ross, D. Shen et al., "*Ccl2*/*Cx3cr1*-deficient mice: an animal model for age-related macular degeneration," *Ophthalmic Research*, vol. 40, no. 3-4, pp. 124–128, 2008.

[142] R. J. Ross, M. Zhou, D. Shen et al., "Immunological protein expression profile in *Ccl2*/*Cx3cr1* deficient mice with lesions similar to age-related macular degeneration," *Experimental Eye Research*, vol. 86, no. 4, pp. 675–683, 2008.

[143] S. J. Clark, R. Perveen, S. Hakobyan et al., "Impaired binding of the age-related macular degeneration-associated complement factor H 402H allotype to Bruch's membrane in human retina," *Journal of Biological Chemistry*, vol. 285, no. 39, pp. 30192–30202, 2010.

[144] J. D. Ding, U. Kelly, M. Landowski et al., "Expression of human complement factor H prevents age-related macular degeneration–like retina damage and kidney abnormalities in aged *Cfh* knockout mice," *The American Journal of Pathology*, vol. 185, no. 1, pp. 29–42, 2015.

[145] Y. H. Kim, S. He, S. Kase, M. Kitamura, S. J. Ryan, and D. R. Hinton, "Regulated secretion of complement factor H by RPE and its role in RPE migration," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 247, no. 5, pp. 651–659, 2009.

[146] M. Chen, J. V. Forrester, and H. Xu, "Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments," *Experimental Eye Research*, vol. 84, no. 4, pp. 635–645, 2007.

[147] J. B. Maller, J. A. Fagerness, R. C. Reynolds, B. M. Neale, M. J. Daly, and J. M. Seddon, "Variation in complement factor 3 is associated with risk of age-related macular degeneration," *Nature Genetics*, vol. 39, no. 10, pp. 1200-1201, 2007.

[148] J. R. W. Yates, T. Sepp, B. K. Matharu et al., "Complement C3 variant and the risk of age-related macular degeneration," *The New England Journal of Medicine*, vol. 357, no. 6, pp. 553–561, 2007.

[149] B. Gold, J. E. Merriam, J. Zernant et al., "Variation in factor B (*BF*) and complement component 2 (*C2*) genes is associated with age-related macular degeneration," *Nature Genetics*, vol. 38, no. 4, pp. 458–462, 2006.

[150] A. E. Hughes, G. M. Mullan, and D. T. Bradley, "Complement factor B polymorphism 32W protects against age-related macular degeneration," *Molecular Vision*, vol. 17, pp. 983–988, 2011.

[151] P. J. Coffey, C. Gias, C. J. McDermott et al., "Complement factor H deficiency in aged mice causes retinal abnormalities and visual dysfunction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 42, pp. 16651–16656, 2007.

[152] C. B. Toomey, U. Kelly, D. R. Saban, and C. Bowes Rickman, "Regulation of age-related macular degeneration-like pathology by complement factor H," *Proceedings of the National Academy of Sciences*, vol. 112, no. 23, pp. E3040–E3049, 2015.

[153] R. L. Ufret-Vincenty, B. Aredo, X. Liu et al., "Transgenic mice expressing variants of complement factor H develop AMD-like retinal findings," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 11, pp. 5878–5887, 2010.

[154] D. Weismann, K. Hartvigsen, N. Lauer et al., "Complement factor H binds malondialdehyde epitopes and protects from oxidative stress," *Nature*, vol. 478, no. 7367, pp. 76–81, 2011.

[155] S. M. Cashman, A. Desai, K. Ramo, and R. Kumar-Singh, "Expression of complement component 3 (C3) from an adenovirus leads to pathology in the murine retina," *Investigative Ophthalmology & Visual Science*, vol. 52, no. 6, pp. 3436–3445, 2011.

[156] C. A. Curcio, M. Johnson, M. Rudolf, and J. D. Huang, "The oil spill in ageing Bruch membrane," *British Journal of Ophthalmology*, vol. 95, no. 12, pp. 1638–1645, 2011.

[157] Z. Cankova, J. D. Huang, H. S. Kruth, and M. Johnson, "Passage of low-density lipoproteins through Bruch's membrane and choroid," *Experimental Eye Research*, vol. 93, no. 6, pp. 947–955, 2011.

[158] A. Thakkinstian, S. Bowe, M. McEvoy, W. Smith, and J. Attia, "Association between apolipoprotein E polymorphisms and age-related macular degeneration: a HuGE review and meta-analysis," *American Journal of Epidemiology*, vol. 164, no. 9, pp. 813–822, 2006.

[159] E. H. Souied, P. Benlian, P. Amouyel et al., "The γ e4 allele of the apolipoprotein E gene as a potential protective factor for exudative age-related macular degeneration," *American Journal of Ophthalmology*, vol. 125, no. 3, pp. 353–359, 1998.

[160] E. Loane, G. J. McKay, J. M. Nolan, and S. Beatty, "Apolipoprotein E genotype is associated with macular pigment optical density," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 5, pp. 2636–2643, 2010.

[161] J. S. L. Tan, P. Mitchell, W. Smith, and J. J. Wang, "Cardiovascular risk factors and the long-term incidence of age-related macular degeneration: the blue mountains eye study," *Ophthalmology*, vol. 114, no. 6, pp. 1143–1150, 2007.

[162] Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group, "Relationship of atherosclerosis in young men to serum lipoprotein cholesterol concentrations and Smoking: A preliminary report from the Pathobiologlcal determinants of atherosclerosis in youth (PDAY) research group," *Journal of Occupational and Environmental Medicine*, vol. 33, no. 8, pp. 841-842, 1990.

[163] A. Cougnard-Grégoire, M. N. Delyfer, J. F. Korobelnik et al., "Elevated high-density lipoprotein cholesterol and age-related macular degeneration: the Alienor study," *PLoS One*, vol. 9, no. 3, article e90973, 2014.

[164] D. G. Vavvas, A. B. Daniels, Z. G. Kapsala et al., "Regression of some high-risk features of age-related macular degeneration (AMD) in patients receiving intensive statin treatment," *eBioMedicine*, vol. 5, pp. 198–203, 2016.

[165] G. Malek, L. V. Johnson, B. E. Mace et al., "Apolipoprotein E allele-dependent pathogenesis: a model for age-related retinal degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 33, pp. 11900–11905, 2005.

[166] L. G. Fritsche, S. Freitag-Wolf, T. Bettecken et al., "Age-related macular degeneration and functional promoter and coding variants of the apolipoprotein E gene," *Human Mutation*, vol. 30, no. 7, pp. 1048–1053, 2009.

[167] M. Kliffen, E. Lutgens, M. J. Daemen, E. D. de Muinck, C. M. Mooy, and P. T. de Jong, "The APO*E3-Leiden mouse as an animal model for basal laminar deposit," *British Journal of Ophthalmology*, vol. 84, no. 12, pp. 1415–1419, 2000.

[168] G. Malek, C.-M. Li, C. Guidry, N. E. Medeiros, and C. A. Curcio, "Apolipoprotein B in cholesterol-containing drusen and basal deposits of human eyes with age-related maculopathy," *The American Journal of Pathology*, vol. 162, no. 2, pp. 413–425, 2003.

[169] M. J. J. Gijbels, M. van der Cammen, L. J. W. van der Laan et al., "Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study," *Atherosclerosis*, vol. 143, no. 1, pp. 15–25, 1999.

[170] L. E. Viiri, K. M. Viiri, E. Ilveskoski et al., "Interactions of functional apolipoprotein E gene promoter polymorphisms with smoking on aortic atherosclerosis," *Circulation: Cardiovascular Genetics*, vol. 1, no. 2, pp. 107–116, 2008.

[171] M. Fujihara, E. Bartels, L. B. Nielsen, and J. T. Handa, "A human apoB100 transgenic mouse expresses human apoB100 in the RPE and develops features of early AMD," *Experimental Eye Research*, vol. 88, no. 6, pp. 1115–1123, 2009.

[172] D. G. Espinosa-Heidmann, J. Sall, E. P. Hernandez, and S. W. Cousins, "Basal laminar deposit formation in APO B100 transgenic mice: complex interactions between dietary fat, blue light, and vitamin E," *Investigative Ophthalmology & Visual Science*, vol. 45, no. 1, pp. 260–266, 2004.

[173] F. B. Sallo, E. Bereczki, T. Csont et al., "Bruch's membrane changes in transgenic mice overexpressing the human biglycan and apolipoprotein b-100 genes," *Experimental Eye Research*, vol. 89, no. 2, pp. 178–186, 2009.

[174] A. Bonen, S. E. Campbell, C. R. Benton et al., "Regulation of fatty acid transport by fatty acid translocase/CD36," *Proceedings of the Nutrition Society*, vol. 63, no. 2, pp. 245–249, 2004.

[175] A. Ibrahimi and N. A. Abumrad, "Role of CD36 in membrane transport of long-chain fatty acids," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 5, no. 2, pp. 139–145, 2002.

[176] S. W. Ryeom, J. R. Sparrow, and R. L. Silverstein, "CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium," *Journal of Cell Science*, vol. 109, pp. 387–395, 1996.

[177] E. Picard, M. Houssier, K. Bujold et al., "CD36 plays an important role in the clearance of oxLDL and associated age-dependent sub-retinal deposits," *Aging*, vol. 2, no. 12, pp. 981–989, 2010.

[178] W. Hu, A. Jiang, J. Liang et al., "Expression of VLDLR in the retina and evolution of subretinal neovascularization in the knockout mouse model's retinal angiomatous proliferation," *Investigative Ophthalmology & Visual Science*, vol. 49, no. 1, pp. 407–415, 2008.

[179] M. Rudolf, B. Winkler, Z. Aherrahou, L. C. Doehring, P. Kaczmarek, and U. Schmidt-Erfurth, "Increased expression of vascular endothelial growth factor associated with accumulation of lipids in Bruch's membrane of LDL receptor knockout mice," *British Journal of Ophthalmology*, vol. 89, no. 12, pp. 1627–1630, 2005.

[180] A. Law and J. Scott, "A cross-species comparison of the apolipoprotein B domain that binds to the LDL receptor," *Journal of Lipid Research*, vol. 31, no. 6, pp. 1109–1120, 1990.

[181] R. W. Mahley, "Apolipoprotein E: cholesterol transport protein with expanding role in cell biology," *Science*, vol. 240, no. 4852, pp. 622–630, 1988.

[182] M. S. Brown and J. L. Goldstein, "Lipoprotein receptors in the liver. Control signals for plasma cholesterol traffic," *The Journal of Clinical Investigation*, vol. 72, no. 3, pp. 743–747, 1983.

[183] M. Rudolf, B. Ivandic, J. Winkler, and U. Schmidt-Erfurth, "Akkumulation von lipidpartikeln in der Bruch-membran von LDL-rezeptor-defizienten Mäusen als modell für die altersbezogene makuladegeneration," *Der Ophthalmologe*, vol. 101, no. 7, pp. 715–719, 2004.

[184] J. R. Heckenlively, N. L. Hawes, M. Friedlander et al., "Mouse model of subretinal neovascularization with choroidal anastomosis," *Retina*, vol. 23, no. 4, pp. 518–522, 2003.

[185] Y. Chen, Y. Hu, K. Lu, J. G. Flannery, and J. X. Ma, "Very low density lipoprotein receptor, a negative regulator of the wnt signaling pathway and choroidal neovascularization," *Journal of Biological Chemistry*, vol. 282, no. 47, pp. 34420–34428, 2007.

[186] L. Yin, Y. Shi, X. Liu et al., "A rat model for studying the biological effects of circulating LDL in the choriocapillaris-BrM-RPE complex," *The American Journal of Pathology*, vol. 180, no. 2, pp. 541–549, 2012.

[187] W. Risau, "Mechanisms of angiogenesis," *Nature*, vol. 386, no. 6626, pp. 671–674, 1997.

[188] P. Carmeliet, "Angiogenesis in health and disease," *Nature Medicine*, vol. 9, no. 6, pp. 653–660, 2003.

[189] E. Moult, W. Choi, N. K. Waheed et al., "Ultrahigh-speed swept-source OCT angiography in exudative AMD," *Ophthalmic Surgery, Lasers and Imaging Retina*, vol. 45, no. 6, pp. 496–505, 2014.

[190] R. Kawasaki, M. Yasuda, S. J. Song et al., "The prevalence of age-related macular degeneration in asians: a systematic review and meta-analysis," *Ophthalmology*, vol. 117, no. 5, pp. 921–927, 2010.

[191] M. S. Pepper, "Role of the matrix metalloproteinase and plasminogen activator–plasmin systems in angiogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 21, no. 7, pp. 1104–1117, 2001.

[192] S. Liekens, E. De Clercq, and J. Neyts, "Angiogenesis: regulators and clinical applications," *Biochemical Pharmacology*, vol. 61, no. 3, pp. 253–270, 2001.

[193] C. Campa, C. Costagliola, C. Incorvaia et al., "Inflammatory mediators and angiogenic factors in choroidal neovascularization: pathogenetic interactions and therapeutic implications," *Mediators of Inflammation*, vol. 2010, Article ID 546826, 14 pages, 2010.

[194] J. A. Wells, R. Murthy, R. Chibber et al., "Levels of vascular endothelial growth factor are elevated in the vitreous of patients with subretinal neovascularisation," *British Journal of Ophthalmology*, vol. 80, no. 4, pp. 363–366, 1996.

[195] U. Schmidt-Erfurth, V. Chong, A. Loewenstein et al., "Guidelines for the management of neovascular age-related macular degeneration by the European Society of Retina Specialists (EURETINA)," *British Journal of Ophthalmology*, vol. 98, no. 9, pp. 1144–1167, 2014.

[196] J. P. Tong and Y. F. Yao, "Contribution of VEGF and PEDF to choroidal angiogenesis: a need for balanced expressions," *Clinical Biochemistry*, vol. 39, no. 3, pp. 267–276, 2006.

[197] K. Ohno-Matsui, I. Morita, J. Tombran-Tink et al., "Novel mechanism for age-related macular degeneration: an equilibrium shift between the angiogenesis factors VEGF and PEDF," *Journal of Cellular Physiology*, vol. 189, no. 3, pp. 323–333, 2001.

[198] D. W. Dawson, O. V. Volpert, P. Gillis et al., "Pigment epithelium-derived factor: a potent inhibitor of angiogenesis," *Science*, vol. 285, no. 5425, pp. 245–248, 1999.

[199] S. B. Bressler, "Introduction: understanding the role of angiogenesis and antiangiogenic agents in age-related macular degeneration," *Ophthalmology*, vol. 116, no. 10, pp. S1–S7, 2009.

[200] P. A. Campochiaro, "Retinal and choroidal neovascularization," *Journal Cell Physiology*, vol. 184, no. 3, pp. 301–310, 2000.

[201] H. Gille, J. Kowalski, B. Li et al., "Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2)," *Journal of Biological Chemistry*, vol. 276, no. 5, pp. 3222–3230, 2001.

[202] H. Zeng, H. F. Dvorak, and D. Mukhopadhyay, "Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) receptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways," *Journal of Biological Chemistry*, vol. 276, no. 29, pp. 26969–26979, 2001.

[203] N. Rahimi, V. Dayanir, and K. Lashkari, "Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells," *Journal of Biological Chemistry*, vol. 275, no. 22, pp. 16986–16992, 2000.

[204] A. N. Witmer, B. C. van Blijswijk, J. Dai et al., "VEGFR-3 in adult angiogenesis," *The Journal of Pathology*, vol. 195, no. 4, pp. 490–497, 2001.

[205] A. N. Witmer, J. Dai, H. A. Weich, G. F. J. M. Vrensen, and R. O. Schlingemann, "Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia," *Journal of Histochemistry & Cytochemistry*, vol. 50, no. 6, pp. 767–777, 2002.

[206] P. Carmeliet and R. K. Jain, "Molecular mechanisms and clinical applications of angiogenesis," *Nature*, vol. 473, no. 7347, pp. 298–307, 2011.

[207] S. J. Ryan, "The development of an experimental model of subretinal neovascularization in disciform macular degeneration," *Transactions of the American Ophthalmological Society*, vol. 77, pp. 707–745, 1979.

[208] V. Lambert, J. Lecomte, S. Hansen et al., "Laser-induced choroidal neovascularization model to study age-related macular degeneration in mice," *Nature Protocols*, vol. 8, no. 11, pp. 2197–2211, 2013.

[209] T. A. Krause, A. F. Alex, D. R. Engel, C. Kurts, and N. Eter, "VEGF-production by CCR2-dependent macrophages contributes to laser-induced choroidal neovascularization," *PLoS One*, vol. 9, no. 4, article e94313, 2014.

[210] Y. M. Paulus, C.-H. Kuo, K. Morohoshi et al., "Serum inflammatory markers after rupture retinal laser injury in mice," *Ophthalmic Surgery, Lasers and Imaging Retina*, vol. 46, no. 3, pp. 362–368, 2015.

[211] L. Berglin, S. Sarman, I. van der Ploeg et al., "Reduced choroidal neovascular membrane formation in matrix metalloproteinase-2–deficient mice," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 1, pp. 403–408, 2003.

[212] D. M. Albert, A. Neekhra, S. Wang et al., "Development of choroidal neovascularization in rats with advanced intense cyclic light–induced retinal degeneration," *Archives of Ophthalmology*, vol. 128, no. 2, pp. 212–222, 2010.

[213] Y. He, K. W. Leung, Y. Ren, J. Pei, J. Ge, and J. Tombran-Tink, "PEDF improves mitochondrial function in RPE cells during oxidative stress," *Investigative Ophthalmology & Visual Science*, vol. 55, no. 10, pp. 6742–6755, 2014.

[214] S. H. Byeon, S. C. Lee, S. H. Choi et al., "Vascular endothelial growth factor as an autocrine survival factor for retinal pigment epithelial cells under oxidative stress via the VEGF-R2/PI3K/Akt," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 2, pp. 1190–1197, 2010.

[215] I. Schmack, L. Berglin, X. Nie et al., "Modulation of choroidal neovascularization by subretinal injection of retinal pigment epithelium and polystyrene microbeads," *Molecular Vision*, vol. 15, pp. 146–161, 2009.

[216] K. Spilsbury, K. L. Garrett, W. Y. Shen, I. J. Constable, and P. E. Rakoczy, "Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization," *The American Journal of Pathology*, vol. 157, no. 1, pp. 135–144, 2000.

[217] J. Baffi, G. Byrnes, C. C. Chan, and K. G. Csaky, "Choroidal neovascularization in the rat induced by adenovirus mediated expression of vascular endothelial growth factor," *Investigative Ophthalmology & Visual Science*, vol. 41, pp. 3582–3589, 2000.

[218] Y. Oshima, S. Oshima, H. Nambu et al., "Increased expression of VEGF in retinal pigmented epithelial cells is not sufficient to cause choroidal neovascularization," *Journal of Cellular Physiology*, vol. 201, no. 3, pp. 393–400, 2004.

[219] Q. Liu and D. A. Muruve, "Molecular basis of the inflammatory response to adenovirus vectors," *Gene Therapy*, vol. 10, no. 11, pp. 935–940, 2003.

[220] F. Wang, K. G. Rendahl, W. C. Manning, D. Quiroz, M. Coyne, and S. S. Miller, "AAV-mediated expression of vascular endothelial growth factor induces choroidal neovascularization in rat," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 2, pp. 781–790, 2003.

[221] P. R. Lowenstein, R. J. Mandel, W.-D. Xiong, K. Kroeger, and M. Castro, "Immune responses to adenovirus and adeno-associated vectors used for gene therapy of brain diseases: the role of immunological synapses in understanding the cell biology of neuroimmune interactions," *Current Gene Therapy*, vol. 7, no. 5, pp. 347–360, 2007.

[222] A. Szade, A. Grochot-Przeczek, U. Florczyk, A. Jozkowicz, and J. Dulak, "Cellular and molecular mechanisms of inflammation-induced angiogenesis," *IUBMB Life*, vol. 67, no. 3, pp. 145–159, 2015.

[223] S. Baatout and N. Cheta, "Matrigel: a useful tool to study endothelial differentiation," *Romanian Journal of Internal Medicine*, vol. 34, no. 3-4, pp. 263–269, 1996.

[224] A. Passaniti, R. M. Taylor, R. Pili et al., "A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor," *Laboratory Investigation*, vol. 67, pp. 519–528, 1992.

[225] D. Shen, R. Wen, J. Tuo, C. M. Bojanowski, and C. C. Chan, "Exacerbation of retinal degeneration and choroidal neovascularization induced by subretinal injection of matrigel in CCL2/MCP-1-deficient mice," *Ophthalmic Research*, vol. 38, no. 2, pp. 71–73, 2006.

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Review Article

New Insights for Cellular and Molecular Mechanisms of Aging and Aging-Related Diseases: Herbal Medicine as Potential Therapeutic Approach

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Aging is a progressive disease affecting around 900 million people worldwide, and in recent years, the mechanism of aging and aging-related diseases has been well studied. Treatments for aging-related diseases have also made progress. For the long-term treatment of aging-related diseases, herbal medicine is particularly suitable for drug discovery. In this review, we discuss cellular and molecular mechanisms of aging and aging-related diseases, including oxidative stress, inflammatory response, autophagy and exosome interactions, mitochondrial injury, and telomerase damage, and summarize commonly used herbals and compounds concerned with the development of aging-related diseases, including *Ginkgo biloba*, *ginseng*, *Panax notoginseng*, *Radix astragali*, *Lycium barbarum*, *Rhodiola rosea*, *Angelica sinensis*, *Ligusticum chuanxiong*, *resveratrol*, *curcumin*, and *flavonoids*. We also summarize key randomized controlled trials of herbal medicine for aging-related diseases during the past ten years. Adverse reactions of herbs were also described. It is expected to provide new insights for slowing aging and treating aging-related diseases with herbal medicine.

1. Introduction

Aging, which can be divided into pathological and physiological aging, is a complex biological process characterized by functional decline of tissues and organs, structural degeneration, and reduced adaptability and resistance, all of which contribute to an increase in morbidity and mortality caused by multiple chronic diseases [1, 2]. As fertility declines and life expectancy increases, the proportion of people aged 60 and older is increasing. According to the UNESA population division, approximately 900 million people are 60 years or older worldwide, and this will increase to 21.5% of the global population by 2050 [3] (see Figure 1). As aging progresses, it increases one's susceptibility to diseases associated with this process, such as vascular aging disorders [4–6], diabetes [7], muscle dysfunction [8, 9], macular degeneration [10], Alzheimer's disease (AD) [11, 12], skin diseases [13], and a series of other diseases [14–18] (see Figure 2). Aging-related diseases pose a serious threat to human health and reduce the quality of life among elderly people. In addition, it has become a global difficulty to clarify the mechanisms of aging, slow the process of aging, reduce the occurrence of aging-related diseases, and maintain that unfading appearance during the aging process.

Aging is a complex process with complicated mechanisms. At present, one of the accepted theories is related to oxidative stress [19–21]. In the process of aerobic metabolism, reactive oxygen species (ROS), including hydroxyl radicals, superoxide anions, and hydrogen peroxide (H_2O_2), can be produced in cells [22–24]. When ROS level exceeds the antioxidant capacity of cells, they react with lipids, proteins, and nucleic acids in cells, resulting in oxidation or peroxide formation. This leads to the destruction of the cell membrane structure, changes in permeability, and a cytotoxic reaction.

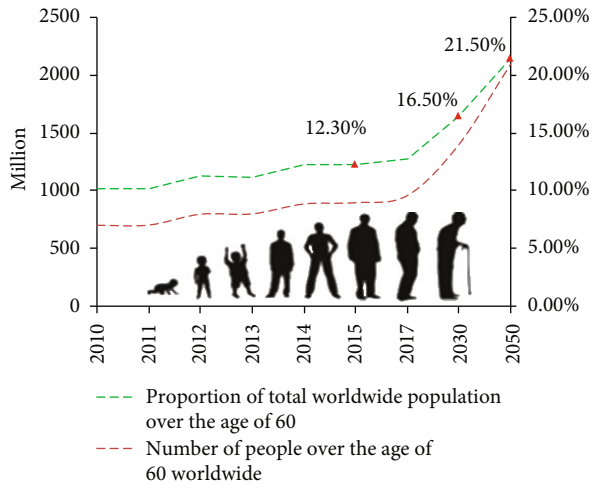

Figure 1: Epidemiological trends of aging worldwide.

High levels of ROS can directly damage DNA, proteins, and lipids in cells, causing a DNA damage response [25] and activation of p38MAPK for p16 upregulation. This leads to cell senescence and development of aging-related diseases [26]. DNA damage response also provides an appropriate trigger for the onset of telomere-induced senescence through the p53 pathway [25, 27, 28]. In addition to oxidative stress, various factors play a role in the aging process. Some reviews have reported the mechanisms of aging [26, 29–32]; however, only one of the mechanisms was examined; for example, some researchers [29, 30] described the role of miRNAs in aging while others [31, 32] placed an emphasis on autophagy.

In this review article, by mainly retrieving PubMed, here, we identify and critically analyzed nearly 10 years of published studies focusing on the mechanisms of aging and aging-related diseases, while summarizing some herbs and compounds that were more extensively used and studied for slowing aging. Compared with the latest published article describing the efficacy, mechanism, and safety of herbal medicine in slowing aging [33], this review is aimed at discussing the cellular and molecular mechanisms of aging from multiple perspectives, also emphasizing the interaction between exosome and autophagy in aging, and discussing age-related diseases and the progress of herbal medicine as potential therapeutic agents for aging and aging-related diseases. The adverse effects of herbs also get our attention in this review.

2. Cell Types Involved in Aging

2.1. Endothelial Cells. Endothelial cells are an essential part of the heart and vasculature [34]. They possess multiple functions through paracrine and endocrine actions, such as regulating vascular tension, maintaining blood circulation, and mediating inflammation, immune response, and neovascularization [35–37]. Endothelial dysfunction caused by endothelial cell senescence is closely linked to the development of aging. Several studies revealed that ROS and inflammation play a role in the apoptosis of endothelial cells [38–41]. Oxidative stress combined with thioredoxin-interacting protein (TXNIP) could activate NOD-like receptor family pyrin domain containing 3 (NLRP3) and inflammatory corpuscles during senescence of endothelial cells. In addition, the production of the proinflammatory cytokine, interleukin-1 (IL-1), which is induced by the activation of NLRP3 inflammatory corpuscles, could promote senescence of endothelial cells [42]. In recent years, it has been well established that autophagy and exosomes play significant roles in the course of a disease [43, 44]. Endothelial dysfunction and impaired autophagic activity are associated with age-related diseases [45]. Exosomes containing harbor miRNAs also participate in the regulation of endothelial function [46]. Studies demonstrated that miR-216a, a molecular component of miRNAs, could be induced during endothelial aging and play an important role in aging-related diseases by regulating autophagy-related genes, such as Beclin1 (BECN1) [47].

2.2. Stem Cells. Stem cells are pluripotent cells characterized as undifferentiated and immature with the ability to self-renew. Stem cell therapy is widely used in clinic, especially in cardiovascular regenerative medicine [48]. Under certain conditions, stem cells can be differentiated into various functional cells, with the potential function of regenerating various tissues and organs [49]. Changes in the cell cycle and a decline in the self-renewal ability of stem cells are closely related to aging. Although some changes in their function are intrinsic [50, 51], more external factors can lead to impairment in their function [52]. Studies have shown that the physiological levels of ROS could regulate the balance between self-renewal and stem cell differentiation [53, 54]. Nevertheless, oxidative stress due to high ROS levels could lead to DNA damage, shortening of telomeres [55], and the onset of premature aging markers, such as prelamin A, the lamin A. Nicotinamide adenine dinucleotide phosphate oxidase isoform 4 (Nox4) component of ROS could be localized to promyelocytic leukemia nuclear bodies (PML-NB) related to prelamin A, which could control the aging of stem cells [56]. Additionally, decline in self-renewal factors contributes to stem cell aging [57].

2.3. Vascular Smooth Muscle Cells. There are evidence suggesting that senescent vascular smooth muscle cells (VSMCs) have been observed in aging-related diseases, such as diabetes mellitus and atherosclerosis [58, 59], which indicate that senescent VSMCs contribute to aging. According to a study by Zhan et al. [60], VSMCs pretreated with the AMPK activator and mammalian target of rapamycin (mTOR) inhibitor could delay the replicative senescence of these cells. They revealed that the AMPK/TSC2/mTOR signaling pathway can regulate the replication and aging of VSMCs, which is mainly manifested as inhibition of the AMPK/TSC2/mTOR pathway which can inhibit the replication and aging of VSMCs. Another study showed that miR-34c-5p downregulation promoted VSM aging through a mechanism that might be mediated by the Bcl-2 modifying factor (BMF), which is a functional target of miR-34c-5p. LncRNAES3 was also found to act as a competing endogenous RNA (ceRNA) of miR-34c-5p to enhance BMF expression [61].

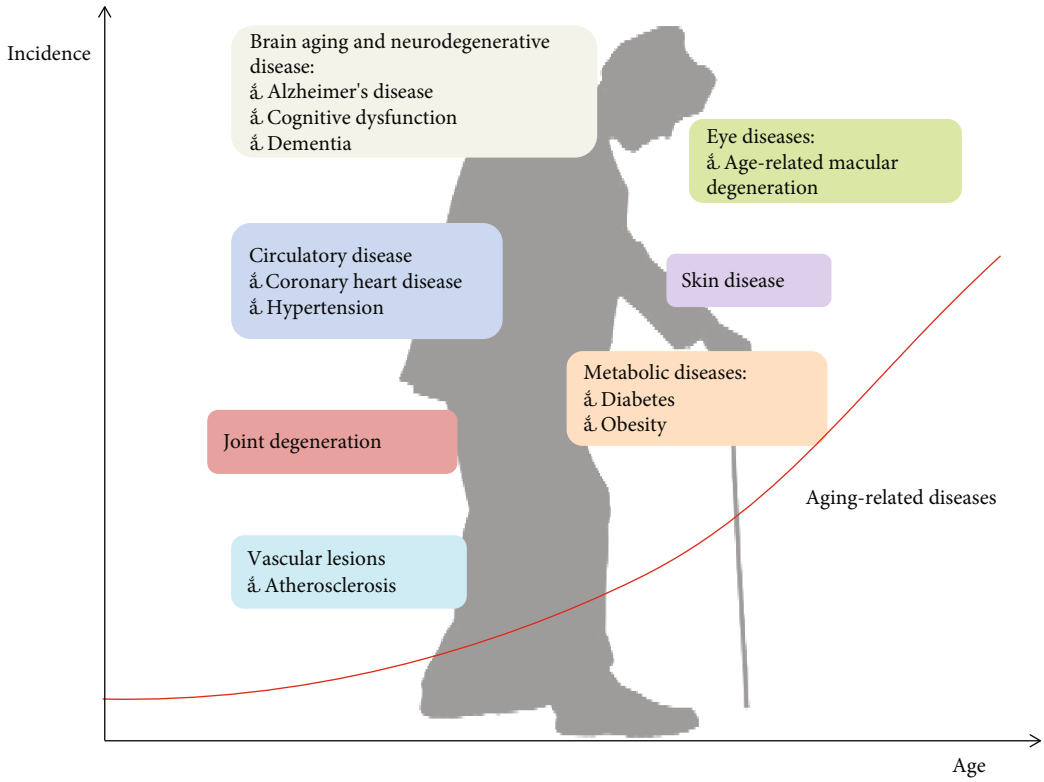

Figure 2: Main aging-related diseases with age and incidence.

3. Molecules or Signal Transduction Pathways in Aging

3.1. Molecules in Aging

3.1.1. MicroRNAs (miRNAs). MicroRNAs (miRNAs, approximately 20-25 nucleotides) are a class of endogenous noncoding RNAs with regulatory functions found in eukaryotes. Recently, miRNAs were found to play an important role in aging [62–82] (see Table 1). According to a study by Du et al. [62], miR-17 extends the lifespan of transgenic mice by upregulating MKP and FoxO3 and downregulating mTOR and JNK through two targets, ADCY5 and IRS1. This study also found that ADCY5 or IRS1 can activate autophagy and inhibit cell aging and apoptosis. Dzakah et al. [63] demonstrated the role of miR-83 in modulating lifespan in Caenorhabditis elegans. Their study found that the deletion of miR-83 extended the lifespan of C. elegans and the expression of miR-83 decreased with age. The life-prolonging effect of miR-83 was achieved by high expression of the transcription factors, daf-16 and din-1. Lyu et al. [64] revealed that the regulation of transforming growth factor-β (TGF-β) signaling promotes senescence via miR-29-induced loss of H4K20me3. Their study found that miR-29 mediated the loss of suv4-20h2, downregulated H4K20me3 expression in mouse fibroblast senescent cells, and promoted cell senescence. Meanwhile, TGF-β accelerated cellular senescence by promoting the miR-29-mediated loss of H4K20me3. Fan et al. [65] observed the role of miR-1292 in cellular senescence of human adipose-derived mesenchymal stem cells (hADSCs). They found that FZD4 downregulation acted as a potential target of miR-1292, leading to overexpression of miR-1292, which promoted hADSC aging and osteogenic differentiation. This event was found to occur via the Wnt/β-catenin signaling pathway. Accumulating evidence suggest that miR-335-3p, which is neuron-enriched, is strongly linked to aging and age-related neurological diseases. Schilling et al. [66] found that statin-associated impairment of cognitive dysfunction is associated with PSD95 decrease, indicating that cholesterol levels are tightly linked to PSD95 levels. According to a study by Raihan et al. [67], overexpression of miR-335-3p, which could suppress cholesterol by inhibiting the expression of 3-hydroxy-3-methylglutaryl-CoA synthase-1 (HMGCS1) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in astrocyte, led to impaired cognitive function and memory. To add, the decrease in cholesterol levels was associated with the decrease in PSD95. When the miR-335-3p expression was reduced in the hippocampal brain of elderly patients, cognitive impairment and synaptic function could be restored in the aging process.

3.1.2. Telomere. Telomeres, composed of the telomere DNA sequence and telomere protein, are nucleoprotein structures located at the end of chromosomes, which control the cell division cycle and maintain the genome's integrity [83]. Studies have shown that decreases in telomere attrition and telomerase activity are two of the main drivers of aging and age-associated damage that lead to cellular senescence [84]. The most well-established driver is the connection between adverse social conditions with DNA damage and accelerated telomere shortening [85, 86]. Epel et al. [87] used standardized questionnaires to assess the previous month's stress

Table 1: MicroRNAs involved in aging-related diseases.

miR type	Model	Function	Target gene	Reference
miR-17	H_2O_2 induced senescent cells	Inhibited mTOR and JNK activation	ADCY5, ISR1	[62]
miR-83	Caenorhabditis elegans	Inhibitory activity of miR-83	din-1, daf-16	[63]
miR-29	Senescent embryonic fibroblast cell	Mediated loss of H4K20me3 promotes senescence	Suv4-20h2	[64]
miR-1292	hADSCs	Accelerated hADSC senescence and restrained osteogenesis	FZD4	[65]
miR-335-3p	Male C57B/6J mice	Reduced cholesterol and impaired memory	Cholesterol	[66, 67]
miR-195	Neonatal mouse cardiomyocyte	Promote apoptosis, causing lipotoxic cardiomyopathy	SIRT1	[68]
miR-126	HUVECs	Regulate high-fat diet-induced endothelial permeability and apoptosis	TGF-β	[69]
miR-138	Aging participants	Regulating the memory function of the elderly	DCP1B	[70]
miR-451	Streptozotocin-induced diabetic mouse heart	Participated in cardiac fibrosis	TGF-β1	[71]
miR-34	Myocardial infarction (MI) in neonatal and adult mice	Its inhibition diminished cell apoptosis	Bcl2, cyclin D1, Sirt1	[72]
miR-146a	Human microvascular endothelial cells (HMVECs)	Ameliorates endothelial inflammation and the progression of atherosclerosis	Receptor-associated factor 6 (TRAF6)	[73]
miR-21	Human umbilical vein ECs	Promoting endothelial inflammation	PPARα	[74]
miR-155	Human nasopharyngeal cancer and cervical cancer cells	Prevention of an age-induced deleterious decrease in autophagy	RHEB, RICTOR, RPS6KB2	[75]
miR-24	H9C2 cardiomyocytes	Attenuate cardiomyocyte apoptosis and myocardial injury	Keap1	[76]
miR-181	Apolipoprotein E-deficient mice	Dampen the inflammatory response in the endothelium	NF-κB	[77]
miR-18a	Naturally aged mice	Regulation of extracellular matrix production during aging cardiomyopathy	CTGF, TSP-1	[78]
miR-377	Old skin tissues	Promotes fibroblast senescence	DNA methyltransferase 1 (DNMT1)	[79]
miR-9-5p	Human neuroblastoma cell line SH-SY5Y	Suppression in cell apoptosis, inflammation, and oxidative stress	SIRT1	[80]
miR-124	Normal human epidermal keratinocytes	Cause skin cell senescence	MEK1, cyclin E1	[81]
miR-15	Human dermal fibroblast	Counteracting senescence-associated mitochondrial dysfunction	SIRT4	[82]

levels of 58 healthy premenopausal women. The control group included women with at least one healthy biological child, and the experimental group included the biological mother of a child with a chronic disease ($n = 39$). Mean telomere length and telomerase activity were measured to evaluate stress-induced changes. The results showed that stress in the experimental group was significantly higher than that in the control group. In addition, women in the experimental group had lower telomerase activity and shorter telomere length than those in the control group. These findings shed light on the cellular level of stress, which can affect one's health by modulating cell aging, possibly leading to the early onset of age-related diseases. Accumulated evidence indicates that DDR-related protein components are found in senescence-associated DNA damage foci (SDFs) [88]. Once ATM/ATR is activated, phosphorylation occurs in Chk1/Chk2, which further acts on effectors such as p53, leading to cell cycle arrest and failure to continue the cell cycle for a certain period of time, ultimately resulting in cell aging and even apoptosis [89, 90]. Further studies have also confirmed that telomere DNA shortening can induce ATM/ATR-mediated DDR and activate the downstream p53-p21 signal transduction pathway, leading to cell senescence [91].

3.1.3. Sirtuins. Sirtuins containing seven different subtypes (SIRT1-SIRT7), which are members of NAD^+ dependent histone deacetylase III, play an important role in cell stress resistance, energy metabolism, apoptosis, and aging [92]. Evidence exists that SIRT1 could deacetylate FOXO, block foxo-dependent transcription and the apoptotic pathway, and promote the survival of senescent cells. This occurs

through an increase in SIRT1 expression with age, suggesting that Sirt1 is involved in longevity [93, 94]. SIRT 2 is closely related to age-related diseases, such as Alzheimer's disease (AD) and Parkinson's [95]. Studies have shown that inhibition of SIRT2 expression could delay the progression of these diseases. In addition, knockout of SIRT2 and SIRT5 could alleviate the neurodegenerative lesion induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The expression of SIRT2 was found to inhibit the dephosphorylation of FOXO3a and increase the level of Bim, leading to apoptosis and acceleration of the process of aging [96]. The mechanism by which SIRT5 deletion reduced apoptosis might be related to the reduction of SOD2 (manganese superoxide dismutase) expression [97]. SIRT3 has been reported to be associated with longevity. It can interact with FOXO3a to remove ROS and inhibit oxidative stress to prolong one's lifespan [98]. In the latest research by Zhang et al. [99], they found that by performing a whole-body knockout of "longevity gene" SIRT6 in nonhuman primates, they could obtain the world's first cynomolgus monkey model of longevity gene knockout, thereby revealing the new role of the SIRT6 gene in regulating embryonic development of primates. They could also elucidate the differences in aging and longevity regulation pathways between primates and rodents, laying an important foundation for research on the mechanisms of human development and aging and the treatment of related diseases [99]. SIRT7 could result in antiaging and prolong life by regulating the repair of the nonhomologous DNA damage to maintain the stable heredity of cells [100].

3.1.4. Klotho Gene. The *Klotho* gene, located on human chromosome 13, contains five exons and exerts antiaging effects. Studies have confirmed that the decrease in *Klotho* expression with an increase in age leads to aging [101]. Ullah and Sun [102] found that lack of the *Klotho* gene reduced the activity of telomerase by modifying the expression of TERF1 and TERT, leading to apoptosis of pluripotent stem cells. Sustained exposure to Wnt accelerated cellular senescence both in vitro and in vivo [103]. However, studies revealed that the tissue and organs of *Klotho*-deficient animals could enhance the Wnt signaling pathway to cause cell senescence [103]. A few other studies showed that *Klotho* downregulation leads to premature aging of human fibroblasts, which might be achieved by regulating the insulin/IGF-1 pathway to upregulate p53 and p21 protein levels [104–106]. According to study by Gao et al. [107], *Klotho* deficiency could downregulate SIRT1, which reduce activities of AMP-activated protein kinase alpha (AMPKα) and endothelial nitric oxide synthase (eNOS), and upregulate NADPH oxidase activity, ultimately leading to aging-related aortic stiffness.

3.1.5. p16, p53/p21. Cell cycle stagnation is the premise of aging [108]. Although cell aging involves a series of gene expression and cell morphological changes, which are not as simple as cycle stagnation, many experiments have confirmed that the increase in p16 or p53/p21 is enough to cause cell aging [89, 109–113]. In mouse embryonic fibroblasts, overexpression of miR-20a increased p16 and upregulated the transcriptional activity of INK4a/ARF, leading to cell senescence [114]. P53 is not only an initiator of cell aging but also a participant in antiaging. These effects of p53 are closely related to its involvement in the regulation of the mTOR pathway, which is closely related to autophagy. P53 can play an antiaging role by inhibiting the activity of mTOR and can also activate mTOR to inhibit the aging process [115]. Meanwhile, p53, through its downstream p53/p21/CDK2 signaling pathway, was found to result in cell cycle arrest and enter the aging state [111]. Studies have found that azithromycin might cause aging of VSMCs by activating the mTOR signaling pathway and increasing the expression of p53/p21/p16. When the activity of mTOR was inhibited, the autophagy level of proteins related to the mTOR signaling pathway increased, leading to a decrease in the expression of p53/p21/p16, thereby delaying the aging of VSMCs [116].

3.2. Signaling Pathway in Aging

3.2.1. Mammalian Target of Rapamycin (mTOR) Pathway. mTOR, activated by growth factors and nutrients, inhibits autophagy and promotes protein synthesis. Over time, mTOR may promote cellular stress, such as protein aggregation, organelle dysfunction, and oxidative stress, which may lead to the accumulation of damage and cell function decline, ultimately promoting the occurrence of age-related diseases [117]. The classical pathway of mTOR is the PI3K/Akt/mTOR signaling pathway. Tan et al. [118] transfected human VSMCs with mTOR siRNA and scrambled siRNA and found that PI3K/Akt/mTOR plays a significant role in VSMC replication and aging, which might be related to the regulation of oxidative stress and telomere function. Additionally, mTOR activation induced stem cell depletion, which reduced tissue repair and aggravated tissue dysfunction. Experimental studies have also shown that by inhibiting the mTOR signaling pathway through gene knockout, rapamycin or dietary restriction can delay aging of various biological models, including yeast, worms, fruit flies, and mice [119].

3.2.2. Nuclear Factor of Activated B-Cell (NF-κB) Signaling Pathway. NF-κB, activation of the transcription factor protein family, is involved in oxidative stress, immunity, inflammation, cell proliferation, apoptosis, and aging of gene transcription regulation. Studies have confirmed that NF-κB has a highly conserved REL homologous domain consisting of 300 amino acids and that its protein family members include p50, p52, REL, REL-A, and REL-B [120]. The NF-κB signaling pathway is activated by senescence-related inflammatory factors. Activated NF-κB enters the nucleus and binds to DNA, thereby participating in cellular immune response [121]. Studies have confirmed that the occurrence of various senile degenerative diseases is closely related to the aging signaling pathway regulated by NF-κB. Postmortem examination revealed an increase in NF-κB activity in brain tissues of Alzheimer disease (AD) patients. In addition, the immunological activity of p65 was detected in neurons and glial cells adjacent to degenerative neurons and senile plaques [122]. The activation of NF-κB is related to the

deposition of β-amyloid (Aβ). Studies have found that Aβ deposition could activate NF-κB in cultured neurons with the formation of NO products related to oxidative stress [123]. Autopsy studies found that the number of NF-κB-positive dopaminergic neurons in the brain of patients with Parkinson's disease was 70 times higher than that of normal people, suggesting that the activation of NF-κB is related to the pathological mechanism of Parkinson's disease [123, 124].

3.2.3. Nuclear Factor-E2-Related Factor 2 (Nrf2) Signaling Pathway. Nrf2 is a key factor of antioxidant activity in cells. When oxidative stress occurs, Nrf2 is transferred to the nucleus to bind with the antioxidant response element (ARE) and regulates the expression of various antioxidant proteins and detoxification enzymes downstream, ultimately playing a role in endogenous protection [125]. Suh et al. [126] found that total Nrf2 protein and the amount of nuclear Nrf2 protein in rat liver cells significantly decline with an increase in aging. As age increases, the antioxidant capacity of ovarian cells decreases, and the imbalance between oxidation and antioxidants causes gradual apoptosis of ovarian cells, which is one of the important causes of ovarian aging. Studies have found that Nrf2 gene knockout can increase the ovary's sensitivity to toxic substances and accelerate the aging of ovaries in mice [127]. Chen et al. [128] found that the upregulation of Nrf2 expression could alleviate oxidative stress and DNA damage and inhibit the p53-p21 p16-rb signaling pathway, thereby slowing cell aging. Nrf2 can regulate mitochondrial biogenesis and kinetics to maintain muscle mass and function, and its deficiency with aging increasingly promotes age-related skeletal muscle mitochondrial dysfunction and muscle atrophy [129, 130]. Study also found that Nrf2 activation could inhibit age-related inflammatory responses and oxidative stress and delay the occurrence of aging and age-related diseases [131]. Activation of Nrf2 also improved learning and memory of aging mice administered with D-galactose (D-gal) [132].

3.2.4. Wnt/β-Catenin Signaling Pathway. The Wnt/β-catenin signaling pathway is an evolutionarily, highly conserved signaling pathway with a wide range of biological functions. Studies found that this pathway plays an important regulatory role in cell aging and its activation could lead to senescence changes in mesenchymal stem cells [133]. Studies have also shown that activation of this pathway could lead to DNA damage response and increase the expression of the p53 protein, which might be one of the important mechanisms for stem cell senescence [134]. The p53/p21 pathway and DNA oxidative damage response have been confirmed to play an important role in the aging process of hematopoietic stem/progenitor cells caused by the Wnt/β-catenin signal pathway [135]. Skin aging is the most important external manifestation of human body aging, and the related components of WNT/β-catenin signal pathway are abnormally overexpressed in aged skin tissues [136]. The WNT/β-catenin signal pathway was found to be enhanced in the aging mouse model, and inhibition of the WNT/β-catenin signal pathway could reverse age-related skeletal muscle regeneration injury [137].

3.2.5. Adenosine Monophosphate Protein Kinase (AMPK) Signaling Pathway. AMPK is a highly conserved cellular energy metabolism regulator that plays an important role in regulating cell growth, proliferation, survival, and energy metabolism [138]. AMPK is involved in the regulation of a series of senescence-related signaling pathways, such as SIRT1 and CRTC-1. Studies have shown that AMPK first enhanced the expression of niacinamide phosphoribose transferase and then increased the intracellular concentration of NAD^+ to activate SIRT1, which then activates the downstream PGC-1, FoxO1, and FoxO3, ultimately interfering with the aging process [139]. Mair et al. [140] identified that CRTC-1 is the phosphorylation site of AMPK/AAK-2 with the nematode model, and AMPK/AAK-2 prevented its nuclear translocation via CRTC-1 phosphorylation, thereby inhibiting the transactivation of CREB transcriptional regulator crh-1 which extended the nematode's lifespan. AMPK activates p53 at certain phosphorylation sites and induces cell cycle arrest, leading to cell aging [141].

4. Aging-Related Diseases and Therapy

4.1. Vascular Aging. With an increase in age, the degeneration of vascular structure and function causes vascular sclerosis, which is called vascular aging. The main manifestations of vascular aging are increased arterial stiffness, pulse wave velocity, systolic blood pressure, and central venous pressure [142]. Vascular aging is a major risk factor for atherosclerosis and cardiovascular disease. Vascular aging mainly includes atherosclerosis and arteriosclerotic cardiovascular disease (ASCVD), such as coronary heart disease, hypertension, stroke, cognitive dysfunction, dementia, and peripheral vascular disease [143].

Studies have shown that decreased vasorin magnified the angiotensin II- (Ang II-) mediated increase in the TGF-β1 signaling cascade and caused vascular remodeling, thus leading to vascular aging [144, 145]. Increased Ang II with age led to activation of its downstream molecules MMP, McP-1, and TGF-β. This pathological change made the aortic wall of the elderly present a proinflammatory profile, which could promote atherosclerosis [146, 147]. Vascular endothelial cell senescence is one of the important pathological changes of vascular aging while oxidative stress is one of the main causes of endothelial senescence. eNOS has a significant effect on cardiovascular protection, and oxygenation should stimulate the decreased expression, resulting in a decrease in NO bioavailability, vascular diastolic dysfunction, and arteriosclerosis, ultimately promoting vascular aging [148]. Vascular endothelial cell aging is identified by ROS, the secretion of inflammatory cytokines, eNOS uncoupling, DNA damage, and telomere dysfunction, leading to obstacles in the structure and function of the cardiovascular system. It is also associated with coronary atherosclerotic heart disease [149, 150]. Studies have shown that atherosclerosis is associated with pathological thickening of vascular intima, loss of vascular smooth muscle cells, lipid deposition, and infiltration of macrophages [151]. Senescence was also found to accelerate atherosclerosis by inducing endoplasmic reticulum stress in VSMCs [152].

Complex functional impairment of cerebral microvessels and astrocytes may lead to neurovascular dysfunction and cognitive decline, which results in aging and age-related neurodegenerative diseases [153].

Early intervention of vascular aging can delay the occurrence of ASCVD and protect target organs. Presently, early intervention of vascular aging mainly includes lifestyle improvement and drug therapy. Caloric restriction and low-sodium diet combined with exercise can delay vascular aging. Meanwhile, active control of cardiovascular risk factors, such as hypertension, diabetes, and hyperlipidemia, can also prevent vascular aging. Drug therapy can target structural components of vascular aging, thus delaying development of aging. These mainly include antihypertensive drugs, statins, and hypoglycemic drugs. Antihypertensive drugs such as angiotensin-converting enzyme inhibitors (ACEI)/angiotensin-receptor antagonists (ARBs) have been shown to delay vascular aging due to their antifibrotic effects. Statins can not only regulate fat but also interfere with the process of vascular aging. Hypoglycemic drugs can increase the sensitivity of insulin, improve blood sugar, prevent the reconstruction of blood vessels, and inhibit inflammation of the tube wall.

4.2. Diabetes Mellitus. Diabetes is closely related to aging, and dysfunction of the pancreatic β cells plays an important role in the occurrence and development of diabetes. Aging of β cells in islets is mainly manifested as a decrease in the number of β cells and reduction in their secretion capacity. The mechanisms between islet cell failure in diabetes and aging are complex. Nonetheless, study found that the expression of autophagy signature proteins, LC3/Atg8 and Atg7, was decreased in aging islet cells. Similarly, the autophagy function of islets in aged rats was found to decrease [154]. Upregulation of P16ink4a/p19ARF expression, decrease in bmi-1 and EZH2 levels, and abnormal regulation of platelet-derived growth factor signals are important factors leading to a decline in the proliferation and insulin secretion of age-related β cells [155, 156]. The main interventions for diabetes include diet control, exercise, weight loss, and combination of hypoglycemic drugs.

4.3. Alzheimer's Disease. Alzheimer's disease (AD) is a neurodegenerative disease that occurs in old age and preold age. Brain aging is the basis and condition for the formation of neurodegenerative diseases. Alzheimer's disease is characterized by amyloid-β protein (Aβ) deposits that form plaques and by hyperphosphorylation of Tau protein that forms tangles of neurons (NFT). Abnormal mitochondria accumulate in neurons, leading to reduced ATP production, large release of oxygen-free radicals, the production of Aβ, and the intensification of Tau protein phosphorylation [157]. Mutations of PSEN 1 and PSEN 2 cause lysosomal dysfunction, and the presence of lysosomal dysfunction leads to a large number of autophagosomes generated by enhanced mitochondrial autophagy, leading to lysosomal overload and further aggravating brain injury [158]. Chronic activation of the NF-κB pathway can cause the transcription of various inflammatory cytokines and promote glial cells to secrete inflammatory cytokines, leading to nerve cell injury and apoptosis [159, 160]. Currently, drugs used in the clinical treatment of AD are mainly noncompetitive N-methyl-D-aspartic acid receptor antagonists (such as memantine) and cholinesterase inhibitors (such as donepezil and galantamine).

4.4. Skin Aging. Skin aging, which is a part of the overall aging of the body, not only affects its appearance but also reduces its function as the body's barrier. This can lead to various diseases, such as depression and self-abasement. Tashiro et al. [161] cultured skin fibroblasts from women of different ages to study the relationship between autophagy and skin aging. They found that the autophagy degradation step was inhibited in skin fibroblasts of elderly donors, leading to the accumulation of autophagosomes. This suggests that the impairment of autophagy function in skin fibroblasts of elderly people may impact the skin's integrity and strength. Some researchers constructed a *Drosophila* model of skin aging and found that the increased expression of the autophagy marker, Atg7, was associated with skin aging [162]. Another study found that exosome miR-30a can regulate the apoptosis of epidermal cells, and its overexpression led to impaired epidermal differentiation by directly targeting AVEN (encodes a caspase inhibitor), IDH1 (encodes isocitrate dehydrogenase, an enzyme of cellular metabolism), and LOX (encodes lysyl oxidase, a regulator of the proliferation/differentiation balance of keratinocytes), inducing severe barrier dysfunction and skin aging [163]. Treatment for skin aging mainly includes oral antioxidant drugs, topical antiaging agents, and photoelectric and acoustic physical technology.

4.5. Aging-Related Macular Degeneration. Age-related macular degeneration (AMD) is one of the major causes of vision impairment in people older than 60 years of age. AMD can be divided into two types: dry AMD (atrophic), accounting for 85 to 90% of AMD cases and is a pattern of atrophy caused by the absence of retinal pigment epithelial cells and photoreceptor cells, and wet AMD (exudative, neovascular), which is caused by bleeding and exudation of neovascularization into the retina pigment epithelium (RPE) and into the sensory layer of the retina. Accumulating evidence suggests that the abnormal function of autophagy is related to the AMD formation. According to a study by Cai et al. [164], activation of mTOCR1 in aging RPE cells led to impaired lysosomal function and decreased autophagy in RPE cells. When the expression level of miR-29 is increased, the activity of mTORC1 is inhibited to enhance autophagy and remove protein aggregates to delay the occurrence of AMD. Another study found that SQSTM1/p62, a marker of autophagy injury, is deposited in the RPE along with the decrease in autophagy, which activates the inflammatory body, impairs protein clearance, and damages RPE cells, leading to AMD formation [165].

5. Herbal Medicines: Promising Therapeutic Agents for the Management of Aging and Aging-Related Diseases

Studies had shown that many herbs had curative effect of slowing aging; selected herbs and compounds that were more

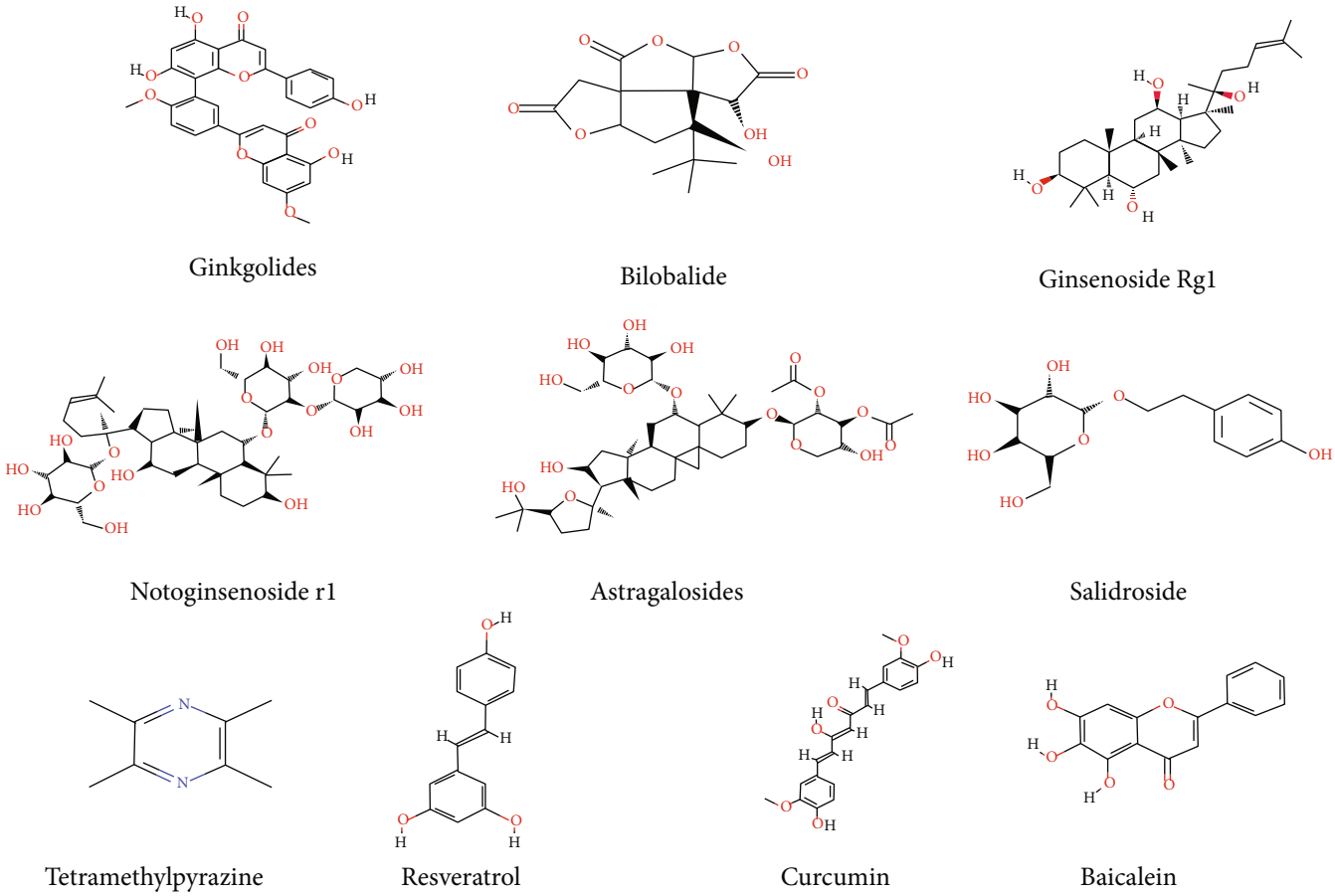

Figure 3: Chemical structural formula of the main active ingredients of herbs and compounds.

extensively used and studied for review include Ginkgo biloba, ginseng, Panax notoginseng, Radix astragali, Lycium barbarum, Rhodiola rosea, Angelica sinensis, Ligusticum chuanxiong, resveratrol, curcumin, and flavonoids. The chemical structural formula of the main active ingredients of herbs and compounds was shown in Figure 3.

5.1. Herbs

5.1.1. Ginkgo biloba (Yinxing). Ginkgo biloba extract (EGb) has definite pharmacological effects of protecting the vascular endothelium, improving insulin resistance, and preventing atherosclerosis [166]. In addition, *EGb* exerts a good intervention in various age-related diseases, such as type 2 diabetes mellitus, dementia, cognitive impairment, and coronary heart disease [167]. The first international expert consensus regarding the clinical application of EGb for the treatment of dementia and moderate cognitive impairment was published in 2019 [168]. Dong et al. [169] pretreated senescent endothelial progenitor cells (EPCs) with 10, 25, and 50 mg/L of EGb and found that it could inhibit the senescence of EPCs and increase the activity of telomerase, especially at the concentration of 25 mg/L. The mechanism whereby EGb inhibited the aging of EPCs may be related to the activation of the PI3k/Akt signaling pathway. Zhou et al. [170] administered EGb-761 to aging mice at different doses of 20, 40, 80, and 100 mg/kg once every 3 days for 12 months and found that EGb could reduce ischemic injury and oxidative stress caused by ischemia in aging mice. Its mechanism might be related to the upregulation of protein phosphatase 2 (PP2A) and reduction in extracellular signal-regulated kinase (ERK) activation. Tian et al. [171] administered EGB to streptozotocin- (STZ-) induced diabetic ApoE$^{-/-}$ mice at doses of 200 and 400 mg/kg/day for 12 weeks and found that EGb could regulate glucose and lipid metabolism, reduce arterial plaque, and upregulate autophagy to relieve endoplasmic reticulum stress (ERS). Its mechanisms might be related to the inhibition of ERS through the restoration of autophagy via the mTOR signaling pathway.

5.1.2. Panax ginseng (Renshen). Ginsenosides are the main active ingredients of *Panax ginseng*. Studies have shown that ginsenosides display plentiful pharmacological effects such as relieving fatigue, improving immunity, slowing aging, inhibiting metastasis of cancer cells, regulating blood glucose, and protecting liver and kidney functions [172].

Aging mice were intraperitoneally injected with the ginsenoside Rg1, at a dose of 20 mg/kg/day for 28 days continuously. Rg1 could retard testis senescence in mice via antioxidation and the downregulation of the p19/p53/p21 signaling pathway [173]. Zhou et al. [174] cultured aging Sca-1+ hematopoietic stem cells in ginsenoside for 6 h and found that ginsenoside could protect hematopoietic stem cells from aging. Its possible mechanisms of action might

involve the regulation of the p16-Rb signaling pathway, the repair of worn telomeres, and maintenance of telomerase activity. Aging mice were fed an experimental diet based on AIN-93G containing 10 g/kg and 30 g/kg ginseng powder for 24 weeks continuously. The results suggested that long-term ginseng feeding could improve aging-related cognitive ability, which was achieved by regulating the cholinergic and antioxidant systems [175]. Other studies found that Rg1 could decrease oxidative stress and downregulate Akt/mTOR signaling to attenuate cognitive impairment in mice and senescence of neural stem cells induced by D-gal [176].

5.1.3. Panax notoginseng (Sanqi). *Panax notoginseng* contains the notoginseng saponins Rh1, Rh2, Rg1, Rg2, Rgb1, and others, with pharmacological actions such as antitumor activity, enhanced learning and memory, hemolysis, hemostasis, antiaging, and antifatigue [177, 178]. Zhou et al. [179] administered Panax notoginseng saponins (PNS) at 10, 30, and 60 mg/kg/day to natural aging mice and found that it could significantly and dose-dependently inhibit the apoptosis of myocardial cells in senescent rats by attenuating oxidative damage. Li et al. [180] pretreated aging H9c2 cells induced by D-gal with different concentrations of total saponins of Panax notoginseng (5, 25, and 50 g/mL) for 4 h. They found that the number of positive cells stained with galactosidase in the total saponins of the Panax notoginseng group was significantly reduced; SOD activity was found to significantly increase while MDA content and ROS fluorescence intensity were significantly decreased. Results suggest that PNS could resist aging of H9c2 cells induced by D-gal by improving their antioxidant capacity and reducing apoptosis.

5.1.4. Radix astragali (Huangqi). *Radix Astragalus* mainly contains astragalus polysaccharides, saponins, flavonoids, and other active components, which have various pharmacological actions such as antioxidation, antiaging, myocardium protection, and enhancement of immune function and hematopoietic function [181].

Ma et al. [182] used different doses (100, 200, 400, and 600 mg/kg) of *astragalus* extract for intervention in the animal model of sustained myocardial ischemia in vivo. They found that *Astragalus* can reduce myocardial injury and protect cardiac function, which are related to the reduction of oxidative damage and free radical generation. Ma et al. [182] also conducted in vitro experiments to interfere with the oxidative stress model of cardiac myocytes using *Astragalus membranaceus* at different concentrations (100, 200, 400, and 600 μg/mL). They found that *Astragalus* could reduce the number of cell apoptosis by attenuating oxidative injury and arresting Ca^{2+} influx to block cell death. Li et al. [183] administered different doses (8, 16, and 32 mg/kg) of astragalosides via the intragastric route to the rat model with learning and memory impairment. They found that astragalosides could improve the learning and memory ability and ameliorate the neurodegenerative lesion of hippocampal CA1, which are related to the reduction of intracerebral amyloid precursor protein (APP) and a-secretase and β-secretase mRNA levels. Astragalus polysaccharides can also protect the mitochondria by scavenging ROS, inhibiting mitochondrial permeability transition (PT), and increasing antioxidant enzyme activity to improve aging in mice [184].

5.1.5. Lycium barbarum (Gouqi). *Lycium barbarum* has pharmacological actions such as regulating immunity, antitumor activity, nervous system function, liver protection, and slow aging process [185]. Hu et al. [186] administered different doses of Chinese wolfberry, via the intragastric route, to a mouse model of AD induced by the combination of $AlCl_3$ and D-gal. They found that the quantity of horizontal and vertical movements increased while AChE and ChAT levels decreased significantly in mice. These events were related to the modulation of the mitochondrial pathway of apoptosis and the cholinergic system. Jeong et al. [187] used goji berry (150 and 300 mg/kg/day) to interfere with aging rats and found that goji berry could elevate the level of testosterone and reduce the expression of cell apoptosis activators, which are associated with its antioxidant action. Yu et al. [188] used *L. barbarum* to interfere with oxygen glucose deprivation and reoxygenation-induced injury of neurons. They found that *L. barbarum* inhibits oxygen glucose deprivation and reoxygenation-induced neuronal cell and autophagic cell death by activating the PI3K/Akt/mTOR pathway.

5.1.6. Rhodiola rosea (Hongjingtian). *Rhodiola rosea* contains alkaloids, flavonoids, glycosides, phenolic compounds, volatile oils, coumarins, steroids, and organic acids, plus small amounts of nonorganic elements, which could protect the heart and brain vessels by exhibiting antifibrosis, antioxidation, anti-inflammatory, antivirus, antiapoptosis, and antifatigue activities [189]. Zhou et al. [190] orally administered *R. rosea* (60 and 120 mg/kg daily) to an atherosclerosis rat model for 9 weeks continuously. The results showed that *R. rosea* could contribute to antiatherosclerosis via lowering blood lipids, antioxidant, and anti-inflammatory activities and by regulating endothelial function. Schriner et al. [191] demonstrated that *R. rosea* could prolong the lifespan of *Drosophila* by perturbing the silent information regulator 2 (SIR2) proteins, insulin and insulin-like growth factor signaling (IIS), and the target of rapamycin (TOR). Furthermore, *R. rosea* could prolong the life of silkworms by improving antioxidant capacity [192].

5.1.7. Angelica sinensis (Danggui). The active components of *Angelica sinensis* mainly include volatile oils (ligustilide, Angelica sinensis ketone), organic acids (ferulic acid, succinic acid, niacin, and azelaic acid), polysaccharides, and flavonoids (ferulic acid, succinic acid, niacin, anisolic acid, and azelaic acid) [193]. Zhang et al. [194, 195] orally administered Angelica polysaccharide (ASP, 200 mg/kg/) to aging mice induced by X-ray whole-body uniform irradiation. HSCs were then separated and purified after mice were sacrificed. The results showed that ASP could significantly reduce the positive rate of SA-β-gal staining and the proportion of G1 phase in the aging group of HSCs, reduce ROS production, downregulate p16 mRNA, and increase the ability of mixed colony formation and T-AOC. Cheng et al. [196] showed that ASP restored cognitive impairment caused by D-gal administration, promoted neural

stem cell (NSC) proliferation, attenuated D-gal-induced NSC senescence, decreased the level of oxidative stress by enhancing antioxidative capacity, and decreased the levels of inflammatory cytokines of NSCs. These events slowed the aging speed by enhancing the antioxidant and anti-inflammatory capacity and downregulating the p53/p21 signaling pathway [197, 198].

5.1.8. Ligusticum chuanxiong (Chuanxiong). *Ligusticum chuanxiong* contains tetramethylpyrazine (TMP), ligustrazine, vanillin, emodin, ferulic acid, and other active ingredients which display various pharmacological actions in the cardiac and cerebrovascular system, nervous system, and respiratory system [199]. Chen et al. [200] demonstrated that TMP at different doses of 1, 3, and 10 mg/kg interfered with 6-ohda-induced Parkinson's disease in mice which confirmed that TMP protects against dopaminergic (DA) neurodegeneration and attenuates DA neuronal apoptosis by activating the PI3K/Akt/GSK3β signaling pathway. Wei and Wang [201] found that ligustrazine alleviated hypoxia-induced HUVEC cell injury, enhanced cell viability, and inhibited cell apoptosis, all of which are related to the upregulation of miR-135b and subsequent activation of JNK/SAPK and PI3K/AKT/mTOR pathways. These events promoted hypoxia-treated HUVEC cell growth. Another study has shown that TMP could inhibit the accumulation of senescent $LepR^+$ mesenchymal stem/progenitor cells in bone marrow, reduce bone loss, and improve the metabolic microenvironment of aging mice via the AMPK-mTOR-Hif1a-VEGF pathway [202]. As a potential treatment, TMP could improve bone diseases related to human age and promote a healthy lifespan.

5.1.9. Other Herbs. Hou et al. [203] selected aging, 24-month-old guinea pigs as the animal experimental models and fed them with a diet containing different doses (75, 100, or 150 mg/kg/day) of water-soluble extract components of *Salvia miltiorrhiza Bunge* for 28 days continuously. The study found a significant decrease in whole blood viscosity and improvement of blood viscosity and viscoelasticity at the dose of 150 mg/kg/day. Park et al. [204] gave old (20-month-old) specific pathogen-free male Sprague-Dawley rats with magnesium lithospermate B, extracted from Salvia at a dose of 2 or 8 mg/kg/day for 16 consecutive days. The results suggested that it reduces the renal damage of oxidative stress in old rats. After the researchers fed the fruit flies a full or dietary restriction diet supplemented with oregano-cranberry (OC) mixture, the study found that OC could extend the lifespan of fruit flies, especially females, while only OC supplementation at the young age interval increased reproduction in females [205, 206].

After 8 weeks of intraperitoneal injection of 100 mg/kg/d d-galactose to establish a rat model of aging with different doses of Ganoderma lucidum extract, it was found that Ganoderma lucidum could delay the progression of AD by regulating DNA methylation [207]. Lobo et al. [208] gave different concentrations (0.5-5.0 mg/mL) of the *Gynostemma pentaphyllum* extract to mouse dermal fibroblasts, which were placed under 8-watt ultraviolet C (UVC) light at a distance of 50 cm to induce oxidative stress. The results showed that *Gynostemma pentaphyllum* extract prolongs viability of mouse dermal fibroblasts damaged by UVC light-induced oxidative stress, especially at 4.5 mg/mL, and it suggested that *Gynostemma pentaphyllum* extract had potential therapeutic effect on dermal cell aging.

5.2. Compounds

5.2.1. Resveratrol. Resveratrol is a natural polyphenol with anticardiovascular, anticancer, antibacterial, anti-inflammatory, antiaging, antineurodegenerative, and other pharmacological effects [209]. Wu et al. [210] used different doses (30 and 100 mg/kg/day) of resveratrol to intervene in mice with premature ovarian aging caused by chemotherapy. They found that resveratrol could improve premature ovarian aging caused by chemotherapy and ameliorate the renewal ability of oogonial stem cells by attenuating oxidative stress injury via Nrf2 activation.

Dehghani et al. [211] used resveratrol combined with calcitriol to intervene in D-gal-induced aging rats. This combination could protect the heart and its antioxidant status by modulating hemodynamic parameters and increasing the serum level of *Klotho*, respectively. Du et al. [212] used resveratrol (5, 10, and 50 μM) to intervene in aging cells and found that it could improve cell activity and increase SOD level by regulating autophagy to achieve delayed aging. Amos et al. [213] intervened in damages to *Drosophila melanogaster* induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) with resveratrol (0, 7.5, 15, 30, 60, and 120 mg/kg diet) and found that it could improve the survival rate, prolong the lifespan, and improve the behavioral defects of *D. melanogaster*; these effects were related to its anti-inflammatory and antioxidant activities.

Posadino et al. [214] treated HUVECs loaded with the ROS probe H2DCF-DA with different concentrations of RES (1–50 μM), and the results showed that low concentrations of RES enhanced PKC activity, promoted DNA synthesis, and reduced apoptosis; high RES concentrations elicit an opposite effect. The results suggested that resveratrol had a biphasic concentration-dependent effect on endothelial cell survival, thus providing a guide for future investigation. Another study by Posadino et al. [215] showed low doses of resveratrol (0.5 μM) effectively acting as an antioxidant agent by significantly reducing the roGFP oxidation state as compared with roGFP-infected control cells. With the increase of resveratrol dose, cell survival and metabolic activity decreased in parallel, suggesting that antioxidant and prooxidation effects were strongly related to dose. In addition, resveratrol was shown to increase skeletal muscle resistance to fatigue in aging mice for the alleviation of age-related skeletal muscle aging [216].

5.2.2. Curcumin. Curcumin, the main component extracted from the rhizome of turmeric and zedoary, has various pharmacological actions, including antiaging, anti-inflammatory, and antioxidant actions [217–219].

Shailaja et al. [220] showed that curcumin could reduce the level of C-reactive protein (CRP) and enhance the level of malondialdehyde (MDA), which play a favorable role in

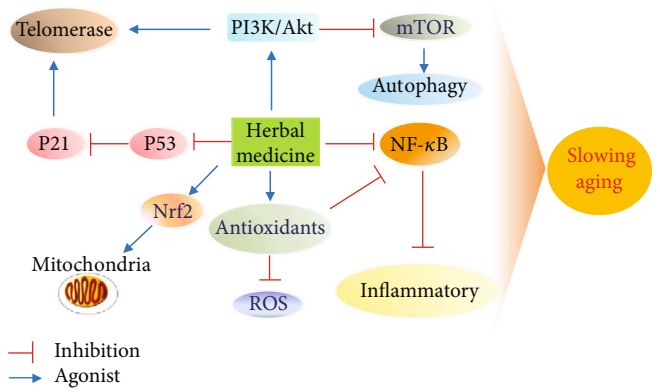

Figure 4: Possible mechanism of herbal medicines in slowing aging.

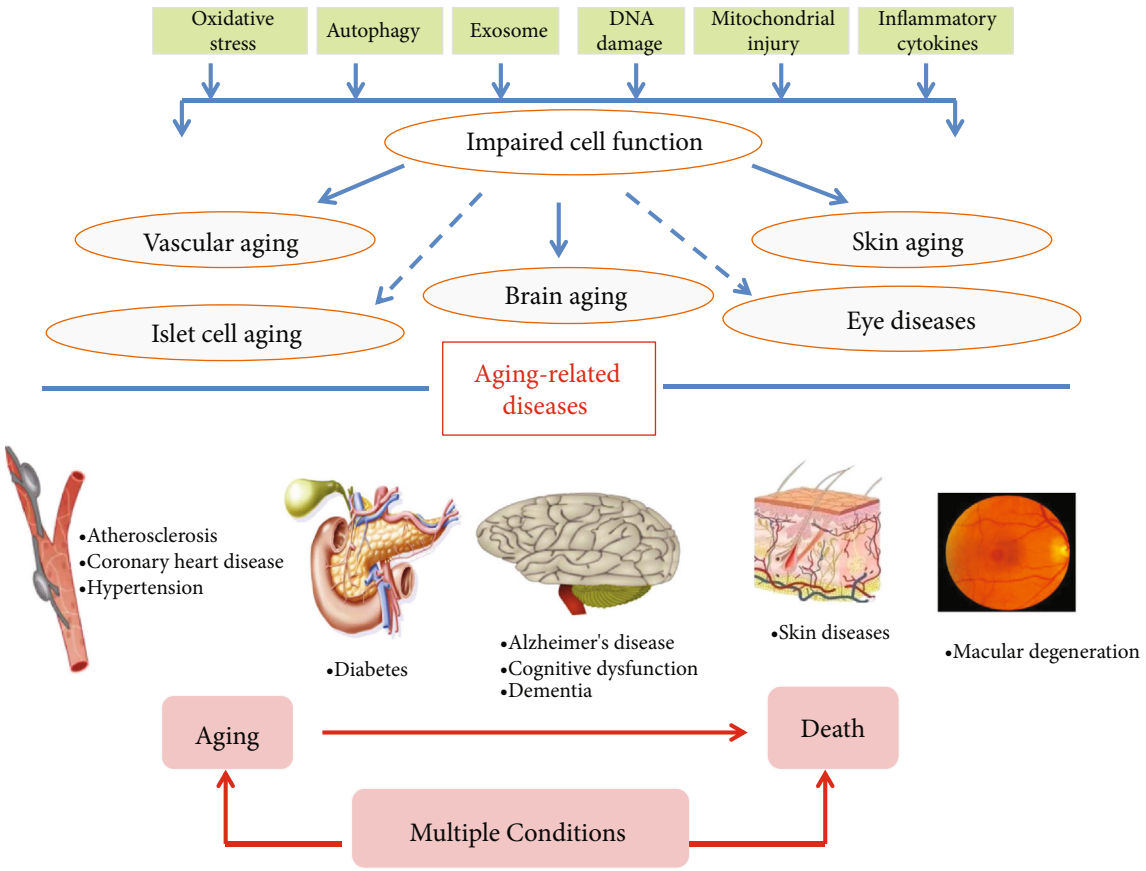

Figure 5: Possible mechanisms of aging and aging-related diseases.

slowing aging by inhibiting the expression of age-related inflammatory cytokines. By using concentrations of 1 μM, 5 μM, 10 μM, and 20 μM, Pirmoradi et al. [221] found the intervention effect of curcumin in rat adipose tissue-derived stem cells (rADSC) in vitro. Their results showed that curcumin could promote the proliferation of rADSC and reduce the senescence of adipose stem cells by promoting TERT gene expression. Hu et al. [222] revealed that curcumin could reduce extracellular matrix degradation and interstitial fibrosis induced by hypertension from modulating covalent histone modification and TIMP1 gene activation, thus protecting against hypertension-related vascular damage. Furthermore, curcumin could prolong the lifespan of *Drosophila* under heat stress conditions by increasing the antioxidant activity and mitigating the effect of heat shock responses [223]. Curcumin could also alleviate aging-related skeletal muscle mass loss and dysfunction [224].

5.2.3. Flavonoids. Flavonoids are a kind of natural polyphenols, mainly including flavonoids, flavanols, flavonoids, anthocyanins, and isoflavones [225]. Studies had shown that flavonoids had definite efficacy in the treatment of age-related neurodegenerative diseases [226], cardiovascular diseases [227, 228], atherosclerosis [229], etc. Some progress has

Table 2: Preclinical studies of herbal medicine for aging-related diseases.

Active ingredients	Dosage	Administration	Model	Possible mechanism	Reference
			In vitro studies		
EGb	10, 25, and 50 mg/L	Pretreatment for 24 h	EPCs cultured on fibronectin-coated culture dishes	Activation of telomerase through the PI3k/Akt signaling pathway	[169]
Ginsenoside Rg1	10 μmol/L	Cultured for 6 h	Aging Sca-1+ hematopoietic cells	Regulating the p16-Rb signaling pathway, repairing worn telomeres, and maintaining telomerase activity	[174]
PNS	5, 25, and 50 μg/mL	Pretreatment for 4 h	D-Galactose induced aging H9c2 cells	Increase antioxidant capacity and reduce apoptosis	[180]
Astragalus membranaceus	100, 200, 400, and 600 μg/mL	Pretreatment for 24 h	Cardiomyocyte model of oxidative stress	Attenuating the oxidative injury and arresting the influx of Ca^{2+} to block cell death	[182]
Lycium barbarum	15, 30, and 60 μg/mL	Pretreatment for 24 h	Primary hippocampal neurons	Activating the PI3K/Akt/mTOR signaling pathway	[188]
Angelica sinensis	—	—	Aging hematopoietic stem cells	Increase in the length of telomere and the activity of telomerase, downregulation of the expression of P53 protein	[194]
Ligustrazine	50, 100, and 200 μM	Pretreated for 24 h	Hypoxia-induced injury of HUVECs	Upregulation of miR-135b and subsequent activation of JNK/SAPK and PI3K/AKT/mTOR pathways	[201]
Gynostemma pentaphyllum extract	0.5-5.0 mg/mL	—	Mouse dermal fibroblasts induced oxidative stress	Reduce oxidative stress	[208]
Resveratrol	5, 10, and 50 μM	Cultured for 24 h	H_2O_2 induced aging of HUVECs	Upregulation of autophagy	[212]
Curcumin	1, 5, 10, and 20 μM	Treatment for 48 h	Rat adipose tissue-derived stem cells	Promoting TERT gene expression	[221]
			In vivo studies		
EGb-761	20,40, 80, and 100 mg/kg	i.g. every 3 days, for 12 months	Aged mice (24 months) of middle cerebral artery occlusion	Upregulation of phosphatase PP2A and diminished extracellular signal-regulated kinase (ERK) activation	[170]
EGb	200, 400 mg/kg/day	i.g. 12 weeks	Streptozotocin-induced diabetic $ApoE^{-/-}$ mice	Inhibiting endoplasmic reticulum stress via restoration of autophagy through the mTOR signaling pathway	[171]
Ginsenoside Rg1	20 mg/kg/day	i.p. 28 days	D-Galactose-induced aging mice	Antioxidation and downregulation of the p19/p53/p21 signaling pathway	[173]
Panax notoginseng saponins	10, 30, and 60 mg/kg/day	i.g. 6 months	Natural aging rats	Attenuating oxidative damage	[179]
Astragalus membranaceus	100, 200, 400, and 600 mg/kg	i.g. twice per day for 7 times	Rat model of persistent myocardial ischemia	Reducing oxidative damage and free radical generation	[182]
Astragalosides	8, 16, and 32 mg/kg	i.g. 14 days	Rats with learning and memory impairment	Downregulate the mRNA levels of APP and β-secretase, decrease expression of APP and $A\beta_{1-40}$ in hippocampus	[183]
Astragalus polysaccharides	100, 200, and 300 mg/kg/d	i.g. 7 weeks	D-Galactose induced aging mice	Scavenging ROS, inhibiting mitochondrial PT, and increasing the activities of antioxidases	[184]
Lycium barbarum	0.5 or 2.0 g/kg	i.g. 4 weeks	A mouse model of AD induced by the combination of AlC_{l3} and D-galactose	Modulation of the mitochondrial pathway of apoptosis and the cholinergic system	[186]

Table 2: Continued.

Active ingredients	Dosage	Administration	Model	Possible mechanism	Reference
Goji berry	150, 300 mg/kg	i.g. 6 weeks	Natural aging rats	Antioxidative stress	[187]
Rhodiola rosea	60, 120 mg/kg	i.g. 9 weeks	Abdominal aorta of atherosclerosis rats	Hypolipemic, antioxidant, and anti-inflammatory activities	[190]
Angelica polysaccharide	140 mg/kg	i.p. 27 days	Aging nestin-GFP mice induced by D-galactose	Enhancing the antioxidant and anti-inflammatory capacity, upregulation of p53/p21 signaling pathway	[196]
Tetramethylpyrazine	1, 3, and 10 mg/kg	i.p. 7 or 14 days	6-OHDA-induced Parkinson's disease mice	Activation of PI3K/Akt/GSK3β signaling pathway	[200]
Resveratrol	30, 100 mg/kg/d	i.g. 2 weeks	Mice with chemotherapy-induced ovarian aging	Attenuating oxidative stress injury by activating Nrf2	[210]
Curcumin	100, 200, and 400 mg/kg/d	i.g. 6 months	Natural aging rats	Suppressing age-related changes in inflammatory indices	[220]
Baicalein	10, 30 mg/kg/day	i.p. 7 days	MPP^+-induced Parkinson's disease mice	Inhibit inflammatory activities and MPP^+-induced apoptosis and autophagy	[231]

Abbreviations: EGb: Ginkgo biloba extract; EPCs: endothelial progenitor cells; HUVECs: human umbilical endothelial vein cells; i.g.: intragastric gavage; i.p.: intraperitoneally injected.

been made in the study of flavonoids in prolonging lifespan [230]. Hung et al. [231] injected 1-methyl-4-phenylpyridinium (MPP^+, a Parkinsonian neurotoxin) into the brains of rats and randomly divided them into three groups which received different does (10, 30 mg/kg/day) of baicalein, a phenolic flavonoid for 7 days. The study found that baicalein could inhibit inflammatory activities and MPP^+-induced apoptosis and autophagy in the nigrostriatal dopaminergic system of the rat brain. The results suggested that baicalein was of therapeutic significance in Parkinson's disease. Studies also showed that flavonoids could exert ameliorative antioxidant capacity and reduce Aβ-induced toxicity in Caenorhabditis elegans, thus prolonging lifespan of Caenorhabditis elegans [232, 233].

6. Adverse Effects of Herbal Medicine

Cianfrocca et al. [234] observed that a 49-year-old man received herbal therapy with Ginkgo biloba (40 mg, 3 times daily) for 2 weeks to improve his cognitive abilities, and the patient complained of two palpitations within a month. The 12-lead ECG had a normal morphology but showed sinus rhythm with frequent ventricular premature beats, and with the withdrawal of ginkgo biloba extract, electrocardiographic evidence of ventricular arrhythmias resolved. Erdle et al. [235] reported allergic reactions in two pediatric patients after inhaling and atomizing *American ginseng* powder, the former with urticaria and respiratory symptoms and the latter with recurrent allergic conjunctivitis, and there was evidence of sensitization to *American ginseng* on skin prick testing (SPT) (13×12 mm wheal). The researchers concluded that excessive oral administration of astragalus could cause allergy, headache, hypertension, or other symptoms; astragalus injection mainly caused fever, shock, and acute asthma [236]. Larramendi et al. [237] carried out a skin test of goji berry on 30 patients with plant food allergy and found that 24 patients showed positive results, which suggested that goji berries are potentially allergenic to people at high risk of food allergies. Chang et al. [238] reevaluated the postmarketing safety of depside salt injection (made from *Radix Salvia miltiorrhiza*) based on the real world and found that most common adverse drug reactions were headache, head distention, dizziness, facial flushing, skin itching, thrombocytopenia, and the reversibility of elevated aspartate transaminase. Chaudhari et al. [239] concluded that curcumin commonly used in dermatologic conditions may cause skin allergies, mainly manifested as contact urticaria.

The safety of drug use is one of the important contents of clinical pharmacology; herbal medicine has drawbacks in this respect. Further studies are needed to completely understand these widely used herbs or compounds and their efficacy in aging-related diseases.

7. Conclusion and Perspectives

Aging and aging-related diseases pose a serious threat to human health and reduce the quality of life of elderly people. Therefore, exploring the mechanisms of aging and against the occurrence of aging-related diseases is of great significance. In this paper, we discuss cellular and molecular mechanisms of aging and aging-related diseases, including oxidative stress, inflammatory response, autophagy and exosome interactions, mitochondrial injury, and telomerase damage (see Figure 4). We also discuss the possible mechanisms of age-related diseases (see Figure 5) and modern medical treatment for diseases related to aging. However, modern medicines result in many adverse reactions when used to treat aging-related diseases. Although drug therapy may improve the symptoms of early AD, they are not effective in patients with advanced AD and are associated with gastrointestinal toxicity. Intravitreal injection of antivascular endothelial growth factor is the most effective way to inhibit angiogenesis and control vascular

Table 3: Published randomized controlled trials of herbal medicines for the treatment of aging-related diseases in humans.

Number	Authors (year)	Targets	Conditions	Age (years)	Name of herb or formula	Dose/duration	Groups	Main outcomes	Adverse reactions
(1)	Liu et al. (2007) [240]	$n = 66$	Aging vascular dementia	≥55	Kangxin capsule (*Fructus lycii*, *Herba epimedii*, *Radix paeoniae alba*, *Radix Salvia miltiorrhiza*, *Fructus crataegi*, *Radix astragali*, *etc.*)	0.9 g once and three times per day, for 1 month	I: compound C: piracetam	CD4, CD4, $CD8^{-1} \uparrow (P < 0.05)$ HIS index, GDS, ET, $E_2 \cdot T^{-1} \downarrow (P < 0.05)$	No adverse reactions were observed
(2)	Zhao et al. (2018) [241]	$n = 140$	Type 2 diabetes mellitus	50-75	Ginkgo leaf tablets Liuwei Dihuang pills	2 Ginkgo leaf tablets and 8 Liuwei Dihuang pills, 3 times a day, for 36 months	I: compound C: placebos	Plasma CML, 8-IsoP levels $\downarrow (P < 0.05)$ FBG, PBG, BP, HbA1c, TC, TG, LDL-C, HDL-C ($P > 0.05$)	Drug reaction
(3)	Kwok et al. (2014) [242]	$n = 165$	Atherosclerosis in postmenopausal	56.0 ± 3.8	DG capsules (Danshen and ginseng)	Two capsules daily, for 12 months	I: compound C: placebos	TC, LDL-C carotid IMT $\downarrow (P < 0.05)$ BP, BMI, Glu ($P > 0.05$)	No adverse reactions were observed
(4)	Dingzhu et al. (2015) [243]	$n = 156$	Carotid atherosclerosis	57.7 ± 4.4	Shoushen granule (*Radix Polygoni multiflori*, *Fructus lycii*, *Crataegus*, and *Radix notoginseng*)	1 tablet once daily for 24 weeks	I: compound C: pravastatin	baPWV, IMTEp, AI, PWV$\beta \downarrow (P < 0.05)$	Not reported
(5)	Lv et al. (2016) [244]	$n = 69$	Type 2 diabetes mellitus	50-80	Naoxintong (*Radix astragali*, *Angelica sinensis*, *Radix paeoniae rubra*, and *Ligusticum wallichii*)	1.2 g per day for 3 months	I: compound C: blank control	HbA1c $\downarrow (P < 0.05)$ Proliferative effects, migration ability, antiapoptotic function of HUVECs $\uparrow (P < 0.05)$ TC, TG, LDL-C, HDL-C ($P > 0.05$)	Not reported
(6)	Akhondzadeh et al. (2003) [245]	$n = 42$	Alzheimer's disease	65-80	Salvia officinalis extract	60 drops daily for 16 weeks	I: compound C: placebos	ADAS-cog, CDR-SB $\downarrow (P < 0.05)$	Vomiting, wheezing, nausea
(7)	Akhondzade et al. (2010) [246]	$n = 46$	Alzheimer's disease	72.65 ± 3.89	Saffron	15 mg twice per day, for 16 weeks	I: compound C: placebos	ADAS-cog, CDR-SB $\downarrow (P < 0.05)$	Dry mouth
(8)	Jia et al. (2014) [247]	$n = 325$	Vascular dementia	64.9 ± 9.1	SaiLuoTong (Ginkgo biloba, ginsenosides, saffron)	360/240 mg daily, for 52 weeks	I: compound C: placebos	VaD Assessment Scale—cognitive subscale scores ($P < 0.05$, 26 weeks)	Mild gastrointestinal intolerance, abnormal alanine aminotransferase, dreaminess

Table 3: Continued.

Number	Authors (year)	Targets	Conditions	Age (years)	Name of herb or formula	Dose/duration	Groups	Main outcomes	Adverse reactions
(9)	Tajadini et al. (2015) [248]	$n = 44$	Alzheimer's disease	>50	Davaie Loban	500 mg, three times daily, for 3 months	I: compound C: placebos	ADAS-cog, CDR-SB ↓ ($P < 0.05$)	Without any adverse drug reaction
(10)	Uno et al. (2005) [249]	$n = 115$	Type 2 diabetes	64 ± 1	Goshajinkigan	7.5 g daily for 1 month	I: combined compound and OHAs C: OHAs	HOMA-R, FBG TC, TG ↓ ($P < 0.05$) HbA1c ($P > 0.05$)	No adverse reactions were observed
(11)	Cho et al. (2009) [250]	$n = 82$	Healthy female	53.6 ± 7.4	Red ginseng root extract mixed with Torilus fructus and Corni fructus	3 g daily for 24 weeks	I: compound C: placebos	Facial wrinkles ↓ Type I procollagen gene, protein expression ↑	Gastrointestinal discomfort

Abbreviations: GDS: Geriatric Depression Scale; HIS: Hachinski Ischemia Scale; ET: endothelin; $E_2{\cdot}T^{-1}$: estradiol (E_2)·testosterone $(T)^{-1}$; CML: carboxymethyl lysine; 8-IsoP: 8-isoprostane; FBG: fasting blood glucose; PBG: postprandial blood glucose; HbA1c: glycosylated hemoglobin; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; IMT: intima-media thickness; GLU: glucose; Ep: pressure-strain elastic modulus; Ac: arterial compliance; AI: augmentation index; PWVβ: pulse wave velocity β; HUVECs: human umbilical vein endothelial cells; ADAS-cog: cognitive subscale of Alzheimer's Disease Assessment Scale; CDR: Clinical Dementia Rating; OHAs: oral hypoglycemic agents.

leakage. However, intravitreal injection has many disadvantages which include risk of infection, the requirement of repeated treatment, and high cost. Most importantly, some patients still experience progressive visual impairment after treatment. Exploring the mechanisms of the multitargeted actions of herbal medicine will therefore help establish novel drugs for the treatment of aging-related diseases. In this review, we initially explored the possible mechanisms of herbal medicines in the treatment of aging and aging-related diseases (Table 2). Through in vivo and in vitro studies, various components of herbal medicine have been found to possess the ability to intervene in aging-related diseases by activating telomerase, increasing antioxidant capacity, reducing apoptosis and anti-inflammatory activities, and regulating aging-related pathways and exosomes. We also summarized the clinical randomized controlled trials (RCTs) of herbal medicine in the treatment of aging-related diseases (Table 3) [240–250]. These trials found that herbal medicine displays certain clinical efficacy in the treatment of age-related diseases such as type 2 diabetes, vascular dementia, AD, and atherosclerosis. A few clinical studies on AMD exist, but this disorder is considered to be related to the special technique used for intravitreal administration when treating macular lesions. Of note, as shown in Table 3, there are some adverse reactions in the clinical use of herbal medicines, including gastrointestinal discomfort, dry mouth, and abnormal alanine aminotransferase [245–247, 250]. Experimental studies had also found that there was a dose-response curve characterized by stimulation at a low dose and inhibition at a high dose. For example, the researchers used different concentrations of the drug to interfere with endothelial cells and found that cell survival rates decreased as the dose of the drug increased [214, 251–253]. This indicates that drugs have the effect of dose-dependent bidirectional regulation. When conducting study, attention should be paid not only to the dose-effect relationship but also to the optimal benefit concentration of drugs. Further analysis of the herbs mentioned in the article found that adverse reactions might occur with herbal treatment, such as palpitations, recurrent allergic conjunctivitis, urticaria and respiratory symptoms, fever, shock, and acute asthma [234–239]. Researchers should analyze the reasons for the adverse reactions and promote the standard and safe use of herbs.

In conclusion, high-quality RCTs should be carried out to observe the effectiveness and safety of herbal medicine in the treatment of aging and aging-related diseases. It is also important that the intervention of integrated traditional Chinese and western medicine be monitored in aging and aging-related diseases.

Abbreviations

ACEI: Angiotensin-converting enzyme inhibitors
AChE: Acetylcholinesterase
ACS: Acute coronary syndrome
AD: Alzheimer's disease
ADCY5: Adenylate cyclase 5
Akt: Protein kinase B
AMD: Age-related macular degeneration
AMPK: Adenosine 5′-monophosphate-activated protein kinase
AMPKα: AMP-activated protein kinase alpha
ARBs: Angiotensin-receptor antagonists
ARF: Auxin response factor
BECN1: Beclin1
ChAT: Choline acetyltransferase
CRTC-1: CREB-regulated transcription coactivator1
EGb: Ginkgo biloba extract
eNOS: Endothelial nitric oxide synthase
EPCs: Endothelial progenitor cells
EZH2: Enhancer of zeste homolog 2
FOXO: Forkhead box class
FZD4: Frizzled class receptor 4
H_2O_2: Hydrogen peroxide
hADSCs: Human adipose-derived mesenchymal stem cells
HMGCR: 3-Hydroxy-3-methylglutaryl-CoA reductase
HMGCS1: 3-Hydroxy-3-methylglutaryl-CoA synthase-1
IL-1: Interleukin-1
INK4: Inhibitors of cyclin-dependent kinase 4
ISR1: Insulin receptor substrate-1
miRNA: MicroRNA
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR: Mammalian target of rapamycin
NAD^+: Nicotinamide adenine dinucleotide
NF-κB: Nuclear factor of activated B-cells
NLRP3: NOD-like receptor family pyrin domain containing 3
NO: Nitric oxide
Nox4: Nicotinamide adenine dinucleotide phosphate oxidase isoform 4
Nrf2: Nuclear factor-E2-related factor 2
NSC: Neural stem cells
PML-NB: Promyelocytic leukemia nuclear bodies
PNS: Panax notoginseng saponins
PSEN: Presenilin
ROS: Reactive oxygen species
SIR1: Sirtuins 1
SOD2: Manganese superoxide dismutase
TGF-β: Transforming growth factor-β
TRAF6: Receptor-associated factor 6
TSC2: Tuberous sclerosis complex 2
TXNIP: Thioredoxin-interacting protein
VSMCs: Vascular smooth muscle cells.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Yanfei Liu put on the reference collection, reference analysis, and writing of the manuscript. Yue Liu and Rui Gao contributed to the topic conception, manuscript revision, and decision to submit for publication and are the cocorresponding authors. Weiliang Weng contributed to reference analysis and helped in the revision of the manuscript.

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References

[1] Q. Bao, J. Pan, H. Qi et al., "Aging and age-related diseases - from endocrine therapy to target therapy," *Molecular and Cellular Endocrinology*, vol. 394, no. 1-2, pp. 115–118, 2014.

[2] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, pp. 1194–1217, 2013.

[3] UNDESA, *Population Division, World Population Prospects the 2015 Revision*, United Nations Department of Economic and Social Affairs, 2015.

[4] M. Barton, M. Husmann, and M. R. Meyer, "Accelerated vascular aging as a paradigm for hypertensive vascular disease: prevention and therapy," *The Canadian Journal of Cardiology*, vol. 32, no. 5, pp. 680–686.e4, 2016.

[5] A. Harvey, A. C. Montezano, R. A. Lopes, F. Rios, and R. M. Touyz, "Vascular fibrosis in aging and hypertension: molecular mechanisms and clinical implications," *The Canadian Journal of Cardiology*, vol. 32, no. 5, pp. 659–668, 2016.

[6] T. W. Buford, "Hypertension and aging," *Ageing Research Reviews*, vol. 26, pp. 96–111, 2016.

[7] A. K. Palmer and J. L. Kirkland, "Aging and adipose tissue: potential interventions for diabetes and regenerative medicine," *Experimental Gerontology*, vol. 86, pp. 97–105, 2016.

[8] C. Domingues-Faria, M. P. Vasson, N. Goncalves-Mendes, Y. Boirie, and S. Walrand, "Skeletal muscle regeneration and impact of aging and nutrition," *Ageing Research Reviews*, vol. 26, pp. 22–36, 2016.

[9] P. Lacolley, V. Regnault, P. Segers, and S. Laurent, "Vascular smooth muscle cells and arterial stiffening: relevance in development, aging, and disease," *Physiological Reviews*, vol. 97, no. 4, pp. 1555–1617, 2017.

[10] W. Ma and W. T. Wong, "Aging changes in retinal microglia and their relevance to age-related retinal disease," in *Retinal Degenerative Diseases*, C. Bowes Rickman, M. LaVail, R. Anderson, C. Grimm, J. Hollyfield, and J. Ash, Eds., vol. 854 of Advances in Experimental Medicine and Biology, pp. 73–78, Springer, Cham, 2016.

[11] C. Cleeland, A. Pipingas, A. Scholey, and D. White, "Neurochemical changes in the aging brain: a systematic review," *Neuroscience & Biobehavioral Reviews*, vol. 98, pp. 306–319, 2019.

[12] I. Maldonado-Lasuncion, M. Atienza, M. P. Sanchez-Espinosa, and J. L. Cantero, "Aging-related changes in cognition and cortical integrity are associated with serum expression of candidate microRNAs for Alzheimer disease," *Cerebral Cortex*, vol. 29, no. 10, pp. 4426–4437, 2019.

[13] A. M. Armenta, E. D. Henkel, and A. M. Ahmed, "Pigmentation disorders in the elderly," *Drugs & Aging*, vol. 36, no. 3, pp. 235–245, 2019.

[14] B. A. Mander, J. R. Winer, and M. P. Walker, "Sleep and human aging," *Neuron*, vol. 94, no. 1, pp. 19–36, 2017.

[15] V. M. Loaiza and A. S. Souza, "An age-related deficit in preserving the benefits of attention in working memory," *Psychology and Aging*, vol. 34, no. 2, pp. 282–293, 2019.

[16] R. Maeso-Díaz, M. Ortega-Ribera, A. Fernández-Iglesias et al., "Effects of aging on liver microcirculatory function and sinusoidal phenotype," *Aging Cell*, vol. 17, no. 6, article e12829, 2018.

[17] J. E. Elliott, C. B. Mantilla, C. M. Pabelick, A. C. Roden, and G. C. Sieck, "Aging-related changes in respiratory system mechanics and morphometry in mice," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 311, no. 1, pp. L167–L176, 2016.

[18] C. Vicini, A. de Vito, G. Iannella et al., "The aging effect on upper airways collapse of patients with obstructive sleep apnea syndrome," *European Archives of Oto-Rhino-Laryngology*, vol. 275, no. 12, pp. 2983–2990, 2018.

[19] M. De Sá Barreto Da Cunha and S. F. Arruda, "Tucum-do-cerrado (*Bactris setosa* Mart.) may promote anti-aging effect by upregulating SIRT1-Nrf2 pathway and attenuating oxidative stress and inflammation," *Nutrients*, vol. 9, no. 11, article 1243, 2017.

[20] I. Liguori, G. Russo, F. Curcio et al., "Oxidative stress, aging, and diseases," *Clinical Interventions in Aging*, vol. 13, pp. 757–772, 2018.

[21] R. Wadhwa, R. Gupta, and P. K. Maurya, "Oxidative stress and accelerated aging in neurodegenerative and neuropsychiatric disorder," *Current Pharmaceutical Design*, vol. 24, no. 40, pp. 4711–4725, 2019.

[22] S. I. Liochev, "Reactive oxygen species and the free radical theory of aging," *Free Radical Biology and Medicine*, vol. 60, pp. 1–4, 2013.

[23] S. G. Rhee, "Cell signaling H_2O_2, a necessary evil for cell signaling," *Science*, vol. 312, no. 5782, pp. 1882-1883, 2006.

[24] L. A. Sena and N. S. Chandel, "Physiological roles of mitochondrial reactive oxygen species," *Molecular Cell*, vol. 48, no. 2, pp. 158–167, 2012.

[25] G. Hewitt, D. Jurk, F. D. M. Marques et al., "Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence," *Nature Communications*, vol. 3, no. 1, p. 708, 2012.

[26] A. Chandrasekaran, M. . P. S. Idelchik, and J. A. Melendez, "Redox control of senescence and age-related disease," *Redox Biology*, vol. 11, pp. 91–102, 2017.

[27] M. Shawi and C. Autexier, "Telomerase, senescence and ageing," *Mechanisms of Ageing and Development*, vol. 129, no. 1-2, pp. 3–10, 2008.

[28] D. Kipling, "Telomeres, replicative senescence and human ageing," *Maturitas*, vol. 38, no. 1, pp. 25–37, 2001.

[29] F. Olivieri, M. Capri, M. Bonafè et al., "Circulating miRNAs and miRNA shuttles as biomarkers: perspective trajectories of healthy and unhealthy aging," *Mechanisms of Ageing and Development*, vol. 165, Part B, pp. 162–170, 2017.

[30] S. Kumar, M. Vijayan, J. S. Bhatti, and P. H. Reddy, "Chapter three - microRNAs as peripheral biomarkers in aging and age-related diseases," *Progress in Molecular Biology and Translational Science*, vol. 146, pp. 47–94, 2017.

[31] A. M. Cuervo and E. Wong, "Chaperone-mediated autophagy: roles in disease and aging," *Cell Research*, vol. 24, no. 1, pp. 92–104, 2014.

[32] A. Plaza-Zabala, V. Sierra-Torre, and A. Sierra, "Autophagy and microglia: novel partners in neurodegeneration and aging," *International Journal of Molecular Sciences*, vol. 18, no. 3, p. 598, 2017.

[33] H. T. Phu, D. T. B. Thuan, T. H. D. Nguyen, A. M. Posadino, A. H. Eid, and G. Pintus, "Herbal medicine for slowing aging and aging-associated conditions: efficacy, mechanisms, and safety," *Current Vascular Pharmacology*, vol. 17, 2019.

[34] C. Sturtzel, "Endothelial cells," in *The Immunology of Cardiovascular Homeostasis and Pathology*, S. Sattler and T. Kennedy-Lydon, Eds., vol. 1003 of Advances in Experimental Medicine and Biology, pp. 71–91, Springer, Cham, 2017.

[35] H. Dib, P. Chafey, G. Clary et al., "Proteomes of umbilical vein and microvascular endothelial cells reflect distinct biological properties and influence immune recognition," *Proteomics*, vol. 12, no. 15-16, pp. 2547–2555, 2012.

[36] S. A. Vielma, R. L. Klein, C. A. Levingston, and M. R. I. Young, "Skewing of immune cell cytokine production by mediators from adipocytes and endothelial cells," *Adipocytes*, vol. 3, no. 2, pp. 126–131, 2014.

[37] E. Suzuki, M. Takahashi, S. Oba, and H. Nishimatsu, "Oncogene- and oxidative stress-induced cellular senescence shows distinct expression patterns of proinflammatory cytokines in vascular endothelial cells," *The Scientific World Journal*, vol. 2013, Article ID 754735, 6 pages, 2013.

[38] J. Zhao, S. Z. Xu, and J. Liu, "Fibrinopeptide A induces C-reactive protein expression through the ROS-ERK1/2/p38-NF-κB signal pathway in the human umbilical vascular endothelial cells," *Journal of Cellular Physiology*, vol. 234, no. 8, pp. 13481–13492, 2019.

[39] W. Wang, C. Shang, W. Zhang et al., "Hydroxytyrosol NO regulates oxidative stress and NO production through SIRT1 in diabetic mice and vascular endothelial cells," *Phytomedicine*, vol. 52, pp. 206–215, 2019.

[40] H. J. Yoon, K. O. Chay, and S. Y. Yang, "Native low density lipoprotein increases the production of both nitric oxide and reactive oxygen species in the human umbilical vein endothelial cells," *Genes & Genomics*, vol. 41, no. 3, pp. 373–379, 2019.

[41] I. Pantsulaia, W. M. Ciszewski, and J. Niewiarowska, "Senescent endothelial cells: potential modulators of immunosenescence and ageing," *Ageing Research Reviews*, vol. 29, pp. 13–25, 2016.

[42] Y. Yin, Z. Zhou, W. Liu, Q. Chang, G. Sun, and Y. Dai, "Vascular endothelial cells senescence is associated with NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activation via reactive oxygen species (ROS)/thioredoxin-interacting protein (TXNIP) pathway," *The International Journal of Biochemistry & Cell Biology*, vol. 84, pp. 22–34, 2017.

[43] Y. Ohsumi, "Historical landmarks of autophagy research," *Cell Research*, vol. 24, no. 1, pp. 9–23, 2014.

[44] L. Barile, T. Moccetti, E. Marban, and G. Vassalli, "Roles of exosomes in cardioprotection," *European Heart Journal*, vol. 38, no. 18, pp. ehw304–ehw309, 2017.

[45] A. Salminen and K. Kaarniranta, "Regulation of the aging process by autophagy," *Trends in Molecular Medicine*, vol. 15, no. 5, pp. 217–224, 2009.

[46] T. Saez, P. de Vos, J. Kuipers, L. Sobrevia, and M. M. Faas, "Fetoplacental endothelial exosomes modulate high d-glucose-induced endothelial dysfunction," *Placenta*, vol. 66, pp. 26–35, 2018.

[47] R. Menghini, V. Casagrande, A. Marino et al., "miR-216a: a link between endothelial dysfunction and autophagy," *Cell Death & Disease*, vol. 5, no. 1, article e1029, 2014.

[48] H. Abou-Saleh, F. A. Zouein, A. el-Yazbi et al., "The march of pluripotent stem cells in cardiovascular regenerative medicine," *Stem Cell Research & Therapy*, vol. 9, no. 1, p. 201, 2018.

[49] G. Kolios and Y. Moodley, "Introduction to stem cells and regenerative medicine," *Respiration*, vol. 85, no. 1, pp. 3–10, 2013.

[50] V. Janzen, R. Forkert, H. E. Fleming et al., "Stem-cell ageing modified by the cyclin-dependent kinase inhibitor $p16^{INK4a}$," *Nature*, vol. 443, no. 7110, pp. 421–426, 2006.

[51] T. A. Rando, "Stem cells, ageing and the quest for immortality," *Nature*, vol. 441, no. 7097, pp. 1080–1086, 2006.

[52] I. M. Conboy, M. J. Conboy, A. J. Wagers, E. R. Girma, I. L. Weissman, and T. A. Rando, "Rejuvenation of aged progenitor cells by exposure to a young systemic environment," *Nature*, vol. 433, no. 7027, pp. 760–764, 2005.

[53] J. M. Ryu, H. J. Lee, Y. H. Jung et al., "Regulation of stem cell fate by ROS-mediated alteration of metabolism," *International Journal of Stem Cells*, vol. 8, no. 1, pp. 24–35, 2015.

[54] Y. L. Guo, S. Chakraborty, S. S. Rajan, R. Wang, and F. Huang, "Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal," *Stem Cells and Development*, vol. 19, no. 9, pp. 1321–1331, 2010.

[55] S. Sart, L. Song, and Y. Li, "Controlling redox status for stem cell survival, expansion, and differentiation," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 105135, 14 pages, 2015.

[56] F. B. Francesca Casciaro, M. Zavatti, J. A. Mccubrey et al., "Nuclear Nox4 interaction with prelamin A is associated with nuclear redox control of stem cell aging," *Aging*, vol. 10, no. 10, pp. 2911–2934, 2018.

[57] M. Boyle, C. Wong, M. Rocha, and D. L. Jones, "Decline in self-renewal factors contributes to aging of the stem cell niche in the *Drosophila* testis," *Cell Stem Cell*, vol. 1, no. 4, pp. 470–478, 2007.

[58] B. A. Monk and S. J. George, "The effect of ageing on vascular smooth muscle cell behaviour-a mini-review," *Gerontology*, vol. 61, no. 5, pp. 416–426, 2015.

[59] H. Yin and J. G. Pickering, "Cellular senescence and vascular disease: novel routes to better understanding and therapy," *The Canadian Journal of Cardiology*, vol. 32, no. 5, pp. 612–623, 2016.

[60] J. K. Zhan, Y. J. Wang, S. Li et al., "AMPK/TSC2/mTOR pathway regulates replicative senescence of human vascular smooth muscle cells," *Experimental and Therapeutic Medicine*, vol. 16, no. 6, pp. 4853–4858, 2018.

[61] X. Z. J. Lin, J. Y. Zhong, Y. J. Wang et al., "lncRNA-ES3/miR-34c-5p/BMF axis is involved in regulating high-glucose-induced calcification_senescence of VSMCs," *Aging*, vol. 11, no. 2, pp. 523–535, 2019.

[62] W. W. Du, W. Yang, L. Fang et al., "miR-17 extends mouse lifespan by inhibiting senescence signaling mediated by MKP7," *Cell Death & Disease*, vol. 5, no. 7, article e1355, 2014.

[63] E. E. Dzakah, A. Waqas, S. Wei et al., "Loss of miR-83 extends lifespan and affects target gene expression in an age-

dependent manner in *Caenorhabditis elegans*," *Journal of Genetics and Genomics*, vol. 45, no. 12, pp. 651–662, 2018.

[64] G. Lyu, Y. Guan, C. Zhang et al., "TGF-β signaling alters H4K20me3 status via miR-29 and contributes to cellular senescence and cardiac aging," *Nature Communications*, vol. 9, no. 1, p. 2560, 2018.

[65] J. Fan, X. An, Y. Yang et al., "miR-1292 targets FZD4 to regulate senescence and osteogenic differentiation of stem cells in TE/SJ/mesenchymal tissue system via the Wnt/β-catenin pathway," *Aging and Disease*, vol. 9, no. 6, pp. 1103–1121, 2018.

[66] J. M. Schilling, W. Cui, J. C. Godoy et al., "Long-term atorvastatin treatment leads to alterations in behavior, cognition, and hippocampal biochemistry," *Behavioural Brain Research*, vol. 267, pp. 6–11, 2014.

[67] O. Raihan, A. Brishti, M. R. Molla et al., "The age-dependent elevation of miR-335-3p leads to reduced cholesterol and impaired memory in brain," *Neuroscience*, vol. 390, pp. 160–173, 2018.

[68] H. Zhu, Y. Yang, Y. Wang, J. Li, P. W. Schiller, and T. Peng, "MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1," *Cardiovascular Research*, vol. 92, no. 1, pp. 75–84, 2011.

[69] X. W. Cheng, Y. F. Wan, Q. Zhou, Y. Wang, and H. Q. Zhu, "MicroRNA-126 inhibits endothelial permeability and apoptosis in apolipoprotein E-knockout mice fed a high-fat diet," *Molecular Medicine Reports*, vol. 16, no. 3, pp. 3061–3068, 2017.

[70] J. Schroder, S. Ansaloni, M. Schilling et al., "MicroRNA-138 is a potential regulator of memory performance in humans," *Frontiers in Human Neuroscience*, vol. 8, p. 501, 2014.

[71] C. Liang, L. Gao, Y. Liu et al., "miR-451 antagonist protects against cardiac fibrosis in streptozotocin-induced diabetic mouse heart," *Life Sciences*, vol. 224, pp. 12–22, 2019.

[72] Y. Yang, H. W. Cheng, Y. Qiu et al., "MicroRNA-34a plays a key role in cardiac repair and regeneration following myocardial infarction," *Circulation Research*, vol. 117, no. 5, pp. 450–459, 2015.

[73] S. Ma, X. Y. Tian, Y. Zhang et al., "E-selectin-targeting delivery of microRNAs by microparticles ameliorates endothelial inflammation and atherosclerosis," *Scientific Reports*, vol. 6, no. 1, article 22910, 2016.

[74] J. Zhou, K. C. Wang, W. Wu et al., "MicroRNA-21 targets peroxisome proliferators-activated receptor-α in an autoregulatory loop to modulate flow-induced endothelial inflammation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 25, pp. 10355–10360, 2011.

[75] G. Wan, W. Xie, Z. Liu et al., "Hypoxia-inducedMIR155is a potent autophagy inducer by targeting multiple players in the MTOR pathway," *Autophagy*, vol. 10, no. 1, pp. 70–79, 2014.

[76] X. Xiao, Z. Lu, V. Lin et al., "MicroRNA miR-24-3p reduces apoptosis and regulates Keap1-Nrf2 pathway in mouse cardiomyocytes responding to ischemia/reperfusion injury," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 7042105, 9 pages, 2018.

[77] X. Sun, S. He, A. K. M. Wara et al., "Systemic delivery of microRNA-181b inhibits nuclear factor-κB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice," *Circulation Research*, vol. 114, no. 1, pp. 32–40, 2014.

[78] G. C. van Almen, W. Verhesen, R. E. W. van Leeuwen et al., "MicroRNA-18 and microRNA-19 regulate CTGF and TSP-1 expression in age-related heart failure," *Aging Cell*, vol. 10, no. 5, pp. 769–779, 2011.

[79] H. F. Xie, Y. Z. Liu, R. Du et al., "miR-377 induces senescence in human skin fibroblasts by targeting DNA methyltransferase 1," *Cell Death & Disease*, vol. 8, no. 3, article e2663, 2017.

[80] Z. Wang, L. Sun, K. Jia, H. Wang, and X. Wang, "miR-9-5p modulates the progression of Parkinson's disease by targeting SIRT1," *Neuroscience Letters*, vol. 701, pp. 226–233, 2019.

[81] M. Harada, M. Jinnin, Z. Wang et al., "The expression of miR-124 increases in aged skin to cause cell senescence and it decreases in squamous cell carcinoma," *Bioscience Trends*, vol. 10, no. 6, pp. 454–459, 2017.

[82] A. Lang, S. Grether-Beck, M. Singh et al., "MicroRNA-15b regulates mitochondrial ROS production and the senescence-associated secretory phenotype through sirtuin 4/SIRT4," *Aging*, vol. 8, no. 3, pp. 484–505, 2016.

[83] K. J. Turner, V. Vasu, and D. K. Griffin, "Telomere biology and human phenotype," *Cell*, vol. 8, no. 1, p. 73, 2019.

[84] H. H. Cheung, X. Liu, L. Canterel-Thouennon, L. Li, C. Edmonson, and O. M. Rennert, "Telomerase protects Werner syndrome lineage-specific stem cells from premature aging," *Stem Cell Reports*, vol. 2, no. 4, pp. 534–546, 2014.

[85] A. O'Donovan, A. J. Tomiyama, J. Lin et al., "Stress appraisals and cellular aging: a key role for anticipatory threat in the relationship between psychological stress and telomere length," *Brain, Behavior, and Immunity*, vol. 26, no. 4, pp. 573–579, 2012.

[86] I. Shalev, T. E. Moffitt, K. Sugden et al., "Exposure to violence during childhood is associated with telomere erosion from 5 to 10 years of age: a longitudinal study," *Molecular Psychiatry*, vol. 18, no. 5, pp. 576–581, 2013.

[87] E. S. Epel, E. H. Blackburn, J. Lin et al., "Accelerated telomere shortening in response to life stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 49, pp. 17312–17315, 2004.

[88] T. Van Nguyen, N. Puebla-Osorio, H. Pang, M. E. Dujka, and C. Zhu, "DNA damage-induced cellular senescence is sufficient to suppress tumorigenesis: a mouse model," *The Journal of Experimental Medicine*, vol. 204, no. 6, pp. 1453–1461, 2007.

[89] G. Lossaint, E. Besnard, D. Fisher, J. Piette, and V. Dulić, "Chk1 is dispensable for G2 arrest in response to sustained DNA damage when the ATM/p53/p21 pathway is functional," *Oncogene*, vol. 30, no. 41, pp. 4261–4274, 2011.

[90] D. Churikov and C. M. Price, "Pot1 and cell cycle progression cooperate in telomere length regulation," *Nature Structural & Molecular Biology*, vol. 15, no. 1, pp. 79–84, 2008.

[91] K. Klement and A. A. Goodarzi, "DNA double strand break responses and chromatin alterations within the aging cell," *Experimental Cell Research*, vol. 329, no. 1, pp. 42–52, 2014.

[92] Y. Wang, J. He, M. Liao et al., "An overview of Sirtuins as potential therapeutic target: structure, function and modulators," *European Journal of Medicinal Chemistry*, vol. 161, pp. 48–77, 2019.

[93] Y. Yang, H. Hou, E. M. Haller, S. V. Nicosia, and W. Bai, "Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation," *The EMBO Journal*, vol. 24, no. 5, pp. 1021–1032, 2005.

[94] L. Bordone, D. Cohen, A. Robinson et al., "SIRT1 transgenic mice show phenotypes resembling calorie restriction," *Aging Cell*, vol. 6, no. 6, pp. 759–767, 2007.

[95] S. Fourcade, L. Morato, J. Parameswaran et al., "Loss of SIRT2 leads to axonal degeneration and locomotor disability associated with redox and energy imbalance," *Aging Cell*, vol. 16, no. 6, pp. 1404–1413, 2017.

[96] L. Liu, A. Arun, L. Ellis, C. Peritore, and G. Donmez, "Sirtuin 2 (SIRT2) enhances 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal damage via deacetylating forkhead box O3a (Foxo3a) and activating Bim protein," *The Journal of Biological Chemistry*, vol. 287, no. 39, pp. 32307–32311, 2012.

[97] L. Liu, C. Peritore, J. Ginsberg, J. Shih, S. Arun, and G. Donmez, "Protective role of SIRT5 against motor deficit and dopaminergic degeneration in MPTP-induced mice model of Parkinson's disease," *Behavioural Brain Research*, vol. 281, pp. 215–221, 2015.

[98] S. Someya, W. Yu, W. C. Hallows et al., "Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction," *Cell*, vol. 143, no. 5, pp. 802–812, 2010.

[99] W. Zhang, H. Wan, G. Feng et al., "SIRT6 deficiency results in developmental retardation in cynomolgus monkeys," *Nature*, vol. 560, no. 7720, pp. 661–665, 2018.

[100] B. N. Vazquez, J. K. Thackray, N. G. Simonet et al., "SIRT7 promotes genome integrity and modulates non-homologous end joining DNA repair," *The EMBO Journal*, vol. 35, no. 14, pp. 1488–1503, 2016.

[101] N. M. Xiao, Y. M. Zhang, Q. Zheng, and J. Gu, "Klotho is a serum factor related to human aging," *Chinese Medical Journal*, vol. 117, no. 5, pp. 742–747, 2004.

[102] M. Ullah and Z. Sun, "Klotho deficiency accelerates stem cells aging by impairing telomerase activity," *The Journals of Gerontology: Series A*, vol. 74, no. 9, pp. 1396–1407, 2019.

[103] H. Liu, M. M. Fergusson, R. M. Castilho et al., "Augmented Wnt signaling in a mammalian model of accelerated aging," *Science*, vol. 317, no. 5839, pp. 803–806, 2007.

[104] R. M. De Oliveira, "Klotho RNAi induces premature senescence of human cells via a p53/p21 dependent pathway," *FEBS Letters*, vol. 580, no. 24, pp. 5753–5758, 2006.

[105] Y. Maekawa, M. Ohishi, M. Ikushima et al., "Klotho protein diminishes endothelial apoptosis and senescence via a mitogen-activated kinase pathway," *Geriatrics & Gerontology International*, vol. 11, no. 4, pp. 510–516, 2011.

[106] H. Miyauchi, T. Minamino, K. Tateno, T. Kunieda, H. Toko, and I. Komuro, "Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway," *The EMBO Journal*, vol. 23, no. 1, pp. 212–220, 2004.

[107] D. Gao, Z. Zuo, J. Tian et al., "Activation of SIRT1 attenuates klotho deficiency-induced arterial stiffness and hypertension by enhancing AMP-activated protein kinase activity," *Hypertension*, vol. 68, no. 5, pp. 1191–1199, 2016.

[108] B. G. Childs, M. Gluscevic, D. J. Baker et al., "Senescent cells: an emerging target for diseases of ageing," *Nature Reviews Drug Discovery*, vol. 16, no. 10, pp. 718–735, 2017.

[109] D. Wu and C. Prives, "Relevance of the p53-MDM2 axis to aging," *Cell Death and Differentiation*, vol. 25, no. 1, pp. 169–179, 2018.

[110] A. Marusyk, L. J. Wheeler, C. K. Mathews, and J. DeGregori, "p53 mediates senescence-like arrest induced by chronic replicational stress," *Molecular and Cellular Biology*, vol. 27, no. 15, pp. 5336–5351, 2007.

[111] Y. Y. Kim, H. J. Jee, J. H. Um, Y. M. Kim, S. S. Bae, and J. Yun, "Cooperation between p21 and Akt is required for p53-dependent cellular senescence," *Aging Cell*, vol. 16, no. 5, pp. 1094–1103, 2017.

[112] M. Gorospe, X. Wang, and N. J. Holbrook, "Functional role of p21 during the cellular response to stress," *Gene Expression*, vol. 7, no. 4-6, pp. 377–385, 1999.

[113] F. Yang, M. Yi, Y. Liu, Q. Wang, Y. Hu, and H. Deng, "Glutaredoxin-1 silencing induces cell senescence via p53/p21/p16 signaling axis," *Journal of Proteome Research*, vol. 17, no. 3, pp. 1091–1100, 2018.

[114] L. Poliseno, L. Pitto, M. Simili et al., "The proto-oncogene LRF is under post-transcriptional control of miR-20a: implications for senescence," *PLoS One*, vol. 3, no. 7, article e2542, 2008.

[115] M. Qi, H. Zhou, S. Fan et al., "mTOR inactivation by ROS-JNK-p53 pathway plays an essential role in psedolaric acid B induced autophagy-dependent senescence in murine fibrosarcoma L929 cells," *European Journal of Pharmacology*, vol. 715, no. 1-3, pp. 76–88, 2013.

[116] J. Y. Sung, K. Y. Lee, J. R. Kim, and H. C. Choi, "Interaction between mTOR pathway inhibition and autophagy induction attenuates adriamycin-induced vascular smooth muscle cell senescence through decreased expressions of p53/p21/p16," *Experimental Gerontology*, vol. 109, pp. 51–58, 2018.

[117] T. Weichhart, "mTOR as regulator of lifespan, aging, and cellular senescence: a mini-review," *Gerontology*, vol. 64, no. 2, pp. 127–134, 2018.

[118] P. Tan, Y. J. Wang, S. Li et al., "The PI3K/Akt/mTOR pathway regulates the replicative senescence of human VSMCs," *Molecular and Cellular Biochemistry*, vol. 422, no. 1-2, pp. 1–10, 2016.

[119] D. W. Lamming, L. Ye, D. M. Sabatini, and J. A. Baur, "Rapalogs and mTOR inhibitors as anti-aging therapeutics," *The Journal of Clinical Investigation*, vol. 123, no. 3, pp. 980–989, 2013.

[120] J. A. Didonato, F. Mercurio, and M. Karin, "NF-κB and the link between inflammation and cancer," *Immunological Reviews*, vol. 246, no. 1, pp. 379–400, 2012.

[121] J. Zhao, X. Li, S. Mcgowan, L. J. Niedernhofer, and P. D. Robbins, "NF-κB activation with aging: characterization and therapeutic inhibition," *Methods in Molecular Biology*, vol. 1280, pp. 543–557, 2015.

[122] L. A. J. O'Neill and C. Kaltschmidt, "NF-κB: a crucial transcription factor for glial and neuronal cell function," *Trends in Neurosciences*, vol. 20, no. 6, pp. 252–258, 1997.

[123] F. Dou, X. Chu, B. Zhang et al., "EriB targeted inhibition of microglia activity attenuates MPP^+ induced DA neuron injury through the NF-κB signaling pathway," *Molecular Brain*, vol. 11, no. 1, p. 75, 2018.

[124] S. Hunot, B. Brugg, D. Ricard et al., "Nuclear translocation of NF-κB is increased in dopaminergic neurons of patients with Parkinson disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 14, pp. 7531–7536, 1997.

[125] H. Zhang, K. J. A. Davies, and H. J. Forman, "Oxidative stress response and Nrf2 signaling in aging," *Free Radical Biology and Medicine*, vol. 88, Part B, pp. 314–336, 2015.

[126] J. H. Suh, S. V. Shenvi, B. M. Dixon et al., "Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione

synthesis, which is reversible with lipoic acid," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 10, pp. 3381–3386, 2004.

[127] X. Meng, H. Chen, G. Wang, Y. Yu, and K. Xie, "Hydrogen-rich saline attenuates chemotherapy-induced ovarian injury via regulation of oxidative stress," *Experimental and Therapeutic Medicine*, vol. 10, no. 6, pp. 2277–2282, 2015.

[128] L. Chen, R. Yang, W. Qiao et al., "1,25-Dihydroxyvitamin D exerts an antiaging role by activation of Nrf2-antioxidant signaling and inactivation of p16/p53-senescence signaling," *Aging Cell*, vol. 18, no. 3, article e12951, 2019.

[129] D. D. Huang, S. D. Fan, X. Y. Chen et al., "Nrf2 deficiency exacerbates frailty and sarcopenia by impairing skeletal muscle mitochondrial biogenesis and dynamics in an age-dependent manner," *Experimental Gerontology*, vol. 119, pp. 61–73, 2019.

[130] Y. Kitaoka, Y. Tamura, K. Takahashi, K. Takeda, T. Takemasa, and H. Hatta, "Effects of Nrf2 deficiency on mitochondrial oxidative stress in aged skeletal muscle," *Physiological Reports*, vol. 7, no. 3, article e13998, 2019.

[131] K. Sahin, C. Orhan, M. Tuzcu et al., "Tomato powder modulates NF-κB, mTOR, and Nrf2 pathways during aging in healthy rats," *Journal of Aging Research*, vol. 2019, Article ID 1643243, 8 pages, 2019.

[132] S. T. Ho, Y. T. Hsieh, S. Y. Wang, and M. J. Chen, "Improving effect of a probiotic mixture on memory and learning abilities in d-galactose-treated aging mice," *Journal of Dairy Science*, vol. 102, no. 3, pp. 1901–1909, 2019.

[133] H. Cui, D. Tang, G. B. Garside et al., "Wnt signaling mediates the aging-induced differentiation impairment of intestinal stem cells," *Stem Cell Reviews and Reports*, vol. 15, no. 3, pp. 448–455, 2019.

[134] Y. Wang, L. Liu, S. K. Pazhanisamy, H. Li, A. Meng, and D. Zhou, "Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells," *Free Radical Biology & Medicine*, vol. 48, no. 2, pp. 348–356, 2010.

[135] D. Y. Zhang, H. J. Wang, and Y. Z. Tan, "Wnt/β-catenin signaling induces the aging of mesenchymal stem cells through the DNA damage response and the p53/p21 pathway," *PLoS One*, vol. 6, no. 6, article e21397, 2011.

[136] C. Zhang, P. Chen, Y. Fei et al., "Wnt/β-catenin signaling is critical for dedifferentiation of aged epidermal cells *in vivo* and *in vitro*," *Aging Cell*, vol. 11, no. 1, pp. 14–23, 2012.

[137] A. Suzuki, R. Minamide, and J. Iwata, "WNT/β-catenin signaling plays a crucial role in myoblast fusion through regulation of nephrin expression during development," *Development*, vol. 145, no. 23, article dev168351, 2018.

[138] R. Ruiz, E. María Pérez-Villegas, and Á. Manuel Carrión, "AMPK function in aging process," *Current Drug Targets*, vol. 17, no. 8, pp. 932–941, 2016.

[139] M. Y. Kim, J. H. Lim, H. H. Youn et al., "Resveratrol prevents renal lipotoxicity and inhibits mesangial cell glucotoxicity in a manner dependent on the AMPK–SIRT1–PGC1α axis in db/db mice," *Diabetologia*, vol. 56, no. 1, pp. 204–217, 2013.

[140] W. Mair, I. Morantte, A. P. C. Rodrigues et al., "Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB," *Nature*, vol. 470, no. 7334, pp. 404–408, 2011.

[141] D. H. Lee, T. H. Lee, C. H. Jung, and Y. H. Kim, "Wogonin induces apoptosis by activating the AMPK and p53 signaling pathways in human glioblastoma cells," *Cellular Signalling*, vol. 24, no. 11, pp. 2216–2225, 2012.

[142] B. Jani and C. Rajkumar, "Ageing and vascular ageing," *Postgraduate Medical Journal*, vol. 82, no. 968, pp. 357–362, 2006.

[143] X. Xu, B. Wang, C. Ren et al., "Recent progress in vascular aging: mechanisms and its role in age-related diseases," *Aging and Disease*, vol. 8, no. 4, pp. 486–505, 2017.

[144] L. Jiang, M. Wang, J. Zhang et al., "Increased aortic calpain-1 activity mediates age-associated angiotensin II signaling of vascular smooth muscle cells," *PLoS One*, vol. 3, no. 5, article e2231, 2008.

[145] G. Pintus, R. Giordo, Y. Wang et al., "Reduced vasorin enhances angiotensin II signaling within the aging arterial wall," *Oncotarget*, vol. 9, no. 43, pp. 27117–27132, 2018.

[146] M. Wang, J. Zhang, L. Q. Jiang et al., "Proinflammatory profile within the grossly normal aged human aortic wall," *Hypertension*, vol. 50, no. 1, pp. 219–227, 2007.

[147] M. Wang, D. Zhao, G. Spinetti et al., "Matrix metalloproteinase 2 activation of transforming growth Factor-β1 (TGF-β1) and TGF-β1–type II receptor signaling within the aged arterial wall," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 7, pp. 1503–1509, 2006.

[148] K. G. Soucy, S. Ryoo, A. Benjo et al., "Impaired shear stress-induced nitric oxide production through decreased NOS phosphorylation contributes to age-related vascular stiffness," *Journal of Applied Physiology*, vol. 101, no. 6, pp. 1751–1759, 2006.

[149] C. Sepulveda, I. Palomo, and E. Fuentes, "Mechanisms of endothelial dysfunction during aging: predisposition to thrombosis," *Mechanisms of Ageing and Development*, vol. 164, pp. 91–99, 2017.

[150] R. P. Brandes, I. Fleming, and R. Busse, "Endothelial aging," *Cardiovascular Research*, vol. 66, no. 2, pp. 286–294, 2005.

[151] F. D. Kolodgie, A. P. Burke, G. Nakazawa, and R. Virmani, "Is pathologic intimal thickening the key to understanding early plaque progression in human atherosclerotic disease?," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 5, pp. 986–989, 2007.

[152] M. R. Hamczyk, R. Villa-Bellosta, V. Quesada et al., "Progerin accelerates atherosclerosis by inducing endoplasmic reticulum stress in vascular smooth muscle cells," *EMBO Molecular Medicine*, vol. 11, no. 4, article e9736, 2019.

[153] S. Tarantini, C. H. T. Tran, G. R. Gordon, Z. Ungvari, and A. Csiszar, "Impaired neurovascular coupling in aging and Alzheimer's disease: contribution of astrocyte dysfunction and endothelial impairment to cognitive decline," *Experimental Gerontology*, vol. 94, pp. 52–58, 2017.

[154] L. Yamani, B. Li, and L. Larose, "Nck1 deficiency improves pancreatic β cell survival to diabetes-relevant stresses by modulating PERK activation and signaling," *Cellular Signalling*, vol. 27, no. 12, pp. 2555–2567, 2015.

[155] A. V. Molofsky, S. G. Slutsky, N. M. Joseph et al., "Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing," *Nature*, vol. 443, no. 7110, pp. 448–452, 2006.

[156] H. Chen, X. Gu, I. H. Su et al., "Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus," *Genes & Development*, vol. 23, no. 8, pp. 975–985, 2009.

[157] F. M. Menzies, A. Fleming, A. Caricasole et al., "Autophagy and neurodegeneration: pathogenic mechanisms and therapeutic opportunities," *Neuron*, vol. 93, no. 5, pp. 1015–1034, 2017.

[158] R. M. Barrientos, M. M. Kitt, L. R. Watkins, and S. F. Maier, "Neuroinflammation in the normal aging hippocampus," *Neuroscience*, vol. 309, pp. 84–99, 2015.

[159] H. Zhang, X. Sun, Y. Xie, J. Zan, and W. Tan, "Isosteviol sodium protects against permanent cerebral ischemia injury in mice via inhibition of NF-κB-mediated inflammatory and apoptotic responses," *Journal of Stroke and Cerebrovascular Diseases*, vol. 26, no. 11, pp. 2603–2614, 2017.

[160] C. Fang, L. Xie, C. Liu et al., "Tanshinone IIA improves hypoxic ischemic encephalopathy through TLR-4-mediated NF-κB signal pathway," *Molecular Medicine Reports*, vol. 18, no. 2, pp. 1899–1908, 2018.

[161] K. Tashiro, M. Shishido, K. Fujimoto et al., "Age-related disruption of autophagy in dermal fibroblasts modulates extracellular matrix components," *Biochemical and Biophysical Research Communications*, vol. 443, no. 1, pp. 167–172, 2014.

[162] C. Scherfer, V. C. Han, Y. Wang, A. E. Anderson, and M. J. Galko, "Autophagy drives epidermal deterioration in a Drosophila model of tissue aging," *Aging*, vol. 5, no. 4, pp. 276–287, 2013.

[163] C. Muther, L. Jobeili, M. Garion et al., "An expression screen for aged-dependent microRNAs identifies miR-30a as a key regulator of aging features in human epidermis," *Aging*, vol. 9, no. 11, pp. 2376–2396, 2017.

[164] J. Cai, H. Zhang, Y. F. Zhang, Z. Zhou, and S. Wu, "MicroRNA-29 enhances autophagy and cleanses exogenous mutant αB-crystallin in retinal pigment epithelial cells," *Experimental Cell Research*, vol. 374, no. 1, pp. 231–248, 2019.

[165] E. Korhonen, N. Piippo, M. Hytti, J. M. T. Hyttinen, K. Kaarniranta, and A. Kauppinen, "SQSTM1/p62 regulates the production of IL-8 and MCP-1 in IL-1β-stimulated human retinal pigment epithelial cells," *Cytokine*, vol. 116, pp. 70–77, 2019.

[166] J. Tian, Y. Liu, Y. Liu, K. Chen, and S. Lyu, "Cellular and molecular mechanisms of diabetic atherosclerosis: herbal medicines as a potential therapeutic approach," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 9080869, 16 pages, 2017.

[167] W. Zuo, F. Yan, B. Zhang, J. Li, and D. Mei, "Advances in the studies of Ginkgo biloba leaves extract on aging-related diseases," *Aging and Disease*, vol. 8, no. 6, pp. 812–826, 2017.

[168] N. Kandiah, P. A. Ong, T. Yuda et al., "Treatment of dementia and mild cognitive impairment with or without cerebrovascular disease: expert consensus on the use of *Ginkgo biloba* extract, EGb 761®," *CNS Neuroscience & Therapeutics*, vol. 25, no. 2, pp. 288–298, 2019.

[169] X. X. Dong, Z. J. Hui, W. X. Xiang, Z. F. Rong, S. Jian, and C. J. Zhu, "Ginkgo biloba extract reduces endothelial progenitor-cell senescence through augmentation of telomerase activity," *Journal of Cardiovascular Pharmacology*, vol. 49, no. 2, pp. 111–115, 2007.

[170] X. Zhou, Y. Qi, and T. Chen, "Long-term pre-treatment of antioxidant Ginkgo biloba extract EGb-761 attenuates cerebral-ischemia-induced neuronal damage in aged mice," *Biomedicine & Pharmacotherapy*, vol. 85, pp. 256–263, 2017.

[171] J. Tian, M. S. Popal, Y. Liu et al., "Ginkgo biloba leaf extract attenuates atherosclerosis in streptozotocin- induced diabetic ApoE-/- mice by inhibiting endoplasmic reticulum stress via restoration of autophagy through the mTOR signaling pathway," *Oxidative Medicine and Cellular Longevity*, vol. 2019, Article ID 8134678, 19 pages, 2019.

[172] C. Mancuso and R. Santangelo, "*Panax ginseng* and *Panax quinquefolius*: from pharmacology to toxicology," *Food and Chemical Toxicology*, vol. 107, Part A, pp. 362–372, 2017.

[173] Z. L. Wang, L. B. Chen, Z. Qiu et al., "Ginsenoside Rg1 ameliorates testicular senescence changes in D-gal-induced aging mice via anti-inflammatory and antioxidative mechanisms," *Molecular Medicine Reports*, vol. 17, no. 5, pp. 6269–6276, 2018.

[174] Y. Zhou, J. Liu, S. Cai, D. Liu, R. Jiang, and Y. Wang, "Protective effects of ginsenoside Rg1 on aging Sca-1$^+$ hematopoietic cells," *Molecular Medicine Reports*, vol. 12, no. 3, pp. 3621–3628, 2015.

[175] M. R. Lee, J. Y. Ma, and C. K. Sung, "Chronic dietary ginseng extract administration ameliorates antioxidant and cholinergic systems in the brains of aged mice," *Journal of Ginseng Research*, vol. 41, no. 4, pp. 615–619, 2017.

[176] L. Chen, H. Yao, X. Chen et al., "Ginsenoside Rg1 decreases oxidative stress and down-regulates Akt/mTOR signalling to attenuate cognitive impairment in mice and senescence of neural stem cells induced by D-galactose," *Neurochemical Research*, vol. 43, no. 2, pp. 430–440, 2018.

[177] C. Xu, W. Wang, B. Wang et al., "Analytical methods and biological activities of Panax notoginseng saponins: recent trends," *Journal of Ethnopharmacology*, vol. 236, pp. 443–465, 2019.

[178] H. Zhao, Z. Han, G. Li, S. Zhang, and Y. Luo, "Therapeutic potential and cellular mechanisms of Panax notoginseng on prevention of aging and cell senescence-associated diseases," *Aging and Disease*, vol. 8, no. 6, pp. 721–739, 2017.

[179] Z. Zhou, J. Wang, Y. Song et al., "Panax notoginseng saponins attenuate cardiomyocyte apoptosis through mitochondrial pathway in natural aging rats," *Phytotherapy Research*, vol. 32, no. 2, pp. 243–250, 2018.

[180] J. Li, L. Yang, J. Z. Wang et al., "Study on protective effect of Panax notoginseng total saponins on H9c2 cells senescence against D-galactose," *Zhong Yao Cai*, vol. 37, no. 8, pp. 1421–1424, 2014.

[181] Z. Guo, Y. Lou, M. Kong, Q. Luo, Z. Liu, and J. Wu, "A systematic review of phytochemistry, pharmacology and pharmacokinetics on Astragali radix: implications for Astragali radix as a personalized medicine," *International Journal of Molecular Sciences*, vol. 20, no. 6, p. 1463, 2019.

[182] X. Ma, K. Zhang, H. Li, S. Han, Z. Ma, and P. Tu, "Extracts from *Astragalus membranaceus* limit myocardial cell death and improve cardiac function in a rat model of myocardial ischemia," *Journal of Ethnopharmacology*, vol. 149, no. 3, pp. 720–728, 2013.

[183] W. Z. Li, W. Y. Wu, D. K. Huang et al., "Protective effects of astragalosides on dexamethasone and A$\beta_{25\text{-}35}$ induced learning and memory impairments due to decrease amyloid precursor protein expression in 12-month male rats," *Food and Chemical Toxicology*, vol. 50, no. 6, pp. 1883–1890, 2012.

[184] X. T. Li, Y. K. Zhang, H. X. Kuang et al., "Mitochondrial protection and anti-aging activity of Astragalus polysaccharides and their potential mechanism," *International Journal of Molecular Sciences*, vol. 13, no. 2, pp. 1747–1761, 2012.

[185] Y. Gao, Y. Wei, Y. Wang, F. Gao, and Z. Chen, "Lycium barbarum: a traditional Chinese herb and a promising anti-aging agent," *Aging and Disease*, vol. 8, no. 6, pp. 778–791, 2017.

[186] X. Hu, Y. Qu, Q. Chu, W. Li, and J. He, "Investigation of the neuroprotective effects of Lycium barbarum water extract in apoptotic cells and Alzheimer's disease mice," *Molecular Medicine Reports*, vol. 17, no. 3, pp. 3599–3606, 2018.

[187] H. C. Jeong, S. H. Jeon, Z. G. Qun et al., "Lycium chinense Mill improves hypogonadism via anti-oxidative stress and anti-apoptotic effect in old aged rat model," *The Aging Male*, pp. 1–10, 2018.

[188] Y. Yu, X. Wu, J. Pu et al., "*Lycium barbarum* polysaccharide protects against oxygen glucose deprivation/reoxygenation-induced apoptosis and autophagic cell death via the PI3K/Akt/mTOR signaling pathway in primary cultured hippocampal neurons," *Biochemical and Biophysical Research Communications*, vol. 495, no. 1, pp. 1187–1194, 2018.

[189] H. Tao, X. Wu, J. Cao et al., "Rhodiola species: a comprehensive review of traditional use, phytochemistry, pharmacology, toxicity, and clinical study," *Medicinal Research Reviews*, vol. 39, no. 5, pp. 1779–1850, 2019.

[190] Q. Zhou, X. Han, R. Li et al., "Anti-atherosclerosis of oligomeric proanthocyanidins from *Rhodiola rosea* on rat model via hypolipemic, antioxidant, anti-inflammatory activities together with regulation of endothelial function," *Phytomedicine*, vol. 51, pp. 171–180, 2018.

[191] S. E. Schriner, K. Lee, S. Truong et al., "Extension of *Drosophila* lifespan by *Rhodiola rosea* through a mechanism independent from dietary restriction," *PLoS One*, vol. 8, no. 5, article e63886, 2013.

[192] C. Chen, J. Song, M. Chen et al., "*Rhodiola rosea* extends lifespan and improves stress tolerance in silkworm, *Bombyx mori*," *Biogerontology*, vol. 17, no. 2, pp. 373–381, 2016.

[193] W.-L. Wei, R. Zeng, C.-M. Gu, Y. Qu, and L.-F. Huang, "*Angelica sinensis* in China-a review of botanical profile, ethnopharmacology, phytochemistry and chemical analysis," *Journal of Ethnopharmacology*, vol. 190, pp. 116–141, 2016.

[194] X. P. Zhang, J. Liu, C. Y. Xu et al., "Effect of Angelica sinensis polysaccharide on expression of telomere, telomerase and P53 in mice aging hematopoietic stem cells," *China Journal of Chinese Materia Medica*, vol. 38, no. 14, pp. 2354–2358, 2013.

[195] X. P. Zhang, Q. X. Wang, B. Chen et al., "Angelica sinensis polysaccharides delay aging of hematopoietic stem cells through inhibitting oxidative damge," *China Journal of Chinese Materia Medica*, vol. 38, no. 3, pp. 407–412, 2013.

[196] X. Cheng, H. Yao, Y. Xiang et al., "Effect of Angelica polysaccharide on brain senescence of Nestin-GFP mice induced by D-galactose," *Neurochemistry International*, vol. 122, pp. 149–156, 2019.

[197] X. Mu, Y. Zhang, J. Li et al., "Angelica sinensis polysaccharide prevents hematopoietic stem cells senescence in D-galactose-induced aging mouse model," *Stem Cells International*, vol. 2017, Article ID 3508907, 12 pages, 2017.

[198] Q. Wang, Y. Huang, C. Qin et al., "Bioactive peptides from *Angelica sinensis* protein hydrolyzate delay senescence in *Caenorhabditis elegans* through antioxidant activities," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 8956981, 10 pages, 2016.

[199] Z. Chen, C. Zhang, F. Gao et al., "A systematic review on the rhizome of *Ligusticum chuanxiong* Hort. (Chuanxiong)," *Food and Chemical Toxicology*, vol. 119, pp. 309–325, 2018.

[200] L. Chen, L. Cheng, X. Wei et al., "Tetramethylpyrazine analogue CXC195 protects against dopaminergic neuronal apoptosis via activation of PI3K/Akt/GSK3β signaling pathway in 6-OHDA-induced Parkinson's disease mice," *Neurochemical Research*, vol. 42, no. 4, pp. 1141–1150, 2017.

[201] S. Wei and H. Wang, "Ligustrazine promoted hypoxia-treated cell growth by upregulation of miR-135b in human umbilical vein endothelial cells," *Experimental and Molecular Pathology*, vol. 106, pp. 102–108, 2019.

[202] B. Gao, X. Lin, H. Jing et al., "Local delivery of tetramethylpyrazine eliminates the senescent phenotype of bone marrow mesenchymal stromal cells and creates an anti-inflammatory and angiogenic environment in aging mice," *Aging Cell*, vol. 17, no. 3, article e12741, 2018.

[203] W.-C. Hou, H.-S. Tsay, H.-J. Liang, T.-Y. Lee, G.-J. Wang, and D.-Z. Liu, "Improving abnormal hemorheological parameters in aging guinea pigs by water- soluble extracts of *Salvia miltiorrhiza* Bunge," *Journal of Ethnopharmacology*, vol. 111, no. 3, pp. 483–489, 2007.

[204] C. H. Park, S. H. Shin, E. K. Lee et al., "Magnesium lithospermate B from *Salvia miltiorrhiza* Bunge ameliorates aging-induced renal inflammation and senescence *via* NADPH oxidase-mediated reactive oxygen generation," *Phytotherapy Research*, vol. 31, no. 5, pp. 721–728, 2017.

[205] S. Zou, J. R. Carey, P. Liedo, D. K. Ingram, B. Yu, and R. Ghaedian, "Prolongevity effects of an oregano and cranberry extract are diet dependent in the Mexican fruit fly (*Anastrepha ludens*)," *The Journals of Gerontology: Series A*, vol. 65A, no. 1, pp. 41–50, 2010.

[206] S. Zou, J. R. Carey, P. Liedo, D. K. Ingram, and B. Yu, "Prolongevity effects of a botanical with oregano and cranberry extracts in Mexican fruit flies: examining interactions of diet restriction and age," *Age*, vol. 34, no. 2, pp. 269–279, 2012.

[207] G. Lai, Y. Guo, D. Chen et al., "Alcohol extracts from Ganoderma lucidum delay the progress of Alzheimer's disease by regulating DNA methylation in rodents," *Frontiers in Pharmacology*, vol. 10, p. 272, 2019.

[208] S. N. Lobo, Y. Q. Qi, and Q. Z. Liu, "The effect of *Gynostemma pentaphyllum* extract on mouse dermal fibroblasts," *ISRN Dermatology*, vol. 2014, Article ID 202876, 6 pages, 2014.

[209] Y.-R. Li, S. Li, and C.-C. Lin, "Effect of resveratrol and pterostilbene on aging and longevity," *BioFactors*, vol. 44, no. 1, pp. 69–82, 2018.

[210] M. Wu, L. Ma, L. Xue et al., "Resveratrol alleviates chemotherapy-induced oogonial stem cell apoptosis and ovarian aging in mice," *Aging*, vol. 11, no. 3, pp. 1030–1044, 2019.

[211] A. Dehghani, Z. Hafizibarjin, R. Najjari, F. Kaseb, and F. Safari, "Resveratrol and 1,25-dihydroxyvitamin D co-administration protects the heart against D-galactose-induced aging in rats: evaluation of serum and cardiac levels of klotho," *Aging Clinical and Experimental Research*, vol. 31, no. 9, pp. 1195–1205, 2019.

[212] L. Du, E. Chen, T. Wu, Y. Ruan, and S. Wu, "Resveratrol attenuates hydrogen peroxide-induced aging through upregulation of autophagy in human umbilical vein endothelial cells," *Drug Design, Development and Therapy*, vol. 13, pp. 747–755, 2019.

[213] A. O. Abolaji, A. O. Adedara, M. A. Adie, M. Vicente-Crespo, and E. O. Farombi, "Resveratrol prolongs lifespan and improves 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced oxidative damage and behavioural deficits in

Drosophila melanogaster," *Biochemical and Biophysical Research Communications*, vol. 503, no. 2, pp. 1042–1048, 2018.

[214] A. M. Posadino, R. Giordo, A. Cossu et al., "Flavin oxidase-induced ROS generation modulates PKC biphasic effect of resveratrol on endothelial cell survival," *Biomolecules*, vol. 9, no. 6, p. 209, 2019.

[215] A. M. Posadino, A. Cossu, R. Giordo et al., "Resveratrol alters human endothelial cells redox state and causes mitochondrial-dependent cell death," *Food and Chemical Toxicology*, vol. 78, pp. 10–16, 2015.

[216] L. Toniolo, P. Fusco, L. Formoso et al., "Resveratrol treatment reduces the appearance of tubular aggregates and improves the resistance to fatigue in aging mice skeletal muscles," *Experimental Gerontology*, vol. 111, pp. 170–179, 2018.

[217] A. A. Momtazi-Borojeni, E. Abdollahi, B. Nikfar, S. Chaichian, and M. Ekhlasi-Hundrieser, "Curcumin as a potential modulator of M1 and M2 macrophages: new insights in atherosclerosis therapy," *Heart Failure Reviews*, vol. 24, no. 3, pp. 399–409, 2019.

[218] M. B. Naeini, A. A. Momtazi, M. R. Jaafari et al., "Antitumor effects of curcumin: a lipid perspective," *Journal of Cellular Physiology*, vol. 234, no. 9, pp. 14743–14758, 2019.

[219] S. Tasneem, B. Liu, B. Li, M. I. Choudhary, and W. Wang, "Molecular pharmacology of inflammation: medicinal plants as anti-inflammatory agents," *Pharmacological Research*, vol. 139, no. 139, pp. 126–140, 2019.

[220] M. Shailaja, K. M. D. Gowda, K. Vishakh, and N. S. Kumari, "Anti-aging role of curcumin by modulating the inflammatory markers in albino Wistar rats," *Journal of the National Medical Association*, vol. 109, no. 1, pp. 9–13, 2017.

[221] S. Pirmoradi, E. Fathi, R. Farahzadi, Y. Pilehvar-Soltanahmadi, and N. Zarghami, "Curcumin affects adipose tissue-derived mesenchymal stem cell aging through TERT gene expression," *Drug Research*, vol. 68, no. 4, pp. 213–221, 2018.

[222] J. Hu, T. Shen, J. Xie, S. Wang, Y. He, and F. Zhu, "Curcumin modulates covalent histone modification and TIMP1 gene activation to protect against vascular injury in a hypertension rat model," *Experimental and Therapeutic Medicine*, vol. 14, no. 6, pp. 5896–5902, 2017.

[223] Y. Chen, X. Liu, C. Jiang et al., "Curcumin supplementation increases survival and lifespan in Drosophila under heat stress conditions," *BioFactors*, vol. 44, no. 6, pp. 577–587, 2018.

[224] C. Receno, C. Liang, D. Korol et al., "Effects of prolonged dietary curcumin exposure on skeletal muscle biochemical and functional responses of aged male rats," *International Journal of Molecular Sciences*, vol. 20, no. 5, p. 1178, 2019.

[225] L. Wen, Y. Jiang, J. Yang, Y. Zhao, M. Tian, and B. Yang, "Structure, bioactivity, and synthesis of methylated flavonoids," *Annals of the New York Academy of Sciences*, vol. 1398, no. 1, pp. 120–129, 2017.

[226] P. Maher, "The potential of flavonoids for the treatment of neurodegenerative diseases," *International Journal of Molecular Sciences*, vol. 20, no. 12, p. 3056, 2019.

[227] A. M. Mahmoud, R. J. Hernández Bautista, M. A. Sandhu, and O. E. Hussein, "Beneficial effects of citrus flavonoids on cardiovascular and metabolic health," *Oxidative Medicine and Cellular Longevity*, vol. 2019, Article ID 5484138, 19 pages, 2019.

[228] D. Maaliki, A. A. Shaito, G. Pintus, A. El-Yazbi, and A. H. Eid, "Flavonoids in hypertension: a brief review of the underlying mechanisms," *Current Opinion in Pharmacology*, vol. 45, pp. 57–65, 2019.

[229] T. De Bruyne, B. Steenput, L. Roth et al., "Dietary polyphenols targeting arterial stiffness: interplay of contributing mechanisms and gut microbiome-related metabolism," *Nutrients*, vol. 11, no. 3, p. 578, 2019.

[230] K. Pallauf, N. Duckstein, and G. Rimbach, "A literature review of flavonoids and lifespan in model organisms," *The Proceedings of the Nutrition Society*, vol. 76, no. 2, pp. 145–162, 2017.

[231] K.-C. Hung, H.-J. Huang, Y.-T. Wang, and A. M.-Y. Lin, "Baicalein attenuates α-synuclein aggregation, inflammasome activation and autophagy in the MPP^+-treated nigrostriatal dopaminergic system in vivo," *Journal of Ethnopharmacology*, vol. 194, pp. 522–529, 2016.

[232] E. Lashmanova, N. Zemskaya, E. Proshkina et al., "The evaluation of geroprotective effects of selected flavonoids in *Drosophila melanogaster* and *Caenorhabditis elegans*," *Frontiers in Pharmacology*, vol. 8, p. 884, 2017.

[233] T. Yang, L. Fang, T. Lin et al., "Ultrasonicated sour Jujube seed flavonoids extract exerts ameliorative antioxidant capacity and reduces Aβ-induced toxicity in *Caenorhabditis elegans*," *Journal of Ethnopharmacology*, vol. 239, article 111886, 2019.

[234] C. Cianfrocca, F. Pelliccia, A. Auriti, and M. Santini, "Ginkgo biloba-induced frequent ventricular arrhythmia," *Italian Heart Journal*, vol. 3, no. 11, pp. 689–691, 2002.

[235] S. C. Erdle, E. S. Chan, H. Yang, B. A. Vallance, C. Mill, and T. Wong, "First-reported pediatric cases of American ginseng anaphylaxis and allergy," *Allergy, Asthma & Clinical Immunology*, vol. 14, no. 1, p. 79, 2018.

[236] S. Lu, K.-j. Chen, Q.-y. Yang, and H.-r. Sun, "Progress in the research of Radix Astragali in treating chronic heart failure: effective ingredients, dose-effect relationship and adverse reaction," *Chinese Journal of Integrative Medicine*, vol. 17, no. 6, pp. 473–477, 2011.

[237] C. H. Larramendi, J. L. García-Abujeta, S. Vicario et al., "Goji berries (Lycium barbarum): risk of allergic reactions in individuals with food allergy," *Journal of Investigational Allergology & Clinical Immunology*, vol. 22, no. 5, pp. 345–350, 2012.

[238] Y. Chang, W. Zhang, Y. Xie et al., "Postmarketing safety evaluation: depside salt injection made from Danshen (*Radix Salviae Miltiorrhizae*)," *Journal of Traditional Chinese Medicine*, vol. 34, no. 6, pp. 749–753, 2014.

[239] S. P. Chaudhari, A. Y. Tam, and J. A. Barr, "Curcumin: a contact allergen," *The Journal of Clinical and Aesthetic Dermatology*, vol. 8, no. 11, pp. 43–48, 2015.

[240] X. Liu, J. Du, J. Cai et al., "Clinical systematic observation of Kangxin capsule curing vascular dementia of senile kidney deficiency and blood stagnation type," *Journal of Ethnopharmacology*, vol. 112, no. 2, pp. 350–355, 2007.

[241] Y. Zhao, X. An, J. Liu et al., "The improvement of oxidative stress by two proprietary herbal medicines in type 2 diabetes," *Complementary Therapies in Medicine*, vol. 40, pp. 120–125, 2018.

[242] T. Kwok, P. C. Leung, C. Lam et al., "A randomized placebo controlled trial of an innovative herbal formula in the prevention of atherosclerosis in postmenopausal women with borderline hypercholesterolemia," *Complementary Therapies in Medicine*, vol. 22, no. 3, pp. 473–480, 2014.

[243] S. Dingzhu, X. Sanli, C. Chuan, S. Rui, and L. Danfei, "Effect of Shoushen granule on arterial elasticity in patients with carotid atherosclerosis: a clinical randomized controlled trial," *Journal of Traditional Chinese Medicine*, vol. 35, no. 4, pp. 389–395, 2015.

[244] P. Lv, X. Tong, Q. Peng et al., "Treatment with the herbal medicine, naoxintong improves the protective effect of high-density lipoproteins on endothelial function in patients with type 2 diabetes," *Molecular Medicine Reports*, vol. 13, no. 3, pp. 2007–2016, 2016.

[245] S. Akhondzadeh, M. Noroozian, M. Mohammadi, S. Ohadinia, A. H. Jamshidi, and M. Khani, "Salvia officinalis extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomized and placebo-controlled trial," *Journal of Clinical Pharmacy and Therapeutics*, vol. 28, no. 1, pp. 53–59, 2003.

[246] S. Akhondzadeh, M. S. Sabet, M. H. Harirchian et al., "Saffron in the treatment of patients with mild to moderate Alzheimer's disease: a 16-week, randomized and placebo-controlled trial," *Journal of Clinical Pharmacy and Therapeutics*, vol. 35, no. 5, pp. 581–588, 2010.

[247] J. Jia, C. Wei, S. Chen et al., "Efficacy and safety of the compound Chinese medicine SaiLuoTong in vascular dementia: a randomized clinical trial," *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, vol. 4, pp. 108–117, 2018.

[248] H. Tajadini, R. Saifadini, R. Choopani, M. Mehrabani, M. Kamalinejad, and A. A. Haghdoost, "Herbal medicine Davaie Loban in mild to moderate Alzheimer's disease: a 12-week randomized double-blind placebo-controlled clinical trial," *Complementary Therapies in Medicine*, vol. 23, no. 6, pp. 767–772, 2015.

[249] T. Uno, I. Ohsawa, M. Tokudome, and Y. Sato, "Effects of Goshajinkigan on insulin resistance in patients with type 2 diabetes," *Diabetes Research and Clinical Practice*, vol. 69, no. 2, pp. 129–135, 2005.

[250] S. Cho, C.-H. Won, D. H. Lee et al., "Red ginseng root extract mixed with Torilus fructus and Corni fructus improves facial wrinkles and increases type I procollagen synthesis in human skin: a randomized, double-blind, placebo-controlled study," *Journal of Medicinal Food*, vol. 12, no. 6, pp. 1252–1259, 2009.

[251] R. Giordo, A. Cossu, V. Pasciu, P. T. Hoa, A. M. Posadino, and G. Pintus, "Different redox response elicited by naturally occurring antioxidants in human endothelial cells," *The Open Biochemistry Journal*, vol. 7, pp. 44–53, 2013.

[252] V. Pasciu, A. M. Posadino, A. Cossu et al., "Akt downregulation by flavin oxidase–induced ROS generation mediates dose-dependent endothelial cell damage elicited by natural antioxidants," *Toxicological Sciences*, vol. 114, no. 1, pp. 101–112, 2010.

[253] A. M. Posadino, A. Cossu, R. Giordo et al., "Coumaric acid induces mitochondrial damage and oxidative-mediated cell death of human endothelial cells," *Cardiovascular Toxicology*, vol. 13, no. 3, pp. 301–306, 2013.

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Review Article

Interplay between the Adaptive Immune System and Insulin Resistance in Weight Loss Induced by Bariatric Surgery

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Low-grade chronic inflammation plays a pivotal role among other pathophysiological mechanisms involved in obesity. Innate and adaptive immune cells undergo systemic proinflammatory polarization that gives rise to an increased secretion of proinflammatory cytokines, which in turn leads to insulin resistance. Bariatric surgery is currently the most effective treatment for obesity, as it brings on significant weight loss, glucose metabolism improvement, and a decrease in systemic inflammation biomarkers. After bariatric surgery, several changes have been reported to occur in adaptive immunity, including reduction in CD4+ and CD8+ T cell counts, a decrease in the Th1/Th2 ratio, an increase in B regulatory cells, and reduction in proinflammatory cytokine secretion. Overall, there seems to be a major shift in several lymphocyte populations from a proinflammatory to an anti-inflammatory phenotype. Furthermore, increased antioxidant activity and reduced lipid and DNA oxidation products have been reported after bariatric surgery in circulating mononuclear cells. This paper highlights the shift in the adaptive immune system in response to weight loss and improved insulin sensitivity, as well as the interplay between immunological and metabolic adaptations as a result of bariatric surgery. Finally, based on data from research, we propose several mechanisms such as changes in adaptive immune cell phenotypes and their by-products, recruitment in adipose tissue, reduced oxidative stress, and modification in metabolic substrate availability as drivers to reduce low-grade chronic inflammation after bariatric surgery in severe obesity.

1. Introduction

Obesity is defined as an excess of body fat. Body mass index (BMI) has been the most widely used parameter to assess and classify the grade of obesity. The World Health Organization defines obesity as a BMI $\geq 30\,kg/m^2$ [1]. In recent decades, obesity prevalence has risen to alarming levels. Global prevalence has increased from 3.2% in men and 6.4% in women in 1975 to 10.8% and 14.9%, respectively, in 2014 [2]. Obesity has been associated with metabolic disorders such as insulin resistance [3], dyslipidemia [4], and nonalcoholic fatty liver disease [5] and with endocrine conditions such as type 2 diabetes mellitus (T2DM) [6], polycystic ovarian syndrome [7], and vitamin D deficiency [8]. Despite a strong epidemiological association indicating an increased risk for obese subjects to suffer metabolic comorbidities, it should be noted that a proportion of the obese population has no manifest disorder (the so-called "metabolically healthy obese"), while also a relatively small but considerable proportion of normal-weight subjects may suffer from the metabolic conditions associated with obesity [9]. Obesity has also been related to autoimmune diseases such as rheumatoid arthritis [10], psoriasis [11], and

systemic lupus erythematosus [12]. Furthermore, obesity increases mortality [13] and diminishes quality of life [14].

The treatment for obesity has proven to be a difficult challenge. Lifestyle changes including a change of diet and an increase in physical activity have been widely approved as the first-line options [15]. Several drugs, indicated as a complementary treatment to lifestyle changes, have proven to be effective in achieving a weight loss of 5% [16, 17]. However, weight regain is a common problem given that only about 50% of the subjects have been found to achieve a weight loss of at least 5% after 8 years of an intensive lifestyle intervention [18]. On the other hand, bariatric surgery has been regarded as the most effective long-term treatment for obesity [19]. According to the clinical guidelines from the American Society for Metabolic and Bariatric Surgery, surgical procedures for weight loss are indicated for patients with a BMI $\geq 40\,kg/m^2$, a BMI $\geq 35\,kg/m^2$ with at least one obesity-associated comorbidity, or a BMI $\geq 30\,kg/m^2$ with either T2DM or metabolic syndrome [20].

Obesity is regarded as a low-grade inflammatory state characterized by an elevation of acute-phase reactants and proinflammatory cytokines. Inflammation is driven by the immune response, which is classified into innate and adaptive immunity. The innate response, mediated by neutrophils and macrophages that rapidly migrate to the inflamed tissues to try to eliminate the offensive agent, is nonspecific but fast-acting. On the other hand, the adaptive response is directed against a specific insult, mediated by T and B lymphocytes, which recognize specific epitopes with high affinity by the T cell receptor (TCR) or by antibody production, respectively. Both responses usually interact with and reinforce each other. For instance, macrophages act as antigen-presenting cells (APC) for T cells, and in turn, T cells secrete proinflammatory cytokines such as interferon-γ, which further activates macrophages [21, 22]. Specifically, the proinflammatory milieu associated with obesity is driven in part by adipose tissue macrophages, which are a component of the innate immune system. However, increasing recent evidence indicates that adaptive immune cells, such as T and B lymphocytes, play a crucial role in the activation and maintenance of such inflammatory state [23, 24]. The purpose of this review is to highlight the alterations in the adaptive immune system that follow bariatric surgery-induced weight loss and the potential underlying mechanisms associated with the inflammatory milieu and the insulin resistance status.

2. Low-Grade Chronic Inflammatory State in Obesity Is Driven by Both Innate and Adaptive Immune Cells

Obesity is associated with a low-grade chronic systemic inflammatory state characterized by elevation of acute-phase proteins such as C-reactive protein (CRP) produced by the liver [25] and IL-6, secreted by adipocytes and adipose tissue macrophages [26–28], as well as by an increase in TNF-α by adipose tissue [29]. Although early studies failed to find a significant increase in circulating TNF-α in patients with obesity [30], recent reports have observed higher serum TNF-α among obese subjects [31]. TNF-α has been shown to be mainly secreted by macrophages [32] that infiltrate adipose tissue creating crown-like structures (CLS) around necrotic adipocytes [33]. The role of adaptive immunity in obesity has been partly described. B lymphocytes have also been found within CLS in human adipose tissue, although their role is not completely understood [34]. In addition, adipose tissue from obese subjects has been found to contain an increased number of both CD4+ and CD8+ T lymphocytes, which also secrete proinflammatory cytokines such as TNF-α and IFN-γ [35]. Furthermore, increased waist circumference has been associated with increased expression of the activation markers CD25 and CD69 in T lymphocytes from adipose tissue [36]. Immune cells in peripheral blood have also been found to be related to inflammatory markers. Th1 cells are well known to secrete IFN-γ, a proinflammatory cytokine, while Th2 cells secrete IL-4, an interleukin with an anti-inflammatory role [23]. Peripheral blood mononuclear cells (PBMCs) have been shown to exhibit a proinflammatory secretory profile in obese subjects [37, 38]. Also, higher levels of the activation factor CD25 in T lymphocytes and increased Th1/Th2 ratios, correlated with insulin resistance assessed by the HOMA index, have been reported in obese subjects compared with lean or healthy overweight controls [39]. Furthermore, BMI has been found to be positively correlated with CD4+ effector memory T lymphocytes and negatively correlated with anti-inflammatory T regulatory (Treg) lymphocytes in severely obese subjects undergoing bariatric surgery [40].

Other specific T lymphocyte populations, such as mucosal-associated invariant T (MAIT) cells, have also been associated with the secretion of proinflammatory mediators. MAIT cells are innate-like T lymphocytes found in peripheral blood, intestinal mucosa, and the liver. Circulating MAIT cell numbers have been found to be reduced in patients with obesity and in those with T2DM. However, secretion of IL-17 by MAIT cells was increased in obese compared with the lean subjects. In addition, secretion of IL-2, granzyme B, and TNF-α was increased in T2DM. Noteworthy, both MAIT and CD8+ cells were found to be more abundant in omental adipose tissue than in peripheral blood in obese patients. These findings suggest a recruitment of MAIT cells by adipose tissue in obese subjects [41]. The polarization toward proinflammatory subpopulations of lymphocytes is a systemic phenomenon that has been observed in several tissues, including the skeletal muscle, liver, and pancreas [42].

The role of innate immunity in the development of obesity-associated low-grade chronic inflammation has been well studied. Mounting evidence indicates that despite the lack of an identified specific antigen, the adaptive immune system also participates in the development of this inflammatory state and exhibits proinflammatory polarization.

3. Inflammation Drives Insulin Resistance

Although a causative relationship between inflammation and insulin resistance is generally not regarded as proven, multiple evidence from studies in animal models and clinical trials that suggest that chronic inflammation may be a mechanism involved in the development of insulin resistance has been

shown [43]. Adipose tissue-secreted TNF-α has been shown to induce insulin resistance in animal models [29] through activation of Janus N kinases (JNK) and serine phosphorylation of insulin receptor substrate (IRS) [44]. Both TNF-α and IL-6 have been associated with incident T2DM in case-control studies [45]. Furthermore, anti-TNF-α treatment has been able to reduce insulin resistance in rheumatoid arthritis [46] and in psoriasis [47]. Kinases have been shown to play a role in insulin resistance. IKKε (IκB kinase ε) and TBK1 (TANK-binding kinase 1) are noncanonical IκB kinases that stimulate IFN-α and IFN-β. While these kinases have been shown to increase in response to a high-fat diet, their inactivation, either by genetic knockout or by pharmacological drugs, has been shown to prevent obesity and insulin resistance in murine models [48, 49]. Furthermore, a novel dual inhibitor of IKKε and TBK1, amlexanox, has been shown to improve glycemic control in T2DM in a subset of patients with increased baseline inflammatory status [50]. In addition, IFN-γ has been shown to decrease insulin sensitivity in human adipose tissue by downregulation of PI3K (fosfatidilinositol-3-kinase), inhibiting the adipocyte secretion of the lipogenic enzymes, fatty acid synthase, and lipoprotein lipase, in response to insulin [35]. A more detailed description of the mechanisms by which inflammatory mediators lead to insulin resistance may be found in the revision by Chen et al. [51]. Besides inflammation, other mechanisms leading to insulin resistance in obesity include mitochondrial dysfunction, hyperinsulinemia through a negative feedback loop, lipotoxicity, endoplasmic reticulum stress, hypoxia, and oxidative stress [52]. Therefore, inflammation is a clinically relevant mechanism, among others, that contributes to insulin resistance associated with obesity and a potential source of pharmacological target for the treatment of insulin resistance and even T2DM in some patients.

4. Insulin Might Induce Changes in an Immune Cell Phenotype

Lymphocytes express both glucose transporter 1 (GLUT1) and GLUT3. Alterations in lymphocyte glucose uptake have been demonstrated in T2DM. Diet-treated subjects recently diagnosed with T2DM were shown to have increased peripheral lymphocyte glucose uptake at 15 minutes but have decreased uptake at 30 and 60 minutes compared with healthy controls. In contrast, insulin-treated patients with longer duration of disease were shown to have increased lymphocyte glucose uptake at all time points [53]. In lymphocytes from healthy subjects, *in vitro* treatment with insulin at 50 mIU/ml was shown to increase lymphocyte glucose uptake at 30 minutes, as well as GLUT3 and GLUT4 expression. These findings demonstrate that peripheral lymphocytes react to insulin stimulation and, therefore, could represent a model for the study of insulin resistance [54].

In addition, in an *in vitro* model, insulin has been described to be promoting anti-inflammatory Th2 differentiation in CD4+ lymphocytes, an effect likely mediated by ERK (extracellular-signal-regulated kinase) phosphorylation. Interestingly, while the insulin receptor was not shown to be detected in resting T cells, it was significantly upregulated upon activation in both CD4+ and CD8+ cells [55]. Furthermore, lymphocytes from obese subjects were not shown to increase pAKT (protein kinase B) intracellular levels or to decrease the Th1/Th2 ratio upon *in vitro* stimulation with supraphysiological concentrations of insulin, as the lymphocytes from lean controls did, indicating that lymphocytes from obese subjects have an impaired response to insulin [39].

These results indicate that lymphocytes respond to insulin stimulation, a fact not widely explored. Furthermore, in conditions characterized by insulin resistance, such as obesity and T2DM, lymphocytes have an impaired response to insulin. Finally, lymphocytes from obese subjects are resistant to insulin-mediated Th2 differentiation *in vitro*. Therefore, Th1 polarization seen in obesity may be partially caused by insulin resistance. Whether this is a relevant mechanism *in vivo* requires further study.

5. Bariatric Surgery Induces Significant Weight Loss

Lifestyle changes have been indicated as the first-line treatment option for people with obesity, but weight loss has proven to be rather small. In the Look AHEAD interventional study, a mean weight loss of 8.5% was achieved after one year of an intensive lifestyle intervention, but after an 8-year follow-up, only 50.3% were able to maintain a 5% weight loss [18]. In another lifestyle change interventional study, a 5% weight loss was shown to be sufficient in order to increase insulin sensitivity in the adipose tissue, liver, and skeletal muscle, but not to reduce systemic inflammation markers. A significant decrease in CRP was proven to decrease only after a progressive weight loss of more than 15%, although no significant changes were seen for IL-6, MCP-1, or white blood cell count [56]. In contrast, bariatric surgery has proven to induce a consistent significant and persistent weight loss. A pooled meta-analysis which included 25 studies concluded that bariatric surgery was more effective than nonsurgical interventions in achieving weight loss after a follow-up of one, two, and three years. Bariatric surgery was proven to achieve a weight loss of at least 20 kg higher compared with nonsurgical interventions [19]. A worldwide study estimated that bariatric surgery overall induces a weight loss of 30.5% at one-year follow-up [57].

Roux-en-Y gastric bypass (RYGB), laparoscopic sleeve gastrectomy (LSG), laparoscopic adjustable gastric banding (LAGB), and biliopancreatic diversion with duodenal switch (BPD-DS) are the most common bariatric surgical procedures (Figure 1). LAGB and LSG are traditionally regarded as merely restrictive methods, since the main effect of the operation is a reduction in gastric capacity. RYGB and BDP-DS induce a reduction in gastric capacity along with a malabsorptive component, given that a partial bypass of the small bowel is generated. The malabsorptive component is far more significant in BPD-DS due to the increased extent of the bypassed small bowel. Beyond their restrictive and malabsorptive mechanisms, LSG, RYGB, and BPD-DS lead to alterations in endocrine mechanisms by modulation of the secretion of gastrointestinal factors, such as ghrelin and

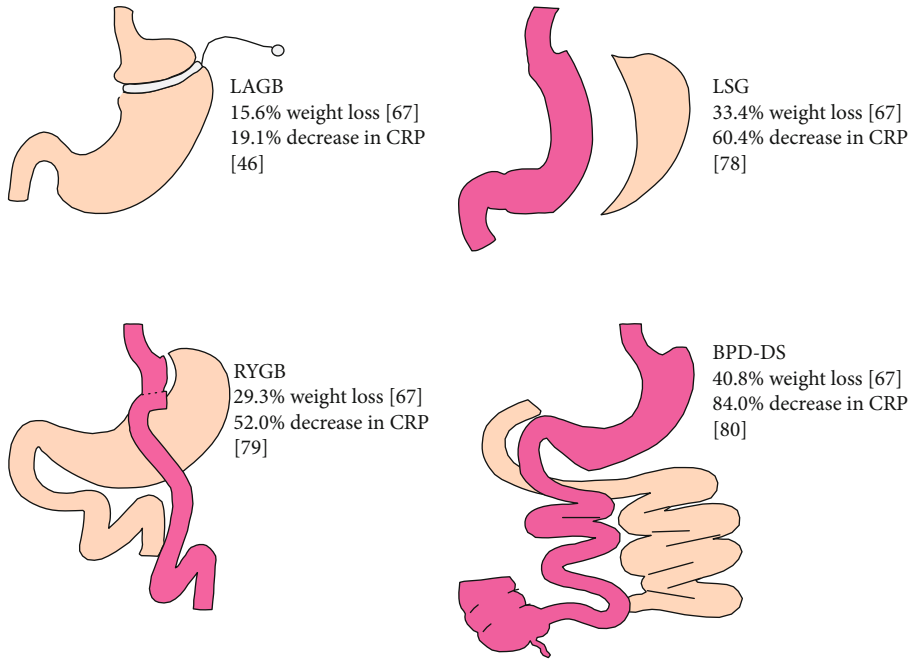

Figure 1: Graphic representation of bariatric surgical procedures to achieve weight loss and a % decrease in CRP. LAGB: laparoscopic adjustable gastric band; LSG: laparoscopic sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; BPD-DS: biliopancreatic diversion with duodenal switch; CRP: C-reactive protein. References in brackets. Further details in text.

glucagon-like peptide 1 (GLP-1), which have an effect on satiety and on glucose metabolism [58, 59].

While RYGB is widely regarded as the "gold standard" of bariatric procedures, BPD-DS is considered more effective, since it induces greater weight loss. However, its increased technical complexity and the higher morbidity risk limit its use. Regarding weight loss with these different procedures, LSG weight loss outcomes are similar to those of RYGB, while LAGB induces a more modest weight loss [58, 59]. In a retrospective study, a weight loss of 15.6%, 29.3%, 33.4%, and 40.8% was achieved after a 3-year follow-up using LAGB, RYGB, LSG, and BPD-DS, respectively [60] (Figure 1). Gastric imbrication or plicature, which involves folding and stitching the stomach into itself to adopt a tubular structure, is a relatively new surgical technique used for weight loss. Although there is little available data, short-term outcomes seem to be similar to those of sleeve gastrectomy [61]. In addition to weight loss, bariatric surgery is known to improve control of obesity-associated comorbidities, particularly T2DM and insulin resistance in the case of RYGB and BPD-DS, due to their endocrine component [59, 62].

6. Bariatric Surgery Is Associated with a Decrease in Systemic Inflammation Assessed by C-reactive Protein

Multiple studies have reported a decrease in CRP levels, a frequently assessed marker of systemic inflammation in the clinical setting after bariatric surgery. CRP has been shown to decrease from 1.15 to 0.34 mg/l at 6 months after either RYGB or LSG in a small cohort of Mexican subjects [63]. CRP was also found to fall from 0.68 to 0.55 mg/dl 3 months after LAGB, associated with a 13.9% weight loss, in 46 subjects [64]. In another study with a smaller sample size ($n = 20$), but a longer follow-up at 3 years, LAGB was shown to induce a weight loss of 30% and CRP was found to decrease from 7.6 to 3.4 mg/l [65]. CRP was found to be reduced from 5.3 to 2.1 U/ml one year after LSG in 30 morbidly obese women in which a 29.3% weight reduction was achieved [66]. Other authors have reported a decrease in CRP from 1.02 to 0.49 mg/dl in 20 obese subjects 3 months after RYGB. Noteworthy, no correlation was found between changes in CRP and changes in BMI. This lack of correlation points to a potential association of CRP with direct markers of fat mass or with caloric deprivation or malabsorption [67]. Finally, CRP was described to decrease from 11.9 mol/l to 4.9 mmol/l at 6 months, with further reduction to 1.9 mmol/l at 12-month follow-up in 70 subjects that underwent BPD-DS. Meanwhile, a 37.1% one-year weight loss was achieved [68]. Overall, all bariatric procedures were found to decrease CRP concentrations, although differences among the procedures might be attributable to the different follow-up periods and the varied baseline levels. It seems, however, that bariatric procedures that induce greater weight loss are also the most effective in reducing CRP levels and systemic inflammation (Table 1). However, other predictors of decreased CRP levels, such as adiposity markers or caloric deprivation, have been proposed.

7. Weight Loss Induced by Bariatric Surgery Is Associated with a Systemic Decrease in Oxidative Stress Final Products

Obesity has also been associated with high levels of oxidative stress in different studies. Obese women were found to have

Table 1: Comparison of changes in systemic C-reactive protein observed after weight loss across different surgical procedures.

Population	Mean age (years)	Intervention	Follow-up (months)	Weight loss (%)	CRP reduction	Reference
36 subjects 88.9% F	37	RYGB/LSG	6	24.3	70.4%	[63]
46 subjects 87% F	40.6	LAGB	3	13.2	19.1%	[64]
20 subjects		LAGB	36	30.3	55.3%	[65]
30 subjects 100% F	40.3	LSG	12	29.3%	60.4%	[66]
70 subjects	41.3	BPD-DS	12	37.1%	84%	[68]

F: female; RYGB: Roux-en-Y gastric bypass; LSG: laparoscopic sleeve gastrectomy; LAGB: laparoscopic adjustable gastric band; BPD-DS: biliopancreatic diversion with duodenal switch; CRP: C-reactive protein.

higher plasmatic concentrations of lipid peroxidation and protein carbonylation markers, compared with normal-weight controls. When measured 6 months after bariatric surgery, the levels of these markers were significantly reduced, although the concentrations did not reach those of the control group [69]. Short-term outcomes after biliopancreatic diversion have demonstrated an increase in glutathione-S-transferase and glutathione reductase activity in plasma, despite a decrease in glutathione peroxidase activity. Lipid peroxidation was found to increase initially 15 days after surgery, but afterwards, a significant decrease that remained below baseline levels 3 months after surgery was shown [70]. After a 1-year follow-up of bariatric surgery patients, a significant reduction in both lipid peroxidation and protein carbonylation, as well as an increase in nonprotein thiols and in whole blood antioxidant enzyme activity, was demonstrated [71]. Altogether, these results indicate an overall reduction in oxidative stress after weight loss induced by bariatric surgery.

Furthermore, bariatric surgery-mediated weight loss has been shown to reduce oxidative stress specifically in PBMCs, mainly composed of lymphocytes. Antioxidant enzyme activity has been reported to increase in PBMCs, while products of lipid and DNA oxidation have been shown to decrease after a one-year follow-up in morbid obese patients that underwent bariatric surgery [72, 73]. Previous work indicates that mitochondrial ROS are necessary for T cell activation [74]. ROS modulation has been reported to reduce cytokine secretion [75, 76]. Therefore, ROS reduction observed after bariatric surgery-induced weight loss should be expected to contribute to decreased proinflammatory cytokine secretion. However, its relevance in this specific clinical context has not been directly addressed.

8. Bariatric Surgery Drives a Shift in Adaptive Immune Cells Characterized by Changes in Lymphocyte Count, Phenotype, and Anti-Inflammatory Marker Secretion

Changes in lymphocyte count and variations in the phenotype, as well as in the secretion of their by-products, have been reported after bariatric surgery-induced weight loss. Shortly after gastric banding, a decrease in the Th1/Th2 ratio together with a reduction in Th1 and/or an increase in Th2 levels has been reported in T2DM and prediabetes, with improvement in glucose metabolism [77, 78]. B lymphocytes have also been shown to shift from an effector to a regulatory phenotype, suppressing proinflammatory cytokine secretion by T lymphocytes after RYGB [79]. In addition, the total number of CD4+ and CD8+ circulating T cells was found to be reduced after laparoscopic greater curvature plication in morbidly obese patients, suggesting a declined level of cell-mediated immune activity [80]. Likewise, a reduction in the count of B, T CD8+, and natural killer (NK) lymphocytes has been described after bariatric surgery in a subset of severely obese subjects with insulin resistance [81]. However, in a small sample of 20 severely obese women, no significant change in T CD4+, T CD8+, B, and NK lymphocyte subpopulations was found after bariatric surgery. When analyzed by the type of surgery, a change in CD4+ T lymphocytes was shown to correlate with changes in BMI in the RYGB subgroup, but not in the LAGB group, suggesting that more pronounced weight loss might be associated with a reduction in CD4+ T cells [82]. Using another procedure, four months after laparoscopic greater curvature plication, a decrease in CD4+ T cells from 38.2% to 29.3%, in CD8+ from 17.3% to 9.5%, and in leptin from 43.01 to 24.8 ng/ml was demonstrated in 20 subjects [80]. However, the association between the observed changes in lymphocyte populations and leptin was not evaluated in the study. Considering these results, a weight loss-associated decrease in the inflammatory phenotype lymphocyte differentiation is induced after the diverse bariatric surgery procedures.

Furthermore, Tfh cells, which play a crucial role in activating and differentiating B lymphocytes, have been shown to reduce the expression of activation markers and the secretion of proinflammatory cytokines after RYGB. Activation markers of Tfh cells were found to be downregulated 3 months after RYGB. The secretion of IFN-γ, IL-2, IL-4, and IL-17 by Tfh cells was also shown to decrease, while no significant change was observed in IL-10. Secretion of IL-10 was described to be higher after 72 hr incubation of Tfh post-RYGB plus staphylococcal enterotoxin B- (SEB-) pulsed autologous B cells compared with that of Tfh pre-RYGB plus SEB-pulsed B cells. In addition, Tfh IL-10+ was shown to promote the differentiation of naïve B cells toward an IL-10- and TGF-β-secreting phenotype, directly mediated

by IL-10 itself. B cells isolated after RYGB were found to express more IL-10 and TGF-β [83]. These findings demonstrate an overall decrease in inflammatory cytokines and an overall increase in anti-inflammatory cytokines secreted by Tfh cells which may have a direct effect on the differentiation of anti-inflammatory B cells. Similarly, in another study, 3 months after the RYGB, B lymphocytes were found to shift from a proinflammatory IL-6+ phenotype to an anti-inflammatory IL-10+ phenotype, while T lymphocytes were shown to reduce the secretion of proinflammatory cytokines IL-17 and IFN-γ. Furthermore, coincubation of B and T cells showed that preoperative B cells stimulated proinflammatory cytokine secretion by T cells, while postoperative B cells inhibited this phenomenon [79]. These findings indicate that bariatric surgery induces changes in the lymphocyte phenotype from proinflammatory to anti-inflammatory that further impact other cell populations to regulate their inflammatory potential.

Lips et al. [84] reported outcomes in inflammatory status in obese and diabetic women 3 months after RYGB. Weight loss was shown to lead to a reduction in systemic CRP, total T cells, and helper T cells, but paradoxically, also to an increase in TNF-α systemic concentration. It is speculated that this later finding may reflect incomplete recovery from the surgical procedure itself and that longer follow-up periods are recommended to get a better picture of systemic inflammation after bariatric surgery [84]. In addition, decreased circulating MAIT cell numbers with surprisingly increased proinflammatory IL-17 secretion have been found in obese subjects compared with healthy controls. The lower numbers of MAIT cells in obese subjects have been attributed to increased activation and infiltration in adipose tissue. After bariatric surgery, the subjects were reported to present increased MAIT cell count in peripheral blood, but with no decrease in IL-17 secretion [41]. The relevance of the changes in MAIT cells and whether they reflect a reduction in their proinflammatory phenotype require further study.

9. Changes in Adaptive Cellular Immunity Elicited by Bariatric Surgery-Induced Weight Loss May Be Linked to Metabolic Improvement

The relationship of lymphocyte changes induced by bariatric surgery-induced weight loss with insulin sensitivity and metabolic alterations is crucial. However, available data is scarce and somewhat controversial, though pointing toward a positive link between them. While some studies have found changes in cell immunity to be related to glucose metabolism, others have not shown such a relationship. Viardot et al. evaluated changes in the immune system induced by weight loss after a 24-week-long restriction diet with laparoscopic gastric banding (LGB) performed at week 12 in obese subjects with either T2DM or impaired glucose tolerance (IGT) and in 10 healthy matched control subjects. The mean weight loss was found to be 5% at week 12 and 13.5% at week 24, while glucose control was described to improve significantly. Weight loss was shown to elicit a decrease in Th1 count and the Th1/Th2 ratio and to reduce the expression of CD69 and CD25 activation markers in lymphocytes. The decrease in CD69 and in the Th1/Th2 ratio was found to be associated with the reduction in BMI, but not with the HOMA-IR index or with fasting glucose [77]. However, other studies have shown a link between adaptive immune changes and metabolic parameters after bariatric surgery. The association between glucose metabolism and both systemic and adipose tissue inflammation was evaluated in 15 subjects with BMI above 35 kg/m^2 and either T2DM or IGT who underwent LGB. Weight loss of 12.5% at week 12 was found to lead to lower fasting glucose levels and to a decrease in the Th1/Th2 ratio. A negative correlation between Th2 levels and fasting glucose concentration was also found [78]. Likewise, in another study, a negative correlation between Tfh IL10+ cell percentage change and BMI, glucose levels, and fat mass percentage was found after RYGB [83]. As inflammation is one of the mechanisms leading to insulin resistance and other obesity-related comorbidities, an increase in the anti-inflammatory lymphocyte subpopulation associated with decreased glucose levels after bariatric surgery might suggest that changes in cellular immunity after weight loss are linked to metabolic improvements. Noteworthy, the absolute numbers of B lymphocytes, CD8+ T lymphocytes, and NK lymphocytes have been reported to decrease after BPD-DS only in insulin-resistant subjects, indicating a potential relationship between these lymphocyte populations and insulin resistance. It is possible that some of these lymphocytes, particularly NK cells, contribute to the development of insulin resistance [81]. On the other hand, another unexplored possibility is that insulin resistance might be the driver responsible for the changes in lymphocyte populations, which would reverse after bariatric surgery-related improvement in insulin sensitivity (Figure 2). Insulin has an effect on lymphocyte differentiation, but the role played by insulin in bariatric surgery-induced weight loss, as well as the potential effects on other lymphocyte populations besides that on CD4+ T cells, requires further study. Changes in lymphocyte populations and cytokine secretion seen after bariatric surgery-induced weight loss are summarized in Table 2.

10. Shift to an Anti-Inflammatory Lymphocyte Phenotype after Bariatric Surgery and Weight Loss: From Insulin Resistance to Immunometabolism

As previously described in this paper, bariatric surgery has been well known to considerably reduce white adipose tissue mass, to improve insulin resistance and T2DM, and to induce a shift from the proinflammatory condition that characterizes obesity to an anti-inflammatory state. However, the mechanism by which weight loss induces activation of immune system cells is not completely understood. We propose three main mechanisms based on reports that describe the relationship between postoperative weight loss and changes in the immune system.

The first mechanism involves the association of adipose tissue and chronic inflammation. The role of adipose tissue

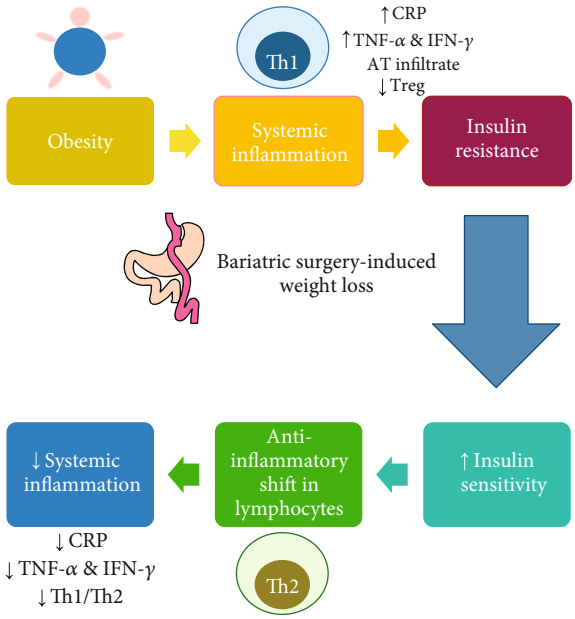

Figure 2: Potential mechanism of improvement in inflammatory status after bariatric surgery-induced weight loss. Obesity induces a systemic inflammatory status characterized by increases in C-reactive protein, proinflammatory cytokines such as TNF-α and IFN-γ, and an inflammatory infiltrate in adipose tissue (AT) and a decrease in circulating Treg lymphocytes. Inflammatory cytokines, among other mechanisms, induce insulin resistance in obesity. Bariatric surgery induces a significant weight loss that is associated with an increase in insulin sensitivity and a decrease in systemic inflammation. An important change in the lymphocyte phenotype is a decrease in the Th1/Th2 ratio after weight loss. It is possible that insulin effect on T cell differentiation may mediate inflammation resolution, at least partially.

in maintaining a low-grade chronic inflammation has been related to physiological stress in adipocytes as they progressively accumulate excessive fat. Increased release of inflammatory cytokines has been shown to elicit infiltration and activation of macrophages within the adipose tissue, which in turn alters the adipocyte secretion of adiponectin, leptin, and resistin. Thus, chronic insulin resistance is ensued and maintained [85]. Additionally, adipocyte death has been shown to induce immune cell activation and to initiate inflammation via macrophage activation [86]. The release of damage-associated molecular patterns (DAMPs) such as HMGB1 can lead to the activation of toll-like receptors (TLRs), and immune activation has also been described [87]. Also, differences in the localization of macrophages within adipose tissue between lean and obese fat mice have been found. In the lean group, interstitially spaced macrophages have been observed, while in the obese groups, macrophages are arranged in CLS [88], suggesting the presence of different subpopulations and paracrine activation. Therefore, the reduction of fat in the adipose tissue generated by bariatric surgery in the obese population would decrease the inflammatory stimulus driven by the interaction of immune cells and adipocytes.

The second mechanism involved in the relationship between postoperative weight loss and changes in the immune system is related to the activation and infiltration of immune cells. The reduction in the number and activation of infiltrating immune cells in the adipose tissue has been found to reduce the secretion of proinflammatory cytokines, such as CRP, IL-6, IL-1β, TNF-α, and IFN-γ, which in turn leads to a shift to an anti-inflammatory state and improves metabolic functions, contributing to weight loss. The interplay between the immune system and its associated effects on the metabolic status is a recent research field known as immunometabolism. Macrophages in lean adipose tissue have been described to have higher levels of arginase-1 and IL-10 expression, which are typical markers of anti-inflammatory M2 macrophages, compared with proinflammatory M1 macrophages found in obesity [89]. From this perspective, bariatric surgery could decrease infiltrating proinflammatory macrophages by reducing the differentiation of macrophages or by reestablishing the M1/M2 cell ratio, leading to a reduction in the infiltration of adaptive immune cells. Adaptive immunity in obesity has been shown to be affected by the infiltration of T and B cells into the adipose tissue, producing even more inflammatory cytokines, as demonstrated in obese mice [42, 90, 91]. After RYGB surgery, B cells have been shown to present a regulatory (IL-10) versus an effector (IL-6) profile and halt their previous role of sustaining T cell inflammation [79]. Bariatric surgery could modulate the increased activation of CD4+ and CD8+ T cells regularly observed in obese conditions and abate the level of both systemic inflammation and adipose tissue inflammation. In consequence, bariatric surgery can decrease or regularize the secretion of proinflammatory cytokines and insulin resistance, which in turn favors and improves glucose metabolism and adipogenesis (Figure 3).

The third mechanism involves the relationship of nutrients and immune cells. Metabolites such as glucose, free fatty acids, glutamine, and succinate are energy resources that can alter macrophage, neutrophil, and T cell functions [92]. Obesity is characterized by a continuous supply of these metabolites. The variations in these nutrient supplies observed after bariatric surgery could modulate the immune system and have been proposed as a key factor in immune differentiation. Bariatric surgery could modulate inflammation directly by decreasing the supply of nutrient metabolites through the inhibition of pathologic immune activation, hence leading to an anti-inflammatory state and improving metabolic functions. In particular, succinate has been shown to induce inflammation in macrophages *via* succinate receptor 1- (SUCNR1, also known as GPR91) mediated amplification of TLR signaling enhancing the secretion of IL-1β [93–94]. The metabolism of circulating fructose, which is mainly absorbed systemically by GLUT2 and GLUT5, has been associated with ATP depletion, oxidative stress, and inflammatory responses [95]. However, the cyclic guanosine-adenosine monophosphate (cGAMP) pathway has been described to be dysregulated, leading to the activation and induction of type I interferon synthesis induced by the STING receptor [87, 96]. Obesity is associated with an increase in T effector memory cells and a decrease in naïve T cells. This polarization in T CD4+ cells is driven by palmitate and is mediated by the PI3K-Akt pathway. The protein mTORc2

Table 2: Summary of changes in lymphocyte populations and cytokine secretion observed after bariatric surgery-induced weight loss.

Population	Intervention	Main findings	Ref
13 subjects BMI ≥ 35 T2D or IGT Age: 35-65	12 weeks of DCR (860-1434 kcal/day) followed by LGB	↓ Th1/Th2 after DCR; changes maintained 12 weeks after LGB	[77]
15 subjects BMI ≥ 35 T2D or IGT Age: 35-65	LGB	↓ T cells and Th1/Th2, associated with lower FG, glucose AUC, and improved insulin secretion	[78]
9 subjects BMI: 35-38 Age: 45-61	RYGB	3 months after: B cells lose the capacity to support production of IL-17 and IFN-γ by T cells	[79]
20 subjects BMI: 37-45 Age: 25-50	Laparoscopic greater curvature plication	4 months after: ↓ CD4+ and CD8+ T cells and leptin	[80]
8 subjects BMI ≥ 40 With T2DM Mean age: 41.3	RYGB	3 months after: Tfh cells secreted: ↓ IFN-γ, IL-2, IL-4, and IL-17 and ↑ IL-10. Tfh IL-10+ promoted Breg cell differentiation and predicted better clinical response	[83]
69 subjects BMI ≥ 35 25% T2DM 30 subjects T2DM 67% obese	RYGB	↑ MAIT cells (potentially explained by ↓ peripheral infiltration); IL-17 remains	[41]
27 women 55.5% obese	RYGB	3 months after: ↓ IR, CRP, leptin, and T cells; ↑ TNF-α	[84]
58 subjects Nondiabetic BMI ≥ 40 63.8% IR Age: 18-60	6 weeks of VLCD followed by BPD	1 year after BPD: ↓ T cells and B cells in IR subjects Correlation: changes in CD8+ and HOMA index	[81]
20 women BMI: 36.4-68.2 Age: 25-90	LGB and RYGB	Correlation: changes from baseline in BMI and CD4+ cells only in the RYGB group at 3 months	[82]

DCR: dietary caloric restriction; BMI: body mass index; T2DM: type 2 diabetes mellitus; IR: insulin resistance; LGB: laparoscopic gastric banding; RYGB: Roux-en-Y gastric bypass; VLCD: very low-calorie diet; BDP: biliopancreatic diversion; ↓: decrease; ↑: increase; FG: fasting glucose; MAIT: mucosal-associated invariant T cells; IR: insulin resistance; CRP: C-reactive protein; Ref: reference.

is involved in the activation of this pathway in an obese animal model [97].

Short-chain fatty acids (SCFAs), by-products of microbial fermentation, can drive the differentiation of T cell subsets. The potential for butyrate and propionate produced by commensal bacteria in the gut to promote Treg cell differentiation has been previously demonstrated [98–100]. Fatty acid oxidation (FAO) has been described to enhance the functions of M2-polarized macrophages. Also, glucose metabolism has been implicated in granulocyte, dendritic cell, and M1-type macrophage activation. Th1 and Th2 immune responses have been suggested to be supported by the oxidation of fatty acids, inducing the development of the CD8+ memory phenotype, M2-like macrophages, and Tregs [101, 102]. Particularly, a rapid supply of ATP has been found to increase bioenergetics requirements in CD8+ T cells, improving their activity. Conversely, abundant lactic acid and glucose deficiency have been shown to impair the function of these cells. Treg cells have been described to depend on oxidative phosphorylation for energy source, as they rely on FAO and glutaminolysis for cell differentiation and proliferation, respectively [103–105] Figure 4. As a result of the weight loss induced by bariatric surgery, the nutrient supply deprivation mechanism is regulated through decreased activation of most immune cells, resulting in a decreased systemic inflammatory state.

Gut microbiota represents a potential link between bariatric surgery and SCFAs. Changes in gut microbiota have been observed in overweight and obese patients. Variations in the bacterial composition of the microbiota and their distribution along the bowel have been associated with metabolic alterations such as insulin resistance, low-grade

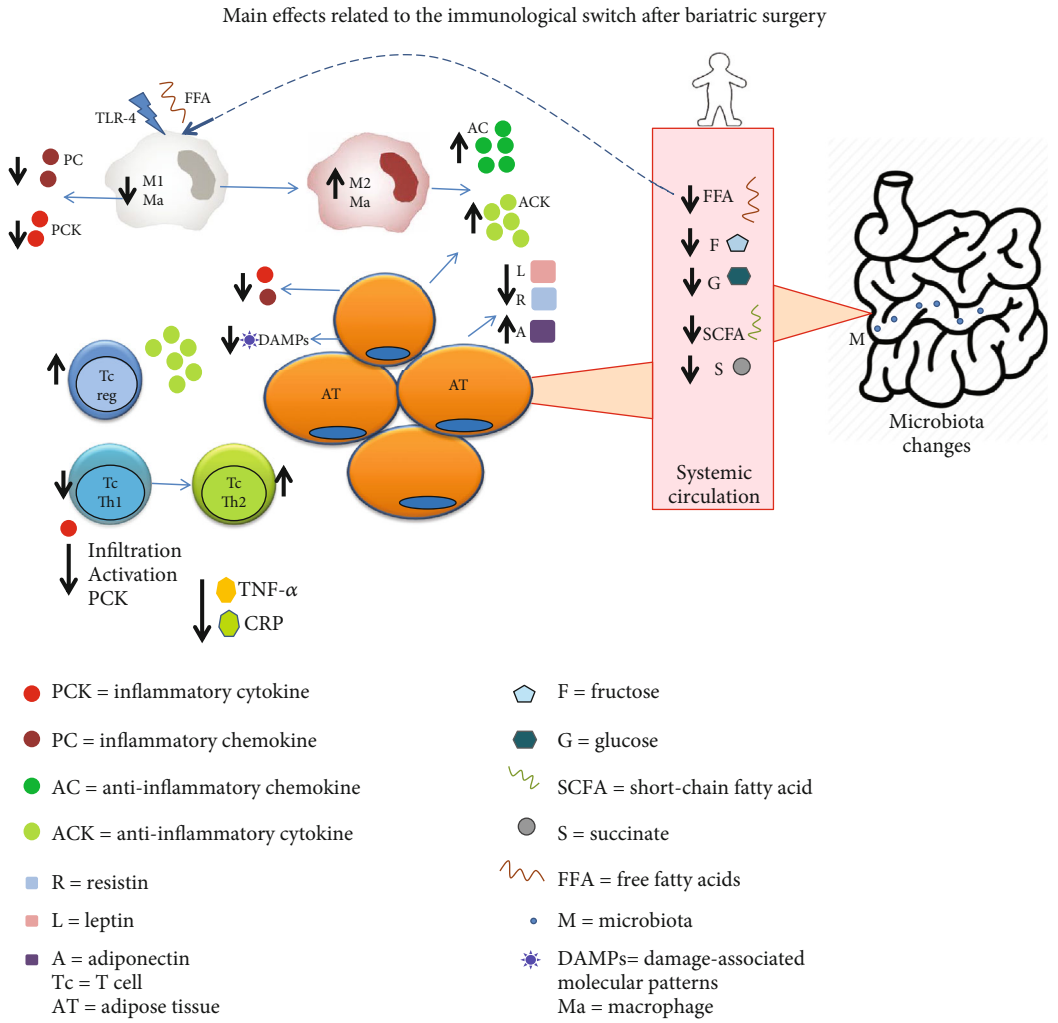

Figure 3: Main effects related to the immunological switch after bariatric surgery. Changes involved after weight loss include several molecular pathways: (i) a decrease in adipocyte proinflammatory mediators, (ii) changes in the activation and recruitment of immune cells in adipose tissue, (iii) modification in nutrient and metabolite absorption, and (iv) alterations in the intestinal microbiota.

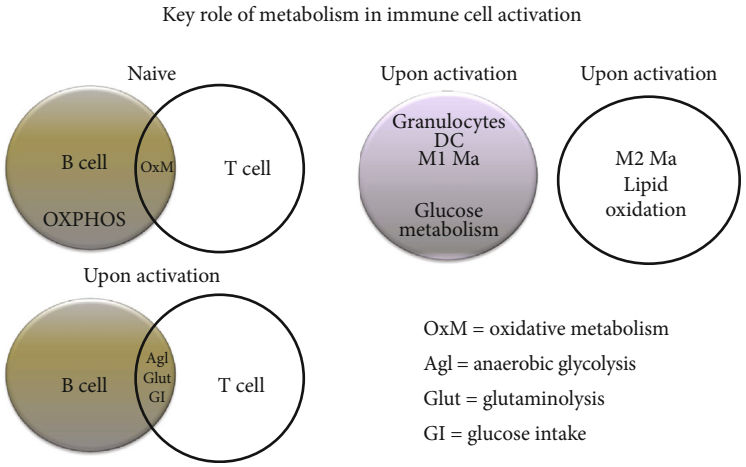

Figure 4: Metabolism modulates immune cell activation. Metabolic differences in *naïve* and activated T and B cells. Differences in energy supply between proinflammatory cells (M1 macrophages (M1 Ma)), dendritic cells (DC), granulocytes, and anti-inflammatory cells (M2 macrophages (M2 Ma)).

inflammation, and adipocyte hypertrophy [106]. Reduced body weight and lower fat deposition have been found in a RYGB rat model compared with a sham group, attributed to a decrease in the absorption of SCFAs. SCFAs have been shown to be stored in the adipose tissue and to function as substrates for lipid and glucose metabolism, as well as immune cell modulators. As a consequence of fewer fatty acids being absorbed and stored in fat tissue, reduction in the secretion adipokines, growth factors, TNF-α, IL-6, leptin, and resistin in fat tissue ensues [107], as well as improvement in the adaptive immune system cells with reduction of both local and systemic inflammations. Transfer of gut microbiota from RYGB-treated mice to nonoperated germ-free animals has been reported to lead to weight loss and changes in specific SCFAs compared with microbiota transfer from sham to surgery animals. Thus, changes in SCFA concentration caused by modification in gut microbiota after bariatric surgery may alter metabolic functions in the host [108]. Diet and obesity have been shown to induce dysbiosis in microbiota favoring immunogenic bacterial products [109, 110]. Thus, immune system modulation after bariatric surgery might respond to changes in microbiota metabolic by-products.

11. Conclusion

Obesity is associated with a systemic low-grade inflammatory state in which cells from the innate and adaptive immune system increase proinflammatory cytokine secretion. Among other potential mechanisms, the inflammatory milieu leads to insulin resistance and metabolic comorbidities. An improvement in this deleterious condition is achieved through weight loss. Currently, bariatric surgery represents the most effective treatment option to induce significant and persistent weight loss. Weight loss induced by bariatric surgery leads to significant changes in adaptive immune cells. Both CD4+ and CD8+ T cell counts are reduced. Also, Tfh increases anti-inflammatory cytokine secretion, which leads to an increase in Breg cells. Anti-inflammatory cytokines, such as IL-10 and TGF-β produced by Breg cells, inhibit the secretion of the proinflammatory cytokines IFN-γ and IL-17 by T cells. Furthermore, a decrease in the Th1/Th2 ratio is also induced after bariatric surgery-mediated weight loss, probably related to improved insulin sensitivity. After bariatric surgery, immune cells develop stronger antioxidant capacity and reduce the level of lipid and DNA oxidation products. Oxidative stress is a known modulator of lymphocyte differentiation, metabolism, and proliferation, which improves after bariatric surgery. Changes in metabolic substrate availability after bariatric surgery, including glucose, succinate, and fatty acids, influence the adaptive immune response after bariatric surgery and still constitute a field that deserves further study. Palmitate, specifically, has been shown to promote T CD4+ effector memory cell differentiation in obesity. Mechanisms involved in bariatric surgery-induced changes in adaptive immunity include weight loss per se, caloric deprivation, substrate availability, insulin sensitivity, fatty acid, and metabolite concentration changes. A better underpinning of the specific influence of each of these mechanisms on immune cell modulation in the context of bariatric surgery-mediated weight loss in the patient with severe obesity represents an open area of study. Further research focused on the interplay between the immune system pathways and the metabolic processes after bariatric surgery may lead to developments for the treatment of obesity and its associated comorbidities.

Conflicts of Interest

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

References

[1] World Health Organization, "Obesity: preventing and managing the global epidemic: report of a WHO consultation," World Health Organization, Geneva, 2000.

[2] NCD Risk Factor Collaboration (NCD-RisC),, "Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants," *The Lancet*, vol. 387, no. 10026, pp. 1377–1396, 2016.

[3] S. R. Preis, J. M. Massaro, S. J. Robins et al., "Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham Heart Study," *Obesity*, vol. 18, no. 11, pp. 2191–2198, 2010.

[4] N. T. Nguyen, C. P. Magno, K. T. Lane, M. W. Hinojosa, and J. S. Lane, "Association of hypertension, diabetes, dyslipidemia, and metabolic syndrome with obesity: findings from the National Health and Nutrition Examination Survey, 1999 to 2004," *Journal of the American College of Surgeons*, vol. 207, no. 6, pp. 928–934, 2008.

[5] E. Fabbrini, S. Sullivan, and S. Klein, "Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications," *Hepatology*, vol. 51, no. 2, pp. 679–689, 2010.

[6] M. L. Ganz, N. Wintfeld, Q. Li, V. Alas, J. Langer, and M. Hammer, "The association of body mass index with the risk of type 2 diabetes: a case–control study nested in an electronic health records system in the United States," *Diabetology and Metabolic Syndrome*, vol. 6, no. 1, p. 50, 2014.

[7] L. Elizondo-Montemayor, C. Hernández-Escobar, E. Lara-Torre, B. Nieblas, and M. Gómez-Carmona, "Gynecologic and obstetric consequences of obesity in adolescent girls," *Journal of Pediatric and Adolescent Gynecology*, vol. 30, no. 2, pp. 156–168, 2017.

[8] L. Elizondo-Montemayor, E. C. Castillo, C. Rodríguez-López et al., "Seasonal variation in vitamin D in association with age, inflammatory cytokines, anthropometric parameters, and lifestyle factors in older adults," *Mediators of Inflammation*, vol. 2017, 14 pages, 2017.

[9] R. P. Wildman, P. Muntner, K. Reynolds et al., "The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999-2004)," *Archives of Internal Medicine*, vol. 168, no. 15, pp. 1617–1624, 2008.

[10] M. D. George and J. F. Baker, "The obesity epidemic and consequences for rheumatoid arthritis care," *Current Rheumatology Reports*, vol. 18, no. 1, p. 6, 2016.

[11] A. R. Setty, G. Curhan, and H. K. Choi, "Obesity, waist circumference, weight change, and the risk of psoriasis in women: Nurses' Health Study II," *Archives of Internal Medicine*, vol. 167, no. 15, pp. 1670–1675, 2007.

[12] S. K. Tedeschi, M. Barbhaiya, S. Malspeis et al., "Obesity and the risk of systemic lupus erythematosus among women in the Nurses' Health Studies," *Seminars in Arthritis and Rheumatism*, vol. 47, no. 3, pp. 376–383, 2017.

[13] K. M. Flegal, B. K. Kit, H. Orpana, and B. I. Graubard, "Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis," *Journal of the American Medical Association*, vol. 309, no. 1, pp. 71–82, 2013.

[14] R. Busutil, O. Espallardo, A. Torres, L. Martínez-Galdeano, N. Zozaya, and Á. Hidalgo-Vega, "The impact of obesity on health-related quality of life in Spain," *Health and Quality of Life Outcomes*, vol. 15, no. 1, 2017.

[15] T. A. Wadden, V. L. Webb, C. H. Moran, and B. A. Bailer, "Lifestyle modification for obesity: new developments in diet, physical activity, and behavior therapy," *Circulation*, vol. 125, no. 9, pp. 1157–1170, 2012.

[16] R. Khera, M. H. Murad, A. K. Chandar et al., "Association of pharmacological treatments for obesity with weight loss and adverse events: a systematic review and meta-analysis," *Journal of the American Medical Association*, vol. 315, no. 22, pp. 2424–2434, 2016.

[17] C. M. Apovian, L. J. Aronne, D. H. Bessesen et al., "Pharmacological management of obesity: an Endocrine Society clinical practice guideline," *The Journal of Clinical Endocrinology and Metabolism*, vol. 100, no. 2, pp. 342–362, 2015.

[18] The Look AHEAD Research Group, "Eight-year weight losses with an intensive lifestyle intervention: the look AHEAD study," *Obesity*, vol. 22, no. 1, pp. 5–13, 2014.

[19] J. Cheng, J. Gao, X. Shuai, G. Wang, and K. Tao, "The comprehensive summary of surgical versus non-surgical treatment for obesity: a systematic review and meta-analysis of randomized controlled trials," *Oncotarget*, vol. 7, no. 26, pp. 39216–39230, 2016.

[20] J. I. Mechanick, A. Youdim, D. B. Jones et al., "Clinical practice guidelines for the perioperative nutritional, metabolic, and nonsurgical support of the bariatric surgery patient–2013 update: cosponsored by American Association of Clinical Endocrinologists, the Obesity Society, and American Society for Metabolic & Bariatric Surgery," *Surgery for Obesity and Related Diseases*, vol. 9, no. 2, pp. 159–191, 2013.

[21] T. Zhou, Z. Hu, S. Yang, L. Sun, Z. Yu, and G. Wang, "Role of adaptive and innate immunity in type 2 diabetes mellitus," *Journal Diabetes Research*, vol. 2018, pp. 1–9, 2018.

[22] D. A. Cronkite and T. M. Strutt, "The regulation of inflammation by innate and adaptive lymphocytes," *Journal of Immunology Research*, vol. 2018, 14 pages, 2018.

[23] T. McLaughlin, S. E. Ackerman, L. Shen, and E. Engleman, "Role of innate and adaptive immunity in obesity-associated metabolic disease," *The Journal of Clinical Investigation*, vol. 127, no. 1, pp. 5–13, 2017.

[24] S. Winer and D. A. Winer, "The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance," *Immunology and Cell Biology*, vol. 90, no. 8, pp. 755–762, 2012.

[25] D. Aronson, P. Bartha, O. Zinder et al., "Obesity is the major determinant of elevated C-reactive protein in subjects with the metabolic syndrome," *International Journal of Obesity*, vol. 28, no. 5, pp. 674–679, 2004.

[26] A. L. Carey, C. R. Bruce, M. Sacchetti et al., "Interleukin-6 and tumor necrosis factor-? are not increased in patients with type 2 diabetes: evidence that plasma interleukin-6 is related to fat mass and not insulin responsiveness," *Diabetologia*, vol. 47, no. 6, 2004.

[27] M. Nikseresht, "Comparison of serum cytokine levels in men who are obese or men who are lean: effects of nonlinear periodized resistance training and obesity," *Journal of Strength and Conditioning Research*, vol. 32, no. 6, pp. 1787–1795, 2018.

[28] K. Eder, N. Baffy, A. Falus, and A. K. Fulop, "The major inflammatory mediator interleukin-6 and obesity," *Inflammation Research*, vol. 58, no. 11, pp. 727–736, 2009.

[29] G. S. Hotamisligil, N. S. Shargill, and B. M. Spiegelman, "Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance," *Science*, vol. 259, no. 5091, pp. 87–91, 1993.

[30] G. S. Hotamisligil, P. Arner, J. F. Caro, R. L. Atkinson, and B. M. Spiegelman, "Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance," *The Journal of Clinical Investigation*, vol. 95, no. 5, pp. 2409–2415, 1995.

[31] F. M. Schmidt, J. Weschenfelder, C. Sander et al., "Inflammatory cytokines in general and central obesity and modulating effects of physical activity," *PLOS ONE*, J. Eckel, Ed., vol. 10, no. 3, article e0121971, 2015.

[32] S. P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante, "Obesity is associated with macrophage accumulation in adipose tissue," *The Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1796–1808, 2003.

[33] S. Cinti, G. Mitchell, G. Barbatelli et al., "Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans," *Journal of Lipid Research*, vol. 46, no. 11, pp. 2347–2355, 2005.

[34] M. E. McDonnell, L. M. Ganley-Leal, A. Mehta et al., "B lymphocytes in human subcutaneous adipose crown-like structures," *Obesity*, vol. 20, no. 7, pp. 1372–1378, 2012.

[35] C. Duffaut, A. Zakaroff-Girard, V. Bourlier et al., "Interplay between human adipocytes and T lymphocytes in Obesity," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 10, pp. 1608–1614, 2009.

[36] R. L. Travers, A. C. Motta, J. A. Betts, A. Bouloumié, and D. Thompson, "The impact of adiposity on adipose tissue-resident lymphocyte activation in humans," *International Journal of Obesity*, vol. 39, no. 5, pp. 762–769, 2015.

[37] V. Catalán, J. Gómez-Ambrosi, A. Rodríguez et al., "Peripheral mononuclear blood cells contribute to the obesity-associated inflammatory state independently of glycemic status: involvement of the novel proinflammatory adipokines chemerin, chitinase-3-like protein 1, lipocalin-2 and osteopontin," *Genes & Nutrition*, vol. 10, no. 3, p. 460, 2015.

[38] D. Dicker, M. A. Salook, D. Marcoviciu, M. Djaldetti, and H. Bessler, "Role of peripheral blood mononuclear cells in the predisposition of obese individuals to inflammation and infection," *Obesity Facts*, vol. 6, no. 2, pp. 146–151, 2013.

[39] A. Viardot, L. K. Heilbronn, D. Samocha-Bonet, F. Mackay, L. V. Campbell, and K. Samaras, "Obesity is associated with

activated and insulin resistant immune cells," *Diabetes/Metabolism Research and Reviews*, vol. 28, no. 5, pp. 447–454, 2012.

[40] C. Agabiti-Rosei, V. Trapletti, S. Piantoni et al., "Decreased circulating T regulatory lymphocytes in obese patients undergoing bariatric surgery," *PLOS ONE*, X. Cheng, Ed., vol. 13, no. 5, article e0197178, 2018.

[41] I. Magalhaes, K. Pingris, C. Poitou et al., "Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients," *The Journal of Clinical Investigation*, vol. 125, no. 4, pp. 1752–1762, 2015.

[42] Y. S. Lee, J. Wollam, and J. M. Olefsky, "An integrated view of immunometabolism," *Cell*, vol. 172, no. 1–2, pp. 22–40, 2018.

[43] M. Blüher, "Adipose tissue inflammation: a cause or consequence of obesity-related insulin resistance?," *Clinical Science*, vol. 130, no. 18, pp. 1603–1614, 2016.

[44] J. Hirosumi, G. Tuncman, L. Chang et al., "A central role for JNK in obesity and insulin resistance," *Nature*, vol. 420, no. 6913, pp. 333–336, 2002.

[45] J. Spranger, A. Kroke, M. Möhlig et al., "Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study," *Diabetes*, vol. 52, no. 3, pp. 812–817, 2003.

[46] A. N. Burska, R. Sakthiswary, and N. Sattar, "Effects of tumour necrosis factor antagonists on insulin sensitivity/-resistance in rheumatoid arthritis: a systematic review and meta-analysis," *PLOS ONE*, M. Nurmohamed, Ed., vol. 10, no. 6, article e0128889, 2015.

[47] T. Pina, S. Armesto, R. Lopez-Mejias et al., "Anti-TNF-α therapy improves insulin sensitivity in non-diabetic patients with psoriasis: a 6-month prospective study," *Journal of the European Academy of Dermatology and Venereology*, vol. 29, no. 7, pp. 1325–1330, 2015.

[48] S.-H. Chiang, M. Bazuine, C. N. Lumeng et al., "The protein kinase IKKepsilon regulates energy balance in obese mice," *Cell*, vol. 138, no. 5, pp. 961–975, 2009.

[49] S. M. Reilly, S.-H. Chiang, S. J. Decker et al., "An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice," *Nature Medicine*, vol. 19, no. 3, pp. 313–321, 2013.

[50] E. A. Oral, S. M. Reilly, A. V. Gomez et al., "Inhibition of IKKε and TBK1 improves glucose control in a subset of patients with type 2 diabetes," *Cell Metabolism*, vol. 26, no. 1, pp. 157–170.e7, 2017.

[51] L. Chen, R. Chen, H. Wang, and F. Liang, "Mechanisms linking inflammation to insulin resistance," *International Journal of Endocrinology*, vol. 2015, Article ID 508409, 9 pages, 2015.

[52] J. Ye, "Mechanisms of insulin resistance in obesity," *Frontiers in Medicine*, vol. 7, no. 1, pp. 14–24, 2013.

[53] P. Piątkiewicz, A. Czech, and J. Tatoń, "Glucose transport in human peripheral blood lymphocytes influenced by type 2 diabetes mellitus," *Archivum Immunologiae et Therapiae Experimentalis (Warsz)*, vol. 55, no. 2, pp. 119–126, 2007.

[54] P. Piatkiewicz, A. Czech, J. Tatoń, and A. Górski, "Investigations of cellular glucose transport and its regulation under the influence of insulin in human peripheral blood lymphocytes," *Endokrynologia Polska*, vol. 61, no. 2, pp. 182–187, 2010.

[55] A. Viardot, S. T. Grey, F. Mackay, and D. Chisholm, "Potential antiinflammatory role of insulin via the preferential polarization of effector T cells toward a T helper 2 phenotype," *Endocrinology*, vol. 148, no. 1, pp. 346–353, 2007.

[56] F. Magkos, G. Fraterrigo, J. Yoshino et al., "Effects of moderate and subsequent progressive weight loss on metabolic function and adipose tissue biology in humans with obesity," *Cell Metabolism*, vol. 23, no. 4, pp. 591–601, 2016.

[57] R. Welbourn, D. J. Pournaras, J. Dixon et al., "Bariatric surgery worldwide: baseline demographic description and one-year outcomes from the second IFSO Global Registry Report 2013–2015," *Obesity Surgery*, vol. 28, no. 2, pp. 313–322, 2018.

[58] J. L. Colquitt, K. Pickett, E. Loveman, and G. K. FramptonEndocrine Disorders Group, "Surgery for weight loss in adults," *Cochrane Database of Systematic Reviews*, C. Metabolic, Ed., no. 8, 2014.

[59] N. T. Nguyen and J. E. Varela, "Bariatric surgery for obesity and metabolic disorders: state of the art," *Nature Reviews. Gastroenterology & Hepatology*, vol. 14, no. 3, pp. 160–169, 2017.

[60] M. Elrefai, T. Hasenberg, S. Diouf, C. Weiß, P. Kienle, and M. Otto, "Quality of life after bariatric surgery: comparison of four different surgical procedures," *Bariatric Surgical Practice and Patient Care*, vol. 12, no. 2, pp. 61–66, 2017.

[61] S. Sharma, M. Narwaria, D. R. Cottam, and S. Cottam, "Randomized double-blinded trial of laparoscopic gastric imbrication v laparoscopic sleeve gastrectomy at a single Indian institution," *Obesity Surgery*, vol. 25, no. 5, pp. 800–804, 2015.

[62] H. Buchwald, R. Estok, K. Fahrbach et al., "Weight and type 2 diabetes after bariatric surgery: systematic review and meta-analysis," *The American Journal of Medicine*, vol. 122, no. 3, pp. 248–256.e5, 2009.

[63] M. E. Rojano-Rodríguez, C. Valenzuela-Salazar, L. E. Cárdenas-Lailson, L. S. Romero Loera, M. Torres-Olalde, and M. Moreno-Portillo, "C-Reactive protein level in morbidly obese patients before and after bariatric surgery," *Revista de Gastroenterología de México*, vol. 79, no. 2, pp. 90–95, 2014.

[64] J. Santos, P. Salgado, C. Santos et al., "Effect of bariatric surgery on weight loss, inflammation, iron metabolism, and lipid profile," *Scandinavian Journal of Surgery*, vol. 103, no. 1, pp. 21–25, 2014.

[65] L. Iaffaldano, C. Nardelli, V. Pilone et al., "Laparoscopic adjustable gastric banding reduces subcutaneous adipose tissue and blood inflammation in nondiabetic morbidly obese individuals," *Obesity Surgery*, vol. 24, no. 12, pp. 2161–2168, 2014.

[66] G. Morshed and S. M. Fathy, "Impact of post-laparoscopic sleeve gastrectomy weight loss on C-reactive protein, lipid profile and CA-125 in morbidly obese women," *Videosurgery and Other Miniinvasive Techniques*, vol. 4, pp. 521–526, 2015.

[67] S. M. Zagorski, N. N. Papa, and M. H. Chung, "The effect of weight loss after gastric bypass on C-reactive protein levels," *Surgery for Obesity and Related Diseases*, vol. 1, no. 2, pp. 81–85, 2005.

[68] S.-M. Caron-Cantin, J. Martin, M. Bastien et al., "Acute and chronic effects of biliopancreatic diversion with duodenal switch surgery on plasma visfatin and apelin levels in patients with severe obesity," *Obesity Surgery*, vol. 23, no. 11, pp. 1806–1814, 2013.

[69] R. C. Horn, G. T. Gelatti, N. C. Mori et al., "Obesity, bariatric surgery and oxidative stress," *Revista da Associação Médica Brasileira*, vol. 63, no. 3, pp. 229–235, 2017.

[70] M. Murri, E. García-Fuentes, J. M. García-Almeida et al., "Changes in oxidative stress and insulin resistance in morbidly obese patients after bariatric surgery," *Obesity Surgery*, vol. 20, no. 3, pp. 363–368, 2010.

[71] R. Schmatz, M. R. Bitencourt, L. D. Patias et al., "Evaluation of the biochemical, inflammatory and oxidative profile of obese patients given clinical treatment and bariatric surgery," *Clinica Chimica Acta*, vol. 465, pp. 72–79, 2017.

[72] L. Monzo-Beltran, A. Vazquez-Tarragón, C. Cerdà et al., "One-year follow-up of clinical, metabolic and oxidative stress profile of morbid obese patients after laparoscopic sleeve gastrectomy. 8-oxo-dG as a clinical marker," *Redox Biology*, vol. 12, pp. 389–402, 2017.

[73] E. E. Bankoglu, F. Seyfried, C. Arnold et al., "Reduction of DNA damage in peripheral lymphocytes of obese patients after bariatric surgery-mediated weight loss," *Mutagenesis*, vol. 33, no. 1, pp. 61–67, 2018.

[74] L. A. Sena, S. Li, A. Jairaman et al., "Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling," *Immunity*, vol. 38, no. 2, pp. 225–236, 2013.

[75] E. Naik and V. M. Dixit, "Mitochondrial reactive oxygen species drive proinflammatory cytokine production," *The Journal of Experimental Medicine*, vol. 208, no. 3, pp. 417–420, 2011.

[76] M. Foster and S. Samman, "Zinc and regulation of inflammatory cytokines: implications for cardiometabolic disease," *Nutrients*, vol. 4, no. 7, pp. 676–694, 2012.

[77] A. Viardot, R. V. Lord, and K. Samaras, "The effects of weight loss and gastric banding on the innate and adaptive immune system in type 2 diabetes and prediabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 6, pp. 2845–2850, 2010.

[78] K. Samaras, A. Viardot, N. K. Botelho, A. Jenkins, and R. V. Lord, "Immune cell-mediated inflammation and the early improvements in glucose metabolism after gastric banding surgery," *Diabetologia*, vol. 56, no. 12, pp. 2564–2572, 2013.

[79] X. Dai, W. Zhao, J. Zhan et al., "B cells present skewed profile and lose the function of supporting T cell inflammation after Roux-en-Y gastric bypass," *International Immunopharmacology*, vol. 43, pp. 16–22, 2017.

[80] S. M. Fathy and G. Morshed, "Peripheral blood lymphocyte subsets (CD4+, CD8+ T cells), leptin level and weight loss after laparoscopic greater curvature plication in morbidly obese patients," *Archives of Medical Science*, vol. 5, no. 5, pp. 886–890, 2014.

[81] M. Ballesteros-Pomar, S. Calleja, R. Díez-Rodríguez et al., "Inflammatory status is different in relationship to insulin resistance in severely obese people and changes after bariatric surgery or diet-induced weight loss," *Experimental and Clinical Endocrinology & Diabetes*, vol. 122, no. 10, pp. 592–596, 2014.

[82] Z. O. Merhi, H. G. Durkin, J. Feldman, J. Macura, C. Rodriguez, and H. Minkoff, "Effect of bariatric surgery on peripheral blood lymphocyte subsets in women," *Surgery for Obesity and Related Diseases*, vol. 5, no. 2, pp. 165–171, 2009.

[83] J. Zhan, L. Huang, H. Ma et al., "Reduced inflammatory responses of follicular helper T cell promote the development of regulatory B cells after Roux-en-Y gastric bypass," *Clinical and Experimental Pharmacology & Physiology*, vol. 44, no. 5, pp. 556–565, 2017.

[84] M. A. Lips, J. B. van Klinken, H. Pijl et al., "Weight loss induced by very low calorie diet is associated with a more beneficial systemic inflammatory profile than by Roux-en-Y gastric bypass," *Metabolism*, vol. 65, no. 11, pp. 1614–1620, 2016.

[85] D. K. Hagman, I. Larson, J. N. Kuzma et al., "The short-term and long-term effects of bariatric/metabolic surgery on subcutaneous adipose tissue inflammation in humans," *Metabolism*, vol. 70, pp. 12–22, 2017.

[86] M. Kuroda and H. Sakaue, "Adipocyte death and chronic inflammation in obesity," *The Journal of Medical Investigation*, vol. 64, no. 3.4, pp. 193–196, 2017.

[87] Q. Chen, X. Guan, X. Zuo, J. Wang, and W. Yin, "The role of high mobility group box 1 (HMGB1) in the pathogenesis of kidney diseases," *Acta Pharmaceutica Sinica B*, vol. 6, no. 3, pp. 183–188, 2016.

[88] A. A. Hill, W. Reid Bolus, and A. H. Hasty, "A decade of progress in adipose tissue macrophage biology," *Immunological Reviews*, vol. 262, no. 1, pp. 134–152, 2014.

[89] C. N. Lumeng, J. L. Bodzin, and A. R. Saltiel, "Obesity induces a phenotypic switch in adipose tissue macrophage polarization," *The Journal of Clinical Investigation*, vol. 117, no. 1, pp. 175–184, 2007.

[90] S. Winer, Y. Chan, G. Paltser et al., "Normalization of obesity-associated insulin resistance through immunotherapy," *Nature Medicine*, vol. 15, no. 8, pp. 921–929, 2009.

[91] D. Frasca, A. Diaz, M. Romero, T. Vazquez, and B. B. Blomberg, "Obesity induces pro-inflammatory B cells and impairs B cell function in old mice," *Mechanisms of Ageing and Development*, vol. 162, pp. 91–99, 2017.

[92] R. M. Loftus and D. K. Finlay, "Immunometabolism: cellular metabolism turns immune regulator," *The Journal of Biological Chemistry*, vol. 291, no. 1, pp. 1–10, 2016.

[93] A. Littlewood-Evans, S. Sarret, V. Apfel et al., "GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis," *The Journal of Experimental Medicine*, vol. 213, no. 9, pp. 1655–1662, 2016.

[94] Q. Zhang, Y. Lou, X.-L. Bai, and T.-B. Liang, "Immunometabolism: a novel perspective of liver cancer microenvironment and its influence on tumor progression," *World Journal of Gastroenterology*, vol. 24, no. 31, pp. 3500–3512, 2018.

[95] D.-M. Zhang, R.-Q. Jiao, and L.-D. Kong, "High dietary fructose: direct or indirect dangerous factors disturbing tissue and organ functions," *Nutrients*, vol. 9, no. 4, p. 335, 2017.

[96] F. Nicoli, S. Paul, and V. Appay, "Harnessing the induction of CD8+ T-cell responses through metabolic regulation by pathogen-recognition-receptor triggering in antigen presenting cells," *Frontiers in Immunology*, vol. 9, p. 9, 2018.

[97] C. Mauro, J. Smith, D. Cucchi et al., "Obesity-induced metabolic stress leads to biased effector memory CD4 + T cell differentiation via PI3K p110δ-Akt-mediated signals," *Cell Metabolism*, vol. 25, no. 3, pp. 593–609, 2017.

[98] N. Arpaia, C. Campbell, X. Fan et al., "Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation," *Nature*, vol. 504, no. 7480, pp. 451–455, 2013.

[99] Y. Furusawa, Y. Obata, S. Fukuda et al., "Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells," *Nature*, vol. 504, no. 7480, pp. 446–450, 2013.

[100] P. M. Smith, M. R. Howitt, N. Panikov et al., "The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis," *Regulate Colonic Treg Cell Homeostasis. Science*, vol. 341, no. 6145, pp. 569–573, 2013.

[101] L. A. J. O'Neill, R. J. Kishton, and J. Rathmell, "A guide to immunometabolism for immunologists," *Nature Reviews. Immunology*, vol. 16, no. 9, pp. 553–565, 2016.

[102] M. Agrawal, P. A. Kern, and B. S. Nikolajczyk, "The immune system in obesity: developing paradigms amidst inconvenient truths," *Current Diabetes Reports*, vol. 17, no. 10, p. 87, 2017.

[103] P. J. Siska and J. C. Rathmell, "T cell metabolic fitness in antitumor immunity," *Trends in Immunology*, vol. 36, no. 4, pp. 257–264, 2015.

[104] R. E. Wawman, H. Bartlett, and Y. H. Oo, "Regulatory T cell metabolism in the hepatic microenvironment," *Frontiers in Immunology*, vol. 8, p. 8, 2018.

[105] U. H. Beier, A. Angelin, T. Akimova et al., "Essential role of mitochondrial energy metabolism in Foxp 3^{+} T-regulatory cell function and allograft survival," *The FASEB Journal*, vol. 29, no. 6, pp. 2315–2326, 2015.

[106] J. Aron-Wisnewsky, E. Prifti, E. Belda et al., "Major microbiota dysbiosis in severe obesity: fate after bariatric surgery," *Gut*, vol. 68, no. 1, pp. 70–82, 2019.

[107] P. Sinclair, D. J. Brennan, and C. W. le Roux, "Gut adaptation after metabolic surgery and its influences on the brain, liver and cancer," *Nature Reviews. Gastroenterology & Hepatology*, vol. 15, no. 10, pp. 606–624, 2018.

[108] A. P. Liou, M. Paziuk, J.-M. Luevano, S. Machineni, P. J. Turnbaugh, and L. M. Kaplan, "Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity," *Science Translational Medicine*, vol. 5, no. 178, p. 178ra41, 2013.

[109] Y. Sanz and A. Moya-Pérez, "Microbiota, inflammation and obesity," in *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, M. Lyte and J. F. Cryan, Eds., pp. 291–317, Springer New York, New York, NY, 2014.

[110] A. Perez-Lopez, J. Behnsen, S.-P. Nuccio, and M. Raffatellu, "Mucosal immunity to pathogenic intestinal bacteria," *Nature Reviews. Immunology*, vol. 16, no. 3, pp. 135–148, 2016.

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Research Article

Pioglitazone Protects Compression-Mediated Apoptosis in Nucleus Pulposus Mesenchymal Stem Cells by Suppressing Oxidative Stress

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Excessive compression, the main cause of intervertebral disc (IVD) degeneration, affected endogenous repair of the intervertebral disc. Pioglitazone (PGZ) is the agonist of peroxisome proliferator-activated receptor γ, which has been widely used in the treatment of diabetes mellitus. The present study aim at investigating whether pioglitazone has protective effects on compression-mediated cell apoptosis in nucleus pulposus mesenchymal stem cells (NP-MSCs) and further exploring the possible underlying mechanism. Our results indicated that the isolated cells satisfied the criteria of MSC stated by the International Society for Cellular Therapy. Besides, our research revealed that pioglitazone could protect cell viability, cell proliferation of NP-MSCs and alleviated the toxic effects caused by compression. The actin stress fibers was suppressed obviously under compression, and pioglitazone alleviated the adverse outcomes. Pioglitazone exerted protective effects on compression-induced NP-MSCs apoptosis according to annexin V/PI double-staining and TUNEL assays. Pioglitazone suppressed compression-induced NP-MSCs oxidative stress, including decreasing compression-induced overproduction of reactive oxygen species (ROS) and malondialdehyde (MDA), and alleviated compression-induced mitochondrial membrane potential (MMP) decrease. Ultrastructure collapse of the mitochondria exhibited a notable improvement by pioglitazone in compression-induced NP-MSCs according to transmission electron microscopy (TEM). Furthermore, the molecular results showed that pioglitazone significantly decreased the expression of apoptosis-associated proteins, including cyto.cytochrome c, Bax, cleaved caspase-9, and cleaved caspase-3, and promoted Bcl-2 expression. These results indicated that pioglitazone alleviated compression-induced NP-MSCs apoptosis by suppressing oxidative stress and the mitochondrial apoptosis pathway, which may be a valuable candidate for the treatment of IVD degeneration.

1. Introduction

Intervertebral disc (IVD) degeneration is a major cause for low back pain (LBP), which has become a global social problem affecting human life and causes a great economic burden on governments [1–3]. At present, the main therapeutic regimens for IVD degeneration are conservative therapy and surgical decompression of the spine, aimed at

alleviating the pain and returning patients to work [4]. However, neither of the strategies is effective for treating IVD degeneration. At present, stem cell therapy has increasingly become a new therapy strategy for the repair of IVD degeneration. Mesenchymal stem cells (MSCs) can differentiate into NP-like cells and promote extracellular matrix (ECM) synthesis, which have exhibited great potential for treating IVD degeneration. Recently, MSCs were found existing in NP tissue, and these NP-MSCs efficiently repaired NP tissue [5, 6]. Many researches showed that the loss of IVD-MSCs number and function was closely related to IVD degeneration [7, 8].

Mechanical stress is an important inducement of IVD degeneration. Generally, the IVD experiences various mechanical loads in daily activities. Chou et al. demonstrated that fluid-induced shear stress changed the expressions of ECM and metalloproteinase genes in IVD cells [9]. Also, under this compression microenvironment, IVD stem cells also suffered from excessive cell death, which made it tough to maintain the number and viability of stem cells in IVDs [10]. Previously, we reported that compression decreased the number and viability of IVD cells, which contributed to IVD degeneration [11]. Recently, we found that compression induced substantial NP-MSCs death [12]. Considering all of the above, seeking an effective pharmaceutical to reduce the NP-MSCs death will be a promising therapy for IVD degeneration.

Pioglitazone, considered as a peroxisome proliferator-activated receptor γ activator, is used to treat diabetes mellitus [13]. It plays potential beneficial roles in inflammation, fat distribution, and lipid and protein metabolism [14–16]. Shen et al. reported that pioglitazone protected pancreatic cells against palmitic acid-induced cytotoxicity through anti-oxidative stress [17]. Recently, researchers reported that pioglitazone alleviated cell apoptosis induced by TNF-α or stress in endothelial cells by altering the Bcl-2 expression and caspase-3 activation [18, 19]. In addition, pioglitazone inhibited advanced glycation end product-induced chondrocyte apoptosis, having a potential therapeutic ability for AGE-induced mouse osteoarthritis with diabetes [20]. However, the influence of pioglitazone on compression-induced NP-MSCs death remains unknown.

Apoptosis as the programmed cell death plays a vital role in eliminating the injured or nonessential cells [21]. Apoptotic signals initiated the mitochondrial dysfunction pathway and caused an increase of intracellular ROS levels, which made oxidative stress occur [22, 23]. Excessive ROS production led to oxidative damage and cell death [24]. ROS could directly impair the mitochondrial function, which then decreased the MMP and released the cytochrome c into the cytosol [25]. The release of cytochrome c was inhibited by the antiapoptotic members of the Bcl-2 family and stimulated by the proapoptotic members, such as Bax. Cytochrome c promoted the formation of the apoptosome by recruiting caspase-9, which then induced caspase-3 activation and triggered the caspase cascade, eventually causing the cell apoptotic death [26–28].

In this research, we used different methods to evaluate the protective role of pioglitazone on compression-induced NP-MSCs apoptosis as well as oxidative stress levels. In addition, we evaluated the role of the mitochondrial pathway in pioglitazone protecting compression-induced NP-MSCs apoptosis by detecting apoptosis-related proteins and mitochondrial function.

2. Material and Methods

2.1. Isolation and Culture of NP-MSCs. All experiments in this study were approved by the Clinical Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. The nucleus pulposus samples were obtained from patients undergoing discectomy for degenerative disc disease. NP-MSCs were isolated and incubated as we reported in our previous studies [12]. In brief, after being washed with normal saline, NP tissues were cut into small pieces and digested with 0.25% type II collagenase for 8 h. And then, the digested tissues were cultured in MSC complete medium (Cyagen, CA, USA) at 37°C and 5% CO_2. The culture medium was changed every three days. The NP-MSCs were purified and cultured from the primary cells. They were seeded in the appropriate culture plates for subsequent experiments when second-passage NP-MSCs reached 80%-90% confluence.

2.2. Surface Marker Identification of NP-MSCs. NP-MSCs were collected and washed with phosphate-buffered saline (PBS). The cells were resuspended and incubated with a solution of antibodies of CD73, CD90, CD105, CD34, and HLA-DR at 37°C for 30 min. After being incubated with the antibodies, the cells were centrifuged at 1500 rpm for 5 min. The labeled cells were rinsed and then marked by secondary antibodies at 37°C for 30 min. Cells were washed twice and resuspended in 200 μl PBS. The labeled cells were examined by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

2.3. Multilineage Differentiation. To assess the multilineage differentiation potential of NP-MSCs, the osteogenic, adipogenic, and chondrogenic differentiations were induced. In brief, NP-MSCs were seeded in culture plates with normal medium. When NP-MSCs reached the appropriate confluence, the conditional medium was changed with human mesenchymal stem cell osteogenic, adipogenic, and chondrogenic differentiation media (Cyagen, CA, USA) respectively according to the manufacturer's protocol. The osteogenic, adipogenic, and chondrogenic differentiation potential of NP-MSCs was assessed by Alizarin Red, Oil Red O, and Alcian blue staining after 21 days, 30 days, and 28 days, respectively. The results were detected by a light microscope (Olympus, Japan).

2.4. Colony Forming Assay. The colony forming assay was detected with NP-MSCs seeded in 6-well plates at 1000 cells/well. After being cultured 10 days, the NP-MSCs were fixed with 4% paraformaldehyde for 20 min. The cells were washed two times with PBS and stained with 1% crystal violet stain (Solarbio, China) for 30 min, then washed two times with PBS. Colonies of NP-MSCs were observed.

2.5. Application of a Compression Apparatus on NP-MSCs. NP-MSCs were cultured in a pressure apparatus to simulate in vivo conditions as we previously described [1, 11, 12, 29–31]. To study the effects of pioglitazone on compression-exposed NP-MSCs, cells were treated with different concentrations of pioglitazone (0 μM, 10 μM, 20 μM, 50 μM, and 100 μM) and exposed to 1.0 MPa compression for 36 h. Then, they were used for subsequent experiments. Control group cells (con) served as a control group in this study.

2.6. CCK-8 Assay. NP-MSCs viability was assessed by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay as described previously [3]. In brief, the cell densities were 5×10^3 cells/well for CCK-8. After being treated, 100 millilitres (ml) of DMEM/F-12 containing 10 ml of CCK-8 solution was added to each well, and the 96-well plates were incubated for 4 h at 37°C. The absorbance was determined with a spectrophotometer at 450 nm (BioTek, Winooski, VT, USA).

2.7. EdU Incorporation Assay. The effect of pioglitazone on compression-mediated NP-MSCs proliferation was evaluated by 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay (Ribobio, China). After being treated with pioglitazone or compression, NP-MSCs were stained with the EdU incorporation assay according to the manufacturer's instructions. After being washed for three times with PBS, EdU staining of NP-MSCs was detected in the dark by a fluorescence microscope (Olympus, Japan).

2.8. Rhodamine-Phalloidin Staining. Rhodamine-labelled phalloidin (Cytoskeleton, Denver, CO) was used to stain the actin filaments in NP-MSCs. Briefly, after being treated, the cells were fixed in 4% formaldehyde for 10 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 15 min. The cells were incubated with rhodamine-phalloidin (100 nM) in the dark for 30 min. Then, the cells were washed two times and incubated with DAPI staining (Boster, China) for 5 min in the dark. After being washed two times with PBS, the cells were observed and photographed by using a confocal microscopy (Nikon A1, Japan).

2.9. Lactate Dehydrogenase Release Assay. Release of lactate dehydrogenase (LDH) was used to assess the effect of pioglitazone on compression-exposed NP-MSCs. Briefly, after being treated, NP-MSCs were incubated with the LDH assay according to the manufacturer's protocol (Beyotime, China). The absorbance at 490 nm was measured using a microplate reader (Biotek, Winooski, VT, USA).

2.10. Live/Dead Assay. We used calcein-AM/PI (Dojindo, Japan) to evaluate the effect of pioglitazone on compression-treated NP-MSCs. After being treated, NP-MSCs were washed two times and then incubated with calcein-AM and propidium iodide (PI) in the dark for 20 min at 37°C. Live cells displayed green fluorescence (calcein-AM-positive) while the dead cells displayed red fluorescence (PI-positive) under the fluorescence microscope. The live/dead cells were detected in the dark by a fluorescence microscope (Olympus, Japan).

2.11. Annexin V/PI Staining. Cell apoptosis was quantified by annexin V/PI staining according to the manufacturer's instructions (KeyGen Biotech, China). After being treated, the NP-MSCs were collected and washed, then a binding buffer was used to resuspend NP-MSCs. 5 μl annexin V and 5 μl PI were used to label NP-MSCs for 15 min, and the cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

2.12. TUNEL Staining. We used TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining to evaluate the cell apoptosis. After being fixed in 4% paraformaldehyde for 25 min at room temperature, NP-MSCs were permeabilized with 0.1% TritonX-100 for 10 min. According to the protocol of the manufacturer (Roche, Germany), the cells were washed with PBS, then incubated with staining at 37°C in the dark. Apoptotic changes were observed under the inverted fluorescence microscope (Olympus, Japan).

2.13. Measurement of ROS Production. Intracellular ROS levels were measured by DCFH-DA assay (Nanjing Jiancheng, China). After being treated, NP-MSCs were collected and washed in PBS, then incubated with DCFH-DA at 37°C for 20 min. The ROS levels were measured using by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

2.14. MDA Assay. The MDA content, which reflect oxidation levels, was quantified using a lipid peroxidation MDA assay kit (Beyotime, China) according to the manufacturer's instructions. The samples and standards were prepared and the OD value was measured at 532 nm. MDA concentrations (nmol/ml) were expressed as nmol/mg protein.

2.15. JC-1 Staining. MMP was measured by using a JC-1 ("5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide") staining kit (Beyotime, China). Briefly, NP-MSCs from every group were collected and washed with PBS, then resuspended in 1 ml culture medium which contained 0.5 ml JC-1 staining fluid. NP-MSCs were incubated with JC-1 staining fluid for 20 min at 37°C with 5% CO_2. The cells were washed twice and resuspended in 0.5 ml staining buffer. The MMP of each group was measured by flow cytometry and expressed as the ratio of red/green fluorescence intensity (Becton Dickinson, Franklin Lakes, NJ, USA). Furthermore, MMP was determined by a fluorescence microscope (Olympus, Japan).

2.16. Transmission Electron Microscopy. Transmission electron microscopy (TEM) was used to examine changes in the ultrastructure of NP-MSCs as described previously [31]. Briefly, NP-MSCs were harvested and washed with PBS. After being pelleted by centrifugation at 1500 rpm for 5 min, the cells were fixed with 2.5% glutaraldehyde for 12 h. Then, the cells were dehydrated in ethanol and infiltrated and embedded. Ultrathin sections were stained

with uranyl acetate and examined under transmission electron microscope (FEI Company, USA).

2.17. Western Blot Analysis. The cells were lysed by using a western and IP cell lysis kit. After NP-MSCs were treated with 100 μM pioglitazone or 1.0 MPa compression, apoptosis-related proteins were detected by western blotting. Also, to determine the cytochrome c of cytosol (cyto.cytochrome c), the cytosol protein of cells was isolated according to the manufacturer's instructions of the Cell Mitochondria Isolation Kit (Beyotime, China). The protein concentration was measured by using the BCA protein assay kit (Beyotime, China). Equal protein amounts (30 μg) were resolved on 10%-12% SDS-PAGE gel and then transferred onto PVDF membranes (Millipore, Burlington, MA, USA), then the membranes were blocked by 5% nonfat milk and then incubated overnight at 4°C with primary antibodies against cytochrome c (1 : 1000, Abcam, USA, ab90529), Bcl-2 (1 : 1000, Abcam, USA, ab59348), Bax (1 : 1000, Abcam, USA, ab32503), cleaved caspase-9 (1 : 1000, Abcam, USA, ab2324), and cleaved caspase-3 (1 : 1000, Abcam, USA, ab2302) overnight at 4°C. After being washed with TBST for three times, the membranes were incubated with secondary antibodies for 1 h at room temperature. Finally, the enhanced chemiluminescence method was used to visualize the proteins.

2.18. Statistical Analysis. GraphPad Prism (GraphPad Software Inc.; La Jolla, CA, USA) was used for statistical analysis. Values are presented as the mean ± standard deviation (SD) from at least three independent experiments. Student's *t*-test and one-way analysis of variance (ANOVA) were used to perform the statistical significance of the differences between two groups and multiple groups, respectively. Bonferroni's post hoc test was used to determine the source of the observed differences. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Identification of NP-MSCs. NP-MSCs were isolated and cultured from IVD. We observed that these cells displayed vortex-style adherent growth and showed a long spindle shape (Figure 1(a)). These cells formed homogeneous colonies (Figure 1(b)). The surface markers on the cells were detected by flow cytometry. NP-MSCs highly expressed surface markers, including CD73, CD90, and CD105. The positive rates of those were more than 95%. NP-MSCs expressed less CD34 and HLA-DR (Figure 1(c)). Furthermore, we assessed the multilineage differentiation potential of NP-MSCs. NP-MSCs formed many visible calcium deposits by Alizarin Red staining after osteogenic induction. After adipogenic differentiation, oil droplets were observed in NP-MSCs by Oil Red O staining. Also, these cells largely produced sulfated proteoglycans after chondrogenic induction by Alcian blue (Figure 1(d)). The results above proved that the isolated cells satisfied the criteria of MSC stated by ISCT. Thus, we successfully isolated and cultured NP-MSCs, and the second-generation cells were used in this study.

3.2. Pioglitazone Alleviated the Inhibitory Effect of Compression on Cell Viability and Cell Proliferation. CCK-8 assay was performed to determine NP-MSCs viability. The results of CCK-8 indicated that pioglitazone had no cytotoxicity on NP-MSCs (Figure 2(a)). We found that compression significantly decreased cell viability. To investigate the protective role of pioglitazone on NP-MSCs, we treated NP-MSCs with different concentrations of pioglitazone. We found that pioglitazone significantly alleviated the inhibitory effect of compression on cell viability. And the protective effect was obvious at the doses of 100 μM (Figure 2(b)). Thus, 100 μM pioglitazone was used in the subsequent experiments. In addition, we used the EdU incorporation assay to detect cell proliferation. The results indicated that the number of EdU-positive cells (red fluorescence) in pioglitazone groups was more than that in the compression groups (Figure 2(c)). These proved that pioglitazone remarkably alleviated the inhibitory effects of compression on cell proliferation.

3.3. Pioglitazone Protected against Compression-Induced Cytotoxicity in NP-MSCs. Rhodamine-phalloidin was used to detect the actin filaments of NP-MSCs. The results displayed that compression obviously broke the actin stress fibers and pioglitazone rescued the adverse outcomes caused by compression (Figure 3(a)). Subsequently, we detected the release of LDH. The results demonstrated that compression significantly enhanced the release of LDH compared with the control group. Pioglitazone significantly reversed the release of LDH induced by compression (Figure 3(b)). To further assess the protective effects of pioglitazone on compression-induced cytotoxicity in NP-MSCs, we used calcein-AM/PI assay to quantify the numbers of live/dead cells under a fluorescence microscope. We found that live cells (green fluorescence) were more in the pioglitazone groups while dead cells (red fluorescence) were more frequent in the compression groups (Figures 3(c)–3(e)). Those results demonstrated that pioglitazone protected against compression-induced cell cytotoxicity in NP-MSCs.

3.4. Protective Effects of Pioglitazone on Compression-Induced Apoptosis of NP-MSCs. To explore the protective effects of pioglitazone on compression-induced apoptosis, we used annexin V/PI double-staining to examine the apoptosis of NP-MSCs. The results of flow cytometry showed that compression induced NP-MSCs apoptosis. Pioglitazone can significantly protect compression-induced apoptosis of NP-MSCs (Figures 4(a) and 4(b)). To further examine the protective effects of pioglitazone, the TUNEL staining was used to investigate apoptotic changes under a fluorescence microscope. TUNEL staining revealed that the TUNEL-positive cells were obviously decreased in the pioglitazone group compared with the compression group (Figures 4(c) and 4(d)). These results showed that pioglitazone exerted protective effects on compression-induced NP-MSCs apoptosis.

3.5. Pioglitazone Reduced Compression-Induced Oxidative Stress in NP-MSCs. Increased levels of oxidative stress caused mitochondrial dysfunction and resulted in cell apoptosis. To

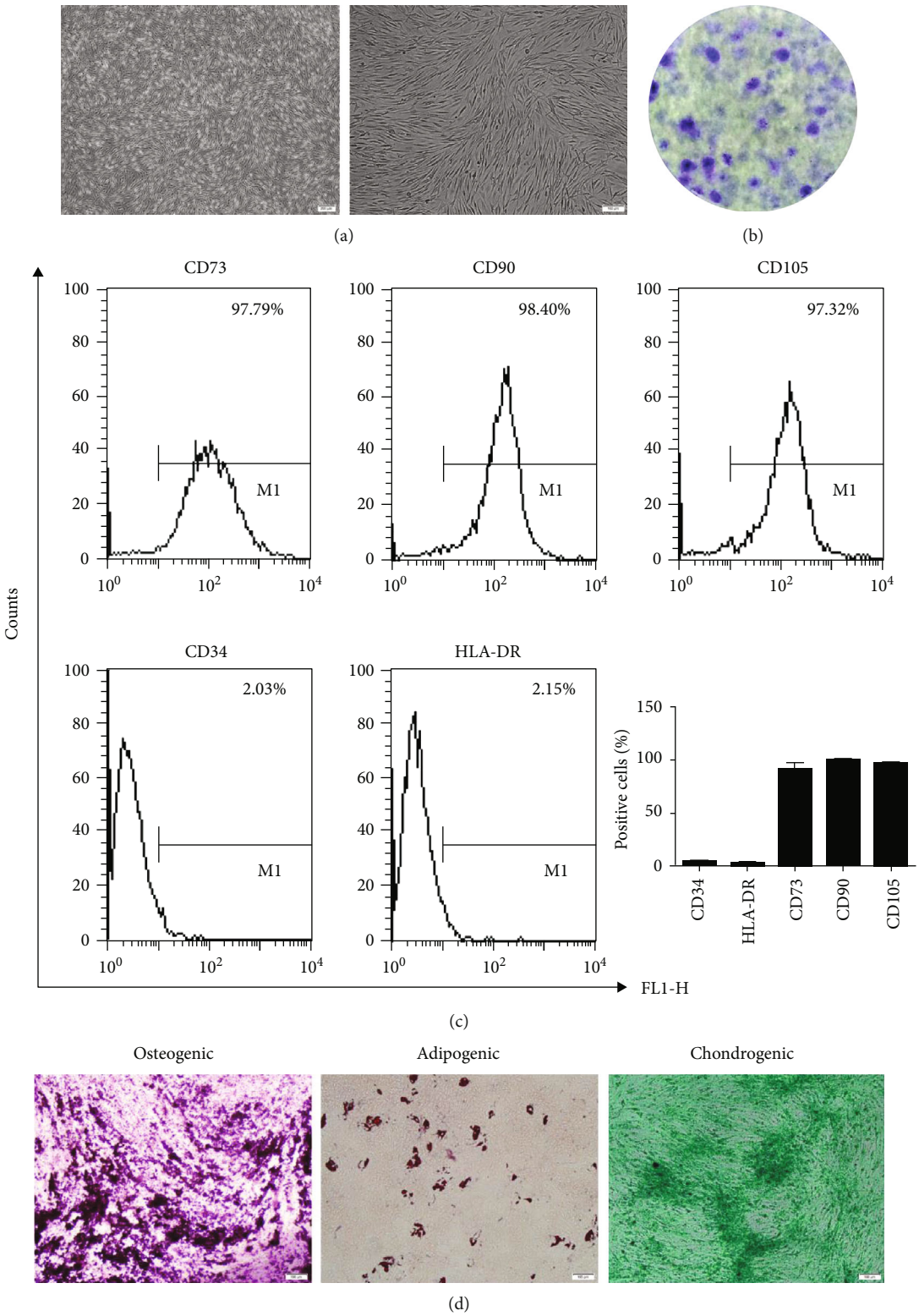


Figure 1: Identification of NP-MSCs. (a) The growth state and morphology of the cells were observed under an optical microscope (40x and 100x). (b) A colony-forming assay was used to observe NP-MSCs-formed homogeneous colonies (1x). (c) Flow cytometry was used to detect the surface markers on the cells, including CD34, CD73, CD90, CD105, and HLA-DR. Quantitative analysis of positive cells in NP-MSCs. (d) Alizarin Red staining, Oil Red O staining, and Alcian blue were used to evaluate osteogenic, adipogenic, and chondrogenic differentiation of NP-MSCs, respectively (100x).

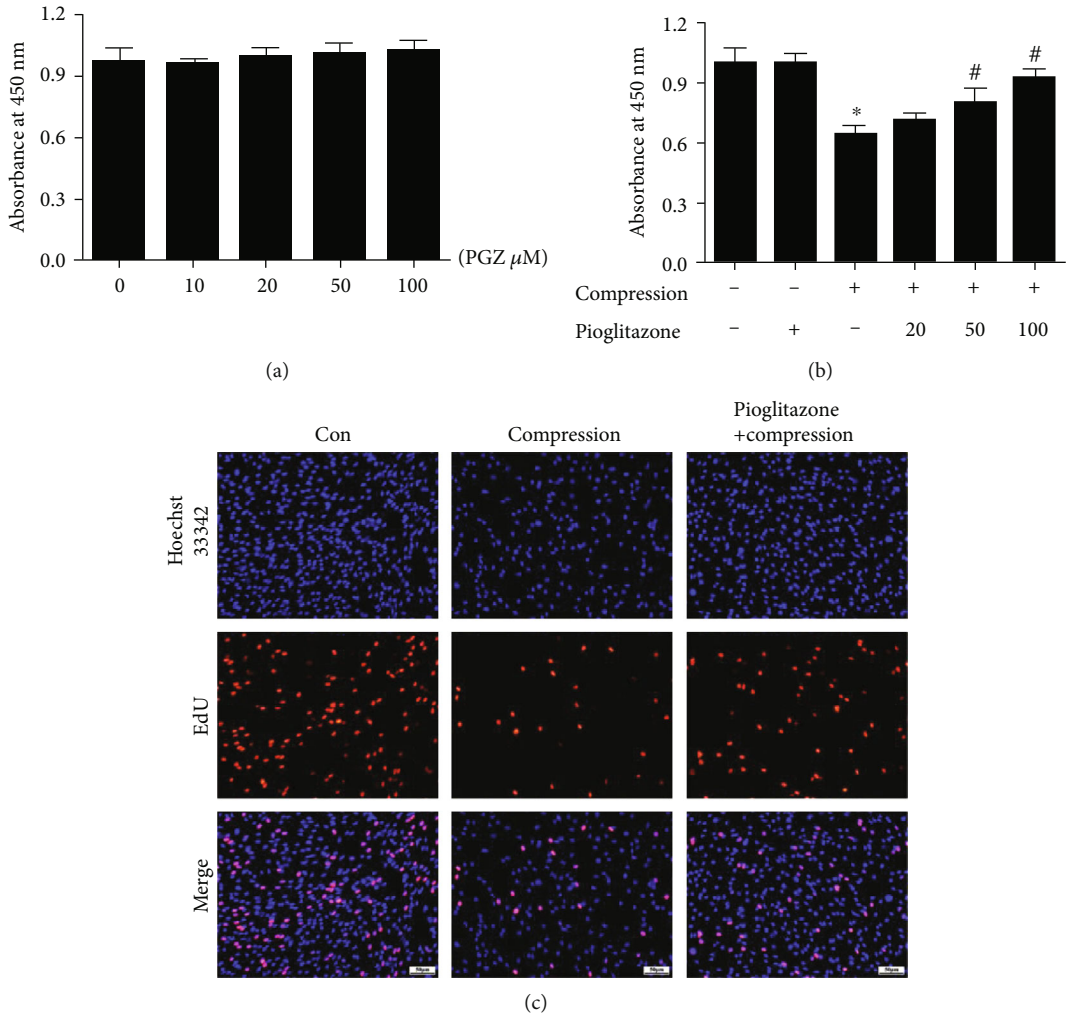


Figure 2: Effects of pioglitazone on NP-MSCs viability and proliferation. (a) The CCK-8 assay was used to evaluate the cell viability with various concentrations of pioglitazone (0, 10, 20, 50, and 100 μM) on NP-MSCs. (b) The CCK-8 assay was used to evaluate the effect of pioglitazone on compression-mediated NP-MSCs viability. Con served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group. (c) Representative micrographs of EdU staining by fluorescence microscopy. The red fluorescence indicates EdU-positive cells (200x).

investigate the effects of pioglitazone on oxidative stress of NP-MSCs, we used DCFH-DA fluorescent probes to detect ROS production to evaluate oxidative stress levels of NP-MSCs. We found that compression significantly increased intracellular ROS production as compared with the control group. Pioglitazone can significantly reduce compression-induced ROS production (Figures 5(a) and 5(b)). In addition, we also assessed the oxidative stress level by the lipid peroxidation MDA assay kit. The data showed that pioglitazone significantly reduced MDA which was high in the compression group (Figure 5(c)). These results showed that pioglitazone alleviated compression-induced oxidative stress levels in NP-MSCs.

3.6. Pioglitazone Alleviated Compression-Induced Mitochondrion Damage in NP-MSCs. Mitochondrion damage is an important factor in the mitochondrial apoptotic pathway. When MMP loss occurred in mitochondrion damage, JC-1 shifted from JC-1 aggregates (red fluorescence) to JC-1 monomers (green fluorescence). To evaluate the protective effects of pioglitazone on compression-induced mitochondrion damage of NP-MSCs, we operated JC-1 staining to detect MMP in NP-MSCs by flow cytometric analysis. We found that compression reduced the red/green ratio in NP-MSCs and pioglitazone significantly increased the ratio of red/green compared with the compression group (Figures 6(a) and 6(b)). Also, we used a fluorescence microscope to observe the protective effects of pioglitazone. In the compression group, NP-MSCs exhibited less red fluorescence but more green fluorescence, while pioglitazone could increase the red fluorescence and reduce the green fluorescence (Figure 6(c)). Meanwhile, we applied TEM to intuitively observe the ultrastructure of NP-MSCs. When NP-MSCs were exposed to compression, the nucleus shrunk and the apoptotic corpuscles appeared. Furthermore, the numbers of mitochondria were reduced

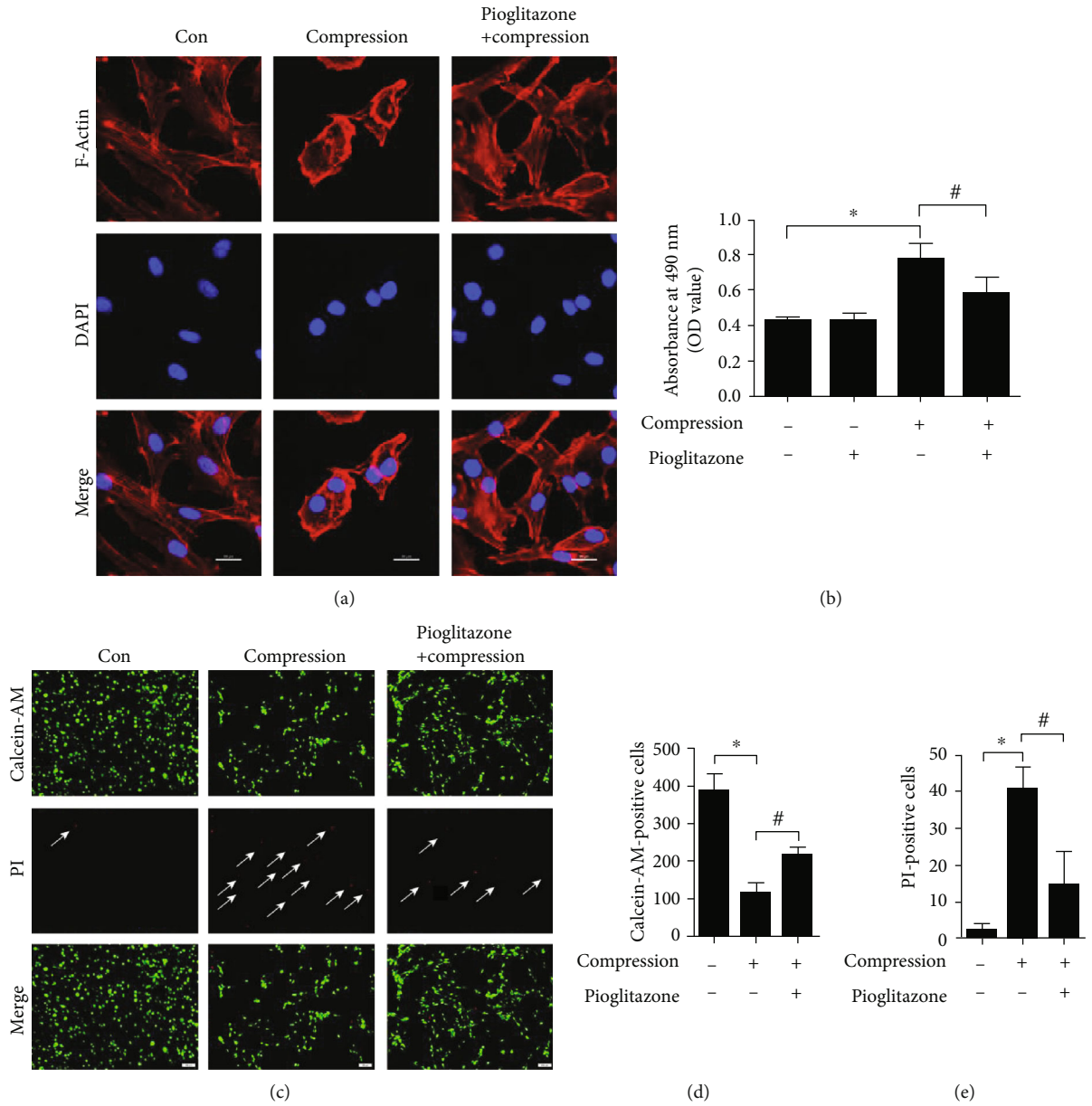


Figure 3: Pioglitazone protected against compression-induced cytotoxicity in NP-MSCs. (a) Rhodamine-phalloidin was used to detect the actin filaments of NP-MSCs. The red fluorescence showed the actin (1000x). (b) The histogram shows the release of LDH. (c) Calcein-AM/PI dye was used to evaluate NP-MSCs damage under a fluorescence microscope. The green fluorescence indicates live cells, and the red fluorescence indicates dead cells (100x). (d) Quantitative analysis of calcein-AM-positive cells. (e) Quantitative analysis of PI-positive cells. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

obviously, and a big lipid droplet appeared in the compression group. Fortunately, pioglitazone can significantly alleviate the damage in the ultrastructure of NP-MSCs. Specifically, the nucleus was relatively normal when compared with that of the compression group, and the numbers and the morphology of mitochondria were improved (Figure 6(d)).

3.7. Effects of Pioglitazone on Mitochondrial Pathway Apoptosis-Related Protein Expression under Compression in NP-MSCs. To investigate the effects of pioglitazone on compression-induced changes of the mitochondrial pathway apoptosis-related protein expression, we used western blotting to detect the expression of apoptosis-related proteins. The results revealed that the expression of the antiapoptotic protein Bcl-2 decreased, while that of the proapoptotic protein Bax increased in the compression group compared with the pioglitazone group. Also, we found that compression increased the release of cytochrome c into the cytosol. The expression levels of cleaved caspase-9 and cleaved caspase-3 of the compression group were higher than those of the control group. However, pioglitazone significantly reversed these effects (Figure 7(a)). Also, we have performed quantitative analysis of the protein levels (Figure 7(b)).

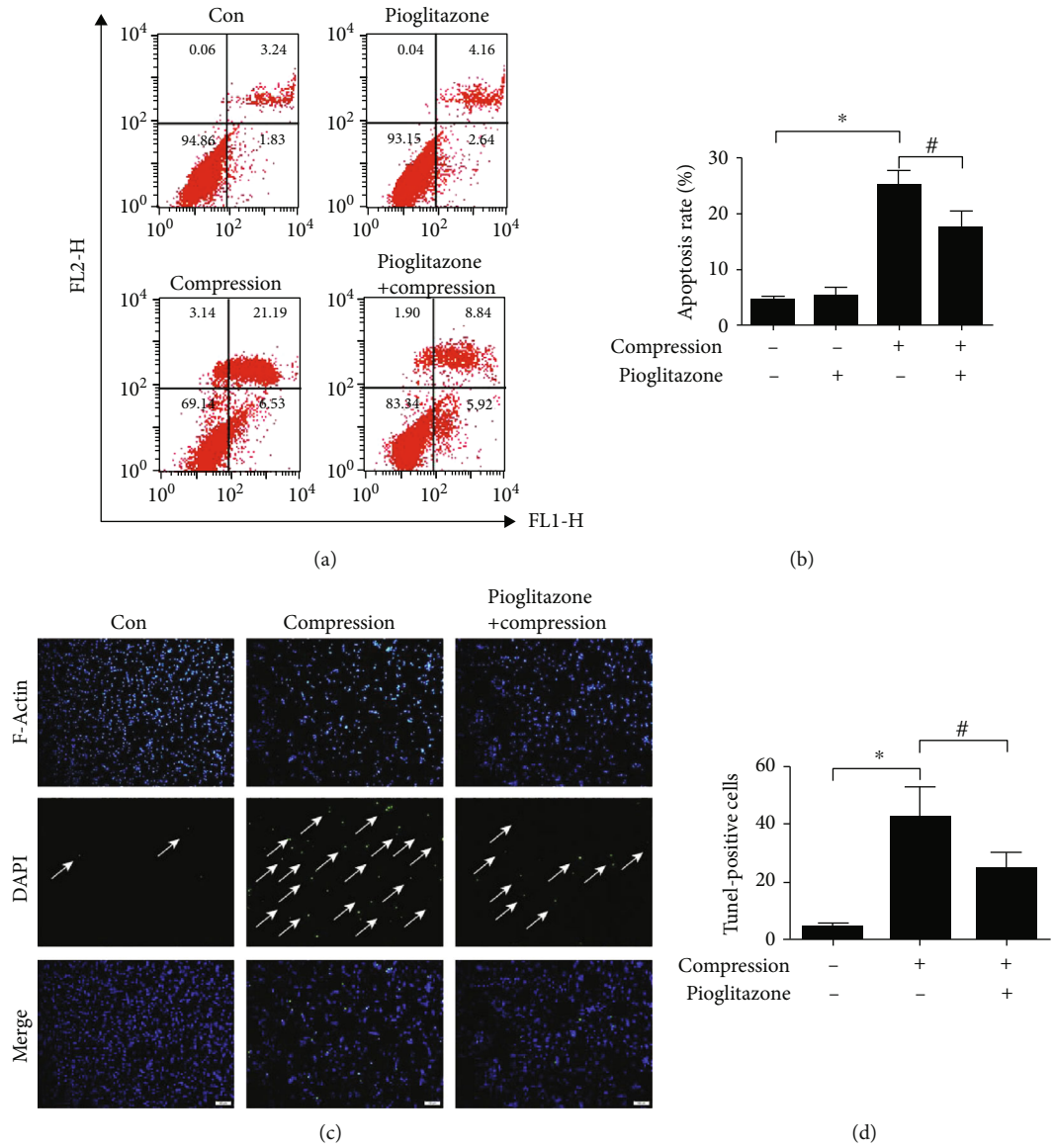


Figure 4: Effects of pioglitazone on NP-MSCs apoptosis. (a) Flow cytometric analysis was used to detect cell apoptosis by annexin V/PI staining. (b) Quantitative analysis of the apoptotic NP-MSCs rate. (c) The TUNEL staining was used to investigate apoptotic changes under a fluorescence microscope (100x). (d) Quantitative analysis of TUNEL-positive cells. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

4. Discussion

In this research, we firstly investigated the effect that pioglitazone could protect NP-MSCs from compression-induced apoptosis and the possible molecular mechanisms. In the research, pioglitazone could reduce the effects of compression on NP-MSCs proliferation and cytotoxicity. Our results revealed that pioglitazone alleviated compression-induced NP-MSCs apoptosis via inhibition of oxidative stress and mitochondrion damage. Also, we found that pioglitazone alleviated the mitochondrial pathway apoptosis-related protein expression caused by compression.

Low back pain due to IVD degeneration increases the global economic burden and affects the quality of life, which has become a global health problem [1–3]. Many recent researches found that NP-MSCs existed naturally in the IVD and these NP-MSCs efficiently repaired NP tissue [6, 8, 32]. NP-MSCs play an important role in IVD endogenous repair, which differentiate into NP-like cells and stimulate disc cells maintaining IVD homeostasis [33]. Furthermore, NP-MSCs are better adapted to the harsh IVD microenvironment than exogenous MSCs such as tolerating hypoxic and hypertonic [7, 34]. In this research, we successfully isolated NP-MSCs from human degenerative IVD. Those cells, highly expressing surface markers CD73, CD90, and CD105 but less CD34 and HLA-DR, had the similar multilineage differentiation potential as BMSCs. Although endogenous NP-MSCs could be an attractive strategy for endogenous repair, it is hard to maintain the number of viable and functional NP-MSCs under an adverse IVD microenvironment.

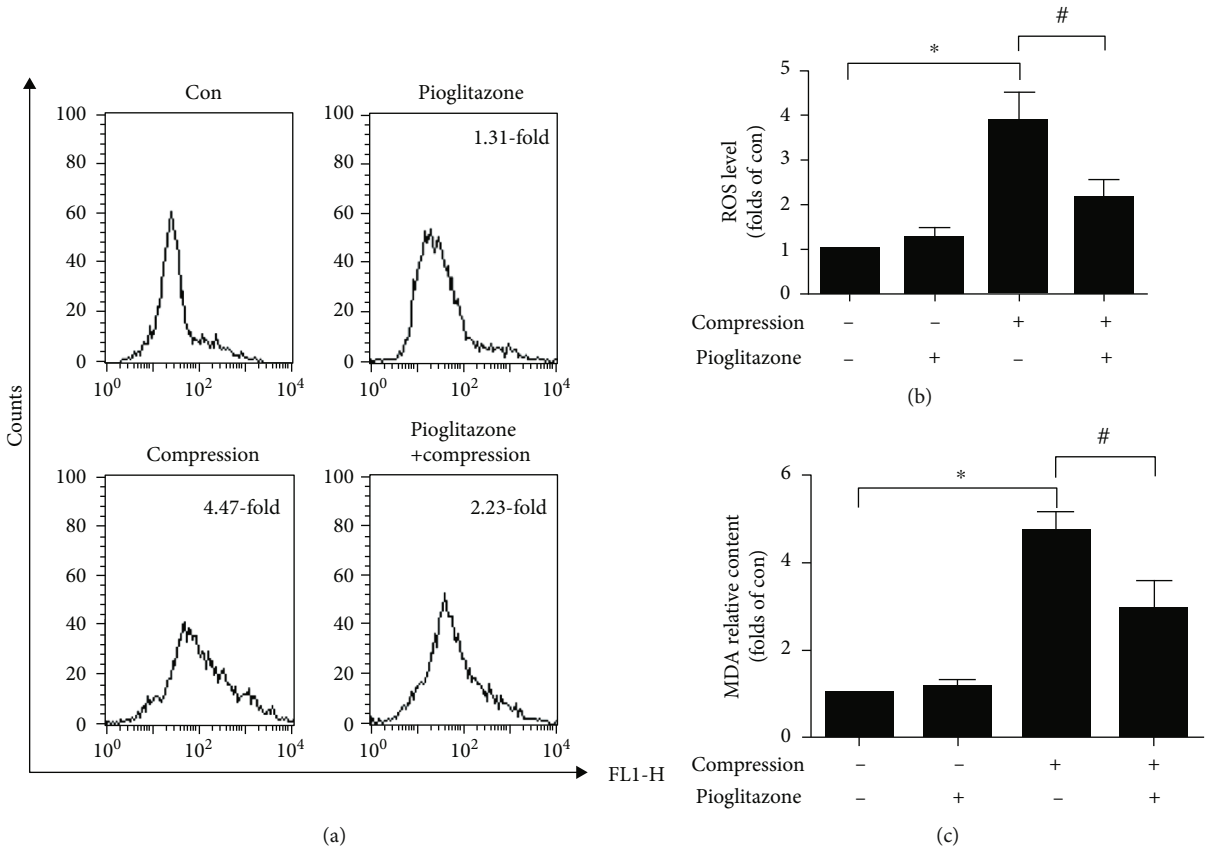


Figure 5: Effect of pioglitazone on compression-induced oxidative stress in NP-MSCs. (a) Flow cytometric analysis was performed to detect intracellular ROS production in NP-MSCs. (b) Quantitative analysis of intracellular ROS production in NP-MSCs. (c) A lipid peroxidation MDA assay kit was used to assess MDA levels. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

Mechanical stress plays a critical role in the progress of IVD degeneration via IVD cell apoptosis [35]. We also reported that compression-induced NP-MSCs apoptosis contributed to IVD degeneration [12]. In this study, our results showed that compression had inhibitory effects on cell viability and proliferation. Also, it caused NP-MSCs apoptosis. Pioglitazone plays potential beneficial roles in inflammation, fat distribution, and lipid and protein metabolism [14–16]. A previous study reported that pioglitazone could attenuate fatty acid-induced oxidative stress and apoptosis in pancreatic cells [36]. Additionally, pioglitazone can also prevent H_2O_2-induced apoptosis of endothelial progenitor cells [37]. Zhang et al. also reported that pioglitazone significantly inhibited AGE-induced chondrocyte apoptosis and degeneration [20]. Interestingly, we found that pioglitazone alleviated the inhibitory effects of compression on cell viability. It also alleviated the inhibitory effects of compression on cell proliferation and cytotoxicity. Furthermore, pioglitazone rescued the breaking of skeleton structure and stress fibers induced by compression. The results of flow cytometry and TUNEL staining showed that pioglitazone had protective effects on compression-induced NP-MSCs apoptosis.

Oxidative stress, caused by high ROS, was widely considered a potent proapoptotic factor. Maintaining a balance between ROS generation and ROS scavenging is essential for the intracellular redox homeostasis. Mitochondria can produce ROS when they are damaged, which will further exacerbate the damage and cause the overproduction of ROS, then induce the occurrence of oxidative stress [38]. Recent studies have indicated that the establishment and progression of IVD degeneration are closely related to ROS products and oxidative stress. Oxidative stress not only accelerates extracellular matrix degradation of IVD but also causes cell apoptosis that leads to the decrease of the cells in the IVD microenvironment, all of the above contribute to IVD degeneration [39]. Ding et al. have reported that the mitochondrial apoptosis pathway participates in NP cell apoptosis induced by ROS overproduction [40]. Therefore, a therapeutic strategy targeting oxidative stress would provide a major method for treating IVD degeneration. Also, intracellular accumulated ROS resulted in releasing cytochrome c from mitochondria to the cytosol and eventually activating caspases, causing cell apoptosis [41–43]. In this study, we used DCFH-DA fluorescent probes to detect ROS production and found that pioglitazone can significantly reduce compression-induced ROS production. Also, the results of the MDA assay showed that pioglitazone alleviated compression-induced oxidative stress levels in NP-MSCs.

Mitochondrion dysfunction plays an important role in cell death [44]. Mitochondria, as a key regulator of cellular processes acting as cellular oxygen sensors [45], not only produce adenosine triphosphate (ATP) but also regulate cell

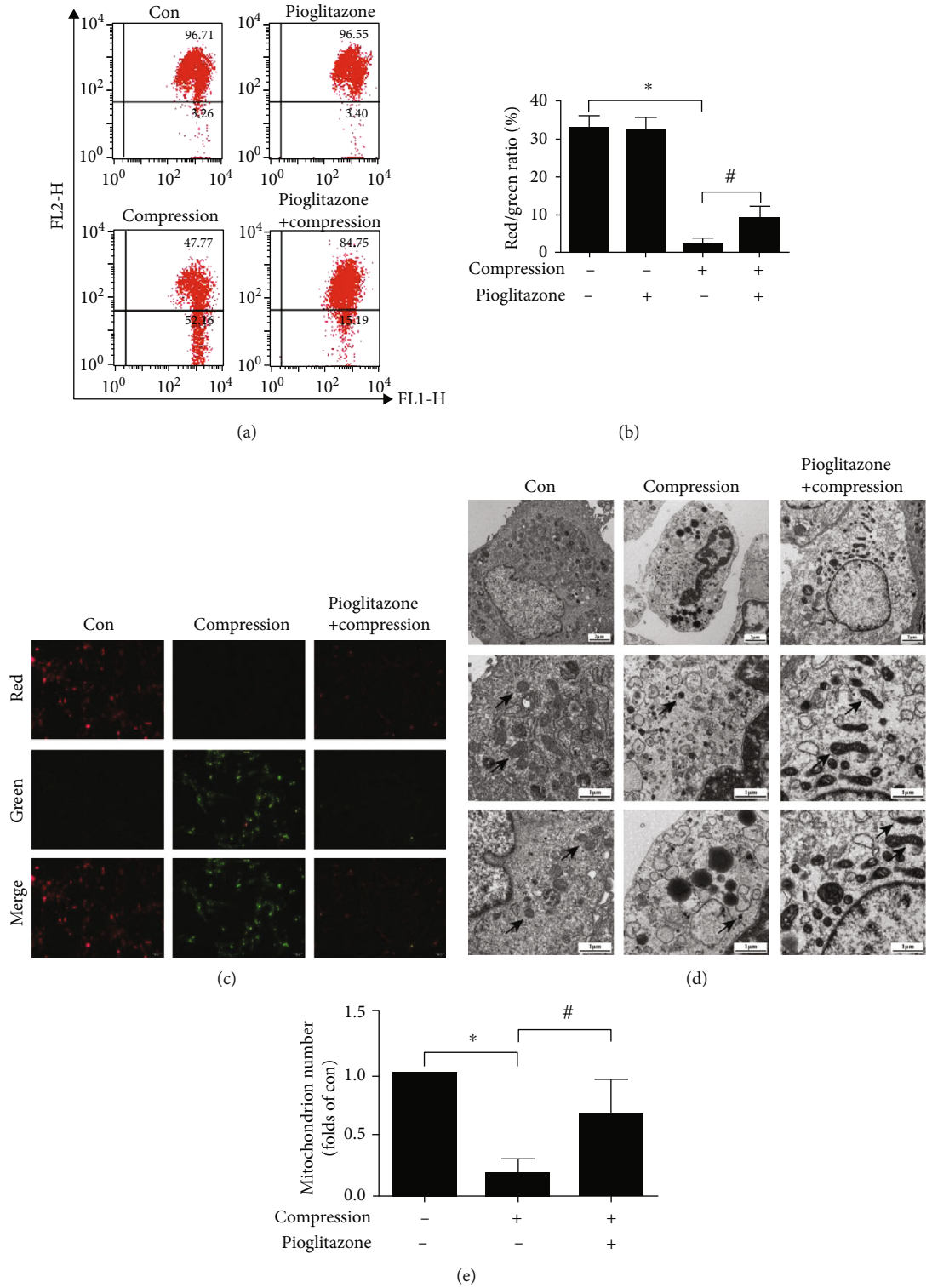
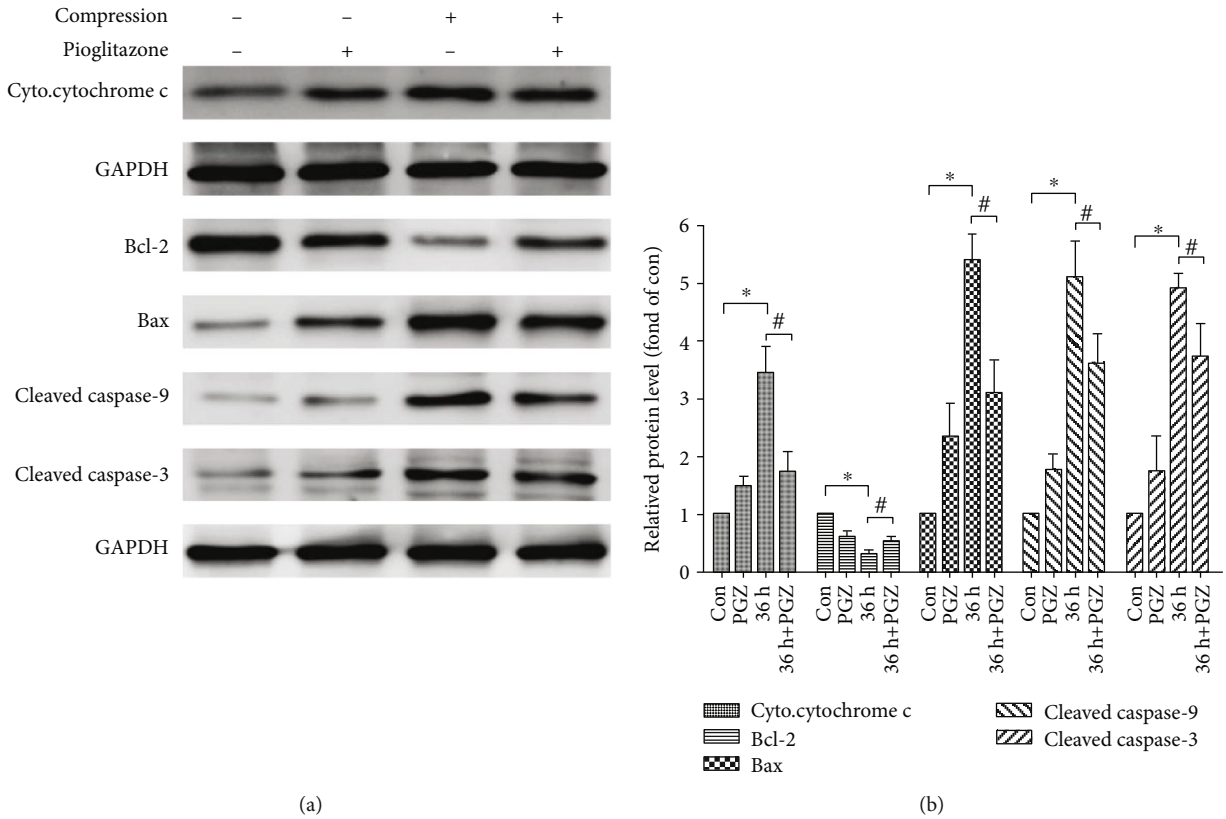


Figure 6: Effect of pioglitazone on compression-induced mitochondrion damage in NP-MSCs. (a) JC-1 staining was used to detect MMP in NP-MSCs by flow cytometric analysis. (b) Quantitative analysis of MMP in NP-MSCs. (c) MMP of NP-MSCs was observed under a fluorescence microscope (200x). (d) The cell nucleus and mitochondria of NP-MSCs were observed by TEM in three groups. Mitochondria were displayed as indicated by the arrowheads (1700x and 5000x). (e) Quantitative analysis of mitochondrion numbers in NP-MSCs. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.



(a) (b)

Figure 7: Effect of pioglitazone on mitochondrial apoptosis-related markers in NP-MSCs. (a) Representative western blotting results for cyto.cytochrome c, Bcl-2, Bax, cleaved caspase-9, and cleaved caspase-3 expression. (b) Quantitative analysis of Bax, cyto.cytochrome c, Bcl-2, cleaved caspase-9, and cleaved caspase-3 protein levels in NP-MSCs. The con group served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the compression group.

proliferation and death [46]. Some evidence shows that cell apoptosis is closely connected with mitochondrial dysfunction, including MMP depolarization, enhanced mitochondrial permeability transition pore (mPTP) opening, and mitochondrial cristae disruption [47]. In recent years, Li et al. found that pioglitazone ameliorated palmitate-induced impairment of mitochondrial morphology and function in beta cells [48]. In addition, a literature from Shokrzadeh et al. reported that pioglitazone could reduce diabetic toxicity induced by streptozocin via improving mitochondrial function [49]. In this research, we found that compression induced the loss of MMP and damaged the number and structure of mitochondria. Pioglitazone significantly reversed these effects induced by compression. These results indicated that pioglitazone has protective roles in compression-induced mitochondrion damage.

The mitochondrial apoptosis pathway is caused by apoptotic signals, including mitochondrion dysfunction. Then, it leads to the loss of MMP and the release of cytochrome c into the cytosol. The release of cytochrome c is suppressed by the antiapoptotic protein Bcl-2 and stimulated by the proapoptotic member Bax, which activated the initiator of the intrinsic apoptotic pathway caspase-9. Then, it cleaved and activated the downstream factor, caspase-3, and triggered apoptosis [40, 50]. In this study, our results indicated that pioglitazone can significantly alleviate the compression-induced expression of cytochrome c, Bax, cleaved caspase-9, and caspase-3. Also, pioglitazone promoted the expression of antiapoptotic protein Bcl-2 compared with the compression group. Such results indicated that pioglitazone exerted a protective role in compression-induced cell apoptosis in NP-MSCs by inhibiting the mitochondrial apoptotic pathway.

Our results were highly reproducible. However, there were three limitations about this study. Firstly, we performed all experiments in vitro, and the conclusions may not be necessarily indicative of what occurs in vivo. Secondly, it is difficult to obtain healthy human NP tissue, so we used NP samples from patients of degenerative disc disease for this research. Thirdly, this study mainly focused on the protective effects of pioglitazone on compression-induced NP-MSCs apoptosis. We will further investigate the functional aspects of NP-MSCs from mesenchymal markers and multilineage differentiation, stem cell-related proteins, and genes in future studies. Also, we will use 3D-culture systems and a hypoxic condition to simulate IVD microenvironment in further studies.

5. Conclusions

In this research, the results indicated that pioglitazone alleviated compression-induced NP-MSC apoptosis by inhibiting oxidative stress and mitochondrial damage. The underlying molecular protective mechanism of pioglitazone

on compression-induced apoptosis involves the mitochondrial pathway. Taken together, these findings may provide a valuable candidate for the treatment of IVD degeneration.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

YQH, LH, and MS conducted the experiments. YCW, WTW, and FD analyzed the results. YQH, LH, and MS wrote the manuscript. KGM, YLL, and YBZ conceived the experiments. GHL, ZWS, XYC, and LMX reviewed the manuscript. All authors read and approved the final manuscript. YQH, LH, MS, and YLL contributed equally to this work.

Acknowledgments

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References

[1] S. Chen, X. Lv, B. Hu et al., "Critical contribution of RIPK1 mediated mitochondrial dysfunction and oxidative stress to compression-induced rat nucleus pulposus cells necroptosis and apoptosis," *Apoptosis*, vol. 23, no. 5-6, pp. 299–313, 2018.

[2] K. Luoma, T. Vehmas, L. Kerttula, M. Grönblad, and E. Rinne, "Chronic low back pain in relation to Modic changes, bony endplate lesions, and degeneration in a prospective MRI study," *European Spine Journal*, vol. 25, no. 9, pp. 2873–2881, 2016.

[3] Y. Hu, Z. Shao, X. Cai et al., "Mitochondrial pathway is involved in advanced glycation end products-induced apoptosis of rabbit annulus fibrosus cells," *Spine*, vol. 44, no. 10, pp. E585–E595, 2019.

[4] J. A. Saal and J. S. Saal, "Intradiscal electrothermal treatment for chronic discogenic low back pain: prospective outcome study with a minimum 2-year follow-up," *Spine*, vol. 27, no. 9, pp. 966–973, 2002.

[5] H. Wang, Y. Zhou, T. W. Chu et al., "Distinguishing characteristics of stem cells derived from different anatomical regions of human degenerated intervertebral discs," *European Spine Journal*, vol. 25, no. 9, pp. 2691–2704, 2016.

[6] L. Qi, R. Wang, Q. Shi, M. Yuan, M. Jin, and D. Li, "Umbilical cord mesenchymal stem cell conditioned medium restored the expression of collagen II and aggrecan in nucleus pulposus mesenchymal stem cells exposed to high glucose," *Journal of Bone and Mineral Metabolism*, vol. 37, no. 3, pp. 455–466, 2018.

[7] H. Li, Y. Tao, C. Liang et al., "Influence of hypoxia in the intervertebral disc on the biological behaviors of rat adipose- and nucleus pulposus-derived mesenchymal stem cells," *Cells, Tissues, Organs*, vol. 198, no. 4, pp. 266–277, 2013.

[8] H. Li, Y. Tao, H. Wang et al., "Biological behavior of human nucleus pulposus mesenchymal stem cells in response to changes in the acidic environment during intervertebral disc degeneration," *Stem Cells and Development*, vol. 26, no. 12, pp. 901–911, 2017.

[9] P. H. Chou, S. T. Wang, M. H. Yen, C. L. Liu, M. C. Chang, and O. K. Lee, "Fluid-induced, shear stress-regulated extracellular matrix and matrix metalloproteinase genes expression on human annulus fibrosus cells," *Stem Cell Research & Therapy*, vol. 7, p. 34, 2016.

[10] K. Ma, S. Chen, Z. Li et al., "Mechanisms of endogenous repair failure during intervertebral disc degeneration," *Osteoarthritis and Cartilage*, vol. 27, no. 1, pp. 41–48, 2019.

[11] S. Chen, L. Zhao, X. Deng et al., "Mesenchymal stem cells protect nucleus pulposus cells from compression-induced apoptosis by inhibiting the mitochondrial pathway," *Stem Cells International*, vol. 2017, Article ID 9843120, 10 pages, 2017.

[12] H. Liang, S. Chen, D. Huang, X. Deng, K. Ma, and Z. Shao, "Effect of compression loading on human nucleus pulposus-derived mesenchymal stem cells," *Stem Cells International*, vol. 2018, Article ID 1481243, 10 pages, 2018.

[13] M. Iwata, T. Haruta, I. Usui et al., "Pioglitazone ameliorates tumor necrosis factor-α–induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator–activated receptor-γ," *Diabetes*, vol. 50, no. 5, pp. 1083–1092, 2001.

[14] L. Djaouti, T. Jourdan, L. Demizieux et al., "Different effects of pioglitazone and rosiglitazone on lipid metabolism in mouse cultured liver explants," *Diabetes/Metabolism Research and Reviews*, vol. 26, no. 4, pp. 297–305, 2010.

[15] A. Hayashi, M. Takemoto, M. Shoji, A. Hattori, K. Sugita, and K. Yokote, "Pioglitazone improves fat tissue distribution and hyperglycemia in a case of Cockayne syndrome with diabetes," *Diabetes Care*, vol. 38, no. 5, article e76, 2015.

[16] M. Peymani, K. Ghaedi, M. S. Hashemi et al., "Ameliorating the effect of pioglitazone on LPS-induced inflammation of human oligodendrocyte progenitor cells," *Cellular and Molecular Neurobiology*, vol. 38, no. 2, pp. 517–527, 2018.

[17] X. Shen, L. Yang, S. Yan et al., "The effect of FFAR1 on pioglitazone-mediated attenuation of palmitic acid-induced oxidative stress and apoptosis in βTC6 cells," *Metabolism*, vol. 63, no. 3, pp. 335–351, 2014.

[18] J. Chen, D. Li, X. Zhang, and J. L. Mehta, "Tumor necrosis factor-α-induced apoptosis of human coronary artery endothelial cells: modulation by the peroxisome proliferator-activated receptor-γ ligand pioglitazone," *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 9, no. 1, pp. 35–41, 2004.

[19] C. Werner, C. Gensch, J. Pöss, J. Haendeler, M. Böhm, and U. Laufs, "Pioglitazone activates aortic telomerase and prevents stress-induced endothelial apoptosis," *Atherosclerosis*, vol. 216, no. 1, pp. 23–34, 2011.

[20] H. B. Zhang, Y. Zhang, C. Chen, Y. Q. Li, C. Ma, and Z. J. Wang, "Pioglitazone inhibits advanced glycation end product-induced matrix metalloproteinases and apoptosis by suppressing the activation of MAPK and NF-κB," *Apoptosis*, vol. 21, no. 10, pp. 1082–1093, 2016.

[21] M. E. Peter, "Programmed cell death: apoptosis meets necrosis," *Nature*, vol. 471, no. 7338, pp. 310–312, 2011.

[22] J. Mo, D. Lin, J. Wang, P. Li, and W. Liu, "Apoptosis in HepG2 cells induced by zinc pyrithione via mitochondrial dysfunction pathway: involvement of zinc accumulation and oxidative stress," *Ecotoxicology and Environmental Safety*, vol. 161, pp. 515–525, 2018.

[23] X. Zhang, M. Wang, S. Teng et al., "Indolyl-chalcone derivatives induce hepatocellular carcinoma cells apoptosis through oxidative stress related mitochondrial pathway in vitro and in vivo," *Chemico-Biological Interactions*, vol. 293, pp. 61–69, 2018.

[24] J. Y. Choe, K. Y. Park, and S. K. Kim, "Oxidative stress by monosodium urate crystals promotes renal cell apoptosis through mitochondrial caspase-dependent pathway in human embryonic kidney 293 cells: mechanism for urate-induced nephropathy," *Apoptosis*, vol. 20, no. 1, pp. 38–49, 2015.

[25] C. Huang, Y. Lin, H. Su, and D. Ye, "Forsythiaside protects against hydrogen peroxide-induced oxidative stress and apoptosis in PC12 cell," *Neurochemical Research*, vol. 40, no. 1, pp. 27–35, 2015.

[26] G. Jia, Q. Wang, R. Wang et al., "Tubeimoside-1 induces glioma apoptosis through regulation of Bax/Bcl-2 and the ROS/-cytochrome C/caspase-3 pathway," *OncoTargets and Therapy*, vol. 8, pp. 303–311, 2015.

[27] Y. Zhao, Z. Jing, J. Lv et al., "Berberine activates caspase-9/cytochrome c-mediated apoptosis to suppress triple-negative breast cancer cells in vitro and in vivo," *Biomedicine & Pharmacotherapy*, vol. 95, pp. 18–24, 2017.

[28] M. Iguchi, M. Hiroi, H. Kanegae, and Y. Ohmori, "Costimulation of murine osteoblasts with interferon-γ and tumor necrosis factor-α induces apoptosis through downregulation of Bcl-2 and release of cytochrome c from mitochondria," *Mediators of Inflammation*, vol. 2018, Article ID 3979606, 10 pages, 2018.

[29] K. G. Ma, Z. W. Shao, S. H. Yang et al., "Autophagy is activated in compression-induced cell degeneration and is mediated by reactive oxygen species in nucleus pulposus cells exposed to compression," *Osteoarthritis and Cartilage*, vol. 21, no. 12, pp. 2030–2038, 2013.

[30] W. Wang, X. Qing, B. Wang, K. Ma, Y. Wei, and Z. Shao, "Tauroursodeoxycholic acid protects nucleus pulposus cells from compression-induced apoptosis and necroptosis via inhibiting endoplasmic reticulum stress," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 6719460, 11 pages, 2018.

[31] S. Chen, X. Lv, B. Hu et al., "RIPK1/RIPK3/MLKL-mediated necroptosis contributes to compression-induced rat nucleus pulposus cells death," *Apoptosis*, vol. 22, no. 5, pp. 626–663, 2017.

[32] R. Lazzarini, S. Guarnieri, G. Fulgenzi et al., "Mesenchymal stem cells from nucleus pulposus and neural differentiation potential: a continuous challenge," *Journal of Molecular Neuroscience*, vol. 67, no. 1, pp. 111–124, 2019.

[33] E. K. Shim, J. S. Lee, D. E. Kim et al., "Autogenous mesenchymal stem cells from the vertebral body enhance intervertebral disc regeneration via paracrine interaction: an in vitro pilot study," *Cell Transplantation*, vol. 25, no. 10, pp. 1819–1832, 2016.

[34] B. Han, H. C. Wang, H. Li et al., "Nucleus pulposus mesenchymal stem cells in acidic conditions mimicking degenerative intervertebral discs give better performance than adipose tissue-derived mesenchymal stem cells," *Cells, Tissues, Organs*, vol. 199, no. 5-6, pp. 342–352, 2014.

[35] Y. J. Kuo, L. C. Wu, J. S. Sun, M. H. Chen, M. G. Sun, and Y. H. Tsuang, "Mechanical stress-induced apoptosis of nucleus pulposus cells: an in vitro and in vivo rat model," *Journal of Orthopaedic Science*, vol. 19, no. 2, pp. 313–322, 2014.

[36] Y. Saitoh, C. Chun-ping, K. Noma, H. Ueno, M. Mizuta, and M. Nakazato, "Pioglitazone attenuates fatty acid–induced oxidative stress and apoptosis in pancreatic β-cells," *Diabetes, Obesity & Metabolism*, vol. 10, no. 7, pp. 564–573, 2008.

[37] C. Gensch, Y. P. Clever, C. Werner, M. Hanhoun, M. Böhm, and U. Laufs, "The PPAR-γ agonist pioglitazone increases neoangiogenesis and prevents apoptosis of endothelial progenitor cells," *Atherosclerosis*, vol. 192, no. 1, pp. 67–74, 2007.

[38] C. Feng, M. Yang, M. Lan et al., "ROS: crucial intermediators in the pathogenesis of intervertebral disc degeneration," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 5601593, 12 pages, 2017.

[39] F. Zhang, X. Zhao, H. Shen, and C. Zhang, "Molecular mechanisms of cell death in intervertebral disc degeneration (review)," *International Journal of Molecular Medicine*, vol. 37, no. 6, pp. 1439–1448, 2016.

[40] F. Ding, Z. W. Shao, S. H. Yang, Q. Wu, F. Gao, and L. M. Xiong, "Role of mitochondrial pathway in compression-induced apoptosis of nucleus pulposus cells," *Apoptosis*, vol. 17, no. 6, pp. 579–590, 2012.

[41] T. Liu, X. M. Chen, J. Y. Sun et al., "Palmitic acid-induced podocyte apoptosis via the reactive oxygen species-dependent mitochondrial pathway," *Kidney & Blood Pressure Research*, vol. 43, no. 1, pp. 206–219, 2018.

[42] X. L. Xue, X. D. Han, Y. Li et al., "Astaxanthin attenuates total body irradiation-induced hematopoietic system injury in mice via inhibition of oxidative stress and apoptosis," *Stem Cell Research & Therapy*, vol. 8, no. 1, p. 7, 2017.

[43] F. Ding, Z. W. Shao, and L. M. Xiong, "Cell death in intervertebral disc degeneration," *Apoptosis*, vol. 18, no. 7, pp. 777–785, 2013.

[44] M. Pietilä, K. Lähteenmäki, S. Lehtonen et al., "Monitoring mitochondrial inner membrane potential for detecting early changes in viability of bacterium-infected human bone marrow-derived mesenchymal stem cells," *Stem Cell Research & Therapy*, vol. 3, no. 6, p. 53, 2012.

[45] C. Andreani, C. Bartolacci, M. Guescini et al., "Combination of coenzyme Q10 intake and moderate physical activity counteracts mitochondrial dysfunctions in a SAMP8 mouse model," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 8936251, 15 pages, 2018.

[46] S. Agarwal, A. Yadav, S. K. Tiwari et al., "Dynamin-related protein 1 inhibition mitigates bisphenol A-mediated alterations in mitochondrial dynamics and neural stem cell proliferation and differentiation," *Journal of Biological Chemistry*, vol. 291, no. 31, pp. 15923–15939, 2016.

[47] H. Zhou, P. Zhu, J. Guo et al., "Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury," *Redox Biology*, vol. 13, pp. 498–507, 2017.

[48] Y. Li, X. Zhang, and N. Tong, "Pioglitazone ameliorates palmitate induced impairment of mitochondrial morphology and function and restores insulin level in beta cells," *Pharmazie*, vol. 68, no. 4, pp. 270–273, 2013.

[49] M. Shokrzadeh, A. Mirshafa, N. Yekta Moghaddam, B. Birjandian, and F. Shaki, "Mitochondrial dysfunction contribute to diabetic neurotoxicity induced by streptozocin in mice: protective effect of Urtica dioica and pioglitazone," *Toxicology Mechanisms and Methods*, vol. 28, no. 7, pp. 499–506, 2018.

[50] M. Shen, Y. Hu, Y. Yang et al., "Betulinic acid induces ROS-dependent apoptosis and S-phase arrest by inhibiting the NF-κB pathway in human multiple myeloma," *Oxidative Medicine and Cellular Longevity*, vol. 2019, Article ID 5083158, 14 pages, 2019.

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Review Article

Iron Redox Chemistry and Implications in the Parkinson's Disease Brain

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The etiology of Parkinson's disease (PD) is linked with cellular inclusions in the *substantia nigra pars compacta* region of the brain that are enriched in the misfolded presynaptic protein α-synuclein (αS) and death of the dopaminergic neurons. Brain iron homeostasis governs both neurotransmission and neurodegeneration; hence, the role of iron in PD progression and neuronal health is apparent. Elevated iron deposits become prevalent in the cerebral region upon aging and even more so in the PD brain. Structural as well as oxidative modifications can result from coordination of αS with redox active iron, which could have functional and/or pathological implications. In this review, we will discuss iron-mediated αS aggregation, alterations in iron metabolism, and the role of the iron-dopamine couple. Moreover, iron interactions with N-terminally acetylated αS, the physiologically relevant form of the human protein, will be addressed to shed light on the current understanding of protein dynamics and the physiological environment in the disease state. Oxidative pathways and biochemical alterations resulting from aberrant iron-induced chemistry are the principal focus of this review in order to highlight the plethora of research that has uncovered this emerging dichotomy of iron playing both functional and disruptive roles in PD pathology.

1. Introduction

Parkinson's disease (PD) is identified as the second most prevalent neurodegenerative disorder in the world, and it is an age-related progressive disease [1]. It has been estimated that approximately 1 million people in the United States alone are suffering from PD, where the majority of that population is over the age of 60. The key symptoms of PD include resting tremors, slow movements, muscle stiffness, and difficulties performing voluntary movements [2]. The main characteristic feature associated with PD is the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), and these neurons are the principal source of dopamine (DA), a neurotransmitter that regulates motor behavior [3]. PD etiology is also linked to the presence of cellular inclusions, known as Lewy bodies (LBs), which have been identified as postmortem hallmarks of PD [4, 5]. The major constituent of LBs is aggregated α-synuclein (αS), an intrinsically unfolded protein predominantly localized in the presynaptic regions of dopaminergic neurons. A tetrameric form of αS has also been identified in human tissues that has been postulated as the inactive storage form of this structurally dynamic protein due to its aggregation resistance [6, 7]. Although the cellular mechanisms and normal functions associated with αS have not yet been completely resolved, the increased levels of αS in the brains of patients with PD suggest a relationship with the neurotransmitter DA [8].

It is well known that metal homeostasis plays an important role in regulating cellular functions [9]. Copper, iron, manganese, and zinc are essential transition metal ions for neurotransmission, enzymatic reactions, and mitochondrial functions regulated by the central nervous system (CNS) [10]. Thus, an imbalance of metal ions has an impact on disease states, among which are neurodegenerative disorders. *In vivo* magnetic resonance imaging (MRI) has highlighted the accumulation of iron in the SNpc region of the PD brain, which disrupts iron circulation pathways to create an imbalance of the metal [11, 12]. In a separate study, meta-analysis of literature data on blood serum iron levels also pinpointed an increase in this metal [13]. However, a direct correlation

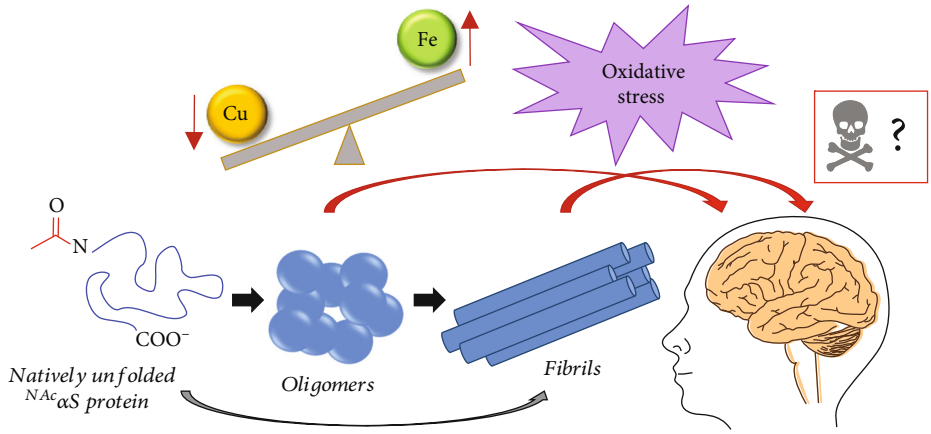

Figure 1: Schematic diagram of αS aggregation.

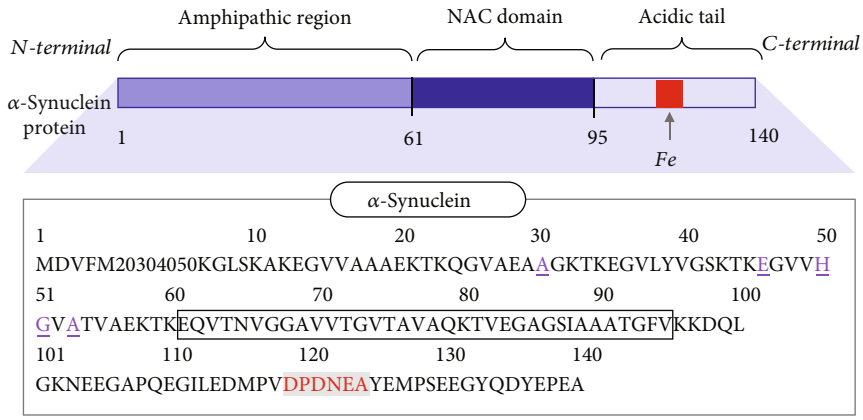

Figure 2: Cartoon representation of the N-terminal region, NAC region (highlighted within the box), and C-terminal region of αS with the primary amino acid sequence shown underneath. The iron binding site is highlighted at the C-terminal region in red text; genetic PD mutations are colored and underlined in purple.

between iron deposition and iron transportation within the brain has not been established in PD patients [14]. Beyond PD, *in vivo* MRI mapping of iron content among neurodegenerative tremulous diseases has also indicated a deposition of iron in the SNpc as compared to healthy controls [15, 16]. It is worth noting that iron dyshomeostasis associated with neurodegeneration is influenced by age, race, and gender. Therefore, iron-sensitive MRI mapping could be a powerful tool to diagnose and differentiate neurodegenerative diseases at an early stage. Evidence for iron accumulation within the SNpc region of the PD brain along with abundant misfolded αS inclusions implies a direct relationship between iron and αS in the pathogenesis of PD [17]. As shown in Figure 1, αS found in LBs exists in the form of highly ordered aggregated species that can be described as oligomers and/or fibrils. Under an oxidatively stressed environment, the aggregation processes of αS are majorly affected by interaction with redox active iron [18].

In this review, structural and biochemical consequences associated with iron-bound αS and alterations in the intracellular iron composition within dopaminergic neurons will be discussed. Key roles of iron will be delineated with respect to both the healthy brain and the PD brain. Cerebral iron levels are often associated with the modulation of dopamine-related biochemical pathways in the brain; therefore, the impact of the iron-dopamine couple and the disruption of biological cascades associated with these two components will be addressed in this review. Moreover, the impact of iron redox chemistry on the αS structure will also be highlighted.

2. α-Synuclein Structure and Iron Coordination

αS, encoded by the SNCA gene, consists of 140 amino acids and contributes approximately 1% of the protein content in cytosol [4, 5]. There are three distinct regions of αS protein (Figure 2). The N-terminal region, which contains 11 imperfect amino acid repeats with a consensus sequence KTKEGV, adopts an amphipathic α-helix conformation upon membrane interaction [8, 19]. The central non-amyloid-β component (NAC region) is involved in aggregation pathways and contains the hydrophobic core of αS. The C-terminal region is an acidic segment rich in negatively charged amino acid residues, namely, aspartic acid (D) and glutamic acid (E), and is highly dynamic. Although the pathogenesis of sporadic PD is mainly driven by the vulnerability of dopaminergic neurons, several genetic point mutations in αS have been identified as associated with PD, including A30P, E46K, H50Q,

G51D, A53E, and A53T (mutation sites are highlighted in Figure 2) [20]. In fact, enhanced aggregation properties have been reported for E46K, H50Q, and A53T as compared to wild-type, suggesting that these single point mutations initiate dramatic structural changes that seed fibrillation. The familial mutant A53T has also been reported to possess increased neurotoxicity upon elevated iron levels in comparison to wild-type αS due to selective loss of DA neurons as well as motor impairment [21].

Postmortem analyses of brain tissues isolated from patients with PD or dementia with Lewy bodies (DLB) have indicated that αS is acetylated at the N-terminus [22, 23]. Thus, it has been confirmed that N-terminally acetylated αS is the physiologically relevant form of the protein [24]. N-terminal acetylation is the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of the first amino acid residue of a protein, as in methionine (M) for αS. Although it is a common posttranslational modification catalyzed by different N-terminal acetyltransferases (NATs) in that approximately 84% of human-derived proteins carry this modification [25–27], the majority of αS research has been carried out with the nonacetylated αS variant. The consequences of this modification are becoming increasingly more apparent with respect to membrane interactions, metal coordination, and the protein folding tendencies [18, 28–31]. The innate structural dynamics and behavioral patterns of αS are strongly influenced by N-terminal capping; for example, this modification can further stabilize the tetrameric structural orientation of αS [7].

The inherent metal binding properties of αS have been vastly studied [9, 10, 32, 33]. Among the prevalent redox active transition metals in the brain, copper has shown the highest binding affinity to αS [34]. Copper levels are diminished in the PD brain, while iron levels are elevated [35]. Thus, iron-αS interactions would be more prevalent under disease conditions. The primary binding site of iron at the C-terminus, ^{119}DPDNEA^{124} (Figure 2), is a motif rich in negatively charged amino acids that will facilitate hard acid/hard base interactions via potential iron-oxygen coupling. The presence of a proline residue can also facilitate protein folding dynamics to form a more stable global configuration upon iron binding. It can also be postulated that binding of iron at the C-terminus of αS (pI of 4.7) causes a neutralization of the negative charges [36]. Such charge pairing can result in electrostatic shielding, which may impact protein folding dynamics by further altering the structure upon protein oxidation, crosslinking, aggregation, etc.

Notably, the metal coordination sites for iron and copper are located at opposite termini, where iron preferentially interacts within the αS C-terminal region, and copper has two separate sites within the N-terminus that are dependent on the copper redox state. Davies et al. have shown that there are only subtle changes in the binding affinity upon iron(III) interaction with copper(II)-saturated αS, indicating that the binding of the two metals is independent from each other [37]. Copper was further proposed as a cofactor for αS due to the measured ferrireductase activity in the presence of an electron accepter such as NADH [37].

The ability of αS to transform iron(III) to iron(II) highlights a ferrireductase activity of αS that could uncover a functional role. Conversely, the overexpression of αS could lead to excessive generation of iron(II) that may eventually result in an oxidative stress environment due to reactive oxygen species (ROS) production mediated by the Fenton reaction. Recently, we also reported evidence to support the ferrireductase activity of αS upon binding to iron(III) under anaerobic conditions as well as an increase in the antiparallel β-sheet composition as is characteristic of αS aggregates formed under oxidizing conditions in the presence of Fe^{II} [18]. On a similar note, Ortega and coworkers have reported that intracellular overexpression of αS in neurons promotes the accumulation of iron in the perinuclear region [38], accentuating that iron binding to αS correlates with αS aggregation and iron deposition as is reported for PD. Detection of iron in these regions could be acknowledged as an indirect biomarker for PD.

3. Iron-Mediated α-Synuclein Aggregation

The typical αS aggregation pathway involves propagation of natively unfolded monomers to higher-ordered oligomeric species which eventually form fibrillar structures [39, 40]. Specifically, the cardinal conformational alteration occurs when the disordered, "random coil" structure of the native protein is transformed into the well-known "β-sheet" structure concentrated in both oligomeric and fibrillar macromolecular aggregates. The orientation patterns of β-sheet (parallel or antiparallel) are distinguishable within certain contexts [41]. Although the *in vivo* toxic form of aggregates is not yet fully identified, antiparallel β-sheets are believed to be a characteristic of the toxic form [41]. The least compact oligomeric intermediates are reported to be toxic due to disruptive cellular functions including cellular leakages generated by membrane pores [42]. Hence, a correlation between oligomers and antiparallel β-sheets is expected. Based on nuclear magnetic resonance (NMR) spectroscopy and high-resolution cryoelectron microscopy techniques, the fold of fibrils is proposed to be parallel β-sheets [43]; however, the antiparallel versus parallel conformations of αS oligomers and/or fibrils have not yet been fully discerned. Traditionally, the mechanistic folding pathway is thought to involve an oligomer state that precedes fibrillation [44]; however, off-pathway aggregation routes have also been reported [18]. Fibrillation pathways via oligomer intermediates have also been thought to progress from antiparallel to parallel β-sheets [45].

The redox activity of metals like copper and iron is linked with oxidative and/or nitrosative stress and contributes as a major factor to the aggregation of αS [18, 30, 31, 46, 47]. For example, rat neuronal cultures rich in αS aggregates have indicated excessive free radical formation, implying oligomer-induced oxidative stress which is also metal-dependent [48]. It has also been suggested that iron(III)-induced SDS-resistant oligomers of αS form pores in the lipid planar bilayer in the presence of ethanol or DMSO, which eventually lead to toxicity as a result of permeability [49, 50]. Previously, it was reported that fibrils

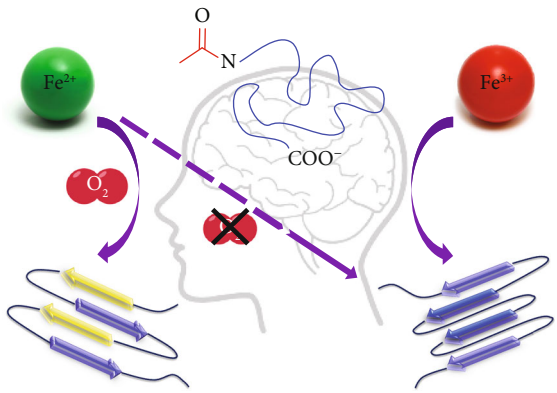

Figure 3: Graphical representation of N-terminally acetylated αS structural consequences upon aggregation mediated by iron-oxygen chemistry.

generated over the course of aggregation with iron(III) adopt a distinctly different morphology when compared to analogous fibrils with Cu(II) [36]. Transition electron microscopy (TEM) analysis of the stoichiometric Fe^{III}-αS aggregates indicated the presence of a short, thick, network-like fibrillar structure without any amorphous material. Growth patterns and morphology of fibrils are a potential biomarker which can aid in understanding structural factors affecting cell viability in neurodegeneration.

Oligomer formation can be coupled with potential membrane interactions related to pore-forming proteins which regulate metal homeostasis [49]. Oligomers generated in the presence of both iron(III) and iron(II) have also been suggested as nonharmful and to instead possess a potential functional role [51–53]. The Xie group has reported that the aggregation of αS induced by iron is dose- and time-dependent [54]. The cell viability assay conducted with SK-N-SH human neuroblastoma cells indicated that the higher the iron concentration (>1 mmol/L) and longer the period of aggregation (>24 h), the more toxicity to cells [54, 55]. Diminished mitochondrial transmembrane potential and elevated ROS production indicated the pivotal role of iron in inducing cytotoxicity. Cell toxicity due to iron-induced αS aggregation was treated by silencing the intracellular expression of αS using siRNA [56]. As it is evident that iron-mediated oxidative stress leads to cytotoxicity, it will be beneficial to look into iron chelators (Section 6 in this review), which could attenuate disease progression.

In order to shed some light on the iron-αS interaction, our group focused on iron-mediated aggregation of αS, marking the first publication to address the relationship of the iron-αS couple on the native N-terminally acetylated form of the protein [18]. A distinct change in the protein conformation was noted demonstrating the iron-oxygen-driven generation of an oligomer-locked iron-αS structure rich in right twisted antiparallel β-sheets (Figure 3). The PD relevance of this oligomeric motif was determined by its positive response to the anti-oligomer A11 polyclonal antibody, which selectively identifies soluble oligomeric epitopes present in common amyloidogenic proteins such as αS (PD), amyloid-β (Alzheimer's disease), IAPP (type II diabetes), and prion protein (Creutzfeldt-Jakob disease or mad cow disease) [57]. Hence, our results highlight the major role of iron redox chemistry in the process of αS oligomerization.

4. Iron Metabolism and Alterations in the PD Brain

Iron plays a functional role in brain biochemistry by acting as a cofactor for tyrosine hydroxylase (TyrH), an enzyme that initiates the conversion of tyrosine to DA in the cytosol [58, 59]. Iron additionally serves as an essential element in various fundamental processes within the CNS, including mitochondrial respiration, DNA synthesis, myelin production, neurotransmission, and metabolism [60]. The innate redox nature of iron is coupled with electron transfer processes. Hence, the redox state of iron, whether ferrous (Fe^{2+}) or ferric (Fe^{3+}) ions, governs the feasibility of various iron-dependent biological functions. Dysfunction or imbalance of the equilibrium between iron(II) and iron(III) ions can disrupt processes due to the generation of ROS as is commonly associated with Fenton chemistry. Therefore, iron homeostasis plays a pivotal role in regulating cellular functions as is briefly illustrated in Figure 4.

Brain iron uptake is mainly driven by the glycoprotein transferrin (Tf), the primary iron transport protein in the CNS [61, 62]. Studies have shown that non-transferrin-bound iron levels are high in the cerebrospinal fluid due to controlled Tf transportation through the blood-brain barrier [63]. Tf possesses high affinity iron(III)-binding sites, and transferrin-bound ferric ions are engulfed into cells with the aid of transferrin receptor-1 (TfR-1) via endocytosis [62]. Newly imported ferric ions are subsequently reduced to ferrous ions and released into the cytosol by divalent metal transporter-1 (DMT-1). Intracellular iron levels are further controlled by iron regulatory proteins (IRP) that act in concert with DMT-1 and TfR-1. In iron-deficient cells, IRPs selectively bind to an iron responsive element (IRE) that facilitates iron uptake by stabilizing the mRNA coding for TfR-1 and DMT-1 [64]. IRE adopts a loop-like structure consisting of 26-30 nucleotides, often present in 3′ or 5′ untranslated regions (3′-UTR or 5′-UTR) of eukaryotic mRNA for iron-dependent translational control. Another iron transporter, transferrin receptor-2 (TfR-2), which does not have an IRE, is also found in the dopaminergic neurons of the SNpc and concentrated more within the mitochondria of these cells [65]. Mitochondrial dysfunction has been accompanied by elevated Tf and TfR-2 levels in PD, suggesting oxidative stress promoted by iron redox chemistry [17, 62, 65].

Translation of ferritin, the main iron storage protein in the body, is also regulated by the availability of intracellular iron, as an IRE is found in the 5′-UTR of Tf mRNA [66]. Interestingly, a region in the 5′-UTR of human αS mRNA is reported to possess a high resemblance to the IRE present in Tf mRNA [67]. A potential IRE motif in αS mRNA suggests the possibility of iron-dependent posttranscriptional regulation of αS protein generation. Polysomal RNA analysis conducted after treatment with iron chelators has confirmed

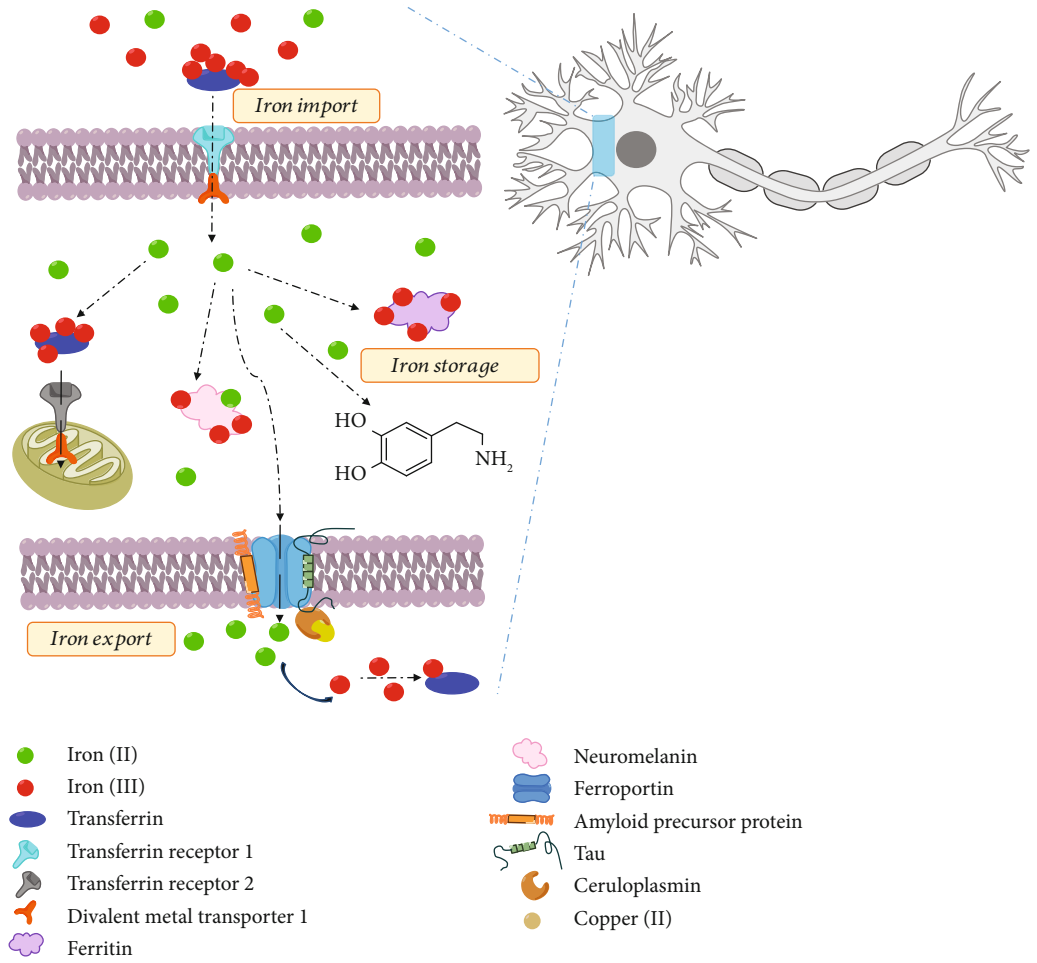

Figure 4: Iron metabolism in the neuron.

that expression of αS is influenced at a translational level by iron availability [66]. Another essential iron storage macromolecule in neurons, neuromelanin, has also been detected to accumulate in the SNpc of PD patients [68]. Both ferritin and neuromelanin possess dense iron cores [69, 70]. In the dopaminergic neurons, the ferric ions are easily reduced to ferrous ions by cytotoxic by-products of DA oxidation [17, 58, 59, 62, 71]. The ability of some cellular components, such as melanin, to reduce iron(III) to iron(II) can stimulate the hydroxyl radical formation mediated by Fenton chemistry [61, 69].

Physiological iron levels are expected to rise with aging; however, a drastic elevation is noted in PD patients [72]. Pathological iron dyshomeostasis affects the progression of PD, resulting from cumulative events that affect the capacity for neuronal survival. Suppressed expression of ferroportin and increased expression of DMT-1 mainly contribute to the elevated levels of iron in the body. Ferroportin, an iron efflux pore, is predominantly responsible for neuronal iron export, yet the efficiency of ferroportin is not governed by cellular iron levels alone [62]. There are several additional factors contributing to inefficient iron export from the neurons. For example, hepcidin is an iron regulatory hormone responsive to iron overload and inflammation. Binding of hepcidin to surface ferroportin impedes iron export via cellular internalization and degradation of ferroportin [73]. The ferroxidase activity of ceruloplasmin, a multicopper oxidase enzyme, can facilitate iron efflux coupled to ferroportin, enabling iron(III) to become readily available for binding with extracellular transferrin [74]. Notably, the low levels of copper in the SNpc as has been reported in PD patients correlate with low activity of ceruloplasmin, thus contributing to the intracellular accumulation of iron [17, 35, 75]. In addition, tau protein interacts with amyloid precursor protein to promote ferroportin-mediated iron export, and reduced levels of both of these neuronal proteins have been reported in PD brains [76]. Hence, the consequences of iron accumulation collectively disrupt cellular pathways that are dependent on our metabolism. Disruption of intracellular homeostasis could potentially be utilized to develop an indirect set of biomarkers for the diagnosis of PD based on various cellular components and proteins that are less commonly linked to PD. However, quantitative statistical analyses based upon external variables, such as age and ethnicity, have to be taken into account to establish a standard biomarker identification system.

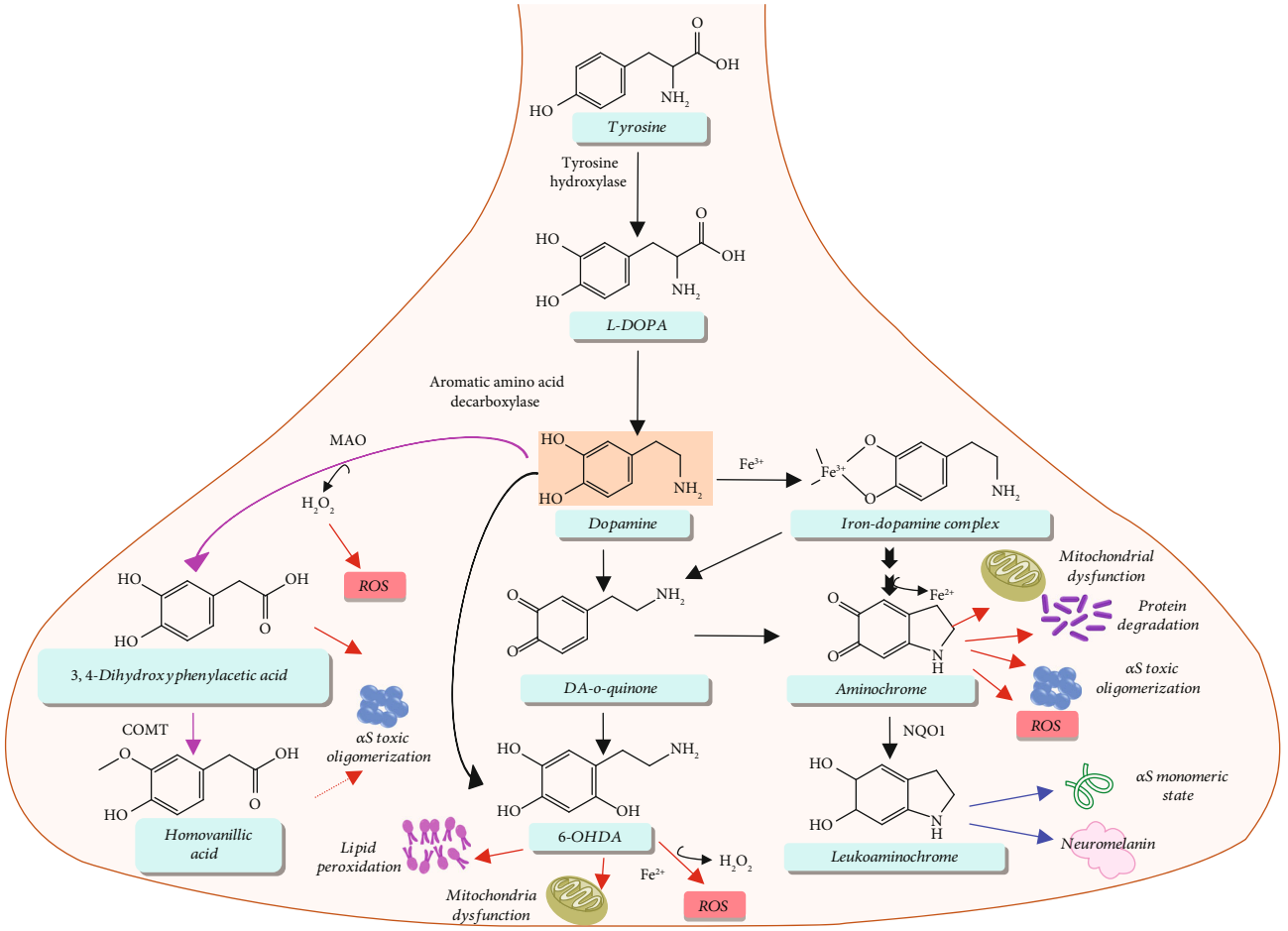

Figure 5: Schematic representation of dopamine metabolism in the synapse and toxic cascades associated with iron.

5. Toxic Consequences of the Interplay between Iron and Dopamine

Biosynthesis of DA is initiated from the amino acid tyrosine following its import into dopaminergic neurons by amino acid transporters (Figure 5) [77]. The rate-limiting step of DA synthesis is the conversion of tyrosine to dihydroxyphenylalanine (L-DOPA), which is driven by TyrH [77]. The active site of TyrH requires an iron atom in its ferrous form as a cofactor; thus, a deficiency of ferrous ions debilitates DA synthesis [77, 78]. The subsequent conversion of L-DOPA to DA is driven by aromatic amino acid decarboxylase (AADC, dopa decarboxylase), and it is selective to L-amino acid substrates [77]. Due to the dependency of the DA metabolic pathway on iron, changes in the redox state balance of Fe(III)/Fe(II) in PD brains and/or alterations in the iron flux can directly impact the health of dopaminergic neurons.

The major metabolites of enzymatic DA degradation are 3,4-dihydroxyphenylacetic acid and homovanillic acid (Figure 5) [79, 80]. DA degradation is prompted by monoamine oxidase (MAO) enzyme which results in 3,4-dihydroxyphenylacetaldehyde and hydrogen peroxide. Both substances are highly reactive and potential candidates for neurotoxicity. Under physiological conditions, 3,4-dihydroxyphenylacetaldehyde is readily oxidized to 3,4-dihydroxyphenylacetic acid, eventually leading to formation of homovanillic acid via catechol-*o*-methyl transferase (COMT) [79]. Elevated levels of both of these metabolites are identified in cerebrospinal fluid in patients with motor disorders or early PD symptoms. Hence, clinical studies emphasize that 3,4-dihydroxyphenylacetic acid and homovanillic acid could be pivotal biomarkers in PD progression [81]. In particular, the highly reactive nature of 3,4-dihydroxyphenylacetaldehyde can initiate hydroxyl radical generation leading to *in vitro* and *in vivo* neurotoxicity [82]. In fact, immunoanalyses have demonstrated that aggregation of αS both *in vitro* and *in vivo* is enhanced by 3,4-dihydroxyphenylacetaldehyde in a dose-dependent manner [83]. Furthermore, the aggregation is provoked by this metabolite to potentially form toxic oligomers [83].

In the presence of ferric ions, DA undergoes oxidation to generate DA-*o*-quinone (Figure 5), which can enter into neurotoxic pathways and eventually promote degeneration of dopaminergic neurons [84]. DA oxidation can be governed by several factors, such as oxygen, inorganic reagents, and redox active metals (primarily manganese, copper, and iron) [85–89]. Iron-facilitated DA oxidation forms another neurotoxic byproduct called 6-hydroxydopamine (6-OHDA) [90, 91], which perturbs mitochondrial functions and consequently promotes acute cell death due to disruption in ATP

synthesis [92]. It also contributes to oxidative stress by producing H_2O_2, eventually triggering lipid peroxidation and cell apoptosis [92].

Conversely, DA oxidation is also an essential step in the synthesis of neuromelanin [84], which is rapidly generated in the presence of iron(III) via formation of an iron-DA complex (Figure 5). While promoting biological reactions, the redox chemistry of iron can simultaneously influence the ROS and reactive nitrosative species (RNS) formation leading to lipid peroxidation, DNA/protein degradation, and ultimately cell death. Neurotoxicity coupled with the iron-DA complex depends upon its cellular uptake. The stable precursor of neuromelanin synthesis, aminochrome, can lead to subsequent ROS generation mediated by Fenton reactions and αS aggregation [93–95]. In addition, aminochrome can progress mitochondrial dysfunction, protein degradation, and oxidative stress in the neurons [96–98]. Toxic oligomerization of αS associated with aminochrome can be prevented by DT-diaphorase (NQO1), a flavoenzyme involved in the quinone reduction pathways [93]. This enzyme attenuated aggregation by stabilizing the monomeric state of αS upon catalyzing the reduction of quinone (aminochrome) to leukoaminochrome. In a separate study, the fibrillization pathway of αS has also been altered to spherical oligomer formation following DA oxidation [94]. The function of αS is also believed to be associated with DA metabolism [99]. DA interaction with αS leads to formation of SDS-resistant soluble oligomeric species upon oxidation of methionine residues in the αS amino acid sequence (Met locations: 1, 5, 116, and 127), consequently thwarting αS fibrillation [100, 101]. Modulation of aggregation pathways associated with αS by DA causes an increase in αS oligomers in the extracellular environment. Even though the disruptive mechanism behind the oligomerization is still under debate, these soluble oligomers are believed to be neurotoxic. Hence, a more detailed analysis of *in vivo* oligomerization and its potential to form cytotoxic membrane pores will be essential to understand the pathophysiology of the disease state.

6. Inhibition of Iron-Mediated Aggregation

Iron accumulation in the brain is implicated in diseases beyond PD, including rare synucleinopathies such as neurodegeneration with brain iron accumulation type 1 (NBIA1), multiple system atrophy (MSA), essential tremor (ET), progressive supranuclear palsy (PSP), and tremor in dystonia [12, 16, 102]. As aforementioned, increasing evidence supports the fact that the aggregation propensity of αS is affected by the redox activity of iron, and exposure to iron and oxygen stimulates a likely toxic oligomeric form of αS [18]. Therefore, inhibition of aggregation and iron chelation as a prevention technique has been briefly addressed in this section.

Baicalein and N′-benzylidene-benzohydrazide (NBB) derivatives, such as 293G02 and 301C09, have been shown to inhibit iron-induced oligomer formation and/or fibrillation (Figure 6). Low concentrations of baicalein have proven to be an excellent candidate to block the aggregation of αS initiated by treatment with organic solvent

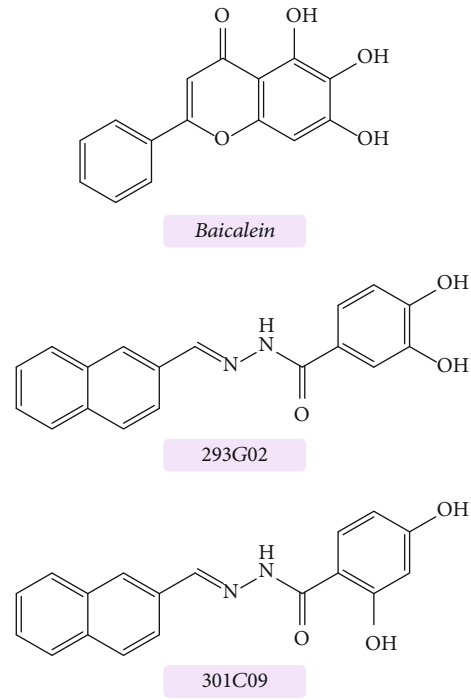

Figure 6: Structures of inhibiting agents.

[49]. The catechol moiety of NBB derivatives governs the inhibitory activity, highlighting a key structure-activity relationship [103, 104]. In particular, the compound 293G02 was highly active at controlling oligomerization inhibition, with cytotoxicity assays indicating a significant reduction in toxicity [104].

Aggregation promoted by iron can also be affected by the presence of redox inactive metal ions under physiological conditions. For example, Golts and coworkers have reported that iron-mediated αS aggregation can be inhibited by the presence of magnesium(II) by negatively modulating the iron(II) affinity [105]. It was suggested that an altered conformation enabled resistance to aggregation rather than competing with the same iron coordination site. Therefore, other metals that can compete for the same binding site in αS could act as potential therapeutic agents by mitigating the harmful effect of iron-promoted oligomerization.

Exposure of iron at early stages of life has been identified as a potential risk factor of PD [106]; however, the toxicity imparted upon early exposure to iron is irreversible even in the presence of a moderate chelator. Hence, the iron-αS interaction window is critical in PD progression as well as in the clinical aspects of disease prevention. Treatment with an iron chelator such as clioquinol has been shown to reduce nigral iron resulting in an increase in the cell viability [107]. In a separate *in vivo* study, treatment of iron-induced αS aggregates with deferiprone, a ferric ion chelator, has displayed improved motor functions in mouse models implying that the clinical application of iron chelation holds promise [108]. Structure-activity relationships among iron chelators will require careful attention as a means to open up new avenues in neurodegenerative drug discovery.

7. Concluding Remarks

Brain iron dyshomeostasis plays a crucial role in neurodegeneration associated with PD. Oxidative and conformational modifications of αS have a clear link to PD etiology, designating this structurally dynamic protein as a major target for therapeutic studies among the research community. The involvement of iron with αS biochemistry has been studied less extensively in comparison to research on copper-αS interactions; however, many studies have begun to address the potential structural and oxidative consequences that lead to αS deposits as a result of iron accumulation in the PD brain. Iron regulation in neurons has already shed light into clinical applications, and new research highlighted in this review may provide an avenue towards future therapeutic studies and/or inspire new biomarkers for PD.

Conflicts of Interest

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

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References

[1] C. Marras, J. C. Beck, J. H. Bower et al., "Prevalence of Parkinson's disease across North America," *NPJ Parkinson's Disease*, vol. 4, no. 1, p. 21, 2018.

[2] S. Sharma, C. S. Moon, A. Khogali et al., "Biomarkers in Parkinson's disease (recent update)," *Neurochemistry International*, vol. 63, no. 3, pp. 201–229, 2013.

[3] S. J. Chinta and J. K. Andersen, "Dopaminergic neurons," *The International Journal of Biochemistry & Cell Biology*, vol. 37, no. 5, pp. 942–946, 2005.

[4] W. S. Kim, K. Kågedal, and G. M. Halliday, "Alpha-synuclein biology in Lewy body diseases," *Alzheimers Research & Therapy*, vol. 6, no. 5-8, p. 73, 2014.

[5] A. D. Stephens, M. Zacharopoulou, and G. S. Kaminski Schierle, "The cellular environment affects monomeric α-synuclein structure," *Trends in Biochemical Sciences*, vol. 44, no. 5, pp. 453–466, 2019.

[6] T. Bartels, J. G. Choi, and D. J. Selkoe, "α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation," *Nature*, vol. 477, no. 7362, pp. 107–110, 2011.

[7] R. D. Fernández and H. R. Lucas, "Isolation of recombinant tetrameric N-acetylated α-synuclein," *Protein Expression and Purification*, vol. 152, pp. 146–154, 2018.

[8] H. A. Lashuel, C. R. Overk, A. Oueslati, and E. Masliah, "The many faces of α-synuclein: from structure and toxicity to therapeutic target," *Nature Reviews Neuroscience*, vol. 14, no. 1, pp. 38–48, 2013.

[9] E. Carboni and P. Lingor, "Insights on the interaction of alpha-synuclein and metals in the pathophysiology of Parkinson's disease," *Metallomics*, vol. 7, no. 3, pp. 395–404, 2015.

[10] H. Kozlowski, M. Luczkowski, M. Remelli, and D. Valensin, "Copper, zinc and iron in neurodegenerative diseases (Alzheimer's, Parkinson's and prion diseases)," *Coordination Chemistry Reviews*, vol. 256, no. 19-20, pp. 2129–2141, 2012.

[11] P. Costa-Mallen, C. Gatenby, S. Friend et al., "Brain iron concentrations in regions of interest and relation with serum iron levels in Parkinson disease," *Journal of the Neurological Sciences*, vol. 378, pp. 38–44, 2017.

[12] J.-H. Lee and M.-S. Lee, "Brain iron accumulation in atypical parkinsonian syndromes: in vivo MRI evidences for distinctive patterns," *Frontiers in Neurology*, vol. 10, 2019.

[13] J. Jiao, H. Guo, Y. He, J. Wang, J. Yuan, and W. Hu, "Meta-analysis of the association between serum iron levels and Parkinson's disease: evidence from 11 publications," *Brain Research*, vol. 1646, pp. 490–493, 2016.

[14] Q. Chen, Y. Chen, Y. Zhang et al., "Iron deposition in Parkinson's disease by quantitative susceptibility mapping," *BMC Neuroscience*, vol. 20, no. 1, p. 23, 2019.

[15] F. Novellino, A. Cherubini, C. Chiriaco et al., "Brain iron deposition in essential tremor: a quantitative 3-tesla magnetic resonance imaging study," *Movement Disorders*, vol. 28, no. 2, pp. 196–200, 2013.

[16] N. Homayoon, L. Pirpamer, S. Franthal et al., "Nigral iron deposition in common tremor disorders," *Movement Disorders*, vol. 34, no. 1, pp. 129–132, 2019.

[17] D. J. Hare and K. L. Double, "Iron and dopamine: a toxic couple," *Brain*, vol. 139, no. 4, pp. 1026–1035, 2016.

[18] D. L. Abeyawardhane, R. D. Fernández, C. J. Murgas et al., "Iron redox chemistry promotes antiparallel oligomerization of α-synuclein," *Journal of the American Chemical Society*, vol. 140, no. 15, pp. 5028–5032, 2018.

[19] H. R. Lucas and J. C. Lee, "Copper(II) enhances membrane-bound α-synuclein helix formation," *Metallomics*, vol. 3, no. 3, pp. 280–283, 2011.

[20] P. Flagmeier, G. Meisl, M. Vendruscolo et al., "Mutations associated with familial Parkinson's disease alter the initiation and amplification steps of α-synuclein aggregation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 37, pp. 10328–10333, 2016.

[21] Z. J. Zhu, K. C. Wu, W. H. Yung, Z. M. Qian, and Y. Ke, "Differential interaction between iron and mutant alpha-synuclein causes distinctive parkinsonian phenotypes in *Drosophila*," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1862, no. 4, pp. 518–525, 2016.

[22] A. Öhrfelt, H. Zetterberg, K. Andersson et al., "Identification of novel α-synuclein isoforms in human brain tissue by using an online NanoLC-ESI-FTICR-MS method," *Neurochemical Research*, vol. 36, no. 11, pp. 2029–2042, 2011.

[23] J. P. Anderson, D. E. Walker, J. M. Goldstein et al., "Phosphorylation of Ser-129 is the dominant pathological modification of α-synuclein in familial and sporadic Lewy body disease," *Journal of Biological Chemistry*, vol. 281, no. 40, pp. 29739–29752, 2006.

[24] F.-X. Theillet, A. Binolfi, B. Bekei et al., *Nature*, vol. 530, no. 7588, pp. 45–50, 2016.

[25] T. Arnesen, P. van Damme, B. Polevoda et al., "Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 20, pp. 8157–8162, 2009.

[26] T. Arnesen, "Towards a Functional Understanding of Protein N-Terminal Acetylation," *PLoS Biology*, vol. 9, no. 5, 2011.

[27] B. Fauvet, M. Fares, F. Samuel et al., "Characterization of semisynthetic and naturally N^{α}-acetylated α-synuclein *in vitro* and in intact cells implications for aggregation and cellular properties of α-synuclein," *Journal of Biological Chemistry*, vol. 287, no. 34, pp. 28243–28262, 2012.

[28] L. Kang, G. M. Moriarty, L. A. Woods, A. E. Ashcroft, S. E. Radford, and J. Baum, "N-Terminal acetylation of α-synuclein induces increased transient helical propensity and decreased aggregation rates in the intrinsically disordered monomer," *Protein Science*, vol. 21, no. 7, pp. 911–917, 2012.

[29] I. Dikiy and D. Eliezer, "N-terminal acetylation stabilizes N-terminal helicity in lipid- and micelle-bound α-synuclein and increases its affinity for physiological membranes," *Journal of Biological Chemistry*, vol. 289, no. 6, pp. 3652–3665, 2014.

[30] D. L. Abeyawardhane, R. D. Fernández, D. R. Heitger, M. K. Crozier, J. C. Wolver, and H. R. Lucas, "Copper induced radical dimerization of α-synuclein requires histidine," *Journal of the American Chemical Society*, vol. 140, no. 49, pp. 17086–17094, 2018.

[31] D. L. Abeyawardhane, D. R. Heitger, R. D. Fernández, A. K. Forney, and H. R. Lucas, "C-Terminal Cu^{II} coordination to α-synuclein enhances aggregation," *ACS Chemical Neuroscience*, vol. 10, no. 3, pp. 1402–1410, 2019.

[32] D. Valensin, S. Dell'Acqua, H. Kozlowski, and L. Casella, "Coordination and redox properties of copper interaction with α-synuclein," *Journal of Inorganic Biochemistry*, vol. 163, pp. 292–300, 2016.

[33] K. Joppe, A.-E. Roser, F. Maass, and P. Lingor, "The contribution of iron to protein aggregation disorders in the central nervous system," *Frontiers in Neuroscience*, vol. 13, p. 15, 2019.

[34] I. Gentile, H. A. Garro, S. Delgado Ocaña et al., "Interaction of Cu(I) with the Met-X_3-Met motif of alpha-synuclein: binding ligands, affinity and structural features," *Metallomics*, vol. 10, no. 10, pp. 1383–1389, 2018.

[35] K. M. Davies, S. Bohic, A. Carmona et al., "Copper pathology in vulnerable brain regions in Parkinson's disease," *Neurobiology of Aging*, vol. 35, no. 4, pp. 858–866, 2014.

[36] Bharathi, S. S. Indi, and K. S. J. Rao, "Copper- and iron-induced differential fibril formation in α-synuclein: TEM study," *Neuroscience Letters*, vol. 424, no. 2, pp. 78–82, 2007.

[37] P. Davies, D. Moualla, and D. R. Brown, "Alpha-synuclein is a cellular ferrireductase," *PLoS One*, vol. 6, no. 1, article e15814, 2011.

[38] R. Ortega, A. Carmona, S. Roudeau et al., "α-synuclein overexpression induces increased iron accumulation and redistribution in iron-exposed neurons," *Molecular Neurobiology*, vol. 53, no. 3, pp. 1925–1934, 2016.

[39] V. N. Uversky, J. Li, and A. L. Fink, "Evidence for a partially folded intermediate in α-synuclein fibril formation," *Journal of Biological Chemistry*, vol. 276, no. 14, pp. 10737–10744, 2001.

[40] S. W. Chen, S. Drakulic, E. Deas et al., "Structural characterization of toxic oligomers that are kinetically trapped during α-synuclein fibril formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 16, pp. E1994–E2003, 2015.

[41] M. S. Celej, R. Sarroukh, E. Goormaghtigh, G. D. Fidelio, J. M. Ruysschaert, and V. Raussens, "Toxic prefibrillar α-synuclein amyloid oligomers adopt a distinctive antiparallel β-sheet structure," *Biochemical Journal*, vol. 443, no. 3, pp. 719–726, 2012.

[42] S. K. Sharma and S. Priya, "Expanding role of molecular chaperones in regulating α-synuclein misfolding; implications in Parkinson's disease," *Cellular and Molecular Life Sciences*, vol. 74, no. 4, pp. 617–629, 2017.

[43] M. Vilar, H. T. Chou, T. Luhrs et al., "The fold of α-synuclein fibrils," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 25, pp. 8637–8642, 2008.

[44] A. L. Fink, "The aggregation and fibrillation of α-synuclein," *Accounts of Chemical Research*, vol. 39, no. 9, pp. 628–634, 2006.

[45] J. I. Gallea, R. Sarroukh, P. Yunes-Quartino, J. M. Ruysschaert, V. Raussens, and M. S. Celej, "Structural remodeling during amyloidogenesis of physiological N^{α}-acetylated α-synuclein," *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, vol. 1864, no. 5, pp. 501–510, 2016.

[46] G. Ferrer-Sueta, N. Campolo, M. Trujillo et al., "Biochemistry of peroxynitrite and protein tyrosine nitration," *Chemical Reviews*, vol. 118, no. 3, pp. 1338–1408, 2018.

[47] R. Burai, N. Ait-Bouziad, A. Chiki, and H. A. Lashuel, "Elucidating the role of site-specific nitration of α-synuclein in the pathogenesis of Parkinson's disease via protein semisynthesis and mutagenesis," *Journal of the American Chemical Society*, vol. 137, no. 15, pp. 5041–5052, 2015.

[48] E. Deas, N. Cremades, P. R. Angelova et al., "Alpha-synuclein oligomers interact with metal ions to induce oxidative stress and neuronal death in Parkinson's disease," *Antioxidants & Redox Signaling*, vol. 24, no. 7, pp. 376–391, 2016.

[49] M. Kostka, T. Högen, K. M. Danzer et al., "Single particle characterization of iron-induced pore-forming α-synuclein oligomers," *Journal of Biological Chemistry*, vol. 283, no. 16, pp. 10992–11003, 2008.

[50] F. Schmidt, J. Levin, F. Kamp, H. Kretzschmar, A. Giese, and K. Botzel, "Single-channel electrophysiology reveals a distinct and uniform pore complex formed by α-synuclein oligomers in lipid membranes," *PLoS One*, vol. 7, no. 8, article e42545, 2012.

[51] K. M. Danzer, S. K. Krebs, M. Wolff, G. Birk, and B. Hengerer, "Seeding induced by α-synuclein oligomers provides evidence for spreading of α-synuclein pathology," *Journal of Neurochemistry*, vol. 111, no. 1, pp. 192–203, 2009.

[52] E. Illes-Toth, M. R. Ramos, R. Cappai, C. Dalton, and D. P. Smith, "Distinct higher-order α-synuclein oligomers induce intracellular aggregation," *Biochemical Journal*, vol. 468, no. 3, pp. 485–493, 2015.

[53] M. Ingelsson, "Alpha-synuclein oligomers—neurotoxic molecules in Parkinson's disease and other Lewy body disorders," *Frontiers in Neuroscience*, vol. 10, 2016.

[54] W. J. Li, H. Jiang, N. Song, and J. X. Xie, "Dose- and time-dependent α-synuclein aggregation induced by ferric iron in SK-N-SH cells," *Neuroscience Bulletin*, vol. 26, no. 3, pp. 205–210, 2010.

[55] W. Li, H. Jiang, N. Song, and J. Xie, "Oxidative stress partially contributes to iron-induced alpha-synuclein aggregation in SK-N-SH cells," *Neurotoxicity Research*, vol. 19, no. 3, pp. 435–442, 2011.

[56] Q. He, N. Song, H. Xu, R. Wang, J. Xie, and H. Jiang, "Alpha-synuclein aggregation is involved in the toxicity induced by ferric iron to SK-N-SH neuroblastoma cells," *Journal of Neural Transmission*, vol. 118, no. 3, pp. 397–406, 2011.

[57] R. Kayed, E. Head, J. Thompson et al., "Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis," *Science*, vol. 300, no. 5618, pp. 486–489, 2003.

[58] J. A. Duce, B. X. Wong, H. Durham, J. C. Devedjian, D. P. Smith, and D. Devos, "Post translational changes to α-synuclein control iron and dopamine trafficking; a concept for neuron vulnerability in Parkinson's disease," *Molecular Neurodegeneration*, vol. 12, no. 1, p. 45, 2017.

[59] J. Sian-Hülsmann, S. Mandel, M. B. H. Youdim, and P. Riederer, "The relevance of iron in the pathogenesis of Parkinson's disease," *Journal of Neurochemistry*, vol. 118, no. 6, pp. 939–957, 2011.

[60] B. X. Wong and J. A. Duce, "The iron regulatory capability of the major protein participants in prevalent neurodegenerative disorders," *Frontiers in Pharmacology*, vol. 5, 2014.

[61] D. Berg, M. Gerlach, M. B. H. Youdim et al., "Brain iron pathways and their relevance to Parkinson's disease," *Journal of Neurochemistry*, vol. 79, no. 2, pp. 225–236, 2001.

[62] D. W. Lee and J. K. Andersen, "Iron elevations in the aging Parkinsonian brain: a consequence of impaired iron homeostasis?," *Journal of Neurochemistry*, vol. 112, no. 2, pp. 332–339, 2010.

[63] T. Moos and E. H. Morgan, "Evidence for low molecular weight, non-transferrin-bound iron in rat brain and cerebrospinal fluid," *Journal of Neuroscience Research*, vol. 54, no. 4, pp. 486–494, 1998.

[64] P. Piccinelli and T. Samuelsson, "Evolution of the iron-responsive element," *RNA*, vol. 13, no. 7, pp. 952–966, 2007.

[65] P. G. Mastroberardino, E. K. Hoffman, M. P. Horowitz et al., "A novel transferrin/TfR2-mediated mitochondrial iron transport system is disrupted in Parkinson's disease," *Neurobiology of Disease*, vol. 34, no. 3, pp. 417–431, 2009.

[66] F. Febbraro, M. Giorgi, S. Caldarola, F. Loreni, and M. Romero-Ramos, "α-synuclein expression is modulated at the translational level by iron," *NeuroReport*, vol. 23, no. 9, pp. 576–580, 2012.

[67] A. L. Friedlich, R. E. Tanzi, and J. T. Rogers, "The 5′ -untranslated region of Parkinson's disease α-synuclein messengerRNA contains a predicted iron responsive element," *Molecular Psychiatry*, vol. 12, no. 3, pp. 222-223, 2007.

[68] L. Zecca, R. Fariello, P. Riederer, D. Sulzer, A. Gatti, and D. Tampellini, "The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease," *FEBS Letters*, vol. 510, no. 3, pp. 216–220, 2002.

[69] M. Fasano, B. Bergamasco, and L. Lopiano, "Modifications of the iron-neuromelanin system in Parkinson's disease," *Journal of Neurochemistry*, vol. 96, no. 4, pp. 909–916, 2006.

[70] L. Zecca, M. Gallorini, V. Schünemann et al., "Iron, neuromelanin and ferritin content in the substantia nigra of normal subjects at different ages: consequences for iron storage and neurodegenerative processes," *Journal of Neurochemistry*, vol. 76, no. 6, pp. 1766–1773, 2001.

[71] K. L. Double, M. Maywald, M. Schmittel, P. Riederer, and M. Gerlach, "In vitro studies of ferritin iron release and neurotoxicity," *Journal of Neurochemistry*, vol. 70, no. 6, pp. 2492–2499, 1998.

[72] A. A. Belaidi and A. I. Bush, "Iron neurochemistry in Alzheimer's disease and Parkinson's disease: targets for therapeutics," *Journal of Neurochemistry*, vol. 139, pp. 179–197, 2016.

[73] E. Nemeth, M. Tuttle, J. Powelson et al., "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization," *Science*, vol. 306, no. 5704, pp. 2090–2093, 2004.

[74] G. Musci, F. Polticelli, and M. C. Bonaccorsi di Patti, "Ceruloplasmin-ferroportin system of iron traffic in vertebrates," *World Journal of Biological Chemistry*, vol. 5, no. 2, pp. 204–215, 2014.

[75] T. Skjørringe, L. B. Møller, and T. Moos, "Impairment of interrelated iron- and copper homeostatic mechanisms in brain contributes to the pathogenesis of neurodegenerative disorders," *Frontiers in Pharmacology*, vol. 3, 2012.

[76] P. Lei, S. Ayton, D. I. Finkelstein et al., "Tau deficiency induces parkinsonism with dementia by impairing APP-mediated iron export," *Nature Medicine*, vol. 18, no. 2, pp. 291–295, 2012.

[77] J. D. Elsworth and R. H. Roth, "Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease," *Experimental Neurology*, vol. 144, no. 1, pp. 4–9, 1997.

[78] Y. Y. Yien and B. H. Paw, "A role for iron deficiency in dopaminergic neurodegeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 13, pp. 3417-3418, 2016.

[79] J. Meiser, D. Weindl, and K. Hiller, "Complexity of dopamine metabolism," *Cell Communication and Signaling*, vol. 11, no. 1, p. 34, 2013.

[80] N. Song and J. Xie, "Iron, dopamine, and α-synuclein interactions in at-risk dopaminergic neurons in Parkinson's disease," *Neuroscience Bulletin*, vol. 34, no. 2, pp. 382–384, 2018.

[81] A. Stefani, M. Pierantozzi, E. Olivola et al., "Homovanillic acid in CSF of mild stage Parkinson's disease patients correlates with motor impairment," *Neurochemistry International*, vol. 105, pp. 58–63, 2017.

[82] S. W. Li, T. S. Lin, S. Minteer, and W. J. Burke, "3,4-Dihydroxyphenylacetaldehyde and hydrogen peroxide generate a hydroxyl radical: possible role in Parkinson's disease pathogenesis," *Molecular Brain Research*, vol. 93, no. 1, pp. 1–7, 2001.

[83] W. J. Burke, V. B. Kumar, N. Pandey et al., "Aggregation of α-synuclein by DOPAL, the monoamine oxidase metabolite of dopamine," *Acta Neuropathologica*, vol. 115, no. 2, pp. 193–203, 2008.

[84] F. A. Zucca, J. Segura-Aguilar, E. Ferrari et al., "Interactions of iron, dopamine and neuromelanin pathways in brain aging and Parkinson's disease," *Progress in Neurobiology*, vol. 155, pp. 96–119, 2017.

[85] W. Linert, E. Herlinger, R. F. Jameson, E. Kienzl, K. Jellinger, and M. B. H. Youdim, "Dopamine, 6-hydroxydopamine, iron, and dioxygen - their mutual interactions and possible implication in the development of Parkinson's disease," *Biochimica et Biophysica Acta-Molecular Basis of Disease*, vol. 1316, no. 3, pp. 160–168, 1996.

[86] M. Jimenez, F. Garcia-Carmona, F. Garcia-Canovas, J. L. Iborra, J. A. Lozano, and F. Martinez, "Chemical intermediates in dopamine oxidation by tyrosinase, and kinetic-studies of the process," *Archives of Biochemistry and Biophysics*, vol. 235, no. 2, pp. 438–448, 1984.

[87] Z. D. Zhou, Y. H. Lan, E. K. Tan, and T. M. Lim, "Iron species-mediated dopamine oxidation, proteasome inhibition, and dopaminergic cell demise implications for iron-

related dopaminergic neuron degeneration," *Free Radical Biology & Medicine*, vol. 49, no. 12, pp. 1856–1871, 2010.

[88] J. Segura-Aguilar and C. Lind, "On the mechanism of the Mn^{3+}-induced neurotoxicity of dopamine: prevention of quinone-derived oxygen-toxicity by DT diaphorase and superoxide-dismutase," *Chemico-Biological Interactions*, vol. 72, no. 3, pp. 309–324, 1989.

[89] I. Paris, A. Dagnino-Subiabre, K. Marcelain et al., "Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in a rat substantia nigra neuronal cell line," *Journal of Neurochemistry*, vol. 77, no. 2, pp. 519–529, 2001.

[90] L. Zhang, G. Yagnik, D. Jiang, S. Shi, P. Chang, and F. Zhou, "Separation of intermediates of iron-catalyzed dopamine oxidation reactions using reversed-phase ion-pairing chromatography coupled in tandem with UV-visible and ESI-MS detections," *Journal of Chromatography B*, vol. 911, pp. 55–58, 2012.

[91] J. Rodriguez-Pallares, J. A. Parga, A. Muñoz, P. Rey, M. J. Guerra, and J. L. Labandeira-Garcia, "Mechanism of 6-hydroxydopamine neurotoxicity: the role of NADPH oxidase and microglial activation in 6-hydroxydopamine-induced degeneration of dopaminergic neurons," *Journal of Neurochemistry*, vol. 103, no. 1, pp. 145–156, 2007.

[92] D. Blum, S. Torch, N. Lambeng et al., "Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease," *Progress in Neurobiology*, vol. 65, no. 2, pp. 135–172, 2001.

[93] P. Munoz, S. Cardenas, S. Huenchuguala et al., "DT-diaphorase prevents aminochrome-induced alpha-synuclein oligomer formation and neurotoxicity," *Toxicological Sciences*, vol. 145, no. 1, pp. 37–47, 2015.

[94] E. H. Norris, B. I. Giasson, R. Hodara et al., "Reversible inhibition of α-synuclein fibrillization by dopaminochrome-mediated conformational alterations," *Journal of Biological Chemistry*, vol. 280, no. 22, pp. 21212–21219, 2005.

[95] M. Bisaglia, S. Mammi, and L. Bubacco, "Kinetic and structural analysis of the early oxidation products of dopamine - analysis of the interactions with α-synuclein," *Journal of Biological Chemistry*, vol. 282, no. 21, pp. 15597–15605, 2007.

[96] R. Xiong, D. Siegel, and D. Ross, "Quinone-induced protein handling changes: implications for major protein handling systems in quinone-mediated toxicity," *Toxicology and Applied Pharmacology*, vol. 280, no. 2, pp. 285–295, 2014.

[97] C. Arriagada, I. Paris, M. J. Sanchez de las Matas et al., "On the neurotoxicity mechanism of leukoaminochrome o-semiquinone radical derived from dopamine oxidation: mitochondria damage, necrosis, and hydroxyl radical formation," *Neurobiology of Disease*, vol. 16, no. 2, pp. 468–477, 2004.

[98] P. Aguirre, P. Urrutia, V. Tapia et al., "The dopamine metabolite aminochrome inhibits mitochondrial complex I and modifies the expression of iron transporters DMT1 and FPN1," *Biometals*, vol. 25, no. 4, pp. 795–803, 2012.

[99] S. Arreguin, P. Nelson, S. Padway, M. Shirazi, and C. Pierpont, "Dopamine complexes of iron in the etiology and pathogenesis of Parkinson's disease," *Journal of Inorganic Biochemistry*, vol. 103, no. 1, pp. 87–93, 2009.

[100] S. L. Leong, C. L. L. Pham, D. Galatis et al., "Formation of dopamine-mediated α-synuclein-soluble oligomers requires methionine oxidation," *Free Radical Biology & Medicine*, vol. 46, no. 10, pp. 1328–1337, 2009.

[101] H.-J. Lee, S. M. Baek, D. H. Ho, J. E. Suk, E. D. Cho, and S. J. Lee, "Dopamine promotes formation and secretion of non-fibrillar alpha-synuclein oligomers," *Experimental and Molecular Medicine*, vol. 43, no. 4, pp. 216–222, 2011.

[102] J. E. Duda, V. M. Y. Lee, and J. Q. Trojanowski, "Neuropathology of synuclein aggregates: new insights into mechanisms of neurodegenerative diseases," *Journal of Neuroscience Research*, vol. 61, no. 2, pp. 121–127, 2000.

[103] U. Bertsch, K. F. Winklhofer, T. Hirschberger et al., "Systematic identification of antiprion drugs by high-throughput screening based on scanning for intensely fluorescent targets," *Journal of Virology*, vol. 79, no. 12, pp. 7785–7791, 2005.

[104] A. S. Hillmer, P. Putcha, J. Levin et al., "Converse modulation of toxic α-synuclein oligomers in living cells by N′-benzylidene-benzohydrazide derivates and ferric iron," *Biochemical and Biophysical Research Communications*, vol. 391, no. 1, pp. 461–466, 2010.

[105] N. Golts, H. Snyder, M. Frasier, C. Theisler, P. Choi, and B. Wolozin, "Magnesium inhibits spontaneous and iron-induced aggregation of α-synuclein," *Journal of Biological Chemistry*, vol. 277, no. 18, pp. 16116–16123, 2002.

[106] N. Ostrerova-Golts, L. Petrucelli, J. Hardy, J. M. Lee, M. Farer, and B. Wolozin, "The A53T α-synuclein mutation increases iron-dependent aggregation and toxicity," *Journal of Neuroscience*, vol. 20, no. 16, pp. 6048–6054, 2000.

[107] J. L. Billings, D. J. Hare, M. Nurjono et al., "Effects of neonatal iron feeding and chronic clioquinol administration on the parkinsonian human A53T transgenic mouse," *ACS Chemical Neuroscience*, vol. 7, no. 3, pp. 360–366, 2016.

[108] E. Carboni, L. Tatenhorst, L. Tönges et al., "Deferiprone rescues behavioral deficits induced by mild iron exposure in a mouse model of alpha-synuclein aggregation," *Neuromolecular Medicine*, vol. 19, no. 2-3, pp. 309–321, 2017.

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Review Article

The Signaling of Cellular Senescence in Diabetic Nephropathy

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Diabetic nephropathy is the leading cause of chronic kidney disease (CKD) in western countries. Notably, it has a rapidly rising prevalence in China. The patients, commonly complicated with cardiovascular diseases and neurologic disorders, are at high risk to progress into end-stage renal disease (ESRD) and death. However, the pathogenic mechanisms of diabetic nephropathy have not been determined. Cellular senescence, which recently has gained broad attention, is thought to be an important player in the onset and development of diabetic nephropathy. In this issue, we generally review the mechanisms of cellular senescence in diabetic nephropathy, which involve telomere attrition, DNA damage, epigenetic alterations, mitochondrial dysfunction, loss of Klotho, Wnt/β-catenin signaling activation, persistent inflammation, and accumulation of uremic toxins. Moreover, we highlight the potential therapeutic targets of cellular senescence in diabetic nephropathy and provide important clues for clinical strategies.

1. Introduction

Diabetic nephropathy (DN) has been the leading cause of CKD and renal failure in developed countries. In the past two decades, the morbidity and mortality of DN have been rising rapidly in the worldwide population [1–4]. Along with the kidney injury, diabetic patients often suffer from multiple complications, such as retinopathy, neuropathy, and cardiovascular diseases. All of them contribute to a high risk of death [5]. Besides health problems in patients, DN also leads to a heavy burden to the society.

Previous reports indicate that the mechanisms of DN involve a multifactorial interaction of metabolic and hemodynamic factors such as high blood glucose, advanced glycation end-products (AGEs), and the renin-angiotensin system (RAS). They further link to the activation of protein kinase C- (PKC-) induced generation of reactive oxygen species (ROS) [6, 7], which further mediates the activation of downstream transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). Thus, the main treatments of DN refer to modulate glycemic and blood pressure through insulin and RAS inhibitors. However, they could only delay the progression of DN but not prevent or cure it. Patients suffering from DN still inevitably reach the stage of ESRD at an alarming rate in both developed and developing countries [8–10]. Hence, the new pathogenic mechanisms except hyperglycemia and hypertension should be determined for a better management of DN. Recently, the emerging role of cellular senescence in DN has attracted a broad attention. However, a comprehensive elucidation has not yet been achieved. In the present review, we will focus on the role of cellular senescence and its related mechanisms in DN. Furthermore, we will explore the potential therapeutic targets of cellular senescence and provide important clues for clinical strategies in the management of DN.

2. DN and Renal Aging

The aging kidneys undergo a wide range of macrostructural changes, such as decreased cortical volume, increased surface roughness, and augmented numbers and sizes of cysts [11], which correspond to the typical microstructural features of glomerulosclerosis, tubular atrophy, interstitial fibrosis, and nephron loss [12]. Cellular aging or cellular senescence is

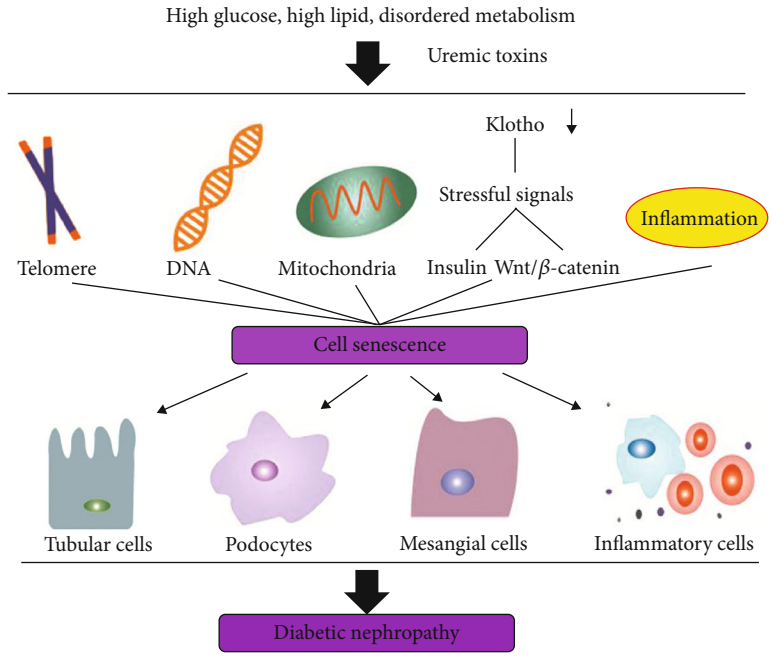

Figure 1: The cellular senescence in diabetic nephropathy.

the critical factor for the process of aging. Although the senescent cells remain viable, they show typical changes with enlarged and flattened cell bodies, apoptosis resistance, increased activity of senescence-associated β-galactosidase (SA-β-gal), and upregulation of cyclin-dependent kinase (CDK) inhibitors including $p16^{INK4A}$, ARF proteins, and p21 [13–16]. Furthermore, senescent cells, with the secretory features known as the senescence-associated secretory phenotype (SASP), could produce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein1 (MCP-1), to greatly affect the neighboring cells [17, 18].

Recent reports show that CKD presents as a clinical model of premature aging. Wang et al. introduced a new concept of CKD-associated secretory phenotype (CASP), which indicates that senescent renal cells could secrete SASP components of various cytokines such as IL-1, IL-6, and TNF-α [19, 20]. Other reports also show that DN is highly associated with accelerated aging in various types of cells such as tubular cells, podocytes, mesangial cells, and endothelial cells [21–23]. Notably, hyperglycemia could directly induce cellular senescence in mesangial [24] and tubular cells [13, 25, 26]. Interestingly, high glucose could also induce macrophages to secrete SASP components, thus promoting the development of a low-grade inflammatory state and cellular senescence [20].

Besides hyperglycemia, the production of AGEs and induction of oxidative stress, chronic persistent inflammation, glucose toxicity, and lipid metabolism disorder under DN disease conditions could cooperatively promote the growing microenvironment for senescent cells [27]. Conversely, senescent cells could accelerate the progression of disease. The studies [28] show a strong association between glomerular expression of $p16^{INK4A}$ and proteinuria. In addition, the excessive SA-β-gal activity and expression of $p16^{INK4A}$ in tubules are positively correlated with interstitial fibrosis, tubular cell atrophy lesions. Of note, tubular cell senescence is intimately associated with BMI and blood glucose level, implicating that controlling cellular senescence plays a critical role in the therapeutics of DN.

There are two main consequences from the accumulation of senescent cells. First, as one might expect, because of permanent cell cycle arrest, cellular senescence may cause a loss of self-repair capacity and regenerative ability [29–32]. These would lead to the exhaustion of renal cells as well as other progenitor or stem cells. A study shows that the number of endothelial progenitor cells is 30%–50% lower in patients with chronic kidney disease than that in healthy subjects [33]. There are a limited reservoir, decreased population, and low renewal efficiency of stem cells in DN-affected kidneys [34, 35], which would certainly accelerate the progression of disease. Second, senescent cells could produce proinflammatory and matrix-synthesizing cytokines, such as IL-6 and TGF-β. These SASP-associated molecules may cause persistent inflammation and fibrosis, as well as stem and renal cell renewal dysfunction in a paracrine and autocrine fashion [17, 36]. Collectively, cellular senescence participates in many pathological processes to accelerate the progression of DN. In this review, we discuss the role of cellular senescence in the pathogenesis of DN, highlight new findings on the mechanisms of cellular senescence (Figure 1), and propose the novel strategies to treat patients with DN by targeting cellular senescence.

3. The Mechanisms of Cellular Senescence in Diabetic Nephropathy

3.1. Telomere Attrition and Cellular Senescence. Telomeres are stretches of repetitive DNA, which are located at the end of each chromosome. Telomeres protect the chromosomes

from degradation or fusion [37, 38]. In repeated cell division, the length of a telomere may gradually shorten due to the lack of telomerase activity, an enzyme that helps to maintain the length of the chromosome.

Telomere shortening could occur in both type 1 and type 2 diabetes [39]. It can be accelerated by inflammation [40, 41], hyperglycemia, AGEs [42], and chronic oxidative stress [43], the main mechanisms of diabetes. Telomere attrition could trigger stress-induced premature senescence (SIPS). As early as 1999, it was found that telomere shortening is displayed in kidney diseases [44]. Consistently, recent studies show that high glucose induces accelerated senescence in proximal tubular cells, which is related to telomere shortening [28]. In both type 1 and type 2 diabetes, chromosomal telomere attrition is associated with renal cell senescence, proteinuria, and the progression of DN [45, 46]. After the prevention of telomere attrition, fenofibrate provides beneficial effects on the treatment of DN [39, 47]. The p53-p21-Rb signaling pathway is involved in the cellular senescence caused by telomere attrition under the condition of high glucose [48]. Besides the kidney parenchymal cells, the telomere of white blood cells in DN patients also displays the state of shortening. Although suggested as a biomarker in coronary heart disease [49], telomere shortening could also be applied in the diagnosis of DN. Supporting findings show that there is a high association between telomere length shortening and the progression of nephropathy through the mass population surveys [50].

3.2. DNA Damage and Cellular Senescence. DNA damage has been thought to be a main cause of cellular senescence since the late 1950s [51]. Besides the "wear and tear damage" during normal aging, there are various stress factors that could directly induce the damage of DNA, such as oxidative stress, ultraviolet (UV) or gamma irradiation [52, 53], chemotherapeutics [54], and hyperproliferation caused by the Ras oncogene [55]. Under diabetic conditions, hyperglycemia-induced generation of ROS and accumulation of AGEs may induce DNA damage and then trigger premature senescence in cells [56]. In type 1 diabetes rat models, AGEs and oxidative stress could induce DNA damage in both glomerular and tubular cells [57].

All of these DNA-damaged stressors could trigger a permanent DNA damage response (DDR) that leads to the activation of phosphatidylinositol 3 kinase-like kinases, such as ataxia telangiectasia-mutated (ATM) or ataxia telangiectasia-mutated and Rad3-related (ATR) kinases. These kinases subsequently activate P53 and its transcriptional target $p21^{CIP1/WAF1}$. In turn, $p21^{CIP1/WAF1}$ inhibits cyclin-dependent kinase 2- (CDK2-) mediated phosphorylation of the retinoblastoma protein (Rb). The hypophosphorylated Rb binds to the E2F transcription factor, preventing E2F through interacting with the transcription machinery. This process ultimately results in permanent cell cycle arrest, i.e., cellular senescence. This pathway is also called ATM/ATR-p21axis [23, 58]. Ultimately, senescent cells exhibit elevated levels of DNA damage response proteins 53BP1 and γH2AX and poor repair capacity for DNA strands. These could induce the restructure of the epigenome (see below), such as CpG methylation patterns [59].

3.3. Epigenetic Alterations and Cellular Senescence. Epigenetics refers to heritable alterations in gene expression and phenotype without involving changes in the DNA sequence. Epigenetic modifications include cytosine methylation of DNA (DNA methylation, DNAme), histone posttranslational modifications (PTMs), and noncoding RNAs [60–63]. Alterations in the epigenome underlie the dynamic switching of chromatin between a transcriptionally silent compact structure (heterochromatin) and an active relaxed structure (euchromatin) that cooperatively regulate gene expression [64].

DNAme occurs primarily, but not exclusively, at the sites of cytosine-guanine (CpG) dinucleotides. DNAme usually represses gene transcription through recruiting repressor complexes or precluding transcription factors [65]. However, the regulatory effects of DNAme can vary from gene repression to gene activation, which largely depends on the genomic contexts of the targeted sequences, such as promoters, gene bodies, enhancers, and repeated sequences [62]. PTMs mainly include histone lysine acetylation (HKAc) and histone lysine methylation (HKme). HKAc activates gene expression, while HKme activates or represses transcription depending on the lysine residue modified and the extent of methylation (mono-, di-, or trimethylation) [64, 65]. Other histone modifications consist of phosphorylation, sumoylation, ubiquitination, ADP-ribosylation, and O-GlcNAcylation [66]. They are also important but not well-studied.

Epigenetic changes could appear in the very early stage of DN. In 5-week-old db/db mice, the expression of Agt, an important component of the renin-angiotensin system, is upregulated by histone H3K9 acetylation [67, 68]. Similarly, the hypomethylation of CpG islands in Claudin-1 gene [67, 68] is also found at a very early stage in diabetes before the increase in albuminuria.

Increasingly, epigenetic changes are thought to be important in the development of DN through the induction of oxidative stress. In a rat model of diabetes, H3K27me at the enhancer site of zeste 2 repressive complex 2 subunit (EZH2) dampens the expression of the endogenous antioxidant inhibitor thioredoxin-interacting protein (TXNIP) via repressing the transcription factor PAX6. The inhibition of EZH2 augments proteinuria, podocytopathy, and renal oxidative stress, through the inhibition of glomerular TXNIP expression [69]. In addition, high glucose-mediated TXNIP expression is coordinated by histone acetylation and methylation in diabetic kidneys [70]. Similarly, in podocytes, high glucose could induce CpG promoter hypomethylation and histone H3 hyperacetylation through the activation of histone GCN5 acetyltransferase, which would drive the expression of p66Shc [71, 72], the key regulator of oxidative stress and player in lifespan shortening [73]. Furthermore, high glucose could also recruit Suv420H2 methyltransferase and LSD1 histone dimethyltransferase to the gene promoter of superoxide dismutase 2 (SOD2), resulting in the downregulation of SOD2, a strong endogenous antioxidant [74].

Epigenetic alterations also play important roles in persistent inflammation and autophagy deficiency, two main mechanisms of cellular senescence. In vascular endothelial cells [75–77] and inflammatory cells, high glucose induces

H3K4me1 activation through SET7 lysine methyltransferase to encode the proinflammatory factor NF-κB [78]. More and more studies show the important roles of noncoding RNAs in the development of DN. In db/db mice, miRNA-125b represses H3k9me3 through the downregulation of Suv39h1 histone methyltransferase, which leads to the upregulation of MCP-1 and IL-6, the two chemoattractants for the differentiation and migration of monocytes and lymphocytes [79]. Another noncoding RNA named miRNA-146a, a well-known anti-inflammatory microRNA, increases in the early stage of DN to protect against inflammation and renal fibrosis through inhibiting the activation of M1 macrophage [80]. Additionally, high glucose induces the activation of histone deacetyltransferase 4 (HDAC4) in podocytes, which leads to the deacetylation and silence of STAT1, a named gene in promoting autophagy to exert renoprotective function [81]. Furthermore, the administration of histone deacetylase (HDAC) inhibitor trichostatin A reduces kidney injury through alleviating the loss of Klotho, a well-known antiaging protein [82]. These studies further confirm the mediating role of epigenetic alterations in cellular senescence.

3.4. Mitochondrial Dysfunction and Cellular Senescence

3.4.1. Mitochondrial Reactive Oxygen Species (mtROS) and Mitochondrial Dysfunction. ROS can be both a trigger and an effector in the aging process and have been viewed as a cause of the aging process since 1996 [83]. Mitochondrial ROS (mtROS) could be driven by external stimuli, such as inflammatory cytokines, growth factors, or environmental toxins. The overproduction of mtROS increases in natural aging and age-related diseases [84]. The production of mtROS originates from the defects in the electron transport chain (ETC), which promotes electron leakage to form superoxide radicals, a key player in cellular senescence and accelerating aging [85]. The overproduced mtROS could damage protein, lipid, and DNA, which further trigger the pathological changes [86]. It is noteworthy that in diabetic nephropathy, the altered metabolic state requires extra demands of ATP, which would accelerate electron leakage to produce excessive ROS. The accumulative ROS in the mitochondrion has a detrimental effect in the integrity and content of mitochondrial DNA (mtDNA), which encodes the subunits of ETC complexes. This would further induce mitochondrial dysfunction and increase the production of ROS [87]. Notably, 30% of diabetic patients are suffering from mitochondrial dysfunction as well as reduced mitochondrial biogenesis, which is tightly associated with the severity of kidney diseases [88].

However, the exact role of ROS in cellular senescence is controversial. Although ROS is commonly considered as a cause of cellular senescence, recent findings show that it also extends Drosophila's lifespan, especially through respiratory complex I reverse electron transport [89]. Other reports also show that low levels of ROS could serve as second messengers to extent lifespan in *Caenorhabditis elegans* [90–92]. Moreover, chemical inhibition of glycolysis or exposure to metabolic poisons that block respiratory complex I (rotenone, paraquat, or piericidin A) or complex III (e.g., antimycin A) also prolong lifespan in *C. elegans* in a ROS-dependent manner [93]. Thus, the role of ROS homeostasis in cell aging under diabetic conditions may need more studies to confirm.

It is noteworthy that the production of mtROS could induce the mutations of mtDNA, which contributes to the aging process. This theory is confirmed by mitochondrial mutator mice, an early aging mice model expressing a proofreading-deficient DNA polymerase POLGγ. In those mice, the accumulated mutations of mtDNA significantly accelerate aging phenotype [94]. Recently, the evidences of mtDNA mutation are shown in DN patients. A study shows that mtDNA mutation G13997A in ND6 gene, a key gene encoding one of the subunits of respiration complex I (NADH dehydrogenase), positively correlates with the development of diabetes and related nephropathy [95]. However, some studies show that mutations of mtDNA are not major contributors to aging, especially in fruit flies [96]. The reason lies in that fruit flies are less sensitive to mtDNA mutations in adulthood than during development. Hence, more studies are needed to prove the correlation between mtDNA mutations and cellular senescence in renal cells.

3.4.2. Mitophagy Impairment and Early Senescence. Mitophagy plays an important role in preserving healthy mitochondria via the removal of altered mitochondria, clearance of protein aggregates. Mitophagy exerts protective functions in inhibiting apoptosis, reducing ROS production, and anti-inflammation [97–99]. It is reported under diabetic condition, more than 50% of renal tubular cells exhibit fragmented mitochondria [100], concomitant with the significant upregulation of mtROS in the renal cortex [101]. These suggest the loss of mitophagy in DN.

Mitophagy depends on the signaling cascade of kinases. The most important kinase is the PTEN-induced putative kinase 1 (PINK1). Upon damage, PINK1 transduces signals to the cytosolic E3 ubiquitin ligase Parkin. Parkin then amplifies the signals of mitophagy by facilitating PINK1-mediated recruitment of optineurin (OPTN) and NDP52 [102, 103]. Optineurin (OPTN) contains an ubiquitin-binding domain with the ability of binding polyubiquitinated cargo and transporting cargo to form autophagosomes [104]. The loss of expression in Parkin correlates with lifespan shortening [105], while the overexpression of Parkin extends longevity [106]. Similarly, the knockdown of PINK1 shortens lifespan and accelerates aging [89]. Interestingly, recent report shows that OPTN is involved in high glucose-induced senescence in renal tubular epithelial cells [25].

A large body of studies show that mitophagy is defective in diabetic kidneys [100], concomitant with mitochondrial abnormalities, overproduced mitochondrial ROS, and reduced expression of PINK and Parkin [107]. Indeed, podocytes show a high rate of baseline autophagy with aging. However, under diabetic status *in vivo* and high glucose conditions *in vitro* [108], the high basal level of autophagy in podocytes is flawed, which facilitates cell injury, glomerular damage, and the progression of kidney diseases. Hence, it can be presumed that in a diabetic setting, defects in mitophagy could induce early senescence in different renal cells and further promote the progression of kidney diseases.

3.5. Falling Levels of Klotho and Dysfunction of the Klotho-FGF-23 Axis. Klotho is an antiaging protein and is predominantly expressed in normal tubular cells. Klotho could act on multiple signals such as insulin and Wnts [109–111] and exerts important protection in kidney function [112]. The decline of Klotho could be seen in an early stage of kidney diseases, and this deficiency is linked to accelerated aging, cellular senescence, vascular calcification, and oxidative stress [113]. However, the underlying mechanisms remain poorly understood. Some reports show Klotho protects against cellular senescence through activating the forkhead transcription factor FOXO, a negative regulator of mtROS generation [114]. Furthermore, Klotho also has endogenous anti-inflammatory effects [115, 116] and antifibrotic properties [109, 117, 118]. Klotho also plays a role in mineral metabolism disorders, which would further affect renal aging. Klotho promotes calcium absorption and phosphate excretion in kidneys [119] and serves as a permissive coreceptor of fibroblast growth factor 23 (FGF-23), the hormone regulating phosphate and vitamin D [120]. This is called the Klotho-FGF-23 axis. The loss of Klotho promotes hyperphosphatemia, a risk factor of senescence process [121] and longevity [122].

Diminished expression of Klotho is a common feature of DN and is observed at the earliest stage of the disease [123, 124]. The loss of Klotho could be associated with multiple mechanisms such as hypermethylation of Klotho gene [125, 126], NF-κB-induced falling level of Klotho gene [127]. Recent studies show that increasing activity of integrin-linked kinase protein (ILK) reduces Klotho gene expression, which leads to cellular senescence in renal cells [128]. Large amounts of studies show that the loss of Klotho links to lifespan shortening, skin and muscle atrophy, osteoporosis, and calcification [129]. Under uremia condition, Klotho retards epithelial cell senescence through decreasing oxidative stress, NF-κB activity, etc. [118]. A survey in humans further confirms that the serum level of Klotho declines with age, and Klotho gene displays single nucleotide polymorphism which correlates with reduced longevity and the pathophysiology of age-related disorders [111].

3.6. Wnt/β-Catenin and Cellular Senescence. Despite being relatively silent in normal adult kidneys, Wnt/β-catenin signaling is reactivated in a wide range of chronic kidney diseases (CKD), such as diabetic nephropathy, obstructive nephropathy, adriamycin nephropathy (ADR), polycystic kidney disease, and chronic allograft nephropathy [130–133]. The canonical Wnt/β-catenin signaling involves β-catenin dephosphorylation in serine/threonine residues, which leads to its translocation to the nucleus, where it binds to transcription factor T-cell factor (TCF)/lymphoid-enhancer binding factor (LEF) to induce the expression of downstream target genes [134–136]. However, in diabetic status, the accumulated intracellular ROS might divert the limited pool of β-catenin from TCF/LEF to forkhead box O- (FOXO-) mediated transcription [137, 138] that leads to insulin deregulation. Notably, the latter plays a pivotal role in the aging process.

Recent evidence suggests that the renin-angiotensin-aldosterone system (RAS) is mediated by Wnt/β-catenin signaling. Zhou et al. reported that the promoter regions of all RAS genes contain putative TCF/LEF-binding sites, and β-catenin induces the binding of LEF-1 to these sites in renal tubular cells. Ectopic β-catenin causes the upregulation of all RAS genes [139]. Notably, RAS activation contributes to renal aging through various mechanisms. Several studies discover that angiotensin II, a key substance of RAS, can induce senescence in renal cells and lead to the development and progression of age-related diseases [140–143]. Ang II induces premature senescence via both STAT3/mTOR-regulated autophagy and the p53/p21 pathway [140], which further drives fibrosis and redox state [144]. Notably, ROS can also induce the expression of $p16^{INK4A}$, a trigger in cell cycle arrest and senescent phenotype, and activate TGF-β1 and NF-κB signaling that could trigger inflammatory reaction in accelerated aging process [145]. Hence, RAS antagonism through administration of ACEI/ARB improves mitochondrial function and exerts antioxidative effects and displays age-retarding benefits [146].

Recently, Luo et al. reported that Wnt9a has a decisive role in driving tubular senescence and renal fibrosis, as well as evoking cell communication between senescent tubular cells and interstitial fibroblasts [147]. Although not studied in their studies, RAS activation is supposed to play a role in Wnt9a-induced cellular senescence. Supporting the presumption, several studies point out the benefits of inhibiting the RAS system in organ aging process. The related mechanisms involve the upregulation of prosurvival nicotinamide phosphoribosyltransferase gene (Nampt) and downregulation of $p16^{INK4A}$ expression [148, 149], as well as the improvement of mitochondrial function [150–152].

3.7. Inflammation and Cellular Senescence. Chronic inflammation is a pathological feature of various CKD. Notably, inflammation serves as an important factor for accelerated aging. Unregulated inflammation has a key role in the pathogenesis and progression of autoimmune diseases such as presenile dementia, osteoporosis, and atherosclerosis [153]. Inflammation is also involved in the pathogenesis of obesity and diabetes and serves as an important mediator of aging [154, 155]. In addition, the senescence-associated decline of the adaptive immune system (immunosenescence) may further aggravate aging phenotypes at the systemic level because of the impaired immune surveillance [156, 157]. Moreover, senescent cells can secrete many proinflammatory factors, such as TNF-α, IL-6, PAI-1, and MCP-1, which may further aggravate inflammation [17, 157]. Recent studies show that AUF1, the mRNA decay factor, links inflammation and aging [158]. Deficiency of AUF1 induces marked cellular senescence and premature aging phenotype.

In diabetic nephropathy, the prominent inflammation is observed even in the beginning and ongoing stage of kidney injury. The upregulation of systemic and local renal inflammation occurs in the early stage of DN. In diabetic status, high glucose, AGEs, and oxidative stress could simultaneously induce the activation of NF-κB, a known transcriptional signature of inflammation [18]. Through recruiting p300, NF-κB triggers the activation of downstream effector iNOS, a promoter of oxidative stress and inflammation that

causes extensive nitrotyrosine (NT) in proteins [159]. Genetic and pharmacological inhibition of NF-κB signaling prevent age-associated diseases [160–162] including diabetic nephropathy [159] and natural aging [163, 164].

Additionally, Nod-like receptor 3 (NLRP3) inflammasome also plays a role in the development of inflammation under diabetic setting [165]. In response to diverse damage-associated molecular patterns (DAMPs) in aging, such as excess glucose, ceramides, amyloids, urate, and cholesterol crystals, NLRP3 inflammasome is intimately correlated with age-related diseases [166]. The NLRP3 inflammasome could activate caspase-1, which stimulates the maturation and secretion of IL-1β and IL-18 through cleaving their precursors [167]. Consequently, these proinflammatory cytokines accelerate the aging process through inhibiting autophagy [168]. Conversely, the impaired autophagy could trigger the accumulation of inflammasome to create a reciprocal activation loop [169]. Interestingly, inflammation could also interplay with many other mechanisms of cellular senescence such as telomere shortening, progressive DNA damage, oxidative stress, and altered epigenetics. These create an intricate network to induce cellular senescence in diabetic nephropathy.

3.8. Uremic Toxins and Cellular Senescence. Uremic toxins are endogenous waste products of metabolism. They are cleared predominantly by the kidneys. Three subgroups of uremic wastes are classified: (i) small water-soluble molecules (MW < 500 Da) such as urea, creatinine, and phosphate; (ii) middle (MW500~5000 Da) and large molecules (MW > 5000 Da) such as parathyroid hormone (PTH), IL-6, fibroblast growth factor 23 (FGF23), AGEs, advanced oxidation protein products (AOPPs), and other peptides; and (iii) small molecules (MW < 500 Da) but with high protein-binding abilities such as indoles, phenols, polyamine, and cresols [170]. Notably, the regular hemodialysis could only remove small molecules with the molecular weight lower than 500 Da. Furthermore, it is also difficult to remove protein-bound uremic toxins such as indoxyl sulfate (IS), p-cresyl sulfate (PCS), and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), due to their high protein-binding capability (90%) to plasma proteins [171, 172]. It is notable that the imbalance of gut microbiome in kidney diseases largely contributes to the formation and retention of uremic toxins [173], which would create a reciprocal activation loop that accelerates the progression of kidney diseases.

Uremic toxins could trigger senescence in various types of renal cells such as the proximal tubular cells [174, 175] and endothelial cells [176] through multiple mechanisms. The most commonly studied pathway is oxidative stress-induced NF-κB signaling [177]. However, other mechanisms are also contributed. Some reports show that uremic wastes could induce mitochondrial dysfunction [178] and hypermethylation in Klotho gene [125], the two main mechanisms of cellular senescence in DN. As the well-known uremic toxins in DN, AGEs account for various mechanisms of cellular senescence. Although their detrimental role is firstly found in the longevity of C. elegans [179, 180], AGEs show the active role in promoting senescence phenotype in multiple organ systems including the kidneys in humans [181]. Studies show that AGEs trigger cellular senescence via oxidative stress-dependent p21 activation [182] and p16 expression [13, 183], inhibition of autophagy [184] through reducing PINK1/Parkin [185], and promotion of inflammation [186] in renal cells and others.

Taken together, the accumulation of uremic toxins would influence cellular senescence nearly in all aspects of mechanisms, which cooperatively and reciprocally promote and accelerate cellular senescence that contributes to the pathogenesis of DN.

4. Therapeutic Potentials

The therapeutic methods for DN nowadays refer to anti-RAS therapy using ACE inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) and glucose control. However, anti-RAS therapy only displays limited efficacy, partly because of the compensatory upregulation of renin expression [187–189]. Due to the metabolic memory of prior exposure to hyperglycemia, a single control of glucose fails to prevent the progression of kidney disease [190, 191]. Notably, several theoretical approaches might be applicable to target the aging process in DN (Figure 2).

It is well known that calorie restriction (CR) reduces oxidative stress and proinflammatory injury. In addition, CR modulates mitochondrial activity and increases the autophagy activity, thereby extending health and lifespan [192–194]. It has been shown that CR protects against cellular senescence through decreasing the expression of $p16^{INK4A}$. Consequently, renal fibrosis is alleviated [192]. Actually, CR inhibits cell senescence through various mechanisms. The most studied is the mammalian target of rapamycin (mTOR) signaling. CR could deactivate mTOR through activating AMP-activated protein kinase (AMPK) [195]. Several kinds of drugs targeting this pathway are effective in the retardation of aging and age-related disease. Rapamycin, the mTOR inhibitor, could protect cellular senescence [196] and phosphate-induced premature aging [197]. While AMPK activator metformin reduces ROS production in podocytes and prevents DN [145]. Interestingly, long-term treatment with rapamycin may improve the quality of mtDNA in aging mice [198]. However, long-term CR has a long way to achieve for clinical practice, especially in DN patients because of the limited daily nutrition. Another promising drug is sirtuin1 (SIRT1) agonist. As a NAD^+-dependent deacetylase, SIRT1 plays an important role in the aging process [199] and age-related phenotypes such as DN [200]. SIRT1 could be induced by CR. The agonists of SIRT1 such as BF175 and resveratrol could greatly ameliorate the pathogenesis of diabetic kidney disease [201]. And BF175 also shows less renal toxicity, suggesting the good prospects.

Theoretically, antioxidants can mitigate ROS-induced damage, such as DNA mutations and protein modifications, as well as delay telomere shortening. However, the advantages of antioxidants are needed to be further clarified in aging-associated diseases [202–205]. Chlorogenic acid could attenuate oxidative stress and inflammation in diabetic nephropathy, possibly through modulating Nrf2/HO-1 and

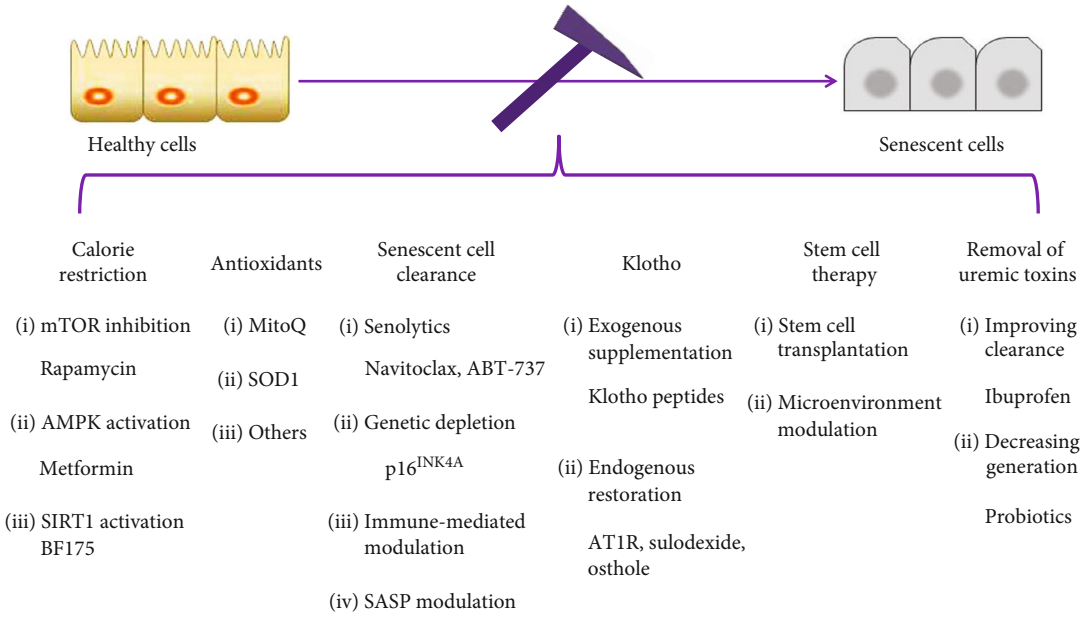

Figure 2: Therapeutic potentials against cellular senescence in diabetic nephropathy.

NF-κB pathways. However, no direct evidences show its effects on aging retardation under the diabetic setting [206]. Oppositely, the administration of mitochondria-targeted antioxidant MitoQ could dramatically ameliorate renal tubular injury in a diabetic mouse model. MitoQ reverses mitophagy through increasing the expression of PINK1 and Parkin [107]. Another important study shows that Cu/Zn-superoxide dismutase 1 (SOD1), a strong endogenous antioxidant, has a new role in aging. Deficiency of SOD1 prolongs lifespan and retards the process of cellular senescence [207]. Overexpression of SOD1 attenuates high glucose-induced endothelial cell senescence [17]. These suggest that SOD1 may act as a promising therapeutic in DN through retarding aging. However, because of side-effects such as tumor genesis and difficulties to control the degree of antioxidation, the reasonable application should be closely noticed in therapy for patients.

Another strategy to retard cellular senescence is the removal of these cells [208] by senolytics, a new kind of drugs with the ability to slow the aging process. Many of these agents could induce the clearance of senescent cells through upregulation of antiapoptosis systems such as the BCL-2 family of proteins (BCL-2, BCL-XL, and BCL-W) [209, 210]. The new senolytic molecules navitoclax and ABT-737 could occupy the inhibitory binding grooves of BCL-2, BCL-XL, and BCL-W, which counteract their antiapoptotic functions and initiate apoptosis in senescent cells [211]. Furthermore, immune surveillance [212] and genetic deletion of senescence-associated factor $p16^{INK4a}$ [213] could also help to enhance the removal of senescent cells.

SASP modulation has also been proposed as a way to slow the aging process. SASP blockade would be another attractive treatment option [214, 215]. However, the interventions of proinflammatory pathways such as NF-κB, p38-MAPK, or MAPK-activated protein kinase 2 could also influence the communications among healthy cells [216]. Notably, a recent finding shows that bromodomain-containing protein 4 (BRD4) inhibitors could modulate SASP with high specificity in senescent cells without influencing healthy subjects [217]. In histones, BRD4 binds to acetylated lysines, which results in the opening of chromatin and the activation of SASP process [217]. BRD4 inhibitors JQ1 and iBET are recently studied and found to be prospective in therapeutic of aging [218].

Restoration of endogenous Klotho or supplementation with exogenous Klotho could be a substitute therapeutic strategy for antiaging. Klotho could block Wnt/β-catenin signaling and its targets such as the renin-angiotensin system (RAS) and modulate the homeostasis of blood phosphate and vitamin D [139, 219]. In the aging process, Klotho gene displays hypermethylation [126]. Notably, Ang II type 1 receptor antagonist (AT1R) losartan could alter the post-translational modifications on histones [220], suggesting a protective role in Klotho gene stability. In addition, some drugs such as sulodexide and osthole may enhance the expression of Klotho to prevent the progression of DN [221]. Considering the side-effects and toxicity of these molecular compounds, the direct supplement of Klotho may be the best way to provide renoprotecion. However, the clinical application of direct Klotho supplement is very limited because Klotho protein is with large molecular weight and complex structure. It is still promising that scientists are developing the small molecular peptides from the binding sites of Klotho with Wnt3a and other Wnts [222], suggesting the prospects for future clinical application.

The latest topics of therapeutics in aging are stem cell therapy [223]. A study shows that the injection of plasma from young mice into the circulation of aged mice induces a more youthful state [224]. Furthermore, early-in-life genetic manipulations could preserve the proliferative ability of the gut stem cells, which leads to lifespan extension [225]. In kidney disease, the supplement of mesenchymal stem cells

(MSC) could prompt functional recovery [226]. Even in aged stem cells, incubation of young blood could still ameliorate the functional deficits [227, 228], suggesting the important role of microenvironment in stem cell renewal. Other studies also show that human umbilical cord-derived mesenchymal stromal cells could ameliorate renal fibrosis and cellular senescence and interestingly increase the expression of Klotho [229]. Regenerative therapies by exogenous stem cell transplantation into damaged tissues could improve natural aging and stress-induced premature senescence (SIPS) [230]. However, the stem cell transplantation in humans is more challenging because of the unpredicted disadvantages such as tumor genesis and needs to be demonstrated repeatedly.

Removal of uremic toxins may be a fundamental resolution strategy for retarding cellular senescence in DN. Some researchers are focusing on the improvement of protein-bound uremic waste clearance through intravenous lipid emulsion [231] or infusion of a binding competitor ibuprofen [232] in conventional dialysis therapies. Although these strategies present benefits, they are still in the primary stage of research. Compared with the controlling in the clearance, to decrease the generation of uremic wastes through the modulation of beneficial gut microbiota members may be more important [233, 234]. It is proved in long-term safety and presents improved removal of BUN when probiotics is supplemented to the therapies for CKD patients [235]. Furthermore, synbiotics, resistant starch, and other dietary fiber could also help to decrease the production of uremic toxins through modifying gut microbial dysbiosis in CKD patients [236], thereby ameliorating oxidative stress and inflammation [237, 238]. However, the formulation of a probiotic bacteria and the detailed therapies in clinic need to be further studied, and the safety is also an important question to be resolved.

5. Conclusion

In summary, we show the multiple mechanisms underscoring cell senescence network in diabetic nephropathy, which involve telomere shortening, DNA damage, epigenetic alterations, mitophagy deficiency, loss of Klotho, Wnt/β-catenin signaling activation, inflammation, and the accumulation of uremic toxins. These factors mutually affect and cooperatively promote cellular senescence in DN. Although some therapeutic methods are promising, the practical application in DN patients needs to be testified by large amounts of animal experiments and preclinical investigations. Notably, the long-term observation of antiaging therapy such as senolytics and stem cell transplantation could not be neglected. Recently, genome editing is attracting the notice of scientists [239, 240]. However, although with the prospective aspects, genome editing would bring out the problems of ethics in humanity. This should be particularly focused. Last, precision medicine therapy for individual DN patient should be considered in the therapeutic strategies of nephrologists. Nevertheless, targeted inhibition of cellular senescence provides important clues for clinical strategies for diabetic nephropathy.

Conflicts of Interest

The authors have no conflict of interests in this paper.

Acknowledgments

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References

[1] S. Bell, E. H. Fletcher, I. Brady et al., "End-stage renal disease and survival in people with diabetes: a national database linkage study," *QJM*, vol. 108, no. 2, pp. 127–134, 2015.

[2] M. Kato and R. Natarajan, "Diabetic nephropathy—emerging epigenetic mechanisms," *Nature Reviews Nephrology*, vol. 10, no. 9, pp. 517–530, 2014.

[3] World Health Organization, *Diabetes—Fact Sheet No. 312*, World Health Organization, Geneva, 2015.

[4] H. J. L. Heerspink, H. H. Parving, D. L. Andress et al., "Atrasentan and renal events in patients with type 2 diabetes and chronic kidney disease (SONAR): a double-blind, randomised, placebo-controlled trial," *Lancet*, vol. 393, no. 10184, pp. 1937–1947, 2019.

[5] S. Rao Kondapally Seshasai, S. Kaptoge, A. Thompson et al., "Diabetes mellitus, fasting glucose, and risk of cause-specific death," *New England Journal of Medicine*, vol. 364, no. 9, pp. 829–841, 2011.

[6] Z. Cao and M. E. Cooper, "Pathogenesis of diabetic nephropathy," *Journal of Diabetes Investigation*, vol. 2, no. 4, pp. 243–247, 2011.

[7] A. Kumar Pasupulati, P. S. Chitra, and G. B. Reddy, "Advanced glycation end products mediated cellular and molecular events in the pathology of diabetic nephropathy," *Biomolecular Concepts*, vol. 7, no. 5-6, pp. 293–309, 2016.

[8] M. E. Cooper, "Diabetes: treating diabetic nephropathy-still an unresolved issue," *Nature Reviews. Endocrinology*, vol. 8, no. 9, pp. 515-516, 2012.

[9] G. C. W. Chan and S. C. W. Tang, "Diabetic nephropathy: landmark clinical trials and tribulations," *Nephrology, Dialysis, Transplantation*, vol. 31, no. 3, pp. 359–368, 2016.

[10] C. Mora-Fernández, V. Domínguez-Pimentel, M. M. de Fuentes, J. L. Górriz, A. Martínez-Castelao, and J. F. Navarro-González, "Diabetic kidney disease: from physiology to therapeutics," *The Journal of Physiology*, vol. 592, no. 18, pp. 3997–4012, 2014.

[11] M. S. Hommos, R. J. Glassock, and A. D. Rule, "Structural and functional changes in human kidneys with healthy aging," *Journal of the American Society of Nephrology*, vol. 28, no. 10, pp. 2838–2844, 2017.

[12] A. D. Rule, H. Amer, L. D. Cornell et al., "The association between age and nephrosclerosis on renal biopsy among healthy adults," *Annals of Internal Medicine*, vol. 152, no. 9, pp. 561–567, 2010.

[13] J. Liu, J. R. Yang, X. M. Chen, G. Y. Cai, L. R. Lin, and Y. N. He, "Impact of ER stress-regulated ATF4/p16 signaling on

the premature senescence of renal tubular epithelial cells in diabetic nephropathy," *American Journal of Physiology. Cell Physiology*, vol. 308, no. 8, pp. C621–C630, 2015.

[14] Y. Marmary, R. Adar, S. Gaska et al., "Radiation-induced loss of salivary gland function is driven by cellular senescence and prevented by IL6 modulation," *Cancer Research*, vol. 76, no. 5, pp. 1170–1180, 2016.

[15] E. A. Georgakopoulou, K. Tsimaratou, K. Evangelou et al., "Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues," *Aging*, vol. 5, no. 1, pp. 37–50, 2012.

[16] G. P. Dimri, X. Lee, G. Basile et al., "A biomarker that identifies senescent human cells in culture and in aging skin in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 20, pp. 9363–9367, 1995.

[17] T. Tchkonia, Y. Zhu, J. van Deursen, J. Campisi, and J. L. Kirkland, "Cellular senescence and the senescent secretory phenotype: therapeutic opportunities," *The Journal of Clinical Investigation*, vol. 123, no. 3, pp. 966–972, 2013.

[18] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, pp. 1194–1217, 2013.

[19] W. J. Wang, G. Y. Cai, and X. M. Chen, "Cellular senescence, senescence-associated secretory phenotype, and chronic kidney disease," *Oncotarget*, vol. 8, no. 38, pp. 64520–64533, 2017.

[20] F. Prattichizzo, V. de Nigris, E. Mancuso et al., "Short-term sustained hyperglycaemia fosters an archetypal senescence-associated secretory phenotype in endothelial cells and macrophages," *Redox Biology*, vol. 15, pp. 170–181, 2018.

[21] N. D'Onofrio, L. Servillo, A. Giovane et al., "Ergothioneine oxidation in the protection against high-glucose induced endothelial senescence: involvement of SIRT1 and SIRT6," *Free Radical Biology & Medicine*, vol. 96, pp. 211–222, 2016.

[22] K. Kitada, D. Nakano, H. Ohsaki et al., "Hyperglycemia causes cellular senescence via a SGLT2- and p21-dependent pathway in proximal tubules in the early stage of diabetic nephropathy," *Journal of Diabetes and its Complications*, vol. 28, no. 5, pp. 604–611, 2014.

[23] J. M. van Deursen, "The role of senescent cells in ageing," *Nature*, vol. 509, no. 7501, pp. 439–446, 2014.

[24] X. Zhang, X. Chen, D. Wu et al., "Downregulation of connexin 43 expression by high glucose induces senescence in glomerular mesangial cells," *Journal of the American Society of Nephrology*, vol. 17, no. 6, pp. 1532–1542, 2006.

[25] K. Chen, H. Dai, J. Yuan et al., "Optineurin-mediated mitophagy protects renal tubular epithelial cells against accelerated senescence in diabetic nephropathy," *Cell Death & Disease*, vol. 9, no. 2, p. 105, 2018.

[26] B. Fu, J. Yang, J. Chen et al., "Preventive effect of Shenkang injection against high glucose-induced senescence of renal tubular cells," *Frontiers in Medicine*, vol. 13, no. 2, pp. 267–276, 2019.

[27] Y. Tamura, K. Takubo, J. Aida, A. Araki, and H. Ito, "Telomere attrition and diabetes mellitus," *Geriatrics & Gerontology International*, vol. 16, Supplement 1, pp. 66–74, 2016.

[28] D. Verzola, M. T. Gandolfo, G. Gaetani et al., "Accelerated senescence in the kidneys of patients with type 2 diabetic nephropathy," *American Journal of Physiology. Renal Physiology*, vol. 295, no. 5, pp. F1563–F1573, 2008.

[29] J. D. Bernet, J. D. Doles, J. K. Hall, K. Kelly Tanaka, T. A. Carter, and B. B. Olwin, "p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice," *Nature Medicine*, vol. 20, no. 3, pp. 265–271, 2014.

[30] B. D. Cosgrove, P. M. Gilbert, E. Porpiglia et al., "Rejuvenation of the muscle stem cell population restores strength to injured aged muscles," *Nature Medicine*, vol. 20, no. 3, pp. 255–264, 2014.

[31] L. García-Prat, M. Martínez-Vicente, E. Perdiguero et al., "Autophagy maintains stemness by preventing senescence," *Nature*, vol. 529, no. 7584, pp. 37–42, 2016.

[32] A. R. Choudhury, Z. Ju, M. W. Djojosubroto et al., "Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation," *Nature Genetics*, vol. 39, no. 1, pp. 99–105, 2007.

[33] A. R. Chade, X. Y. Zhu, J. D. Krier et al., "Endothelial progenitor cells homing and renal repair in experimental renovascular disease," *Stem Cells*, vol. 28, no. 6, pp. 1039–1047, 2010.

[34] J. Bartek and Z. Hodny, "Old blood stem cells feel the stress," *Nature*, vol. 512, no. 7513, pp. 140-141, 2014.

[35] T. Niccoli and L. Partridge, "Ageing as a risk factor for disease," *Current Biology*, vol. 22, no. 17, pp. R741–R752, 2012.

[36] Y. Ovadya and V. Krizhanovsky, "Senescent cells: SASPected drivers of age-related pathologies," *Biogerontology*, vol. 15, no. 6, pp. 627–642, 2014.

[37] L. M. Colgin, K. Baran, P. Baumann, T. R. Cech, and R. R. Reddel, "Human POT1 facilitates telomere elongation by telomerase," *Current Biology*, vol. 13, no. 11, pp. 942–946, 2003.

[38] E. H. Blackburn, "Switching and signaling at the telomere," *Cell*, vol. 106, no. 6, pp. 661–673, 2001.

[39] S. S. I. Sutanto, S. V. McLennan, A. C. Keech, and S. M. Twigg, "Shortening of telomere length by metabolic factors in diabetes: protective effects of fenofibrate," *Journal of Cell Communication and Signaling*, 2019.

[40] A. O'Donovan, M. S. Pantell, E. Puterman et al., "Cumulative inflammatory load is associated with short leukocyte telomere length in the Health, Aging and Body Composition Study," *PLoS One*, vol. 6, no. 5, article e19687, 2011.

[41] J. J. Carrero, P. Stenvinkel, B. Fellström et al., "Telomere attrition is associated with inflammation, low fetuin-A levels and high mortality in prevalent haemodialysis patients," *Journal of Internal Medicine*, vol. 263, no. 3, pp. 302–312, 2008.

[42] J. M. J. Houben, H. J. J. Moonen, F. J. van Schooten, and G. J. Hageman, "Telomere length assessment: biomarker of chronic oxidative stress?," *Free Radical Biology & Medicine*, vol. 44, no. 3, pp. 235–246, 2008.

[43] D. J. Kurz, S. Decary, Y. Hong, E. Trivier, A. Akhmedov, and J. D. Erusalimsky, "Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells," *Journal of Cell Science*, vol. 117, no. 11, pp. 2417–2426, 2004.

[44] B. J. Jennings, S. E. Ozanne, M. W. Dorling, and C. N. Hales, "Early growth determines longevity in male rats and may be related to telomere shortening in the kidney," *FEBS Letters*, vol. 448, no. 1, pp. 4–8, 1999.

[45] F. Fyhrquist, A. Tiitu, O. Saijonmaa, C. Forsblom, P. H. Groop, and on behalf of the FinnDiane Study Group, "Telomere length and progression of diabetic nephropathy in patients with type 1 diabetes," *Journal of Internal Medicine*, vol. 267, no. 3, pp. 278–286, 2010.

[46] M. J. Sampson and D. A. Hughes, "Chromosomal telomere attrition as a mechanism for the increased risk of epithelial cancers and senescent phenotypes in type 2 diabetes," *Diabetologia*, vol. 49, no. 8, pp. 1726–1731, 2006.

[47] J. E. Noonan, A. J. Jenkins, J. X. Ma, A. C. Keech, J. J. Wang, and E. L. Lamoureux, "An update on the molecular actions of fenofibrate and its clinical effects on diabetic retinopathy and other microvascular end points in patients with diabetes," *Diabetes*, vol. 62, no. 12, pp. 3968–3975, 2013.

[48] D. W. Cao, C. M. Jiang, C. Wan et al., "Upregulation of MiR-126 delays the senescence of human glomerular mesangial cells induced by high glucose via telomere-p53-p21-Rb signaling pathway," *Current Medical Science*, vol. 38, no. 5, pp. 758–764, 2018.

[49] J. Bhattacharyya, K. Mihara, D. Bhattacharjee, and M. Mukherjee, "Telomere length as a potential biomarker of coronary artery disease," *The Indian Journal of Medical Research*, vol. 145, no. 6, pp. 730–737, 2017.

[50] M. Mazidi, P. Rezaie, A. Covic et al., "Telomere attrition, kidney function, and prevalent chronic kidney disease in the United States," *Oncotarget*, vol. 8, no. 46, pp. 80175–80181, 2017.

[51] G. Failla, "The aging process and cancerogenesis," *Annals of the New York Academy of Sciences*, vol. 71, no. 6, pp. 1124–1140, 1958.

[52] E. Shaulian, M. Schreiber, F. Piu, M. Beeche, E. F. Wagner, and M. Karin, "The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest," *Cell*, vol. 103, no. 6, pp. 897–908, 2000.

[53] K. Webley, J. A. Bond, C. J. Jones et al., "Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage," *Molecular and Cellular Biology*, vol. 20, no. 8, pp. 2803–2808, 2000.

[54] H. Ge, S. Ni, X. Wang et al., "Dexamethasone reduces sensitivity to cisplatin by blunting p53-dependent cellular senescence in non-small cell lung cancer," *PLoS One*, vol. 7, no. 12, article e51821, 2012.

[55] R. Di Micco, M. Fumagalli, A. Cicalese et al., "Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication," *Nature*, vol. 444, no. 7119, pp. 638–642, 2006.

[56] H. Lans and J. H. Hoeijmakers, "Genome stability, progressive kidney failure and aging," *Nature Genetics*, vol. 44, no. 8, pp. 836–838, 2012.

[57] H. Al-Hussaini and N. Kilarkaje, "Trans-resveratrol mitigates type 1 diabetes-induced oxidative DNA damage and accumulation of advanced glycation end products in glomeruli and tubules of rat kidneys," *Toxicology and Applied Pharmacology*, vol. 339, pp. 97–109, 2018.

[58] J. Campisi, "Aging, cellular senescence, and cancer," *Annual Review of Physiology*, vol. 75, no. 1, pp. 685–705, 2013.

[59] S. Horvath, "DNA methylation age of human tissues and cell types," *Genome Biology*, vol. 14, no. 10, p. R115, 2013.

[60] T. Kouzarides, "Chromatin modifications and their function," *Cell*, vol. 128, no. 4, pp. 693–705, 2007.

[61] A. Portela and M. Esteller, "Epigenetic modifications and human disease," *Nature Biotechnology*, vol. 28, no. 10, pp. 1057–1068, 2010.

[62] P. A. Jones, "Functions of DNA methylation: islands, start sites, gene bodies and beyond," *Nature Reviews. Genetics*, vol. 13, no. 7, pp. 484–492, 2012.

[63] V. J. Peschansky and C. Wahlestedt, "Non-coding RNAs as direct and indirect modulators of epigenetic regulation," *Epigenetics*, vol. 9, no. 1, pp. 3–12, 2014.

[64] B. D. Strahl and C. D. Allis, "The language of covalent histone modifications," *Nature*, vol. 403, no. 6765, pp. 41–45, 2000.

[65] A. P. Bird and A. P. Wolffe, "Methylation-induced repression–belts, braces, and chromatin," *Cell*, vol. 99, no. 5, pp. 451–454, 1999.

[66] V. W. Zhou, A. Goren, and B. E. Bernstein, "Charting histone modifications and the functional organization of mammalian genomes," *Nature Reviews. Genetics*, vol. 12, no. 1, pp. 7–18, 2011.

[67] T. Marumo, S. Yagi, W. Kawarazaki et al., "Diabetes induces aberrant DNA methylation in the proximal tubules of the kidney," *Journal of the American Society of Nephrology*, vol. 26, no. 10, pp. 2388–2397, 2015.

[68] K. Hasegawa, S. Wakino, P. Simic et al., "Renal tubular Sirt1 attenuates diabetic albuminuria by epigenetically suppressing Claudin-1 overexpression in podocytes," *Nature Medicine*, vol. 19, no. 11, pp. 1496–1504, 2013.

[69] F. S. Siddiqi, S. Majumder, K. Thai et al., "The histone methyltransferase enzyme enhancer of zeste homolog 2 protects against podocyte oxidative stress and renal injury in diabetes," *Journal of the American Society of Nephrology*, vol. 27, no. 7, pp. 2021–2034, 2016.

[70] Y. de Marinis, M. Cai, P. Bompada et al., "Epigenetic regulation of the thioredoxin-interacting protein (TXNIP) gene by hyperglycemia in kidney," *Kidney International*, vol. 89, no. 2, pp. 342–353, 2016.

[71] F. Paneni, P. Mocharla, A. Akhmedov et al., "Gene silencing of the mitochondrial adaptor $p66^{Shc}$ suppresses vascular hyperglycemic memory in diabetes," *Circulation Research*, vol. 111, no. 3, pp. 278–289, 2012.

[72] F. Bock, K. Shahzad, H. Wang et al., "Activated protein C ameliorates diabetic nephropathy by epigenetically inhibiting the redox enzyme $p66^{Shc}$," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 2, pp. 648–653, 2013.

[73] M. Giorgio, E. Migliaccio, F. Orsini et al., "Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis," *Cell*, vol. 122, no. 2, pp. 221–233, 2005.

[74] Q. Zhong and R. A. Kowluru, "Epigenetic modification of Sod2 in the development of diabetic retinopathy and in the metabolic memory: role of histone methylation," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 1, pp. 244–250, 2013.

[75] A. el-Osta, D. Brasacchio, D. Yao et al., "Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia," *The Journal of Experimental Medicine*, vol. 205, no. 10, pp. 2409–2417, 2008.

[76] D. Brasacchio, J. Okabe, C. Tikellis et al., "Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail," *Diabetes*, vol. 58, no. 5, pp. 1229–1236, 2009.

[77] J. Okabe, C. Orlowski, A. Balcerczyk et al., "Distinguishing hyperglycemic changes by Set7 in vascular endothelial cells," *Circulation Research*, vol. 110, no. 8, pp. 1067–1076, 2012.

[78] F. Paneni, S. Costantino, R. Battista et al., "Adverse epigenetic signatures by histone methyltransferase Set7 contribute to vascular dysfunction in patients with type 2 diabetes mellitus," *Circulation. Cardiovascular Genetics*, vol. 8, no. 1, pp. 150–158, 2015.

[79] L. M. Villeneuve, M. Kato, M. A. Reddy, M. Wang, L. Lanting, and R. Natarajan, "Enhanced levels of microRNA-125b in vascular smooth muscle cells of diabetic db/db mice lead to increased inflammatory gene expression by targeting the histone methyltransferase Suv39h1," *Diabetes*, vol. 59, no. 11, pp. 2904–2915, 2010.

[80] K. Bhatt, L. L. Lanting, Y. Jia et al., "Anti-inflammatory role of microRNA-146a in the pathogenesis of diabetic nephropathy," *Journal of the American Society of Nephrology*, vol. 27, no. 8, pp. 2277–2288, 2016.

[81] X. Wang, J. Liu, J. Zhen et al., "Histone deacetylase 4 selectively contributes to podocyte injury in diabetic nephropathy," *Kidney International*, vol. 86, no. 4, pp. 712–725, 2014.

[82] W. Lin, Q. Zhang, L. Liu, S. Yin, Z. Liu, and W. Cao, "Klotho restoration via acetylation of peroxisome proliferation–activated receptor γ reduces the progression of chronic kidney disease," *Kidney International*, vol. 92, no. 3, pp. 669–679, 2017.

[83] M. J. Forster, A. Dubey, K. M. Dawson, W. A. Stutts, H. Lal, and R. S. Sohal, "Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 10, pp. 4765–4769, 1996.

[84] V. A. Morais, D. Haddad, K. Craessaerts et al., "PINK1 loss-of-function mutations affect mitochondrial complex I activity via NdufA10 ubiquinone uncoupling," *Science*, vol. 344, no. 6180, pp. 203–207, 2014.

[85] G. Barja, "Updating the mitochondrial free radical theory of aging: an integrated view, key aspects, and confounding concepts," *Antioxidants & Redox Signaling*, vol. 19, no. 12, pp. 1420–1445, 2013.

[86] K. E. Conley, D. J. Marcinek, and J. Villarin, "Mitochondrial dysfunction and age," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 10, no. 6, pp. 688–692, 2007.

[87] D. Edgar, I. Shabalina, Y. Camara et al., "Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice," *Cell Metabolism*, vol. 10, no. 2, pp. 131–138, 2009.

[88] K. Sharma, B. Karl, A. V. Mathew et al., "Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease," *Journal of the American Society of Nephrology*, vol. 24, no. 11, pp. 1901–1912, 2013.

[89] F. Scialò, A. Sriram, D. Fernández-Ayala et al., "Mitochondrial ROS produced via reverse electron transport extend animal lifespan," *Cell Metabolism*, vol. 23, no. 4, pp. 725–734, 2016.

[90] S. J. Lee, A. B. Hwang, and C. Kenyon, "Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity," *Current Biology*, vol. 20, no. 23, pp. 2131–2136, 2010.

[91] W. Yang and S. Hekimi, "A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans," *PLoS Biology*, vol. 8, no. 12, article e1000556, 2010.

[92] W. Yang and S. Hekimi, "Two modes of mitochondrial dysfunction lead independently to lifespan extension in *Caenorhabditis elegans*," *Aging Cell*, vol. 9, no. 3, pp. 433–447, 2010.

[93] S. Schmeisser, S. Priebe, M. Groth et al., "Neuronal ROS signaling rather than AMPK/sirtuin-mediated energy sensing links dietary restriction to lifespan extension," *Molecular Metabolism*, vol. 2, no. 2, pp. 92–102, 2013.

[94] K. L. DeBalsi, K. E. Hoff, and W. C. Copeland, "Role of the mitochondrial DNA replication machinery in mitochondrial DNA mutagenesis, aging and age-related diseases," *Ageing Research Reviews*, vol. 33, pp. 89–104, 2017.

[95] O. Hashizume, A. Shimizu, M. Yokota et al., "Specific mitochondrial DNA mutation in mice regulates diabetes and lymphoma development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 26, pp. 10528–10533, 2012.

[96] T. E. S. Kauppila, A. Bratic, M. B. Jensen et al., "Mutations of mitochondrial DNA are not major contributors to aging of fruit flies," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 115, no. 41, pp. E9620–e9629, 2018.

[97] A. Diot, K. Morten, and J. Poulton, "Mitophagy plays a central role in mitochondrial ageing," *Mammalian Genome*, vol. 27, no. 7-8, pp. 381–395, 2016.

[98] S. Aggarwal, P. Mannam, and J. Zhang, "Differential regulation of autophagy and mitophagy in pulmonary diseases," *American Journal of Physiology. Lung Cellular and Molecular Physiology*, vol. 311, no. 2, pp. L433–L452, 2016.

[99] Q. Liang and S. Kobayashi, "Mitochondrial quality control in the diabetic heart," *Journal of Molecular and Cellular Cardiology*, vol. 95, pp. 57–69, 2016.

[100] M. Zhan, I. M. Usman, L. Sun, and Y. S. Kanwar, "Disruption of renal tubular mitochondrial quality control by *Myo*-inositol oxygenase in diabetic kidney disease," *Journal of the American Society of Nephrology*, vol. 26, no. 6, pp. 1304–1321, 2015.

[101] K. Guo, J. Lu, Y. Huang et al., "Protective role of PGC-1α in diabetic nephropathy is associated with the inhibition of ROS through mitochondrial dynamic remodeling," *PLoS One*, vol. 10, no. 4, article e0125176, 2015.

[102] T. Wauer, M. Simicek, A. Schubert, and D. Komander, "Mechanism of phospho-ubiquitin-induced PARKIN activation," *Nature*, vol. 524, no. 7565, pp. 370–374, 2015.

[103] M. Lazarou, D. A. Sliter, L. A. Kane et al., "The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy," *Nature*, vol. 524, no. 7565, pp. 309–314, 2015.

[104] Y. C. Wong and E. L. F. Holzbaur, "Temporal dynamics of PARK2/parkin and OPTN/optineurin recruitment during the mitophagy of damaged mitochondria," *Autophagy*, vol. 11, no. 2, pp. 422–424, 2015.

[105] J. C. Greene, A. J. Whitworth, I. Kuo, L. A. Andrews, M. B. Feany, and L. J. Pallanck, "Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 4078–4083, 2003.

[106] A. Rana, M. Rera, and D. W. Walker, "Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 21, pp. 8638–8643, 2013.

[107] L. Xiao, X. Xu, F. Zhang et al., "The mitochondria-targeted antioxidant MitoQ ameliorated tubular injury mediated by mitophagy in diabetic kidney disease via Nrf2/PINK1," *Redox Biology*, vol. 11, pp. 297–311, 2017.

[108] L. Fang, Y. Zhou, H. Cao et al., "Autophagy attenuates diabetic glomerular damage through protection of hyperglycemia-induced podocyte injury," *PLoS One*, vol. 8, no. 4, article e60546, 2013.

[109] Y. Xu and Z. Sun, "Molecular basis of klotho: from gene to function in aging," *Endocrine Reviews*, vol. 36, no. 2, pp. 174–193, 2015.

[110] H. Olauson, M. G. Vervloet, M. Cozzolino, Z. A. Massy, P. Ureña Torres, and T. E. Larsson, "New insights into the FGF23-klotho axis," *Seminars in Nephrology*, vol. 34, no. 6, pp. 586–597, 2014.

[111] G. D. Dalton, J. Xie, S. W. An, and C. L. Huang, "New insights into the mechanism of action of soluble klotho," *Frontiers in Endocrinology*, vol. 8, p. 323, 2017.

[112] D. A. Drew, R. Katz, S. Kritchevsky et al., "Association between soluble klotho and change in kidney function: the health aging and body composition study," *Journal of the American Society of Nephrology*, vol. 28, no. 6, pp. 1859–1866, 2017.

[113] H. Olauson and T. E. Larsson, "FGF23 and klotho in chronic kidney disease," *Current Opinion in Nephrology and Hypertension*, vol. 22, no. 4, pp. 397–404, 2013.

[114] G. J. P. L. Kops, T. B. Dansen, P. E. Polderman et al., "Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress," *Nature*, vol. 419, no. 6904, pp. 316–321, 2002.

[115] Y. Zhao, S. Banerjee, N. Dey et al., "Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)536 phosphorylation," *Diabetes*, vol. 60, no. 7, pp. 1907–1916, 2011.

[116] P. Buendía, J. Carracedo, S. Soriano et al., "Klotho prevents NFκB translocation and protects endothelial cell from senescence induced by uremia," *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, vol. 70, no. 10, pp. 1198–1209, 2015.

[117] H. Sugiura, T. Yoshida, S. Shiohira et al., "Reduced klotho expression level in kidney aggravates renal interstitial fibrosis," *American Journal of Physiology. Renal Physiology*, vol. 302, no. 10, pp. F1252–F1264, 2012.

[118] M. Sopjani, M. Rinnerthaler, J. Kruja, and M. Dermaku-Sopjani, "Intracellular signaling of the aging suppressor protein klotho," *Current Molecular Medicine*, vol. 15, no. 1, pp. 27–37, 2015.

[119] M. C. Hu, M. Shi, J. Zhang et al., "Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule," *The FASEB Journal*, vol. 24, no. 9, pp. 3438–3450, 2010.

[120] I. Urakawa, Y. Yamazaki, T. Shimada et al., "Klotho converts canonical FGF receptor into a specific receptor for FGF23," *Nature*, vol. 444, no. 7120, pp. 770–774, 2006.

[121] M. Ohnishi and M. S. Razzaque, "Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging," *The FASEB Journal*, vol. 24, no. 9, pp. 3562–3571, 2010.

[122] M. Kuro-o, "A potential link between phosphate and aging–lessons from klotho-deficient mice," *Mechanisms of Ageing and Development*, vol. 131, no. 4, pp. 270–275, 2010.

[123] O. Asai, K. Nakatani, T. Tanaka et al., "Decreased renal α-klotho expression in early diabetic nephropathy in humans and mice and its possible role in urinary calcium excretion," *Kidney International*, vol. 81, no. 6, pp. 539–547, 2012.

[124] M. F. Cheng, L. J. Chen, and J. T. Cheng, "Decrease of klotho in the kidney of streptozotocin-induced diabetic rats," *Journal of Biomedicine & Biotechnology*, vol. 2010, Article ID 513853, 7 pages, 2010.

[125] G. H. Young and V. C. Wu, "KLOTHO methylation is linked to uremic toxins and chronic kidney disease," *Kidney International*, vol. 81, no. 7, pp. 611-612, 2012.

[126] C. Y. Sun, S. C. Chang, and M. S. Wu, "Suppression of klotho expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation," *Kidney International*, vol. 81, no. 7, pp. 640–650, 2012.

[127] J. A. Moreno, M. C. Izquierdo, M. D. Sanchez-Niño et al., "The inflammatory cytokines TWEAK and TNFα reduce renal klotho expression through NFκB," *Journal of the American Society of Nephrology*, vol. 22, no. 7, pp. 1315–1325, 2011.

[128] N. Troyano-Suárez, M. del Nogal-Avila, I. Mora et al., "Glucose oxidase induces cellular senescence in immortal renal cells through ILK by downregulating *klotho* gene expression," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 416738, 13 pages, 2015.

[129] M. Kuro-o, "Klotho as a regulator of oxidative stress and senescence," *Biological Chemistry*, vol. 389, no. 3, pp. 233–241, 2008.

[130] W. He, Y. S. Kang, C. Dai, and Y. Liu, "Blockade of Wnt/β-catenin signaling by paricalcitol ameliorates proteinuria and kidney injury," *Journal of the American Society of Nephrology*, vol. 22, no. 1, pp. 90–103, 2011.

[131] T. Zhou, X. He, R. Cheng et al., "Implication of dysregulation of the canonical wingless-type MMTV integration site (WNT) pathway in diabetic nephropathy," *Diabetologia*, vol. 55, no. 1, pp. 255–266, 2012.

[132] E. Heikkila, J. Juhila, M. Lassila et al., "β-catenin mediates adriamycin-induced albuminuria and podocyte injury in adult mouse kidneys," *Nephrology, Dialysis, Transplantation*, vol. 25, no. 8, pp. 2437–2446, 2010.

[133] L. Xiao, D. Zhou, R. J. Tan et al., "Sustained activation of Wnt/β-catenin signaling drives AKI to CKD progression," *Journal of the American Society of Nephrology*, vol. 27, no. 6, pp. 1727–1740, 2016.

[134] R. T. Moon, B. Bowerman, M. Boutros, and N. Perrimon, "The promise and perils of Wnt signaling through β-catenin," *Science*, vol. 296, no. 5573, pp. 1644–1646, 2002.

[135] M. Bienz and H. Clevers, "Armadillo/β-catenin signals in the nucleus–proof beyond a reasonable doubt?," *Nature Cell Biology*, vol. 5, no. 3, pp. 179–182, 2003.

[136] F. J. T. Staal and H. Clevers, "Tcf/Lef transcription factors during T-cell development: unique and overlapping functions," *The Hematology Journal*, vol. 1, no. 1, pp. 3–6, 2000.

[137] S. C. Manolagas and M. Almeida, "Gone with the Wnts: β-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism," *Molecular Endocrinology*, vol. 21, no. 11, pp. 2605–2614, 2007.

[138] M. A. Essers, L. M. de Vries-Smits, N. Barker, P. E. Polderman, B. M. Burgering, and H. C. Korswagen, "Functional interaction between β-catenin and FOXO in oxidative stress signaling," *Science*, vol. 308, no. 5725, pp. 1181–1184, 2005.

[139] L. Zhou, Y. Li, S. Hao et al., "Multiple genes of the renin-angiotensin system are novel targets of Wnt/β-catenin

signaling," *Journal of the American Society of Nephrology*, vol. 26, no. 1, pp. 107–120, 2015.

[140] S. Yang, D. Sun, L. Wang et al., "The role of STAT3/mTOR-regulated autophagy in angiotensin II-induced senescence of human glomerular mesangial cells," *Cellular Signalling*, vol. 53, pp. 327–338, 2019.

[141] S. Jiao, F. Meng, J. Zhang, X. Yang, X. Zheng, and L. Wang, "STAT1 mediates cellular senescence induced by angiotensin II and H_2O_2 in human glomerular mesangial cells," *Molecular and Cellular Biochemistry*, vol. 365, no. 1-2, pp. 9–17, 2012.

[142] S. Jiao, X. Zheng, X. Yang, J. Zhang, and L. Wang, "Losartan inhibits STAT1 activation and protects human glomerular mesangial cells from angiotensin II induced premature senescence," *Canadian Journal of Physiology and Pharmacology*, vol. 90, no. 1, pp. 89–98, 2012.

[143] H. Zhou, B. Huang, J. Du, and L. Wang, "Role of the JAK2/-STAT pathway and losartan in human glomerular mesangial cell senescence," *Molecular Medicine Reports*, vol. 3, no. 3, pp. 393–398, 2010.

[144] S. J. Sangaralingham, B. H. Wang, L. Huang et al., "Cardiorenal fibrosis and dysfunction in aging: imbalance in mediators and regulators of collagen," *Peptides*, vol. 76, pp. 108–114, 2016.

[145] M. Y. Lee, Y. Wang, and P. M. Vanhoutte, "Senescence of cultured porcine coronary arterial endothelial cells is associated with accelerated oxidative stress and activation of NFκB," *Journal of Vascular Research*, vol. 47, no. 4, pp. 287–298, 2010.

[146] E. M. V. de Cavanagh, F. Inserra, and L. Ferder, "Angiotensin II blockade: a strategy to slow ageing by protecting mitochondria?," *Cardiovascular Research*, vol. 89, no. 1, pp. 31–40, 2010.

[147] C. Luo, S. Zhou, Z. Zhou et al., "Wnt9a promotes renal fibrosis by accelerating cellular senescence in tubular epithelial cells," *Journal of the American Society of Nephrology*, vol. 29, no. 4, pp. 1238–1256, 2018.

[148] A. Benigni, P. Cassis, and G. Remuzzi, "Angiotensin II revisited: new roles in inflammation, immunology and aging," *EMBO Molecular Medicine*, vol. 2, no. 7, pp. 247–257, 2010.

[149] A. Benigni, D. Corna, C. Zoja et al., "Disruption of the Ang II type 1 receptor promotes longevity in mice," *The Journal of Clinical Investigation*, vol. 119, no. 3, pp. 524–530, 2009.

[150] E. M. de Cavanagh, B. Piotrkowski, N. Basso et al., "Enalapril and losartan attenuate mitochondrial dysfunction in aged rats," *The FASEB Journal*, vol. 17, no. 9, pp. 1096–1098, 2003.

[151] E. M. V. de Cavanagh, F. Inserra, M. Ferder, and L. Ferder, "From mitochondria to disease: role of the renin-angiotensin system," *American Journal of Nephrology*, vol. 27, no. 6, pp. 545–553, 2007.

[152] R. Vajapey, D. Rini, J. Walston, and P. Abadir, "The impact of age-related dysregulation of the angiotensin system on mitochondrial redox balance," *Frontiers in Physiology*, vol. 5, p. 439, 2014.

[153] S. A. Brod, "Unregulated inflammation shortens human functional longevity," *Inflammation Research*, vol. 49, no. 11, pp. 561–570, 2000.

[154] T. Nitta, H. Kanoh, K. I. Inamori, A. Suzuki, T. Takahashi, and J. I. Inokuchi, "Globo-series glycosphingolipids enhance Toll-like receptor 4-mediated inflammation and play a pathophysiological role in diabetic nephropathy," *Glycobiology*, vol. 29, no. 3, pp. 260–268, 2019.

[155] N. Barzilai, D. M. Huffman, R. H. Muzumdar, and A. Bartke, "The critical role of metabolic pathways in aging," *Diabetes*, vol. 61, no. 6, pp. 1315–1322, 2012.

[156] S. G. Deeks, "HIV infection, inflammation, immunosenescence, and aging," *Annual Review of Medicine*, vol. 62, no. 1, pp. 141–155, 2011.

[157] I. Pantsulaia, W. M. Ciszewski, and J. Niewiarowska, "Senescent endothelial cells: potential modulators of immunosenescence and ageing," *Ageing Research Reviews*, vol. 29, pp. 13–25, 2016.

[158] A. R. Pont, N. Sadri, S. J. Hsiao, S. Smith, and R. J. Schneider, "mRNA decay factor AUF1 maintains normal aging, telomere maintenance, and suppression of senescence by activation of telomerase transcription," *Molecular Cell*, vol. 47, no. 1, pp. 5–15, 2012.

[159] Y. Li, X. Li, K. He et al., "C-peptide prevents NF-κB from recruiting p300 and binding to the *inos* promoter in diabetic nephropathy," *The FASEB Journal*, vol. 32, no. 4, pp. 2269–2279, 2018.

[160] J. S. Tilstra, A. R. Robinson, J. Wang et al., "NF-κB inhibition delays DNA damage-induced senescence and aging in mice," *The Journal of Clinical Investigation*, vol. 122, no. 7, pp. 2601–2612, 2012.

[161] F. G. Osorio, C. Barcena, C. Soria-Valles et al., "Nuclear lamina defects cause ATM-dependent NF-κB activation and link accelerated aging to a systemic inflammatory response," *Genes & Development*, vol. 26, no. 20, pp. 2311–2324, 2012.

[162] A. S. Adler, S. Sinha, T. L. A. Kawahara, J. Y. Zhang, E. Segal, and H. Y. Chang, "Motif module map reveals enforcement of aging by continual NF-κB activity," *Genes & Development*, vol. 21, no. 24, pp. 3244–3257, 2007.

[163] I. Lazaro, A. Oguiza, C. Recio et al., "Targeting HSP90 ameliorates nephropathy and atherosclerosis through suppression of NF-κB and STAT signaling pathways in diabetic mice," *Diabetes*, vol. 64, no. 10, pp. 3600–3613, 2015.

[164] J. Wu, T. J. Guan, S. Zheng et al., "Inhibition of inflammation by pentosan polysulfate impedes the development and progression of severe diabetic nephropathy in aging C57B6 mice," *Laboratory Investigation*, vol. 91, no. 10, pp. 1459–1471, 2011.

[165] R. Zhou, A. S. Yazdi, P. Menu, and J. Tschopp, "A role for mitochondria in NLRP3 inflammasome activation," *Nature*, vol. 469, no. 7329, pp. 221–225, 2011.

[166] E. L. Goldberg and V. D. Dixit, "Drivers of age-related inflammation and strategies for healthspan extension," *Immunological Reviews*, vol. 265, no. 1, pp. 63–74, 2015.

[167] K. Schroder and J. Tschopp, "The inflammasomes," *Cell*, vol. 140, no. 6, pp. 821–832, 2010.

[168] A. Salminen, K. Kaarniranta, and A. Kauppinen, "Inflammaging: disturbed interplay between autophagy and inflammasomes," *Aging*, vol. 4, no. 3, pp. 166–175, 2012.

[169] S. Giacoppo, P. Bramanti, and E. Mazzon, "Triggering of inflammasome by impaired autophagy in response to acute experimental Parkinson's disease: involvement of the PI3K/Akt/mTOR pathway," *NeuroReport*, vol. 28, no. 15, pp. 996–1007, 2017.

[170] F. Duranton, G. Cohen, R. de Smet et al., "Normal and pathologic concentrations of uremic toxins," *Journal of*

the American Society of Nephrology, vol. 23, no. 7, pp. 1258–1270, 2013.

[171] A. Pletinck, G. Glorieux, E. Schepers et al., "Protein-bound uremic toxins stimulate crosstalk between leukocytes and vessel wall," *Journal of the American Society of Nephrology*, vol. 24, no. 12, pp. 1981–1994, 2013.

[172] J. Jansen, J. Jankowski, P. R. Gajjala, J. F. M. Wetzels, and R. Masereeuw, "Disposition and clinical implications of protein-bound uremic toxins," *Clinical Science*, vol. 131, no. 14, pp. 1631–1647, 2017.

[173] R. Poesen, K. Windey, E. Neven et al., "The influence of CKD on colonic microbial metabolism," *Journal of the American Society of Nephrology*, vol. 27, no. 5, pp. 1389–1399, 2016.

[174] H. Shimizu, D. Bolati, A. Adijiang et al., "Senescence and dysfunction of proximal tubular cells are associated with activated p53 expression by indoxyl sulfate," *American Journal of Physiology. Cell Physiology*, vol. 299, no. 5, pp. C1110–C1117, 2010.

[175] H. Shimizu, M. Yisireyili, F. Nishijima, and T. Niwa, "Stat3 contributes to indoxyl sulfate-induced inflammatory and fibrotic gene expression and cellular senescence," *American Journal of Nephrology*, vol. 36, no. 2, pp. 184–189, 2012.

[176] M. Yu, Y. J. Kim, and D. H. Kang, "Indoxyl sulfate-induced endothelial dysfunction in patients with chronic kidney disease via an induction of oxidative stress," *Clinical Journal of the American Society of Nephrology*, vol. 6, no. 1, pp. 30–39, 2011.

[177] W. C. Shen, C. J. Liang, T. M. Huang et al., "Indoxyl sulfate enhances IL-1β-induced E-selectin expression in endothelial cells in acute kidney injury by the ROS/MAPKs/NFκB/AP-1 pathway," *Archives of Toxicology*, vol. 90, no. 11, pp. 2779–2792, 2016.

[178] C. Y. Sun, M. L. Cheng, H. C. Pan, J. H. Lee, and C. C. Lee, "Protein-bound uremic toxins impaired mitochondrial dynamics and functions," *Oncotarget*, vol. 8, no. 44, pp. 77722–77733, 2017.

[179] M. Morcos, X. du, F. Pfisterer et al., "Glyoxalase-1 prevents mitochondrial protein modification and enhances lifespan in Caenorhabditis elegans," *Aging Cell*, vol. 7, no. 2, pp. 260–269, 2008.

[180] A. Schlotterer, G. Kukudov, F. Bozorgmehr et al., "C. elegans as model for the study of high glucose- mediated life span reduction," *Diabetes*, vol. 58, no. 11, pp. 2450–2456, 2009.

[181] R. D. Semba, E. J. Nicklett, and L. Ferrucci, "Does accumulation of advanced glycation end products contribute to the aging phenotype?," *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, vol. 65A, no. 9, pp. 963–975, 2010.

[182] J. Liu, K. Huang, G. Y. Cai et al., "Receptor for advanced glycation end-products promotes premature senescence of proximal tubular epithelial cells via activation of endoplasmic reticulum stress-dependent p21 signaling," *Cellular Signalling*, vol. 26, no. 1, pp. 110–121, 2014.

[183] S. Yamagishi, N. Nakamura, M. Suematsu, K. Kaseda, and T. Matsui, "Advanced glycation end products: a molecular target for vascular complications in diabetes," *Molecular Medicine*, vol. 21, Supplement 1, pp. S32–S40, 2015.

[184] Z. Zha, J. Wang, X. Wang, M. Lu, and Y. Guo, "Involvement of PINK1/Parkin-mediated mitophagy in AGE-induced cardiomyocyte aging," *International Journal of Cardiology*, vol. 227, pp. 201–208, 2017.

[185] M. Shi, S. Yang, X. Zhu et al., "The RAGE/STAT5/autophagy axis regulates senescence in mesangial cells," *Cellular Signalling*, vol. 62, article 109334, 2019.

[186] Y. J. Chen, D. C. Chan, C. K. Chiang et al., "Advanced glycation end-products induced VEGF production and inflammatory responses in human synoviocytes via RAGE-NF-κB pathway activation," *Journal of Orthopaedic Research*, vol. 34, no. 5, pp. 791–800, 2016.

[187] T. Nakamura, N. Fujiwara, Y. Kawagoe, T. Sugaya, Y. Ueda, and H. Koide, "Effects of telmisartan and enalapril on renoprotection in patients with mild to moderate chronic kidney disease," *European Journal of Clinical Investigation*, vol. 40, no. 9, pp. 790–796, 2010.

[188] X. Tan, W. He, and Y. Liu, "Combination therapy with paricalcitol and trandolapril reduces renal fibrosis in obstructive nephropathy," *Kidney International*, vol. 76, no. 12, pp. 1248–1257, 2009.

[189] Z. Zhang, Y. Zhang, G. Ning, D. K. Deb, J. Kong, and Y. C. Li, "Combination therapy with AT1 blocker and vitamin D analog markedly ameliorates diabetic nephropathy: blockade of compensatory renin increase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 41, pp. 15896–15901, 2008.

[190] Z. Chen, F. Miao, A. D. Paterson et al., "Epigenomic profiling reveals an association between persistence of DNA methylation and metabolic memory in the DCCT/EDIC type 1 diabetes cohort," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 21, pp. E3002–E3011, 2016.

[191] M. A. Reddy and R. Natarajan, "Epigenetics in diabetic kidney disease," *Journal of the American Society of Nephrology*, vol. 22, no. 12, pp. 2182–2185, 2011.

[192] Y. C. Ning, G. Y. Cai, L. Zhuo et al., "Short-term calorie restriction protects against renal senescence of aged rats by increasing autophagic activity and reducing oxidative damage," *Mechanisms of Ageing and Development*, vol. 134, no. 11-12, pp. 570–579, 2013.

[193] G. Lopez-Lluch and P. Navas, "Calorie restriction as an intervention in ageing," *The Journal of Physiology*, vol. 594, no. 8, pp. 2043–2060, 2016.

[194] X. M. Xu, Y. C. Ning, W. J. Wang et al., "Anti-inflammaging effects of long-term caloric restriction via overexpression of SIGIRR to inhibit NF-κB signaling pathway," *Cellular Physiology and Biochemistry*, vol. 37, no. 4, pp. 1257–1270, 2015.

[195] K. Inoki, J. Kim, and K. L. Guan, "AMPK and mTOR in cellular energy homeostasis and drug targets," *Annual Review of Pharmacology and Toxicology*, vol. 52, no. 1, pp. 381–400, 2012.

[196] R. Iglesias-Bartolome, V. Patel, A. Cotrim et al., "mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis," *Cell Stem Cell*, vol. 11, no. 3, pp. 401–414, 2012.

[197] M. Kawai, S. Kinoshita, K. Ozono, and T. Michigami, "Inorganic phosphate activates the AKT/mTORC1 pathway and shortens the life span of an αKlotho-deficient model," *Journal of the American Society of Nephrology*, vol. 27, no. 9, pp. 2810–2824, 2016.

[198] J. Bielas, A. Herbst, K. Widjaja et al., "Long term rapamycin treatment improves mitochondrial DNA quality in aging mice," *Experimental Gerontology*, vol. 106, pp. 125–131, 2018.

[199] Y. Guan, S. R. Wang, X. Z. Huang et al., "Nicotinamide mononucleotide, an NAD^+ precursor, rescues age-associated susceptibility to AKI in a Sirtuin 1-dependent manner," *Journal of the American Society of Nephrology*, vol. 28, no. 8, pp. 2337–2352, 2017.

[200] S. Kume, T. Uzu, K. Horiike et al., "Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney," *The Journal of Clinical Investigation*, vol. 120, no. 4, pp. 1043–1055, 2010.

[201] Q. Hong, L. Zhang, B. Das et al., "Increased podocyte Sirtuin-1 function attenuates diabetic kidney injury," *Kidney International*, vol. 93, no. 6, pp. 1330–1343, 2018.

[202] V. Conti, V. Izzo, G. Corbi et al., "Antioxidant supplementation in the treatment of aging-associated diseases," *Frontiers in Pharmacology*, vol. 7, p. 24, 2016.

[203] H. Shevalye, S. Lupachyk, P. Watcho et al., "Prediabetic nephropathy as an early consequence of the high-calorie/-high-fat diet: relation to oxidative stress," *Endocrinology*, vol. 153, no. 3, pp. 1152–1161, 2012.

[204] N. Al-Waili, H. Al-Waili, T. Al-Waili, and K. Salom, "Natural antioxidants in the treatment and prevention of diabetic nephropathy; a potential approach that warrants clinical trials," *Redox Report*, vol. 22, no. 3, pp. 99–118, 2017.

[205] M. Tavafi, "Diabetic nephropathy and antioxidants," *Journal of Nephropathology*, vol. 2, no. 1, pp. 20–27, 2013.

[206] L. Bao, J. Li, D. Zha et al., "Chlorogenic acid prevents diabetic nephropathy by inhibiting oxidative stress and inflammation through modulation of the Nrf2/HO-1 and NF-κB pathways," *International Immunopharmacology*, vol. 54, pp. 245–253, 2018.

[207] Y. Zhang, A. Unnikrishnan, S. S. Deepa et al., "A new role for oxidative stress in aging: the accelerated aging phenotype in *Sod1*$^{-/-}$ mice is correlated to increased cellular senescence," *Redox Biology*, vol. 11, pp. 30–37, 2017.

[208] D. J. Baker, T. Wijshake, T. Tchkonia et al., "Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders," *Nature*, vol. 479, no. 7372, pp. 232–236, 2011.

[209] J. Chang, Y. Wang, L. Shao et al., "Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice," *Nature Medicine*, vol. 22, no. 1, pp. 78–83, 2016.

[210] R. Yosef, N. Pilpel, R. Tokarsky-Amiel et al., "Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL," *Nature Communications*, vol. 7, no. 1, article 11190, 2016.

[211] C. Tse, A. R. Shoemaker, J. Adickes et al., "ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor," *Cancer Research*, vol. 68, no. 9, pp. 3421–3428, 2008.

[212] B. G. Childs, M. Gluscevic, D. J. Baker et al., "Senescent cells: an emerging target for diseases of ageing," *Nature Reviews. Drug Discovery*, vol. 16, no. 10, pp. 718–735, 2017.

[213] D. J. Baker, B. G. Childs, M. Durik et al., "Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan," *Nature*, vol. 530, no. 7589, pp. 184–189, 2016.

[214] F. Prattichizzo, V. de Nigris, L. la Sala, A. D. Procopio, F. Olivieri, and A. Ceriello, ""Inflammaging" as a druggable target: a senescence-associated secretory phenotype-centered view of type 2 diabetes," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 1810327, 10 pages, 2016.

[215] M. Xu, T. Tchkonia, H. Ding et al., "JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 46, pp. E6301–E6310, 2015.

[216] D. Alimbetov, T. Davis, A. J. C. Brook et al., "Suppression of the senescence-associated secretory phenotype (SASP) in human fibroblasts using small molecule inhibitors of p38 MAP kinase and MK2," *Biogerontology*, vol. 17, no. 2, pp. 305–315, 2016.

[217] N. Tasdemir, A. Banito, J.-S. Roe et al., "BRD4 connects enhancer remodeling to senescence immune surveillance," *Cancer Discovery*, vol. 6, no. 6, pp. 612–629, 2016.

[218] X. Dong, X. Hu, J. Chen, D. Hu, and L. F. Chen, "BRD4 regulates cellular senescence in gastric cancer cells via E2F/miR-106b/p21 axis," *Cell Death & Disease*, vol. 9, no. 2, p. 203, 2018.

[219] L. Zhou, Y. Li, D. Zhou, R. J. Tan, and Y. Liu, "Loss of klotho contributes to kidney injury by derepression of Wnt/β-catenin signaling," *Journal of the American Society of Nephrology*, vol. 24, no. 5, pp. 771–785, 2013.

[220] M. A. Reddy, P. Sumanth, L. Lanting et al., "Losartan reverses permissive epigenetic changes in renal glomeruli of diabetic db/db mice," *Kidney International*, vol. 85, no. 2, pp. 362–373, 2014.

[221] W. C. Kan, J. Y. Hwang, L. Y. Chuang et al., "Effect of osthole on advanced glycation end products-induced renal tubular hypertrophy and role of klotho in its mechanism of action," *Phytomedicine*, vol. 53, pp. 205–212, 2019.

[222] S. B. Mirza, R. Ekhteiari Salmas, M. Q. Fatmi, and S. Durdagi, "Discovery of klotho peptide antagonists against Wnt3 and Wnt3a target proteins using combination of protein engineering, protein-protein docking, peptide docking and molecular dynamics simulations," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 32, no. 1, pp. 84–98, 2017.

[223] C. Compagnucci and E. Bertini, "The potential of iPSCs for the treatment of premature aging disorders," *Genome Biology*, vol. 18, no. 11, p. 2350, 2017.

[224] S. A. Villeda, K. E. Plambeck, J. Middeldorp et al., "Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice," *Nature Medicine*, vol. 20, no. 6, pp. 659–663, 2014.

[225] B. Biteau, J. Karpac, S. Supoyo, M. DeGennaro, R. Lehmann, and H. Jasper, "Lifespan extension by preserving proliferative homeostasis in Drosophila," *PLoS Genetics*, vol. 6, no. 10, article e1001159, 2010.

[226] A. Eirin and L. O. Lerman, "Mesenchymal stem cell treatment for chronic renal failure," *Stem Cell Research & Therapy*, vol. 5, no. 4, p. 83, 2014.

[227] I. M. Conboy, M. J. Conboy, A. J. Wagers, E. R. Girma, I. L. Weissman, and T. A. Rando, "Rejuvenation of aged progenitor cells by exposure to a young systemic environment," *Nature*, vol. 433, no. 7027, pp. 760–764, 2005.

[228] S. A. Villeda, J. Luo, K. I. Mosher et al., "The ageing systemic milieu negatively regulates neurogenesis and cognitive function," *Nature*, vol. 477, no. 7362, pp. 90–94, 2011.

[229] C. E. Rodrigues, J. M. C. Capcha, A. C. de Bragança et al., "Human umbilical cord-derived mesenchymal stromal cells protect against premature renal senescence resulting from oxidative stress in rats with acute kidney injury," *Stem Cell Research & Therapy*, vol. 8, no. 1, p. 19, 2017.

[230] M. Ullah and Z. Sun, "Stem cells and anti-aging genes: double-edged sword-do the same job of life extension," *Stem Cell Research & Therapy*, vol. 9, no. 1, p. 3, 2018.

[231] Y. Shi, Y. Zhang, H. Tian et al., "Improved dialytic removal of protein-bound uremic toxins by intravenous lipid emulsion in chronic kidney disease rats," *Nephrology Dialysis Transplantation*, 2019.

[232] M. Madero, K. B. Cano, I. Campos et al., "Removal of protein-bound uremic toxins during hemodialysis using a binding competitor," *Clinical Journal of the American Society of Nephrology*, vol. 14, no. 3, pp. 394–402, 2019.

[233] M. Rossi, K. Klein, D. W. Johnson, and K. L. Campbell, "Pre-, pro-, and synbiotics: do they have a role in reducing uremic toxins? A systematic review and meta-analysis," *International Journal of Nephrology*, vol. 2012, Article ID 673631, 20 pages, 2012.

[234] B. K. I. Meijers, V. de Preter, K. Verbeke, Y. Vanrenterghem, and P. Evenepoel, "p-Cresyl sulfate serum concentrations in haemodialysis patients are reduced by the prebiotic oligofructose-enriched inulin," *Nephrology, Dialysis, Transplantation*, vol. 25, no. 1, pp. 219–224, 2010.

[235] N. Ranganathan, P. Ranganathan, E. A. Friedman et al., "Pilot study of probiotic dietary supplementation for promoting healthy kidney function in patients with chronic kidney disease," *Advances in Therapy*, vol. 27, no. 9, pp. 634–647, 2010.

[236] M. Rossi, D. W. Johnson, M. Morrison et al., "Synbiotics easing renal failure by improving gut microbiology (SYNERGY): a randomized trial," *Clinical Journal of the American Society of Nephrology*, vol. 11, no. 2, pp. 223–231, 2016.

[237] N. D. Vaziri, S. M. Liu, W. L. Lau et al., "High amylose resistant starch diet ameliorates oxidative stress, inflammation, and progression of chronic kidney disease," *PLoS One*, vol. 9, no. 12, article e114881, 2014.

[238] T. L. Sirich, N. S. Plummer, C. D. Gardner, T. H. Hostetter, and T. W. Meyer, "Effect of increasing dietary fiber on plasma levels of colon-derived solutes in hemodialysis patients," *Clinical Journal of the American Society of Nephrology*, vol. 9, no. 9, pp. 1603–1610, 2014.

[239] R. Han, L. Li, A. P. Ugalde et al., "Functional CRISPR screen identifies AP1-associated enhancer regulating FOXF1 to modulate oncogene-induced senescence," *Genome Biology*, vol. 19, no. 1, p. 118, 2018.

[240] X. Liu, L. Wei, Q. Dong et al., "A large-scale CRISPR screen and identification of essential genes in cellular senescence bypass," *Aging*, vol. 11, no. 12, pp. 4011–4031, 2019.

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Research Article

N^{ω}-(Carboxymethyl)arginine Is One of the Dominant Advanced Glycation End Products in Glycated Collagens and Mouse Tissues

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Advanced glycation end products (AGEs) accumulate in proteins during aging in humans. In particular, the AGE structure N^{ω}-(carboxymethyl)arginine (CMA) is produced by oxidation in glycated collagen, accounting for one of the major proteins detected in biological samples. In this study, we investigated the mechanism by which CMA is generated in collagen and detected CMA in collagen-rich tissues. When various protein samples were incubated with glucose, the CMA content, detected using a monoclonal antibody, increased in a time-dependent manner only in glycated collagen, whereas the formation of N^{ϵ}-(carboxymethyl)lysine (CML), a major antigenic AGE, was detected in all glycated proteins. Dominant CMA formation in glycated collagen was also observed by electrospray ionization-liquid chromatography-tandem mass spectrometry (LC-MS/MS). During incubation of glucose with collagen, CMA formation was enhanced with increasing glucose concentration, whereas it was inhibited in the presence of dicarbonyl-trapping reagents and a metal chelator. CMA formation was also observed upon incubating collagen with glyoxal, and CMA was generated in a time-dependent manner when glyoxal was incubated with type I–IV collagens. To identify hotspots of CMA formation, tryptic digests of glycated collagen were applied to an affinity column conjugated with anti-CMA. Several CMA peptides that are important for recognition by integrins were detected by LC-MS/MS and amino acid sequence analyses. CMA formation on each sequence was confirmed by incubation of the synthesized peptides with glyoxal and ribose. LC-MS detected CMA in the mouse skin at a higher level than other AGEs. Furthermore, CMA accumulation was greater in the human aorta of older individuals. Overall, our study provides evidence that CMA is a representative AGE structure that serves as a useful index to reflect the oxidation and glycation of collagen.

1. Introduction

Incubation of proteins with glucose leads to the generation of advanced glycation end products (AGEs) from the Maillard reaction through the formation of Schiff bases and Amadori products [1]. The levels of AGE-modified proteins increase during the normal aging process [2]; however, this increase is markedly accelerated in a diabetic condition with sustained hyperglycemia. N^{ϵ}-(Carboxymethyl)lysine (CML), one of the major antigenic AGE structures, accumulates in several tissue proteins, including the kidneys of patients with diabetic nephropathy [3] and chronic renal failure [4], atherosclerotic lesions of arterial walls [5], amyloid fibrils in hemodialysis-related amyloidosis [6], and actinic elastosis of photoaged skin [7]. Pentosidine [8], one of the fluorescent AGE structures generated under oxidative conditions, accumulates in

the blood and in long-lived tissue proteins such as collagens and lens protein. Methylglyoxal is generated through the Embden-Meyerhof and polyol pathways and reacts with proteins to form N^{ε}-(carboxyethyl)lysine (CEL) [9] and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) [10]. Accordingly, detection of CEL and MG-H1 in biological samples is used as evidence for the modification of proteins by methylglyoxal.

Recent studies have demonstrated that AGEs are generated from glucose as well as from carbonyl compounds such as glyoxal, methylglyoxal [11], glucosone [12], and glycolaldehyde [13], which are generated from the Embden-Meyerhof pathway, glucose and lipid oxidation, and inflammation, followed by rapid reaction with proteins. In addition, many reports have suggested that these carbonyl compounds play an important role in the pathogenesis of several diseases other than diabetes mellitus. For example, Arai et al. [14] reported that 20% of schizophrenia cases showed carbonyl stress with high-plasma pentosidine and low-serum pyridoxal despite no physical complications such as diabetes mellitus and renal dysfunction. Among these psychiatric patients with carbonyl stress, several of the patients with schizophrenia were found to harbor mutations in the glyoxalase 1 (*GLO*1) gene with consequent reduction of enzymatic activity, demonstrating that carbonyl stress may also participate in the schizophrenia pathogenesis. Nonenzymatic cross-links and AGE formation in bone collagen deteriorate bone toughness, postyield properties, and ductility. Moreover, Mitome et al. [15] reported that pentosidine in bone collagen was remarkably increased in dialysis patients and was inversely correlated with the bone formation rate/bone volume ratio and mineral apposition rate. This result suggests that modification of collagen by AGEs is strongly associated with disorders of bone metabolism in dialysis patients.

N^{ω}-(Carboxymethyl)arginine (CMA) is an acid-labile AGE structure that was discovered in the enzymatic hydrolysate of glycated collagen [16]. Subsequently, CMA was also detected in human serum by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and its serum level in diabetic patients was found to be higher than that in normal subjects [17]. However, an internal standard was not used in this study, and the CMA content in tissues remains unclear because of its acid instability; thus, little is known about the CMA level in organs. Collagens are one of the major proteins in the human body and play an important role in several biological functions such as formation of the extracellular matrix, which comprises the bulk of the skin and confers strength and resiliency [18], and contributes to the mechanical properties and metabolism of bone [19]. Thus, measurement of the CMA content may be a useful tool to evaluate the degeneration of collagens. To clarify the biological significance of CMA, we investigated the mechanism by which CMA is generated in glycated collagen. Furthermore, to determine the CMA content in tissues, a preparation method for tissue samples was developed and measurement was performed using LC-MS/MS along with an internal standard.

Table 1: Synthesized collagen peptides.

Protein	Position	Sequence
Collagen	556-564	$(GPO)_4$**GMOGERGGO** $(GPO)_4$
Collagen	757-765	$(GPO)_4$**GPOGERGAO** $(GPO)_4$
Collagen	274-282	$(GPO)_4$**GPRGERGEA** $(GPO)_4$
HSA	405-413	$(GPO)_4$**NALLVRYTK** $(GPO)_4$

2. Materials and Methods

2.1. Materials. Bovine collagen types I and II were purchased from Koken Co. (Tokyo, Japan), and bovine collagen types III and IV were purchased from Nitta Gelatin Co. (Osaka, Japan). Human serum albumin (HSA) was donated by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) and was defatted using charcoal treatment as described by Chen [20]. Ribonuclease (RNase) A, human hemoglobin, D-glucose, glyoxal, and S-Carboxymethyl-L-cysteine (CMC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide were purchased from Pierce (Rockford, IL, USA). Horseradish peroxidase- (HRP-) conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard Perry Laboratories (Gaithersburg, MD, USA). All other chemicals were of the best grade available from commercial sources. Standard AGEs and isotope-labeled amino acids were purchased from PolyPeptide Laboratories (Strasbourg, France) and Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA), respectively. The synthesized peptides (Table 1) were purchased from AnyGen Co. Ltd. (Gwangju, Korea).

2.2. Preparation of CMA. CMA was synthesized by the reaction of L-arginine and glyoxal, as described by Odani et al. [17] with minor modifications. In brief, L-arginine (100 mM) was incubated with glyoxal (100 mM) in a 1.0 M sodium hydrate solution for 1 week at 37°C, since approximately 50% of the L-arginine, estimated using the peak area obtained from ESI-LC-MS/MS analysis, was unmodified after the 2-day incubation. The mixture was then neutralized with 1.0 M hydrochloric acid. The reaction products were applied to a Dowex 50W (100-200 mesh, H1 form) column (30 × 170 mm) equilibrated with distilled water, and CMA was then eluted with 10% pyridine. The eluate was evaporated to yield a residue, which was chromatographed on a silica gel column (20 × 200 mm) with 100% methanol. The fraction containing CMA was subjected to MS and nuclear magnetic resonance (NMR) measurements to confirm its structure. [$^{13}C_6$] CMA was also synthesized from [$^{13}C_6$] arginine (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA).

2.3. In Vitro Peptides and Protein Modification. Type I collagen (1.5 mg/mL) was incubated with 200 mM glucose in a 100 mM sodium phosphate buffer (pH 7.4) at 37°C for up to 8 weeks. The same experiment was conducted with HSA, RNase, hemoglobin, immunoglobulin, and low-density

lipoprotein (LDL). Gelled collagenous samples were added to a disruption tube with 6.0 mm stainless steel beads and homogenized on a Shakemaster (Biomedical Science, Japan), followed by determination of protein concentration using a bicinchoninic acid assay (Pierce, Rockford, IL, USA). The CMA or CML content of the samples was determined by enzyme-linked immunosorbent assay (ELISA) [21] and instrumental analysis [22, 23] as described below. Ribose gelatin was also prepared by incubating 30 mM ribose with gelatin (2 mg/mL) in a 200 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 week. Furthermore, each synthesized peptide (2 mg/mL) described in Table 1 was also incubated with 1 mM glyoxal or 30 mM ribose in a 200 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 week.

2.4. Elisa. ELISA was performed as described previously [21] using the previously described monoclonal anti-CMA antibody [24]. In brief, each well of a 96-well microtiter plate was coated with 100 μL of the indicated concentration of sample in PBS and incubated for 2 h. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer), and then blocked with 0.5% gelatin in phosphate-buffered saline (PBS) for 1 h. After washing three times, the wells were incubated for 1 h with 100 μL of monoclonal anti-CMA (3F5; 1 μg/mL) and monoclonal anti-CML (6D12; 0.5 μg/mL) antibodies. After washing thrice, the wells were incubated with HRP-conjugated anti-mouse IgG, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 100 μL of 1.0 M sulfuric acid, and the absorbance was read at 492 nm on a micro-ELISA plate reader. CMA contents were also measured by competitive ELISA. Each well of a 96-well microtiter plate was coated with 100 μL of CMA-conjugated Keyhole limpet hemocyanin (KLH) in PBS and incubated for 2 h, and the wells were blocked with 0.5% gelatin in PBS for 1 h, followed by three washes with washing buffer. 60 μL of 3F5 (2 μg/mL) and 60 μL of samples were preincubated for 1 h, and sample aliquots (100 μL) were added to each well and incubated for 1 h. After three washes with washing buffer, the wells were incubated with HRP-conjugated anti-mouse IgG and developed as described above.

2.5. Formation of Arginine Adduct with High Concentration of Glyoxal. N^{α}-Acetyl-L-arginine (10 mM) was incubated with different concentrations of glyoxal (0.1-100 mM) in PBS (pH 7.4) at 37°C for 2 days, and the elemental composition was analyzed by quadrupole time-of-flight mass spectrometry equipped with an electrospray ionization source (ESI-QTOF) using a compact mass spectrometer (Bruker Daltonics, Bremen, Germany). The reaction mixture (100 μL) was mixed with 900 μL of distilled water containing 0.1% trifluoroacetic acid (TFA). The samples were passed over a Strata-X-C column (Phenomenex, Torrance, CA, USA) as described previously [22, 23] with minor modification. Thus, the column was prewashed with 1 mL of methanol and equilibrated with 1 mL of 0.1% TFA. The final concentrations of the samples for the elemental composition analysis and qualitative analysis with positive-mode ESI-QTOF were 100 μg/mL and 2 μg/mL, respectively. For the elemental composition analysis with positive-mode ESI-QTOF, the capillary voltage was 4.5 kV and the ionization source temperature was 200°C. A collision-induced dissociation was performed using nitrogen as the collision gas at a pressure of 1.6 bar. Data were acquired with a stored mass range of 50–1000 (m/z). Detected ions were analyzed by SmartFormula manually (Bruker Daltonics, Bremen, Germany). For qualitative analysis with positive-mode ESI-QTOF, LC was conducted on a ZIC®-HILIC column (150 × 2.1 mm, 5 μm) (Merck Millipore, Billerica, MA, USA). The mobile phase was collected using solvent A (distilled water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate was 0.2 mL/min, and the column was kept at 40°C. The retention time for the N^{α}-acetyl CMA dimer was approximately 12 min. The structure was detected by electrospray ionization and positive ion mass spectrometry. The target ion of 549.2627 ± 0.005 (m/z) was measured for the analysis of the N^{α}-acetyl-L-arginine adduct with glyoxal in samples incubated with glyoxal.

2.6. Measurement of AGE Contents by ESI-LC-MS/MS. The contents of CMA and other AGEs in the samples were measured by electrospray ionization- (ESI-) LC-MS/MS using a TSQ Quantiva triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described previously [22, 23] with minor modification. In brief, 3 μL of glycated proteins (3 μg protein) and 17 μL of distilled water were mixed with 20 μL of a 200 mM sodium borate buffer (pH 9.1) and reduced by the addition of 2 μL of $NaBH_4$ (1 M $NaBH_4$ in 0.1 N NaOH) at room temperature for 4 h. Standard 10 pmol of [$^{13}C_6$] CMA and [$^{2}H_2$] CML (PolyPeptide Laboratories, Strasbourg, France) and 5 nmol of [$^{13}C_6$] lysine (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA) and [$^{13}C_6$] arginine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) were added to the pellets, and samples were hydrolyzed with 1 mL of 6 N HCl at 100°C for 24 h. CMA is degraded through acid hydrolysis in the same manner as [$^{13}C_6$] CMA; thus, the amount of CMA can be quantitated based on the amount ratio (CMA and [$^{13}C_6$] CMA) and area ratio (233 (m/z) and 239 (m/z)) [25]. As the lysine content in collagen and albumin differs, the AGE content in glycated proteins *in vitro* was expressed as pmol AGEs/30 ng proteins. Furthermore, the lysine-derived AGEs were normalized by the lysine content, and arginine-derived AGEs were normalized by the arginine content; thus, the data are expressed as CML mmol/mol Lys and CMA mmol/mol Arg, respectively.

The AGE contents in mouse skin were analyzed by ESI-LC-MS/MS. All animal experiments were confirmed by Tokai University (approval number: 161076). Experiments were undertaken in compliance with the Guidelines for the Care and Use of Animals for Scientific Purposes at Tokai University (established April 1, 2007). ddy mice were purchased from Kyudo (Kumamoto, Japan) and were housed in a pathogen-free barrier facility (12 h light-dark cycle) and fed a normal rodent chow diet (CLEA Japan, Tokyo, Japan). At 20 weeks, the animals were euthanized under pentobarbital anesthesia. Skin specimens were immediately

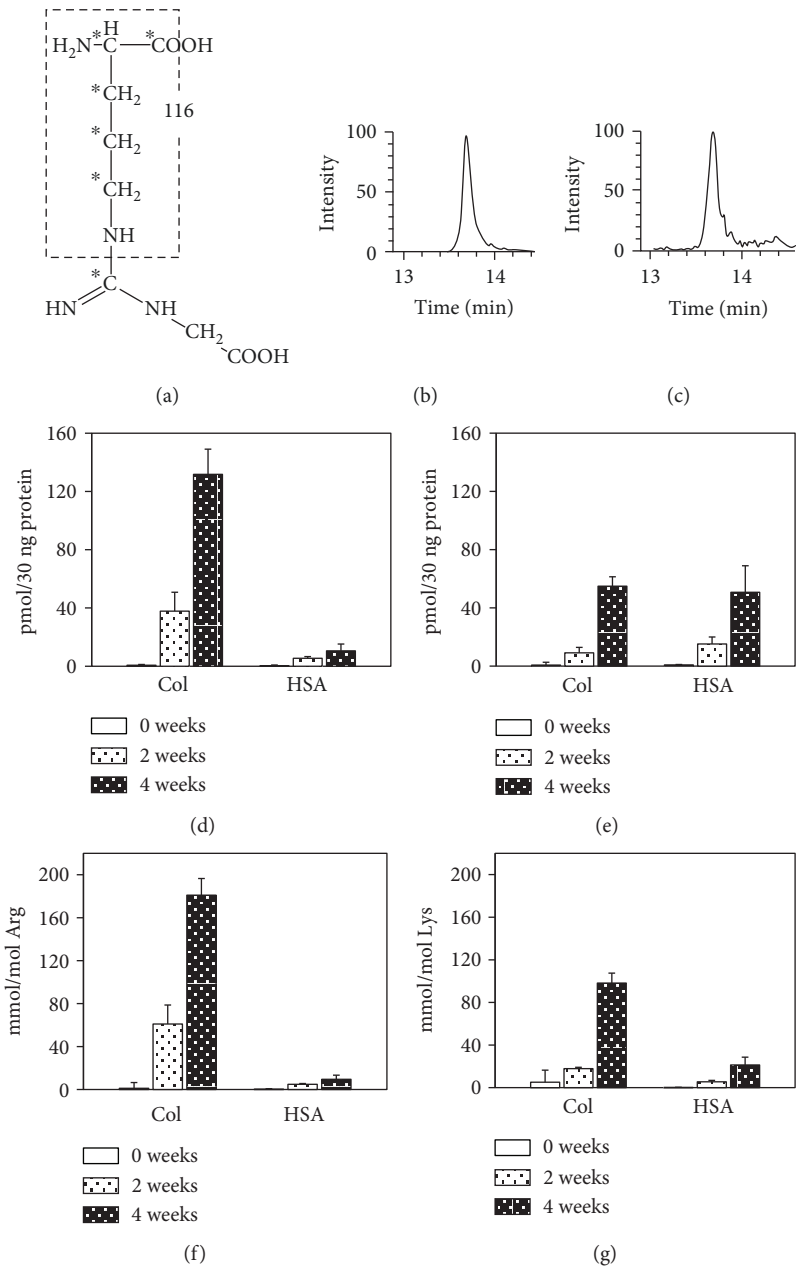


Figure 1: Measurement of CMA in glycated proteins by ESI-LC-MS/MS. (a) 13C in [$^{13}C_6$] CMA is indicated by an asterisk. Type I collagen and HSA were incubated with glucose at 37°C for up to 4 weeks. The parent ions of CMA and [$^{13}C_6$] CMA were 233 (*m*/*z*) and 239 (*m*/*z*), respectively, and peaks of the fragment ion of 116 (*m*/*z*) for CMA (b) and 121 (*m*/*z*) for [$^{13}C_6$] CMA (c) were detected in the samples of glycated collagen. The amounts of CMA (d) and CML (e) were normalized by the amounts of proteins in glycated proteins. The amount of CMA (f) was normalized by the arginine content, and CML (g) was normalized by the lysine content.

frozen and stored at −80°C until use. For measurement of AGEs, 5 mg of minced mouse skins (dry weight) and 100 μL distilled water were mixed with 100 μL of a 200 mM sodium borate buffer (pH 9.1) and reduced by adding 10 μL of 1 M $NaBH_4$ at room temperature for 4 h. The samples were centrifuged at 3,000 rpm, the supernatant was discarded, and the skin was hydrolyzed with 1 mL of 6 N HCl at 100°C for 2 h. One portion of the hydrolyzed samples (0.1 mg) was then transferred to a different tube followed by addition of the standard [$^{13}C_6$] CMA (Figure 1(a)), [$^{2}H_2$] CML, [$^{2}H_4$] N^{ϵ}-(carboxyethyl)lysine (CEL), [$^{2}H_3$] N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) (PolyPeptide Laboratories, Strasbourg, France), [$^{13}C_6$] lysine, and [$^{13}C_6$] arginine to the pellets, which were further hydrolyzed with 1 mL of 6 N HCl at 100°C for 22 h. The following steps were conducted in the same manner as described for the *in vitro* samples. CEL and MG-H1 of AGEs were also measured in addition to CML and CMA. The retention times for CEL and MG-H1 were 12–14 min. The parent ions of CEL and [$^{2}H_4$] CEL were 219 (*m*/*z*) and 223 (*m*/*z*), respectively. Fragment ions of 130 (*m*/*z*) and 134 (*m*/*z*) from

each parent ion were measured for the analysis of CEL and [$^2H_4$] CEL in samples. The parent ions of MG-H1 and [$^2H_3$] MG-H1 were 229 (m/z) and 232 (m/z), respectively. Fragment ions of 114 (m/z) and 117 (m/z) from each parent ion were measured for the analysis of MG-H1 and [$^2H_3$] MG-H1 in the samples. The AGE content in mouse skin *in vivo* was expressed as nmol AGEs/μg mouse skin. The lysine-derived AGEs were normalized by the lysine content, and the arginine-derived AGEs were normalized by the arginine content; the data are also expressed as CML and CEL mmol/mol Lys and CMA and MG-H1 mmol/mol Arg, respectively.

2.7. Isolation of CMA-Modified Peptides from Glyoxal-Modified Collagen by Affinity Chromatography. The hybridoma-producing 3F5 was inoculated into the peritoneal cavities of Balb/c mice to obtain ascetic fluid. One milliliter of protein G Sepharose (GE Healthcare, Sweden) was saturated with a 50 mM borate buffer (pH 8.2), and then 1 mL of ascites was mixed with an equal volume of borate buffer and then applied to protein G Sepharose. After the column was washed with 10 mL of borate buffer, the antibody was conjugated to Sepharose with 1 mL of 25 mM dimethyl pimelimidate (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. The reaction was terminated by adding 0.2 M monoethanolamine (Sigma-Aldrich, St. Louis, MO, USA). To identify the CMA-modified peptides in glycated collagen, type III collagen (1.5 mg/mL) was incubated with glyoxal (1 mM) at 37°C for 1 week. Then, 0.5 mg of glyoxal collagen was further incubated at 60°C for 5 min to denature the collagen structure, followed by digestion with trypsin (10 μg/mL) at 37°C for 12 h. The reaction was terminated by the addition of a 1/50 volume of a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and then applied to the 3F5-conjugated affinity column. The column was washed with 10 mL of PBS, and CMA peptides were then eluted with 0.2 M glycine-HCl (pH 3.0). The adsorbed fraction was concentrated and analyzed using ESI-hybrid triple quadrupole/-linear ion trap mass spectrometry (ESI-QTRAP) on a 3200 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA). The instrument was coupled to an Agilent 1200 Series HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA).

2.8. Detection of CMA Peptide by ESI-QTRAP. The CMA-modified peptide samples isolated as described above were analyzed by ESI-QTRAP [26].

2.9. MS/MS Database Search. Peptide identification was accomplished using ProteinPilot software 4.0 (AB Sciex) with the Paragon™ algorithm. The acquired MS/MS spectra were searched against the UniProtKB/Swiss-Prot database (release 2011_08. 2011-07-27) for *Bos taurus* species (5857 protein entries). The search parameters included digestion by trypsin, biological modification ID focus, and 95% protein confidence threshold. We defined the confidence threshold of the identified peptides as 90%. The carboxymethylation of arginine (+58) and lysine (+58) were added to the search criteria of posttranslational modifications. The probabilities of proline and lysine hydroxylation were set higher than the defaults for collagen analysis.

2.10. Sequence Confirmation by a Protein Sequencer. The CMA peptide sequence was confirmed by an N-terminal amino acid sequence analysis. The tryptic digest of the glyoxal-modified type III collagen was applied to the CMA affinity column as described above. The adsorbed peptide fraction was loaded onto an Ascentis Express C18 HPLC column (Supelco, Bellefonte, PA, USA), and the CMA peptide-containing fraction was collected. This sample was analyzed by a Procise 492 protein sequencer (Applied Biosystems, Invitrogen Co., Carlsbad, CA, USA) in the pulsed liquid mode.

2.11. Tissue Samples and Immunohistochemical Analysis. We evaluated paraffin-embedded thoracic aortas from autopsies of 10 patients (five elderly and five young patients). Informed written consent was obtained from the families after the death of all patients, and the study design was approved by the Institutional Review Board of Kumamoto University in accordance with the World Medical Association Declaration of Helsinki. Autopsies were performed at Kumamoto University Hospital between 2000 and 2017 (approval no. 2224). After sectioning (3 μM thickness), paraffin-embedded aorta tissues were used for immunostaining with anti-CMA (3F5) antibody. The sections were subsequently treated with an HRP-conjugated secondary antibody (Nichirei, Tokyo, Japan), and the reactions were visualized with diaminobenzidine.

2.12. Hematoxylin and Eosin (H&E) and Azan Staining. Three-micrometer-thick sections of paraffin-embedded thoracic aortas were subjected to H&E staining (Mayer's hematoxylin staining, followed by eosin Y staining) and Azan staining (Mordant, Mallory's azocarmine G solution, 5% phosphotungstic acid solution, and Mallory's aniline blue orange G stain solution) according to routine procedures.

3. Results

3.1. Immunoreactivity of Monoclonal Anti-CMA Antibody (3F5). The immunoreactivity of 3F5 was determined using noncompetitive and competitive ELISA. As shown in Figure 2(a), 3F5 significantly reacted with CMA-conjugated HSA and CMA-conjugated keyhole limpet hemocyanin (KLH) in a dose-dependent manner, whereas its reactivity with arginine-conjugated HSA and KLH was negligible. To examine the specificity of the antibody, we next performed competitive ELISA. Immunoreaction of 3F5 to CMA-conjugated HSA was significantly inhibited by the free form of CMA, whereas the reactivities of free CML and *S*-(carboxymethyl)cysteine (CMC) were negligible (Figure 2(b)). These results indicated that the antibody 3F5 specifically recognizes CMA.

3.2. CMA Formation in Glycated Proteins. CMA formation in various glycated protein samples was examined using the antibody 3F5. Glycated proteins were prepared by incubation with 200 mM glucose at 37°C for up to 8 weeks, and the CMA

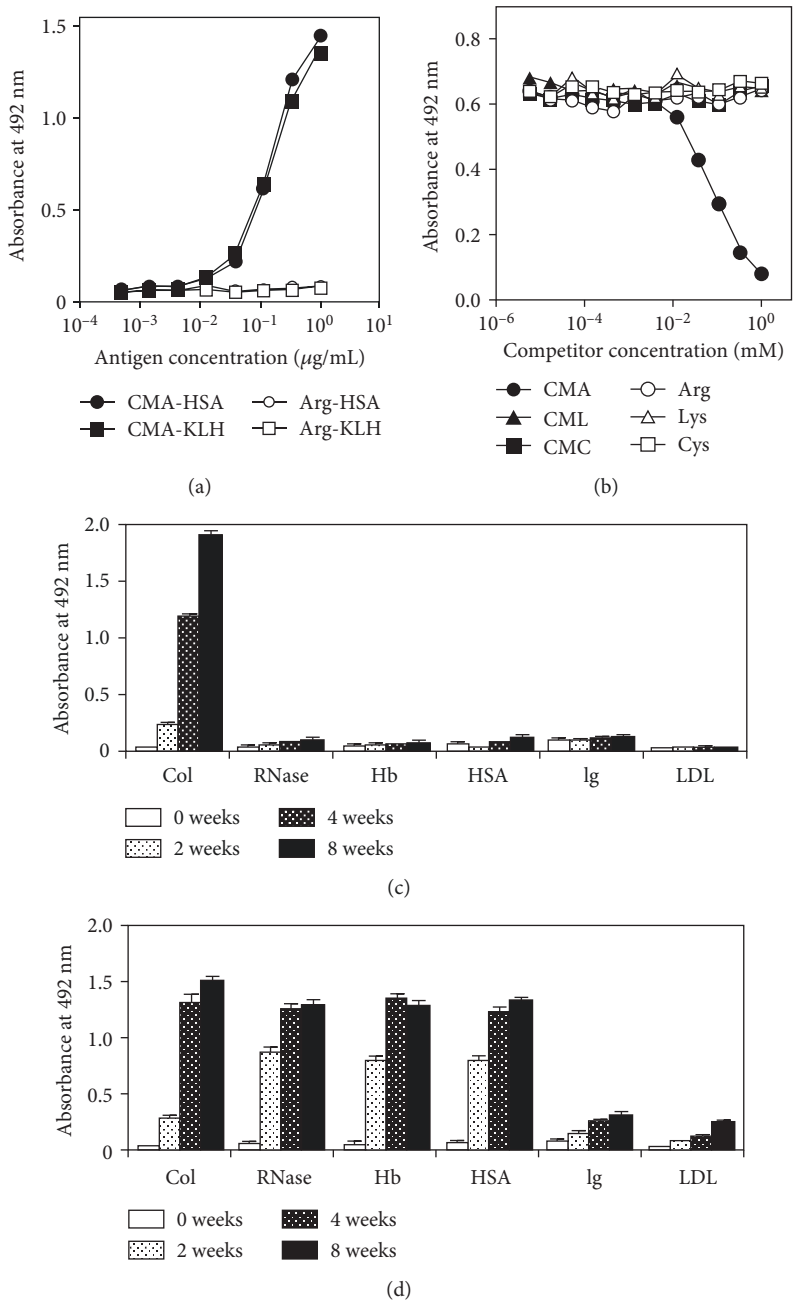


Figure 2: Immunoreactivity of monoclonal anti-CMA antibody (3F5). (a) Each well coated with the indicated concentration of the test sample was reacted with 3F5 (1 μg/mL). The antibody bound to the wells was detected by HRP-conjugated anti-mouse IgG. (b) Specificity of the anti-CMA antibody (3F5). Each well was coated with 0.1 mL of 1 μg/mL CMA-conjugated KLH and blocked with 0.5% gelatin. 60 μL of 3F5 (2 μg/mL) and 60 μL of the samples were preincubated for 1 h, and sample aliquots (100 μL) were added to each well and incubated for 1 h. The antibody bound to the wells was detected as described above. (c) CMA and (d) CML formations in glycated protein samples. Type I collagen (Col), HSA, RNase, hemoglobin (Hb), immunoglobulin (Ig), and LDL were incubated with 200 mM glucose at 37°C for up to 8 weeks, and the CMA or CML content of the samples was determined by ELISA.

or CML content of the samples was then determined by ELISA. The CMA content in glycated collagen was found to increase in a time-dependent manner, whereas CMA formation was not observed in the other proteins (Figure 2(c)). In contrast, CML formation was observed in glycated collagen as well as in RNase, hemoglobin, HSA, immunoglobulin, and LDL in a time-dependent manner (Figure 2(d)).

3.3. CMA Detection by ESI-LC-MS/MS. As shown in Figure 1(a), the parent ions of CMA and [$^{13}C_6$] CMA were 233 (m/z) and 239 (m/z), respectively, and fragment ions of 116 (m/z) and 121 (m/z) were detected to measure CMA and [$^{13}C_6$] CMA. Fragment ion peaks of 116 (m/z) for CMA (Figure 1(b)) and 121 (m/z) for [$^{13}C_6$] (Figure 1(c)) were detected in the glycated collagen.

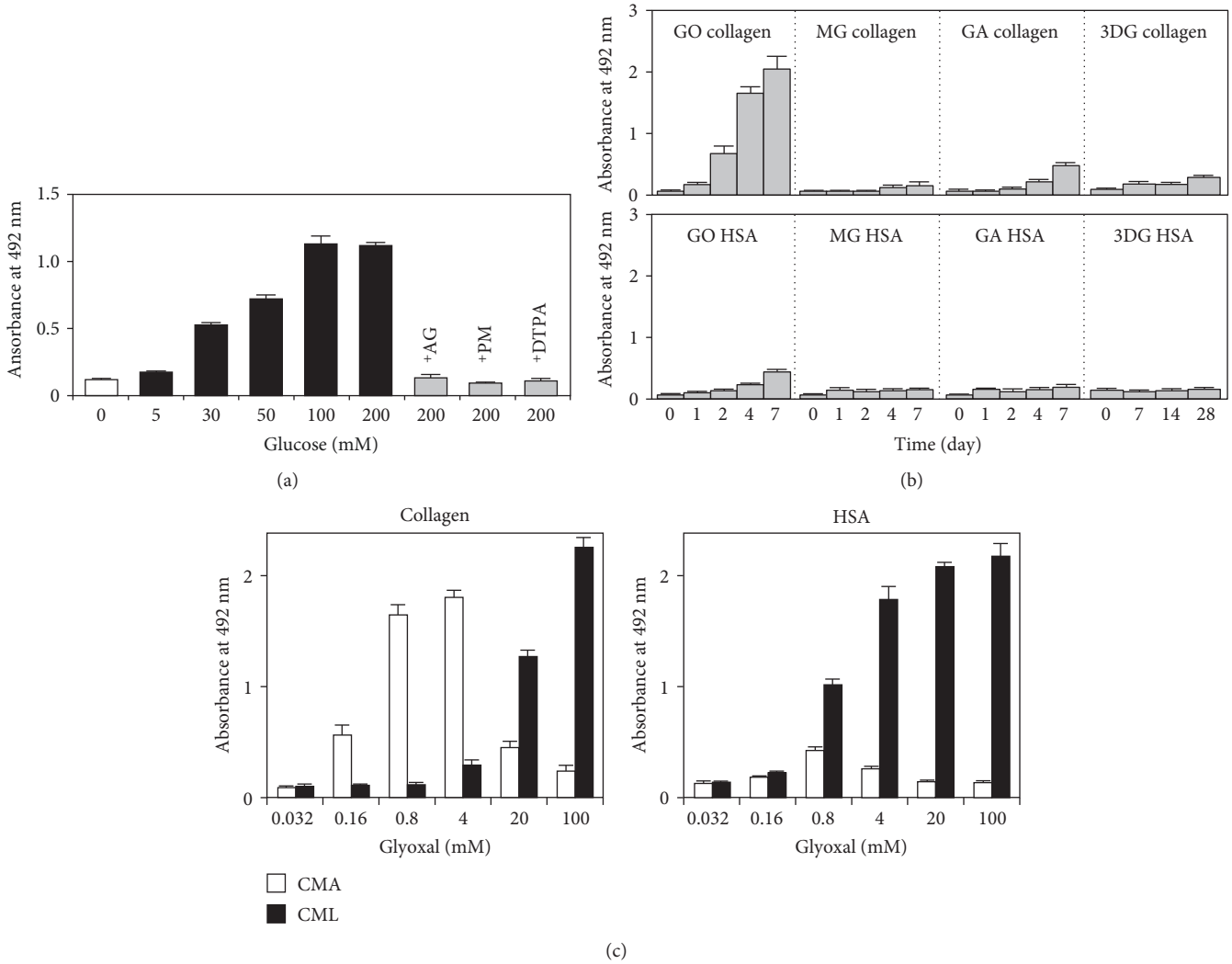


Figure 3: Effect of intermediate aldehydes on CMA formation. (a) Dose-dependent effect of glucose on CMA formation in glycated collagen. Type I collagen (1.5 mg/mL) was incubated with the indicated concentration of glucose at 37°C for 4 weeks. Aminoguanidine (AG), pyridoxamine (PM), and diethylenetriaminepentaacetic acid (DTPA) (1 mM each) were added before glucose addition. (b) CMA formation of aldehyde-modified collagen and HSA. Glyoxal- (GO-), methylglyoxal- (MG-), and glycolaldehyde- (GA-) modified collagen and HSA were prepared by incubation with 1 mM of these aldehydes for up to 7 days, and 3-deoxyglucosone- (3DG-) modified collagen and HSA were also prepared by incubation with 30 mM 3DG for up to 28 days. (c) Dose-dependent effect of glyoxal on CMA and CML formation in collagen. Type I collagen and HSA (1.5 mg/mL each) were incubated with the indicated concentrations of glyoxal at 37°C for 7 days, and CMA and CML contents were determined by ELISA.

The amounts of CMA and CML were normalized by the amounts of proteins as in ELISA. As a result, the change in CMA and CML levels showed a similar tendency as detected with ELISA. Thus, although the CMA level in unmodified collagen and HSA was below detectable levels (<0.04 pmol), the CMA contents in collagens increased in a time-dependent manner, whereas the CMA levels slightly increased in glycated HSA (Figure 1(d)). The levels of CML increased by the glycation of both collagens and HSA (Figure 1(e)).

The yield of CMA was higher than that of CML (Figures 1(f) and 1(g)), and CMA was preferentially generated in collagens (Figure 1(f)). Although CML was generated both in collagens and HSA molecules, its levels in HSA were estimated to be lower than those in collagens (Figure 1(g)) given that the number of lysines differed between albumin and collagen molecules.

3.4. Effect of Intermediate Aldehydes on CMA Formation. The dose-dependent effect of glucose on CMA formation in glycated collagen was then measured. As shown in Figure 3(a), the CMA content increased with increasing glucose concentration and CMA formation was observed even in 30 mM glucose. This result suggested that CMA might be generated in glycated collagen under physiological glucose concentrations. Furthermore, CMA formation was significantly inhibited in the presence of the metal ion chelator diethylenetriaminepentaacetic acid (DTPA) [27] and with aldehyde-trapping reagents such as aminoguanidine [28–30] and pyridoxamine [31] (Figure 3(a)).

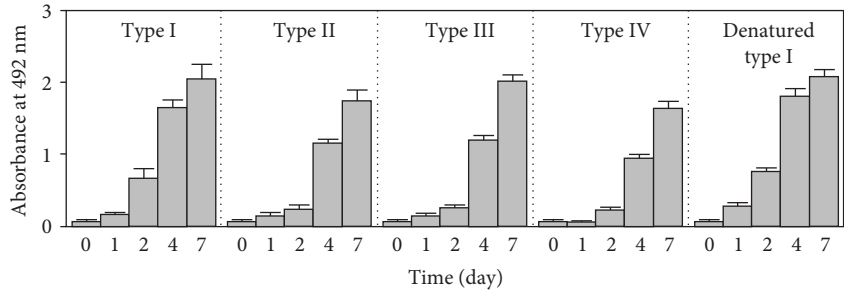


Figure 4: CMA formation in different types of collagen (I, II, III, and IV) and heat-denatured type I collagen. Four types of collagen (I, II, III, and IV) and heat-denatured type I collagen (denatured by heating at 60°C for 15 min) were incubated with 1 mM glyoxal at 37°C for up to 7 days, and the CMA content was determined by ELISA.

These results suggested that intermediate aldehydes may play a role in CMA formation during the incubation of collagen with glucose. To investigate which intermediate aldehydes play a role in CMA formation, collagen was incubated with reactive aldehydes such as glyoxal, methylglyoxal, glycolaldehyde, and 3-deoxyglucosone followed by determination of the CMA content by ELISA. As shown in Figure 3(b), the yield of CMA in glyoxal-modified collagen was the highest among the aldehydes tested. In contrast, only slight CMA formation was observed in glyoxal-modified HSA (Figure 3(b)). These results indicated that glyoxal is an important precursor for CMA formation during collagen glycation.

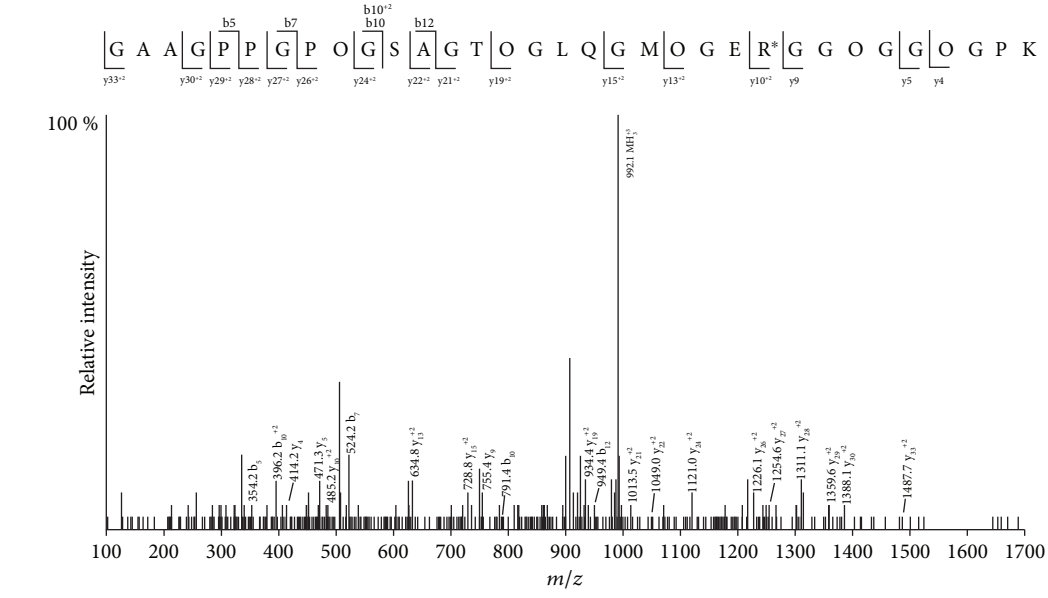
3.5. Dose-Dependent Effect of Glyoxal on CMA and CML Formation in Collagen. We next examined the dose-dependent effect of glyoxal on CMA formation. Collagen and HSA were incubated with the indicated concentrations of glyoxal at 37°C for 7 days, and the CMA or CML content was determined by ELISA. As shown in Figure 3(c), the CMA content in glyoxal-modified collagen increased in a dose-dependent manner up to 4 mM glyoxal, after which it decreased in a dose-dependent manner. In contrast, the CML content increased in a dose-dependent manner up to 100 mM glyoxal in both collagen and HSA (Figure 3(c)). The incubation of N^{α}-acetyl-L-arginine with high concentrations of glyoxal (above 10 mM) generated a parent ion of 549.2637 ± 0.005 (m/z) with an elemental composition of $C_{20}H_{37}N_8O_{10}$, possessing two CMA molecules (CMA dimer). Furthermore, we observed fragment ions of the CMA dimer: 491.2560 ± 0.005 (m/z) for the de-acetylated CMA dimer; 275.1354 ± 0.005 (m/z) for N^{α}-acetyl-CMA; and 217.1299 ± 0.005 (m/z) for N^{α}-acetyl-L-arginine, which demonstrated that CMA formed dimers under high concentrations of glyoxal. Interestingly, at low concentrations of glyoxal, CMA was preferentially generated in collagen over CML. These results indicate that CMA may be generated in collagen under physiological concentrations of glyoxal and that among the AGE structures determined, CMA was dominant in glycated collagen.

3.6. CMA and CML Formation in Different Types of Collagen (I–IV) and Heat-Denatured Type I Collagen. To compare CMA and CML formation in different types of collagen, several types of collagens were incubated with 1 mM glyoxal at 37°C for up to 7 days and the CMA or CML content was determined by ELISA. As shown in Figure 4, the CMA content increased time-dependently in all types of collagen, and no remarkable difference in CMA formation was observed among the collagen types. Furthermore, CMA formation in denatured type I collagen (denatured by heating at 60°C for 15 min before glyoxal incubation) was almost equal to that detected in nondenatured type I collagen (Figure 4). These results suggested that the amino acid sequence of collagen may play an important role in the CMA formation of collagen rather than the three-dimensional structure of collagen.

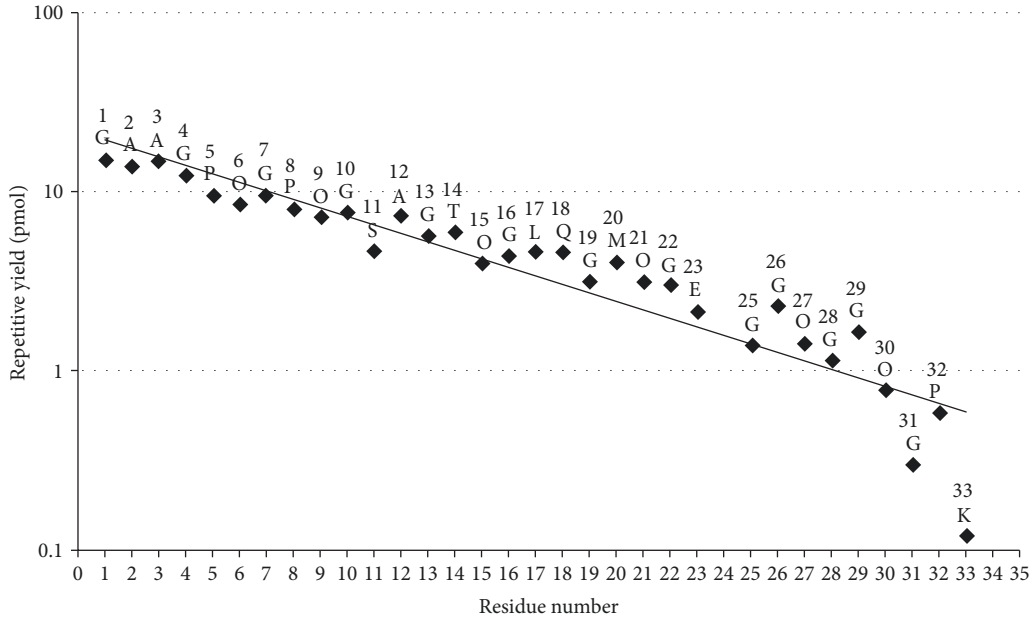
3.7. Identification of CMA Peptides in Glyoxal-Modified Type III Collagen. To identify the CMA-modified peptides in glycated collagen, the tryptic digest of glyoxal-modified type III collagen was applied to the CMA affinity column, because glyoxal was found to be the dominant precursor for CMA formation (Figure 3(b)). The adsorbed peptide fraction was analyzed by ESI-hybrid triple quadrupole/linear ion trap mass spectrometry (QTRAP). As shown in Figure 5(a), one arginine-containing peptide with mass addition equivalent to carboxymethylation (+58) was detected, composed of 33 amino acids, consistent with residues 538–570 of bovine type III collagen. Next, this peptide was isolated with the same HPLC system used in ESI-QTRAP and analyzed with the 491 Protein Sequencer. Similar to the data of LC-MS/MS analysis, the sequence corresponded to residues 538–570 of bovine type III collagen, and only R561 was estimated as an unknown amino acid (Figure 5(b)), possibly because of carboxymethylation. Furthermore, the tryptic digest of glyoxal-modified type III collagen without an affinity column was also analyzed by ESI-QTRAP, and 13 peptides were detected (Table 2).

The numbering of residues begins with the triple-helical portion of the chain. The first residue corresponds to residue 15 of P04258 (type III collagen alpha 1 chain, *Bos taurus*). O represents hydroxyproline and R* represents CMA. The confidence threshold of the identified peptides was set at 90%.

3.8. CMA Formation in Collagen Peptides. To confirm CMA formation in collagen, several collagen peptides (Table 1) were synthesized and CMA formation was measured. The sequence of bovine type III collagen corresponding to residues 538–570 was selected since this sequence was detected



(a)



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
G	A	A	G	P	O	G	P	O	G	S	A	G	T	O	G	L	Q	G	M	O	G	E	*	G	G	O	G	G	O	G	P	K

(b)

Figure 5: Identification of CMA peptide. (a) MS/MS spectrum of ESI-QTRAP of the CMA peptide purified with immunoaffinity column chromatography. The only CMA peptide observed after immunoaffinity purification was identified as GAAG POGPOGSAGTOGLQGMOGER*GGOGGOGPK at 992.11 (m/z) ($z = 3^+$, O represents hydroxyproline and R* represents CMA). (b) The repetitive yield of amino acids in each step of the Edman degradation. The immunoaffinity-purified peptide was also analyzed using the 491 Protein Sequencer. The identified sequence was the same as that obtained by LC-MS/MS analysis, except for an unreadable 24th residue (asterisk).

with or without affinity purification of glyoxal-modified collagen. The type III collagen sequences containing GXXGER motifs that are recognized by integrin were also selected, and both the N- and C-termini of four peptides composed of nine residues were coupled with $(GPO)_4$ in order to form a collagen-like helical structure [32, 33]. In

Table 2: Identification of CMA peptides in glyoxal-modified type III collagen.

Position	CMA site	Sequence of identified peptide	m/z	z	M.W	Identified
226-258	243	K.GEMGPAGIOGAOGLIGA**R***GPOGPOGTNGVPGOR.G	778.63	4	3110.52	Day 7
277-291	279	R.GE**R***GEAGSOGIAGPK.G	486.23	3	1455.70	Day 1
297-321	315	K.DGSOGEOGANGLOGAAGE**R***GVOGFR.G	815.36	3	2443.08	Day 7
318-333	321	V.OGF**R***GPAGANGLOGEK.G	538.93	3	1613.78	Day 7
334-348	339	K.GPOGD**R***GGOGPAGPR.G	478.89	3	1433.66	Day 7
423-440	426	K.NGE**R***GGOGGOGPQGPAGK.N	560.59	3	1678.77	Day 7
514-537	525	R.GPOGAGGPOGP**R***GGAGPOGPEGGK.G	695.32	3	2082.97	Day 7
538-570	561	K.GAAGPOGPOGSAGTOGLQGMOGE**R***GGOGGOGPK.G	992.11	3	2973.34	Day 7
588-609	591	K.DGP**R***GPTGPIGPOGPAGQOGDK.G	705.67	3	2114.00	Day 7
610-630	624	K.GESGAOGVOGIAGP**R***GGOGER.G	660.98	3	1979.93	Day 7
754-786	762	K.GDSGPOGE**R***GAOGPQGPPGAOGPLGIAGLTGAR.G	1008.82	3	3023.47	Day 7
763-798	786	R.GAOGPQGPOGAPGPLGIAGLTGA**R***GLAGPOGMOGAR.G	813.16	4	3248.98	Day 1, day 7
787-807	798	R.GLAGPOGMOGA**R***GSOGPQGIK.G	669.99	3	2006.98	Day 7
813-842	822	K.OGPSGQNGE**R***GPOGPQGLOGLAGTAGEOGR.D	963.12	3	2886.35	Day 7
862-890	864	K.GD**R***GENGSPGAOGAOGHOGPOGPVGPAGK.S	667.30	4	2665.21	Day 1, day 7

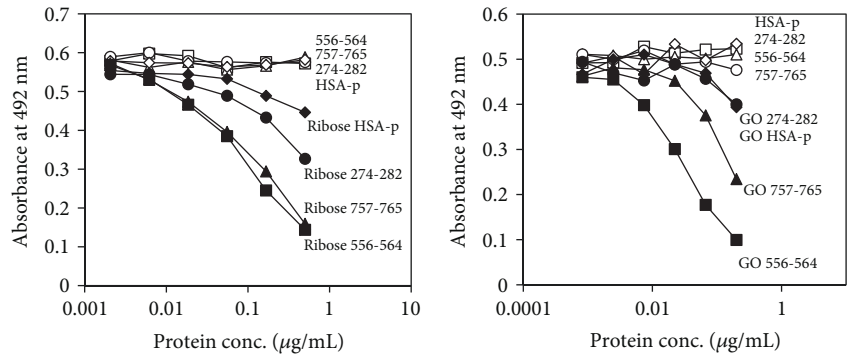


Figure 6: CMA formation in a collagen-like peptide. Synthesized peptides as shown in Table 1 were incubated with ribose (a) or glyoxal (b), and CMA formation was determined by competitive ELISA.

addition, the amount of CMA formation in these collagen peptides was compared with the HSA peptide reported as the hotspot of reactive aldehyde-modification in HSA [34]. As shown in Figures 6(a) and 6(b), CMA formation in ribose- and glyoxal-modified collagen peptides, especially in the 556-564 and 757-765 peptides, was higher than that in the HSA peptide. Furthermore, 274-282 also generated CMA, albeit weakly.

Both the N- and C-termini of the four peptides composed of nine residues were coupled with $(GPO)_4$ in order to form a collagen-like helical structure.

3.9. Measurement of AGEs in Mouse Skin. To clarify the presence of CMA in tissues, the AGE contents were measured by ESI-LC-MS/MS. As shown in Figure 7(a), the levels of CMA in the mouse skin were the highest among all measured AGEs such as CML, CEL, and MG-H1, when the AGE contents were normalized by the total amount of proteins. In particular, the level of CMA was 5-fold higher than that of CML. In contrast, when lysine-derived AGEs were normalized by the lysine content and arginine-derived AGEs were normalized by the arginine content, the level of CMA was similar to that of the other AGEs (Figure 7(b)).

3.10. CMA Accumulation in the Human Aorta. We measured the CMA accumulation in human thoracic aorta tissues, which are not generally recognized inflammation sites. Interestingly, CMA accumulation was detected by immunostaining at higher levels in the samples from elderly subjects compared to those from younger subjects (Figure 8). Furthermore, the sites of CMA accumulation were detected in the collagen layer by Azan staining (Figure 8), suggesting that the CMA accumulates at the collagen site in the aorta and that CMA accumulation is correlated with aging.

4. Discussion

As the measurement of chemically stable AGE structures such as CML and pentosidine is comparatively easier than that of unstable AGEs, CML and pentosidine are typically

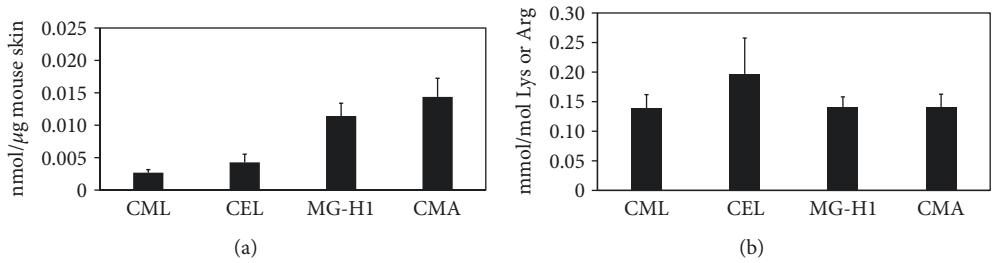


Figure 7: Measurement of AGEs in the mouse skin by ESI-LC-MS/MS. The AGE contents in the skin of mice ($n = 9$) were measured after hydrolysis with 6 N HCl at 100°C for 24 h. The amounts of AGEs were normalized by the dry weight (a), whereas the lysine-derived AGE contents were normalized by the lysine content and arginine-derived AGEs were normalized by the arginine content (b).

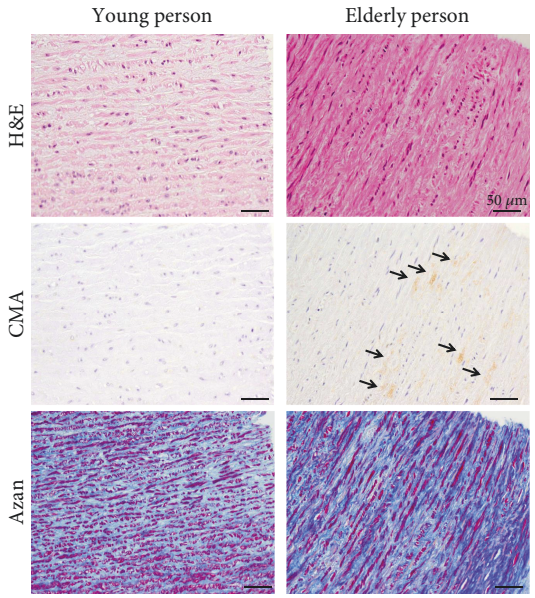


Figure 8: Immunohistochemistry and Azan staining of the human aorta. CMA accumulation was investigated by immunohistochemical analysis using autopsy samples from both young and elderly patients. Accumulation of CMA was detected in tissue sections of the aorta by immunostaining. The arterial collagen fibers were detected by Azan staining.

used as markers for determining the AGE content *in vivo*. The discovery of CMA was delayed because of difficulties in analysis. However, unstable AGEs may possess unique features. To clarify the biological significance of CMA, we generated a monoclonal antibody against CMA (3F5) and developed a detection system using ESI-LC-MS/MS and showed that CMA was generated in collagen during incubation with glucose and that glyoxal, an intermediate aldehyde, plays a role in CMA formation. The concentration of glucose used for the production of AGE-modified proteins varies across studies. Higashi et al. produced AGE-modified proteins by incubating bovine serum albumin with 1600 mM glucose for 40 weeks [35], while Schmidt et al. used 250 mM glucose-6-phosphate [36]; in both cases, highly modified AGE proteins were obtained. We previously reported that only highly modified AGE protein prepared with a supraphysiological concentration of glucose could be recognized by scavenger receptors such as SR-A, SR-B1, and CD36 [37], demonstrating that using the physiological concentration of glucose is important to clarify the biological significance of AGEs.

It is widely believed that the nonenzymatic modification of proteins by AGEs progresses nonspecifically and that their formation correlates with the incubation period with sugars and their concentration. Therefore, human and bovine serum albumins are widely used for glycation research, since this protein is enriched in lysine and arginine and is relatively inexpensive [12, 34, 38, 39]. We previously compared the formation of AGEs by changing dicarbonyl compounds [40, 41]; however, it remained unclear whether the type of protein affects the generation of AGEs. Interestingly, in the present study, both ELISA and ESI-LC-MS/MS showed dominant CMA formation in glycated collagen, demonstrating that CMA is generated in collagens during incubation with carbohydrates.

As CML was observed in collagen and other proteins during glucose incubation, preferential CMA formation in glycated collagen could not be explained simply by the difference in the reactivity of glucose with proteins. CMA formation in denatured collagen during incubation with 1 mM glyoxal was almost equal to that in nondenatured collagen, suggesting that a collagen-like amino acid sequence may play an important role in the CMA formation of collagen. In fact, the collagen-specific sequence was identified as the hotspot of CMA formation in glycated collagen using ESI-QTRAP. Furthermore, the peptide sequence 538–570 of bovine type III collagen was synthesized because this peptide could be detected with or without affinity chromatography. Collagens are composed of unique repeated GXX sequences. In the present study, 100% of the 15 detected CMA peptides were composed of GXR* sequences (where R* represents CMA), whereas human albumin, which generates a lower amount of CMA, has only one GXR sequence. Among the GXR* sequences, the second amino acid was acidic such as glutamic acid (in peptide 277-291) and aspartic acid (in peptide 334-348) or neutral such as alanine (in peptide 226-258), phenylalanine (in peptide 318-333), or proline (in peptide 514-537), whereas the basic amino acid sequences such as GRR, GKR, and GHR were not present in the identified CMA peptides. These results indicate a lack of regularity in the carboxymethylation of the third amino acid residue (R) and the type of the second

amino acid on the GXR sequence. Furthermore, the amount of CMA formation in these collagen peptides containing the GXR sequence was compared with that of the HSA peptide, which was reported as the hotspot of reactive aldehyde-modification in HSA [34]. As a result, CMA formation in the synthesized collagen peptides was significantly higher than that in HSA peptides. These hotspot regions include the GXXGER sequence, which was reported as the integrin ($\alpha 2\beta 1$) recognition site [42]. These results also demonstrated that a collagen-like amino acid sequence plays an important role in CMA formation in collagen and that the difference in amino acid sequence may be a reason for the preferential generation of CMA in glycated collagen.

Dobler and colleagues [43] demonstrated that arginine residues in the RGD and GFOGER motifs on collagen sequences are modified by methylglyoxal to form a hydroimidazolone derivative. Taken together, their report and our present observation demonstrate that collagen-specific sequences are susceptible to modification by dicarbonyls.

The inhibition of CMA formation by dicarbonyl-trapping reagents during incubation of collagen with glucose and the predominant CMA formation from glyoxal demonstrate that glyoxal plays a role in generating important intermediates for CMA formation. Furthermore, CMA formation was increased with increasing glyoxal concentration but was inhibited at a concentration greater than 4 mM. In contrast, CML formation increased in accordance with the glyoxal concentration. Although CMA was detected by ELISA on collagen modified with glyoxal (1 mM), the reactivity disappeared after the sample was further incubated with 100 mM glyoxal, since supraphysiological concentrations of glyoxal formed a dimer of CMA (data not shown).

CMA formation was higher than that of CML under low concentrations of glyoxal, which was supported by our previous observation. Thus, glucose and glycolaldehyde predominantly modify lysine residues more than arginine residues, whereas glyoxal and methylglyoxal modify arginine residues more than lysine residues [40], demonstrating that CMA may be the predominant AGE formed from glyoxal *in vivo*. Therefore, to demonstrate the physiological content, CMA levels in the skin of mice were determined by ESI-LC-MS/MS. The CMA content in the mouse tissue was similar to that of other AGEs when the levels of lysine-derived and arginine-derived AGEs were normalized to lysine and arginine contents, respectively. However, the same tendency of AGE levels in glycated collagen *in vitro* was also observed in physiological samples. Thus, the level of CMA per tissue protein was the highest among the measured AGEs and was 5-fold higher than that of CML. Moreover, higher CMA accumulation was detected in the aortas from elderly subjects compared to that from aortas of younger subjects, suggesting that CMA accumulation is increased with aging in collagen-rich tissues. Taken together, our study demonstrates that CMA is generated in glycated collagen *in vitro* and in collagen-rich tissues such as the mouse skin and human aorta. Although Thornalley et al. [11] measured the AGE content in biological samples by enzymatic hydrolysis, multiple preparation steps are required in this method. In the present study, CMA levels in the mouse skin were measured after acid hydrolysis. Cotham et al. [44] reported that glyoxal generates not only CMA but also cyclic structures such as hydro-imidazolinones (G-H1, G-H2, and G-H3). Our preliminary study demonstrated that approximately 90% of the CMA was cyclized, with 6 N HCl at 100°C for 24 h. CMA content was quantitated using an internal standard based on the principle reported by Shaw et al. [25] (data not shown).

Surprisingly, since collagen produced more CML as well as CMA compared to albumin, collagens are likely to be glycated proteins. As collagens are one of the most abundant proteins and are involved in many physiologically important functions, specific detection of CMA may play an important role in the elucidation of the relationship between the glycation and denaturation of collagens.

Abbreviations

AGEs:	Advanced glycation end products
CMA:	N^{ω}-(Carboxymethyl)arginine
CML:	N^{ϵ}-(Carboxymethyl)lysine
CMC:	*S*-(Carboxymethyl)cysteine
MG-H1:	N^{δ}-(5-Hydro-5-methyl-4-imidazolon-2-yl)-ornithine
CEL:	N^{ϵ}-(Carboxyethyl)lysine
ESI-LC-MS/MS:	Liquid chromatography-tandem mass spectrometry equipped with an electrospray ionization source
ESI-QTRAP:	Hybrid triple quadrupole/linear ion trap mass spectrometer equipped with an electrospray ionization source
HSA:	Human serum albumin
DTPA:	Diethylenetriaminepentaacetic acid.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Supplementary Materials

Supplementary Information: formation of arginine adduct with high concentration of glyoxal. *(Supplementary Materials)*

References

[1] M. X. Fu, K. J. Wells-Knecht, J. A. Blackledge, T. J. Lyons, S. R. Thorpe, and J. W. Baynes, "Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction," *Diabetes*, vol. 43, no. 5, pp. 676–683, 1994.

[2] E. B. Frye, T. P. Degenhardt, S. R. Thorpe, and J. W. Baynes, "Role of the Maillard reaction in aging of tissue proteins. Advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins," *The Journal of Biological Chemistry*, vol. 273, no. 30, pp. 18714–18719, 1998.

[3] H. Makino, K. Shikata, K. Hironaka et al., "Ultrastructure of nonenzymatically glycated mesangial matrix in diabetic nephropathy," *Kidney International*, vol. 48, no. 2, pp. 517–526, 1995.

[4] K. Yamada, Y. Miyahara, K. Hamaguchi et al., "Immunohistochemical study of human advanced glycosylation end-products (AGE) in chronic renal failure," *Clinical Nephrology*, vol. 42, no. 6, pp. 354–361, 1994.

[5] S. Kume, M. Takeya, T. Mori et al., "Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody," *The American Journal of Pathology*, vol. 147, no. 3, pp. 654–667, 1995.

[6] T. Miyata, O. Oda, R. Inagi et al., "Beta 2-microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis," *The Journal of Clinical Investigation*, vol. 92, no. 3, pp. 1243–1252, 1993.

[7] K. Mizutari, T. Ono, K. Ikeda, K. Kayashima, and S. Horiuchi, "Photo-enhanced modification of human skin elastin in actinic elastosis by N^{ϵ}-(carboxymethyl)lysine, one of the glycoxidation products of the Maillard reaction," *The Journal of Investigative Dermatology*, vol. 108, no. 5, pp. 797–802, 1997.

[8] D. R. Sell and V. M. Monnier, "Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process," *The Journal of Biological Chemistry*, vol. 264, no. 36, pp. 21597–21602, 1989.

[9] R. Nagai, Y. Fujiwara, K. Mera, K. Yamagata, N. Sakashita, and M. Takeya, "Immunochemical detection of N^{ϵ}-(carboxyethyl)-lysine using a specific antibody," *Journal of Immunological Methods*, vol. 332, no. 1-2, pp. 112–120, 2008.

[10] N. Ahmed, P. J. Thornalley, J. Dawczynski et al., "Methylglyoxal-derived hydroimidazolone advanced glycation end-products of human lens proteins," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 12, pp. 5287–5292, 2003.

[11] P. J. Thornalley, A. Langborg, and H. S. Minhas, "Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose," *Biochemical Journal*, vol. 344, no. 1, pp. 109–116, 1999.

[12] R. Nagai, Y. Unno, M. C. Hayashi et al., "Peroxynitrite induces formation of N^{ϵ}-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite," *Diabetes*, vol. 51, no. 9, pp. 2833–2839, 2002.

[13] R. Nagai, C. M. Hayashi, L. Xia, M. Takeya, and S. Horiuchi, "Identification in human atherosclerotic lesions of GA-pyridine, a novel structure derived from glycolaldehyde-modified proteins," *The Journal of Biological Chemistry*, vol. 277, no. 50, pp. 48905–48912, 2002.

[14] M. Arai, H. Yuzawa, I. Nohara et al., "Enhanced carbonyl stress in a subpopulation of schizophrenia," *Archives of General Psychiatry*, vol. 67, no. 6, pp. 589–597, 2010.

[15] J. Mitome, H. Yamamoto, M. Saito, K. Yokoyama, K. Marumo, and T. Hosoya, "Nonenzymatic cross-linking pentosidine increase in bone collagen and are associated with disorders of bone mineralization in dialysis patients," *Calcified Tissue International*, vol. 88, no. 6, pp. 521–529, 2011.

[16] K. Iijima, M. Murata, H. Takahara, S. Irie, and D. Fujimoto, "Identification of N^{ω}-carboxymethylarginine as a novel acid-labile advanced glycation end product in collagen," *The Biochemical Journal*, vol. 347, no. 1, pp. 23–27, 2000.

[17] H. Odani, K. Iijima, M. Nakata et al., "Identification of N^{ω}-carboxymethylarginine, a new advanced glycation end product in serum proteins of diabetic patients: possibility of a new marker of aging and diabetes," *Biochemical and Biophysical Research Communications*, vol. 285, no. 5, pp. 1232–1236, 2001.

[18] L. Rittie and G. J. Fisher, "Natural and sun-induced aging of human skin," *Cold Spring Harbor Perspectives in Medicine*, vol. 5, no. 1, article a015370, 2015.

[19] M. Saito and K. Marumo, "Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus," *Osteoporosis International*, vol. 21, no. 2, pp. 195–214, 2010.

[20] R. F. Chen, "Removal of fatty acids from serum albumin by charcoal treatment," *The Journal of Biological Chemistry*, vol. 242, no. 2, pp. 173–181, 1967.

[21] K. Mera, R. Nagai, N. Haraguchi et al., "Hypochlorous acid generates N^{ϵ}-(carboxymethyl)lysine from Amadori products," *Free Radical Research*, vol. 41, no. 6, pp. 713–718, 2007.

[22] J. Shirakawa, S. Arakawa, T. Tagawa et al., "Salacia chinensis L. extract ameliorates abnormal glucose metabolism and improves the bone strength and accumulation of AGEs in type 1 diabetic rats," *Food & Function*, vol. 7, no. 6, pp. 2508–2515, 2016.

[23] H. Sugawa, R. Ohno, J. Shirakawa et al., "Eucommia ulmoides extracts prevent the formation of advanced glycation end products," *Food & Function*, vol. 7, no. 6, pp. 2566–2573, 2016.

[24] K. Mera, Y. Fujiwara, M. Otagiri, N. Sakata, and R. Nagai, "Immunological detection of N^{ω}-(carboxymethyl)arginine by a specific antibody," *Annals of the New York Academy of Sciences*, vol. 1126, no. 1, pp. 155–157, 2008.

[25] J. N. Shaw, J. W. Baynes, S. R. Thorpe et al., "N^{ϵ}-(carboxymethyl)lysine (CML) as a biomarker of oxidative stress in long-lived tissue proteins," *Methods in Molecular Biology*, vol. 186, pp. 129–137, 2002.

[26] Y. Taga, M. Kusubata, K. Ogawa-Goto, and S. Hattori, "Development of a Novel Method for Analyzing Collagen *O*-glycosylations by Hydrazide Chemistry," *Molecular & Cellular Proteomics*, vol. 11, no. 6, article M111.010397, 2012.

[27] D. L. Price, P. M. Rhett, S. R. Thorpe, and J. W. Baynes, "Chelating activity of advanced glycation end-product inhibitors," *The Journal of Biological Chemistry*, vol. 276, no. 52, pp. 48967–48972, 2001.

[28] T. Soulis, M. E. Cooper, D. Vranes, R. Bucala, and G. Jerums, "Effects of aminoguanidine in preventing experimental

diabetic nephropathy are related to the duration of treatment," *Kidney International*, vol. 50, no. 2, pp. 627–634, 1996.

[29] J. M. Forbes, T. Soulis, V. Thallas et al., "Renoprotective effects of a novel inhibitor of advanced glycation," *Diabetologia*, vol. 44, no. 1, pp. 108–114, 2001.

[30] H. P. Hammes, S. Martin, K. Federlin, K. Geisen, and M. Brownlee, "Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 24, pp. 11555–11558, 1991.

[31] M. Colzani, D. de Maddis, G. Casali, M. Carini, G. Vistoli, and G. Aldini, "Reactivity, selectivity, and reaction mechanisms of aminoguanidine, hydralazine, pyridoxamine, and carnosine as sequestering agents of reactive carbonyl species: a comparative study," *ChemMedChem*, vol. 11, no. 16, pp. 1778–1789, 2016.

[32] L. Yao, M. He, D. Li, J. Tian, H. Liu, and J. Xiao, "Terminal aspartic acids promote the self-assembly of collagen mimic peptides into nanospheres," *RSC Advances*, vol. 8, no. 5, pp. 2404–2409, 2018.

[33] Y. Feng, G. Melacini, J. P. Taulane, and M. Goodman, "Acetyl-terminated and template-assembled collagen-based polypeptides composed of Gly-pro-Hyp sequences. 2. Synthesis and conformational analysis by circular dichroism, ultraviolet absorbance, and optical rotation," *Journal of the American Chemical Society*, vol. 118, no. 43, pp. 10351–10358, 1996.

[34] N. Ahmed and P. J. Thornalley, "Peptide mapping of human serum albumin modified minimally by methylglyoxal *in vitro* and *in vivo*," *Annals of the New York Academy of Sciences*, vol. 1043, no. 1, pp. 260–266, 2005.

[35] T. Higashi, H. Sano, T. Saishoji et al., "The receptor for advanced glycation end products mediates the chemotaxis of rabbit smooth muscle cells," *Diabetes*, vol. 46, no. 3, pp. 463–472, 1997.

[36] A. M. Schmidt, M. Vianna, M. Gerlach et al., "Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface," *The Journal of Biological Chemistry*, vol. 267, no. 21, pp. 14987–14997, 1992.

[37] R. Nagai, K. Mera, K. Nakajou et al., "The ligand activity of AGE-proteins to scavenger receptors is dependent on their rate of modification by AGEs," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1772, no. 11-12, pp. 1192–1198, 2007.

[38] K. Motomura, Y. Fujiwara, N. Kiyota et al., "Astragalosides isolated from the root of astragalus radix inhibit the formation of advanced glycation end products," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 17, pp. 7666–7672, 2009.

[39] T. W. Lo, M. E. Westwood, A. C. McLellan, T. Selwood, and P. J. Thornalley, "Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with Nα-acetylarginine, Nα-acetylcysteine, and Nα-acetyllysine, and bovine serum albumin," *The Journal of Biological Chemistry*, vol. 269, no. 51, pp. 32299–32305, 1994.

[40] R. Nagai, K. Matsumoto, X. Ling, H. Suzuki, T. Araki, and S. Horiuchi, "Glycolaldehyde, a reactive intermediate for advanced glycation end products, plays an important role in the generation of an active ligand for the macrophage scavenger receptor," *Diabetes*, vol. 49, no. 10, pp. 1714–1723, 2000.

[41] K. Mera, K. Takeo, M. Izumi, T. Maruyama, R. Nagai, and M. Otagiri, "Effect of reactive-aldehydes on the modification and dysfunction of human serum albumin," *Journal of Pharmaceutical Sciences*, vol. 99, no. 3, pp. 1614–1625, 2010.

[42] P. R.-M. Siljander, S. Hamaia, A. R. Peachey et al., "Integrin activation state determines selectivity for novel recognition sites in fibrillar collagens," *The Journal of Biological Chemistry*, vol. 279, no. 46, pp. 47763–47772, 2004.

[43] D. Dobler, N. Ahmed, L. Song, K. E. Eboigbodin, and P. J. Thornalley, "Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification," *Diabetes*, vol. 55, no. 7, pp. 1961–1969, 2006.

[44] W. E. Cotham, T. O. Metz, P. L. Ferguson et al., "Proteomic analysis of arginine adducts on glyoxal-modified ribonuclease," *Molecular & Cellular Proteomics*, vol. 3, no. 12, pp. 1145–1153, 2004.

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Review Article

Nutrients in the Prevention of Alzheimer's Disease

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Alzheimer's disease (AD) is a disease caused by the complex interaction of multiple mechanisms, some of which are still not fully understood. To date, pharmacological treatments and supplementation of individual nutrients have been poorly effective in terms of the prevention and treatment of AD, while alternative strategies based on multimodal approaches (diet, exercise, and cognitive training) seem to be more promising. In this context, the focus on dietary patterns rather than on single food components could be more useful in preventing or counteracting the pathological processes typical of AD, thanks to the potential synergistic effects of various nutrients (neuronutrients). The aim of this narrative review is to summarize the currently existing preclinical and clinical evidence regarding the Mediterranean diet (MeDi), the Dietary Approaches to Stop Hypertension (DASH) diet, and the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet, which are three dietary patterns with well-known anti-inflammatory and antioxidant properties. Recently, they have been related to brain protection and AD prevention, perhaps thanks to their high content of neuroprotective bioactive compounds. Similarly, intermittent fasting (IF) or calorie restriction (CR) is emerging as interesting approaches that seem to promote hippocampal neurogenesis, activate adaptive stress response systems, and enhance neuronal plasticity, thus leading to motor and cognitive improvements in animal models of AD and hopefully also in human beings.

1. Introduction

Alzheimer's disease (AD) is a disease of later life, affecting one in four people 85 years of age or over, and the incidence is expected to rise in the coming years, with 131.5 million estimated cases by 2050 [1]. To date, approved drugs have shown modest clinical benefits in delaying the neurodegenerative process, and currently, the AD epidemic is facing two major challenges. Both the lack of disease modifying drugs and the need to delay cognitive-related disability and frailty trajectories highlight the necessity to develop nonpharmacological strategies to stop this ever-increasing global burden. In line with this, previous studies, including the milestone Rotterdam study, have pointed out the relevance of nutrition in counteracting brain neurodegeneration [2].

Although several nutritional approaches have been considered as possible alternatives to the currently existing drugs for AD, this line of research has only been partially explored and has not resulted in any solid evidence [3, 4].

The neuropathology and etiology of AD indicate that a complex series of molecular mechanisms is involved, including the amyloid hypothesis, mitochondrial dysfunction, oxidative stress, and brain neuroinflammation as the mainstream molecular pathways [5]. In turn, the neuroinflammatory cascade may be synergistically associated with immunosenescence and gut dysbiosis, intercepting the trajectories of the aging brain and the progression to dementia [6].

Recent evidence has indicated that epigenetics could help shed light on such a complex neurodegenerative pattern. DNA methylation, histone modifications, and microRNAs

are the principal epigenetic mechanisms involved in AD pathophysiology. In line with this, nutrition is believed to be a modifiable environmental factor that seems to strongly impact on AD pathology by modulating its phenotypic expression [7, 8].

Therefore, recent literature reports have underlined the protective role of a number of individual food components, including micro- and macronutrients in the prevention and management of AD [9, 10]. Several researchers have explored the role of single food components, as well as lifestyle habits and inappropriate diets in facilitating the development of AD and its clinical progression. By virtue of the role of cardiovascular risk factors in the onset of AD [11], nutritional approaches targeting insulin resistance, dyslipidemia, and oxidative stress have been found to ameliorate the related clinical conditions, such as diabetes, metabolic syndrome, and dyslipidemia [12–14].

In keeping with the understanding of the complex interplay between nutrition and AD, a multinutrient approach has also been developed which is based on the rationale that multiple dietary molecules can interact in a synergistic manner to modulate several AD molecular hallmarks.

Namely, omega-3 fatty acids, vitamin B and E, choline, and uridine have provided the rationale for improving effectiveness in AD prevention and clinical management. However, to date, no clinical evidence that this putative nutritional supplementation prevents AD onset or progression has been reported [15]. Based on the current findings, it is unlikely that a single food component or a multinutrient supplementation actually represents the right way to prevent the development of AD or slow down its progression.

However, the interactions among several individual nutrients seem to provide the ground for effectiveness with respect to dementia prevention in older adults.

Another important aspect is that all the theoretical evidence that has been collected so far shows a series of pitfalls that hamper practical concepts and the clinical transferability of results.

The main limitations that prevent bench to bedside translation may be related to the experimental conditions and the pharmacological concentrations of the specific food components that hardly mimic human dietary intake and daily recommended doses. Although animal studies seem to be promising, few and contradictory results have been observed in human trials [16]. Moreover, the heterogeneity of the study designs and the paucity of large scale clinical epidemiological and observational studies on the causal link between nutrition and AD make the results even more difficult to understand.

Besides these issues, strategies that focus on dietary patterns rather than on an approach based on individual foods or nutrients seem to provide a unifying conceptual framework between nutrition and AD; the various components or "neuronutrients" included in a good dietary pattern can offer potential synergistic and neuroprotective effects [16–18].

Adopting this as our starting point, our review will summarize the latest developments regarding the use of dietary patterns in older adults as a way to prevent AD.

Thus, studies whose outcomes include "cognitive functions" or "global cognitive performance" or the incidence of a generic "cognitive decline" or "cognitive impairment" will not be taken into consideration.

2. Mediterranean Diet (MeDi) and AD Prevention

The Mediterranean diet (MeDi) would appear to be promising for AD prevention, including the earlier predementia stages. Indeed, the MeDi diet, based on traditional eating habits in Greece, Southern Italy, and other Mediterranean regions, albeit with regional differences, is characterized by high consumption of fruits and vegetables, cereals, legumes, olive oil, nuts, and seeds as the major source of fats, moderate consumption of fish, low to moderate consumption of dairy products and alcohol (wine), and low intake of red and processed meats (see Table 1). It can be considered a nutritional model for healthy dietary habits since it contains all the essential nutrients including monounsaturated fatty acids (mainly in olive oil), polyunsaturated fatty acids (in fatty fish), antioxidants (e.g., allium sulphur compounds, anthocyanins, beta-carotene-flavonoids, catechins, carotenoids, indoles, or lutein), vitamins (A, B1, 6, 9, 12, D, and E), and minerals (magnesium, potassium, calcium, iodine, zinc, and selenium) [19]. Growing evidence indicates the neuroprotective potential of the MeDi, thus supporting the rationale that adherence to this dietary pattern can be a preventative approach towards reducing the risk of cognitive decline, mild cognitive impairment (MCI), and AD [20, 21].

2.1. Epidemiological Evidence (See Table 2). Two cross-sectional studies [22, 23] showed an inverse correlation between the Mediterranean diet and AD in older American and Australian adults. In the first study [22], which was performed on a cohort of elderly American subjects living in New York, the MeDi score (which is a 9-point scale developed on the basis of the eating habits of a Greek population, with higher scores indicating greater adherence) was the main predictor of AD status in logistic regression models adjusted for potential confounders for both AD (age, sex, ethnicity, education, apolipoprotein E genotype, caloric intake, smoking, medical comorbidity index, and body mass index) and for vascular risk factors (dyslipidemia, hypertension, and coronary heart disease) that should be considered possible mediators in the pathogenesis of AD. Higher adherence to the MeDi was associated with a significantly lower risk of AD, considering MeDi adherence both as a continuous and a categorical variable [24]. In the second cross-sectional study, Gardener et al. [23] replicated these results in an Australian population of older adults participating in the Australian Imaging, Biomarkers and Lifestyle Study of Ageing (AIBL) study. As compared to healthy controls, subjects with a diagnosis of MCI or AD had a lower mean MeDi score, and every 1-unit increase in the MeDi score was associated with a 19–26% decrease in the odds of being in the AD category.

Several prospective studies have been published in the last 15 years examining the role of the MeDi diet in reducing the risk of dementia and AD. Numerous US population-

Table 1: Type and frequency of consumption of foods of the various dietary patterns having a role in AD prevention.

Dietary pattern	Characteristics	
	Moderate-to-high consumption	Low consumption
Mediterranean diet (MeDi)	Whole-grain cereals (1-2 s every main meal) Vegetables (≥2 s/every main meal) Fruits (1-2 s/every main meal) Olive oil (every main meal) Olives/nuts/seeds (1-2 s/every day) Low-fat diaries (2 s/every day) Herbs/spices/garlic/onions (every day) Eggs (2-4 s/week) White meat (2 s/week) Fish/seafood (≥2 s/week) Potatoes (≤3 s/week) Legumes (≥2 s/week) (Red) wine	Added salt Red meat (<2 s/week) Processed meat (<1 s/week) Sweets (≤2 s/week)
Dietary Approaches to Stop Hypertension (DASH)	Whole-grain products (every day) Vegetables (every day) Fruits (every day) Diary (moderate-to-high consumption) Poultry (2 s/week) Fish/seafood (1-2 s/week) Legumes (2 s/week)	Saturated fats Total fats Salt (sodium) Sweetened beverages Red and processed meats
Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND)	Green leafy vegetables (≥6 s/week) Other vegetables (≥1 s/day) Nuts (≥5 s/week) Berries (≥2 s/week) Beans (≥3 s/week) Whole grains (≥3 s/day) Fish (≥1 s/week) Poultry (≥2 s/week) Olive oil (primary oil used) (Red) wine (1 glass/day)	Red meats (<4 s/week) Butter and stick margarines Cheese (<1 s/week) Pastries and sweets (<5 s/week) Fried or fast food (<1 s/week)

based studies having a median follow-up of 3–5.4 years revealed that greater adherence to the MeDi was associated with a reduced risk of AD [25–28], a lower risk of developing AD in patients with MCI [29], and lower mortality in AD patients, suggesting a possible role of MeDi in modulating not only the pathogenetic pathways but also the subsequent course of AD [30]. A more recent longitudinal study conducted in a Greek population as part of the Hellenic Longitudinal Investigation of Ageing and Diet (HELIAD) [31] evaluated adherence to the MeDi pattern using a more complicated score, i.e., the Mediterranean Dietary Score (MedDietScore) [32]. The authors of the HELIAD study found that each unit increase in the MedDietScore was associated with a 10% decrease in the odds of dementia.

Unlike the previously mentioned studies, three other prospective studies found no association. In a French study, MeDi adherence was not associated with a risk of incident dementia or AD as a continuous or as a categorical variable [33]. In another study, Roberts et al. reported a 25% reduced risk of MCI or dementia in subjects in the upper tertile of the MeDi score at baseline, but this association did not reach statistical significance, possibly due to the relatively short follow-up (median follow − up = 2.2 years) [34]. In the third one, Olsson et al. found no correlation between MeDi adherence and the risk of AD or all-type dementia in a cohort of 1,138 elderly Swedish men followed-up for 12 years [35].

Several systematic reviews and meta-analyses of both case-control and longitudinal studies confirmed the association between higher adherence to MeDi and a reduced risk of stroke, depression, and neurodegenerative diseases (cognitive decline, dementia, MCI, AD, and Parkinson's disease), albeit with some contradictory results [36–41]. Several factors can at least partly explain these differences in results. The first one is the use of different methods for evaluating eating habits (0 to 9 score, 0 to 55 score, and others): these scores are usually validated in a specific population having specific characteristics, eating habits, and culture. Therefore, they cannot easily be applied to other populations, especially non-Mediterranean ones, such as the Americans or Australians. As already discussed with regard to the assessment of MeDi adherence, the two most commonly used scores are the Trichopoulou's 0 to 9 score [24] and the Panagiotakos's 0 to 55 score [32]. The use of these two scoring systems has been extensively reported in the literature and both have proven to be reliable and valid tools for assessing adherence to the Mediterranean diet, but they are both based on the typical eating habits of the Greek population, so it is difficult to apply these scores to non-Mediterranean populations. Moreover, there is broad heterogeneity in the study characteristics,

Table 2: Summary of the clinical studies that investigate the role of the three dietary patterns (MeDi, DASH, and MIND) in counteracting cognitive decline, incidence of dementia, and/or AD and AD-related mortality.

Authors (year)	Type of diet	Study design	Study population	Results	Follow-up/length of intervention	Reference
Scarmeas et al. (2006)	MeDi	Cross-sectional	Elderly Americans (NY)	Higher adherence to the MeDi was associated with lower risk for AD (odds ratio, 0.76; 95% confidence interval, 0.67-0.87; P.001)	NA	[22]
Gardener et al. (2012)	MeDi	Cross-sectional (AIBL) study	Elderly Australians	Compared with healthy controls, subjects with AD and MCI had a lower mean MeDi score ($P < 0.001$ and <0.05, respectively); each additional unit in the MeDi score was associated with 13–19% lower odds of being in the MCI category, and 19–26% lower odds of being in the AD category	NA	[23]
Scarmeas et al. (2006b)	MeDi	Cohort	Elderly Americans (NY)	Higher adherence to the MeDi was associated with significantly lower risk for development of AD. Each additional unit of the MeDi score was associated with 9 to 10% less risk for development of AD	4 years	[25]
Scarmeas et al. (2009)	MeDi	Cohort	Elderly Americans (NY)	Both higher Mediterranean-type diet adherence and higher physical activity were independently associated with reduced risk for AD	5.4 years	[26]
Gu et al. (2010)	MeDi	Cohort	Elderly Americans (NY)	Significant association between MeDi score and reduction in risk of AD: compared to those in the lowest tertile of MeDi, subjects in the highest tertile had a 34% less risk of developing AD (p-for-trend = 0.04)	3.8 years	[27]
Morris et al. (2015)	MeDi DASH MIND	Cohort	Elderly Americans (Chicago)	Participants in both the middle and the highest tertiles of MIND scores had a statistically significant reduction in AD rate compared to those in the lowest tertile (53% and 35% reduction, respectively). Subjects with the highest adherence to the MeDi and DASH had a 54% and 39% lower risk of developing AD, respectively, compared to those in the lowest tertile (HR = 0.46, 95% CI 0.26, 0.79)	4.5 years	[28]
Scarmeas et al. (2009b)	MeDi	Cohort	Elderly Americans (NY)	Significant association between MeDi adherence and MCI conversion to AD, with a 48% less risk of developing AD (HR: 0.52; 95% CI: 0.30, 0.91; $P = 0.02$) for highest vs. lowest tertile on MeDi score	4.3 years	[29]
Scarmeas et al. (2007)	MeDi	Cohort	Elderly Americans (NY)	Higher adherence to the MeDi is associated with lower mortality in AD patients	4.4 years	[30]
Anastasiou et al. (2017)	MeDi	Cross-sectional	Elderly Greeks	Adherence to MeDi is positively associated with a decreased likelihood of dementia and better	NA	[31]

Table 2: Continued.

Authors (year)	Type of diet	Study design	Study population	Results	Follow-up/length of intervention	Reference
				cognitive performance in many domains, especially memory		
Martinez-Lapiscina et al. (2013)	MeDi	RCT (PREDIMED)	Individuals at high CV risk (from Spain)	A dietary intervention with MeDi enriched with either EVOO or nuts appears to improve cognition compared with a low-fat diet	6.5 years	[43]
Valls-Pedret et al. (2015)	MeDi	RCT (PREDIMED)	Individuals at a high CV risk (from Spain)	A MeDi supplemented with EVOO or nuts is associated with improved composite measures of cognitive function	4.1 years	[44]
Knight et al. (2016)	MeDi	RCT (MedLey)	Elderly Australians	No evidence of a beneficial effect of a MeDi intervention on cognitive function among healthy older adults	6 months	[45]
Marseglia et al. (2018)	MeDi	RCT (NU-AGE)	Five European populations	Improved cognitive performance in both the active and the control groups, with no additional diet-related cognitive improvements	1 year	[46]
Tangney et al. (2014)	MeDi DASH	Cohort	Older Americans (Chicago)	A 1-unit difference in DASH score and in MedDietScore are associated with a slower rate of global cognitive decline by 0.007 standardized units (standard error of estimate = 0.003, $P = 0.03$) and by 0.002 standardized units (standard error of estimate = 0.001, $P = 0.01$), respectively	4.1 years	[67]
Berendsen et al. (2017)	DASH	Cohort	Older American women	Long-term adherence to the DASH diet is associated with better average cognitive function but not with change in cognitive function over the follow-up period	6 years	[69]
Haring et al. (2016)	MeDi DASH	Cohort	Older American women	No association between aMED and DASH scores and incidence of MCI or dementia in older women generally or in those with hypertension	9.1 years	[70]
Smith et al. (2010)	DASH	RCT (ENCORE)	Overweight and sedentary individuals (USA)	Slight improvements in psychomotor speed after the intervention with the DASH diet	4 months	[71]
McEvoy CT et al. (2017)	MeDi MIND	Cross-sectional	Older U.S. adults	Greater adherence to the MeDi and MIND diet is independently associated with better cognitive function and lower risk of cognitive impairment	NA	J Am Geriatr Soc. (2017) 65:1857–1862

such as the mean age of subjects, the duration of follow-up, and the high number of neuropsychological tests used for the diagnosis of MCI and/or AD.

2.2. Randomized Controlled Trials (RCTs). Only few RCTs (PREDIMED, MedLey, and NU-AGE) have assessed the effects of a Mediterranean dietary pattern on cognition in older adults both in Mediterranean and non-Mediterranean countries (see Table 2).

The first RCT to evaluate the effects of long-term MeDi intervention on cognitive function and to shed some light on the role of dietary patterns in counteracting the neurodegenerative process was carried out on a subcohort of the well-known multicenter PREDIMED trial, which was a milestone in establishing the strong preventive role of the MeDi in individuals at a high cardiovascular risk [42]. The nutritional intervention of PREDIMED consisted in a typical MeDi supplemented with extravirgin olive oil or mixed nuts (foods with antioxidant and anti-inflammatory properties) compared to a control low-fat diet. Martinez-Lapiscina and colleagues [43] enrolled 522 subjects and assessed the overall cognitive performance at study completion alone, after 6.5 years. They reported a significant difference in mean Mini-Mental State Examination (MMSE) and Clock Drawing Test (CDT) scores in both intervention groups versus the low-fat control group. There were two main limitations in this study: (1) the lack of evaluation of cognitive performance at the beginning of the study, which did not allow to evaluate changes in cognitive functions over time and (2) the insufficient statistical power to demonstrate a protective effect of MeDi on dementia development, given the small number of total incident cases that were observed during the follow-up.

After a few years, Valls-Pedret et al. [44] published the first MeDi intervention trial demonstrating a positive effect on cognition of the MeDi supplemented with either nuts or extravirgin olive oil. In this PREDIMED substudy, cognitive performance was evaluated both pre- and postintervention, thus enabling the researchers to detect any significant cognitive improvement in the participants allocated to the MeDi intervention groups, who were assessed after a median of 4.1 years.

The MedLey study was the first RCT conducted in older non-Mediterranean adults. A total of 137 subjects were randomly assigned to either a MeDi or a control diet (their usual diet) for six months. This study did not find any significant beneficial effects of a MeDi intervention on cognitive functions (executive functioning, speed of processing, memory visual-spatial ability, and overall age-related cognitive performance) among healthy older adults, perhaps because of the short duration of the intervention, the relatively limited number of participants, or the "Australianization" of the MeDi (i.e., based on the Australian foods and habits, rather than on the traditional MeDi), resulting in smaller nutritional intervention differences between the intervention groups and the control group [45].

The NU-AGE trial (NCT01754012), a recent 1-year multicenter RCT, was carried out in both Mediterranean (including Italy) and non-Mediterranean European countries and allocated a total of 1,279 healthy older adults to two parallel groups. The intervention group followed the NU-AGE diet, which consisted of a culturally adapted and individually tailored Mediterranean-like diet on the basis of the specific dietary recommendations for older adults in the various countries involved in the study. One year later, at the end of the study, all participants showed improvements in their cognitive performance but the differences between the two groups did not reach statistical significance [46]. Nonetheless, the authors highlighted that the participants in the intervention group with the highest adherence to the NU-AGE diet showed a significant improvement in episodic memory, the impairment of which is a core feature of AD [47].

To date, results from RCTs are mostly nonsignificant, with small effect sizes and little or no benefit of the MeDi for incident cognitive impairment or dementia, as also documented by a recent systematic review of RCTs by Radd-Vagenas et al. [48]. RCTs with a longer duration and higher number of participants are needed to establish whether adherence to the MeDi can help prevent (or delay) the onset of AD and dementia.

2.3. Mediterranean Diet and Brain Imaging. AD is a neurodegenerative disease characterized by typical changes in the brain that can be detected by a variety of imaging modalities, including structural and functional Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET). In recent years, cross-sectional and longitudinal studies have shown the relationship between lifestyle and dietary habits and the morphological, structural, functional, and metabolic modifications of the brain regions that undergo neurodegeneration in the preclinical and clinical phases of AD.

2.3.1. Mediterranean Diet and Structural Brain Modifications. Growing evidence, derived mainly from MRI studies, suggests an association between higher MeDi adherence and preservation of brain structures, in particular in the so-called "Regions-of-Interest" (ROIs) which are vulnerable to the neurodegenerative changes typical of AD. Higher MeDi adherence was associated with a significantly greater thickness of 3 ROIs (orbitofrontal cortex, entorhinal cortex, and posterior cingulate cortex of the left hemisphere) in a cross-sectional study performed on an American population of cognitively normal middle-aged participants [49]. Similar findings were reported by two other cross-sectional American studies in which the greatest benefit in terms of brain structure preservation was attributed to the higher intake of fish and legumes and lower intake of meat [50, 51]. More recently, Karstens et al. found that higher adherence to the Mediterranean pattern was associated with better learning and memory performance and larger bilateral dentate gyrus volumes after adjusting for a number of confounding factors [52].

Conversely, several longitudinal studies reported a negative effect on brain structures related to lower adherence to the MeDi [53] or adherence to an unhealthy Western dietary pattern [54], while a Swedish longitudinal study including 194 cognitively healthy elderly individuals found no

association between the total MeDi score and the brain volumes perhaps due to the intrinsic limitations of the study design [55].

In conclusion, gray matter atrophy and reduced cortical thickness in the medial temporal lobe are a typical early feature of AD, and the association of MeDi adherence with greater gray matter volumes in AD regions may indicate some direct involvement of this type of dietary pattern in preventing or slowing the neurodegeneration and the consequent neuronal loss that is typical of this disease. More recently, Pelletier and colleagues reported a positive association between higher MeDi adherence and a general pattern of preserved white matter microstructure with no relation to gray matter volumes, suggestive of alternative mechanisms partly independent of AD-related neurodegeneration, possibly including vascular pathways [56]. The beneficial effect of the MeDi on vascular risk factors (e.g., lipid profile, blood pressure, insulin resistance, adiposity, inflammation, and oxidative stress) is in fact well established [57–60].

2.3.2. Mediterranean Diet and Functional Brain Modifications. The AD brain is characterized by several metabolic changes that can also be found in other neurodegenerative diseases and in normally aging brains and represent nonspecific biomarkers of impairment of neuronal activity and synaptic transmission. These metabolic and functional modifications can be detected by PET, in particular, fluorodeoxyglucose- (FDG-) PET and Pittsburgh Compound B- (PiB-) PET which allow us to evaluate changes in cerebral glucose metabolism (an indicator of neuronal network activity) and the degree of beta-amyloid ($A\beta$) deposition in brain regions known to be involved in AD. Studies concerning the association between MeDi adherence (and in general, adherence to a healthy dietary pattern) and functional neuroimaging outcomes are limited.

Two cross-sectional studies in the American population found an association between higher adherence to a MeDi pattern and lower 11C-PiB PET scan measurements of $A\beta$ deposition as well as higher glucose metabolism as observed by FDG-PET scans [61, 62]. Higher MeDi adherence was also associated with reduced cerebral $A\beta$ accumulation over time (up to 3 years) in a longitudinal study performed by Rainey-Smith et al. on older Australian adults classified as "$A\beta$ accumulators" and thus considered to be on the way to AD [63]. However, the association between the Mediterranean dietary pattern and amyloid deposition or cortical thickness has not been confirmed in all reported studies. In a longitudinal study that involved 70 middle-aged participants living in New York, lower adherence to a Mediterranean-style diet was associated with faster decline in glucose metabolism in the posterior cingulate cortex (an early site of cerebral glucose utilization decline in AD and a well-established predictor of the progression from mild cognitive impairment to AD) and marginally in the frontal cortex, although without any significant changes in amyloid deposition or cortical thickness [64].

In conclusion, the neuroprotective effects of the MeDi may also lie in its ability to preserve brain metabolic activity and glucose metabolism in key brain regions for AD.

3. DASH Diet and AD Prevention

The DASH diet, which stands for Dietary Approaches to Stop Hypertension, promoted by the National Heart, Lung, and Blood Institute (NHLBI), is a dietary pattern that was first developed to identify dietary factors affecting blood pressure. The DASH diet is high in fruits, vegetables, nuts, whole-cereal products, low-fat dairy products, fish, and poultry, all of which are rich in blood pressure-deflating nutrients like potassium, calcium, "lean proteins," minerals, and fiber (see Table 1). DASH also discourages the intake of foods like red and processed meats, full-fat dairy foods, and tropical oils, as well as sugar-sweetened beverages and sweets; therefore, it is designed to be low in sodium, saturated fats, total fats, and cholesterol [65]. This type of dietary pattern has been shown to protect against many cardiovascular risk factors that play a role in the development of dementia and AD (such as high blood pressure or LDL cholesterol), at least in part by modulating the pathological processes that characterize the physiopathology of AD (oxidative stress, inflammation, and insulin resistance) [66]. DASH and MeDi share many food components (i.e., whole-grains, vegetables, and nuts), but there are also some differences, such as the frequency of consumption of low-fat dairy products (moderate-to-high intake and low consumption, respectively).

To date, only a few studies have evaluated the potential effects of the DASH diet on cognitive functions or on the prevention of AD and other types of dementia.

3.1. Epidemiological Evidence. As already stated with regard to the Mediterranean diet, higher adherence to the DASH diet has been associated with slower rates of cognitive decline and reduced incidence of AD [67–69] (see Table 2).

In the study of Morris and colleagues [68], only the highest tertiles of the DASH and MeDi scores were significantly associated with a lower rate of incident AD (39% and 54% reduction, respectively).

Berendsen et al. [69] found a positive association between long-term adherence to the DASH diet and better cognitive function in older American women participating in the Nurses' Health Study, regardless of apolipoprotein E $\varepsilon 4$ allele status, but not with slower cognitive decline during the course of follow-up (6 years). On the contrary, another study which included only older American women, the Women's Health Initiative Memory Study (WHIMS), reported that DASH scores were not associated with incidence of MCI or dementia [70].

3.2. RCTs. The only RCT which examined the potential effects of the DASH diet on neurocognitive functioning was performed some years ago by Smith and colleagues [71] (see Table 2). In the ENCORE trial 124 subjects with high blood pressure were randomized to the DASH diet alone or DASH combined with a behavioral weight management program including exercise and calorie restriction (CR), or to a "usual diet" control group. After 4 months of intervention, psychomotor function improved in both DASH interventions regardless of weight strategies, but only the group which underwent a combination of DASH diet with aerobic

exercise and calorie restriction showed a significant improvement in neurocognitive function (executive memory and learning functions) [71].

4. MIND Diet and AD Prevention

The MIND diet (Mediterranean-DASH Intervention for Neurodegenerative Delay) was developed some years ago by researchers at Rush University Medical Center in Chicago, IL, and Harvard School of Public Health in Boston, MA, as a hybrid of the Mediterranean-DASH diet. The MIND diet is based on 10 brain-healthy foods (leafy green vegetables, other vegetables, nuts, berries, beans, whole grains, fish, poultry, olive oil, and wine) and 5 unhealthy foods (red meats, butter and stick margarines, cheese, pastries and sweets, and fried or fast food), all of which have a strong scientific rationale in the field of nutritional prevention of dementia [72, 73] (see Table 1). Compared to the MeDi, the MIND diet includes a lower consumption of fish, usually 1 serving/week, as does the DASH diet. This relatively low level of fish consumption reflects the findings of prospective epidemiological studies, such as the Rotterdam study which examined the role of fish intake in AD prevention [74–76]. As stated above, leafy green vegetables, in addition to other types of vegetables, are recommended on the basis of several epidemiological studies that reported a significant association between high consumption and slower cognitive decline [77, 78]. Berries represent a separate category in the MIND diet, reflecting strong associations between the consumption of this type of fruit and brain health. Many studies have documented the beneficial effects of various types of berries, including potent anti-inflammatory and antioxidant activity in cell models of neurotoxicity [79, 80]. Moreover, *in vivo* studies on animals supplemented with a berry-enriched diet reported improvements in motor coordination, cognitive performance (spatial memory, learning), hippocampal plasticity, and age-related neuronal loss [81–83]. A unique characteristic of berries is the high content of polyphenols such as flavonoids (anthocyanins and flavonols), which are the main compounds responsible for the antioxidant and anti-inflammatory characteristics of berries [84, 85]. The association between a high intake of berries and flavonoids and slower rates of cognitive decline have also been reported in humans [86], confirming the results that were observed in experimental studies.

4.1. Epidemiological Evidence. Two papers published by Morris and other researchers from Rush University (Chicago, IL, USA) clearly demonstrated the superiority of the MIND diet compared to both the MeDi and the DASH diet in slowing the rates of cognitive decline [28] and in reducing the risk of incident AD or dementia [68]. The MIND diet score was linearly and statistically significantly associated with a lower risk of developing AD (see Table 2). Subjects in both the middle and the highest tertiles of MIND scores had a statistically significant reduction in AD rates compared to those in the lowest tertile, while only those in the highest tertile of MeDi and DASH scores were significantly associated with a lower rate of incident AD [68]. To date, no randomized controlled trials have been published evaluating the effect of the MIND diet on the prevention of AD, but there are two ongoing studies testing the effects of an intervention with the MIND diet on cognitive decline and brain neurodegeneration, the results of which will be reported in the coming years (http://www.clinicaltrials.gov/NCT02817074, http://www.clinicaltrials.gov/NCT03585907).

5. Dietary Patterns and the Brain: The Underlying Mechanisms

The clinical evidence that has been collected in recent years suggests that the dietary patterns described above, namely, the Mediterranean diet, the DASH diet, and more recently the MIND diet, are able to modify the natural history of neurodegenerative pathologies, in particular AD, thereby preventing their development or slowing down their progression. But what is the basis of the favorable neuroprotective effects of these dietary patterns? What are the links between diet and the human brain? Are there any other factors that modulate the effects of diet on the brain?

The strength of these dietary approaches lies in their multifactorial composition. In fact, nutrient-dense foods can interact with each other with potentially synergistic effects on different metabolic and cellular signaling pathways, leading to neuroprotection and maintaining brain health. But there are considerable methodological difficulties in conducting *in vitro* and *in vivo* studies to document the effects of such dietary patterns on the brain, due to the intrinsic complexity of a whole diet, the micro- and macronutrient composition, the importance of food cooking methods, and at least for the Mediterranean diet, the undeniable aspect of conviviality that characterizes this dietary lifestyle. All of these aspects are obviously difficult to reproduce and quantify in experimental models.

To date, the precise mechanisms underlying the neuroprotective benefits associated with the MeDi and the other dietary patterns are not fully understood, even if it is widely accepted that they play a role in counteracting several biological processes implicated in the pathogenesis of AD, e.g., oxidative stress, neuroinflammation, neurovascular dysfunctions and hypoperfusion, disruption of the gut-brain axis, and impairment of hippocampal neurogenesis. It is also possible that these dietary patterns might influence Aβ or Tau metabolism, even if evidence concerning these mechanisms were largely obtained from animal studies and require further assessment and confirmation [87, 88]. At a systemic level, they can also indirectly improve cognition by reducing cardiovascular risk factors such as lipid levels, blood glucose, and blood pressure [89, 90].

5.1. Neurovascular Dysfunctions. Cerebral blood flow regulation is essential for normal brain function. AD is known to be associated with early neurovascular dysfunction and damage to small arteries, arterioles, and brain capillaries via both Aβ-independent (such as hypoxia and/or ischemia) and Aβ-dependent pathways (Aβ angiopathy). These changes, together with the loss of integrity of the blood-brain barrier

(BBB), play a part in disease pathogenesis and lead to neuronal and synaptic dysfunction, neurodegeneration, and cognitive impairment [91, 92]. Several risk factors can contribute to neurovascular dysfunction, for example, genetic factors (APOE4 genotype), vascular factors (hypertension and diabetes mellitus), and environmental factors (for example, pollution). Hypertension is a well-known risk factor for AD, and animal studies have shown impairment in acetylcholine-dependent and endothelium-dependent vasodilation with a consequent reduction of cerebral blood flow after several stimuli [93]. Chronically elevated blood pressure can also enhance Aβ-induced neurovascular dysfunction, promote β-secretase activity, and lead to amyloidogenic processing of the amyloid precursor protein (APP), with further damage to small arteries and arterioles, rupture of the vessel wall, and microhemorrhages [93].

Hence, the importance and the possible preventive and neuroprotective role of a diet such as the DASH diet, which thanks to its reduced content of sugars, sweets/pastries, and sodium can act positively on some of the triggers of neurovascular dysregulation (hypertension and hyperglycemia/insulin resistance) [94]. In addition to limiting the intake of such detrimental foods and nutrients, increasing evidence supports the regular consumption of flavonoid-rich foods, which are associated with better endothelial function, tissue perfusion, and enhanced neuronal viability. For example, the flavonoids contained in cocoa powder (mainly epicatechin) act directly on the endothelium of brain vessels enhancing the activity of endothelial nitric oxide synthase (eNOS) and thereby increasing vasodilatation and cerebrovascular perfusion [95].

5.2. Oxidative Stress. The imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses has been shown to contribute significantly to the pathogenesis and progression of AD [96] and is known to be associated with oxidative damage to DNA, proteins, and lipidic components of neurons and with subsequently impaired synaptic activity and neuronal apoptotic death [96]. Several antioxidant compounds can modulate signaling cascades involving protein and lipid kinases and downstream partners, resulting in the inhibition of neuronal apoptosis induced by toxic products such as ROS [97].

All three dietary patterns discussed in this review are exceptionally rich in foods with a high antioxidant content. Leafy green vegetables and other vegetables, citrus fruits, nuts, red wine, berries, and olive oil all provide a large amount of vitamins, polyphenols, and carotenoids that can prevent and/or repair the damage caused by free radicals (e.g., superoxide, $O_2^{\cdot -}$ and nitric oxide, $NO^{\cdot}$) and by proinflammatory cytokines (e.g., IL-1β and TNF-α) that are produced by activated microglial cells in response to oxidative damage. One of the main components of the Mediterranean diet, as well as the main source of fat in this diet, is extravirgin olive oil (EVOO), which contains mainly oleic acid and to a lesser extent linoleic acid. In addition, EVOO contains hundreds of bioactive compounds including triterpenes (i.e., squalene), biophenols (hydroxytyrosol, tyrosol, and oleuropein), pigments (carotenoids, xanthophylls, and lutein), and vitamin E (tocopherols), whose beneficial effects have been shown in several studies [98, 99]. The low (and nontoxic) level of oxidized forms of EVOO polyphenols in plasma and tissues can activate the Nrf2 pathway and other adaptive stress response systems leading to the upregulation of the endogenous antioxidant and detoxification enzymes and thus rendering the cells "protected" against more dangerous and chronic oxidative stress stimuli (hormesis) [100]. Recent studies have shown that hydroxytyrosol, oleuropein, and oleacein can activate the Nrf2 pathway both *in vitro* and *in vivo* [101, 102].

5.3. Neuroinflammation. Microglial cells are the first and most important immune defense of the brain. While proper microglial function is essentially required for scavenging plaques, damaged molecules, and infectious agents, microglial hyperactivation is a well-established hallmark of neuroinflammation and one of the main actors in AD pathogenesis [103]. In the AD process, the excessive production and deposition of the Aβ peptides trigger an innate immune response and consequently an aberrant production of ROS, proinflammatory cytokine, and chemokine secretion, and degradation of the neuroprotective factors, such as retinoids, involved in promoting adult neurogenesis in the hippocampus [104, 105]. This chronic inflammatory response contributes to disease progression and severity, further boosts Aβ production and deposition, and ultimately leads to neuronal death [106, 107]. Several exogenous or endogenous factors can exacerbate the innate immune response mounted by Aβ-exposed microglia, including genetic factors (for example, TREM2 mutation [108] and APOE4 genotype [109]), traumatic brain injuries, diabetes [110], and obesity [111]. Diet is known to modulate the immune system, and a healthy diet rich in nutrients and bioactive compounds with anti-inflammatory and antioxidant properties can help to counteract the neuroinflammatory process.

Fruits, vegetables, whole grains, and other plant foods provide a wide range of phytochemicals, vitamins, minerals, and fibers with well-established antioxidant anti-inflammatory properties. Phytochemicals are bioactive plant-derived compounds that include various subgroups (phenolics, alkaloids, organosulfur compounds, phytosterols, and carotenoids). One example is Ferulic Acid (FA), an antioxidant with free radical scavenging activity but also antiamyloidogenic properties, as documented in a number of *in vitro* [112, 113] and *in vivo* studies [114, 115] in transgenic mouse models of AD. Resveratrol, another phenolic compound, is mainly found in the skin of many edible plant species, such as mulberries, grapes, peanuts, and pomegranates, as well as in red wine [116]. There are a multitude of actions that have been attributed to resveratrol: inhibition of Tau and Aβ plaque synthesis [117, 118], downregulation of prooxidative stress proteins [119] and increased levels of heme-oxygenase-1 (HO-1) [120] and SIRT-1, and inhibition of neuroinflammation [121, 122].

Long chain omega-3 polyunsaturated fatty acids ($n-3$ PUFAs) are also important [123]. Docosahexaenoic acid (DHA) and Eicosapentaenoic Acid (EPA), two main types

of $n-3$ PUFAs, are abundantly present in seafood and fish oil. *In vitro* experiments showed that EPA and/or DHA administration decreases the expression of proinflammatory factors, such as inducible NO synthase (iNOS), cyclooxygenase (COX) 2, interleukin-1β (IL-1β), IL-6, Tumor Necrosis Factor-α (TNF-α), and Nuclear Factor-κB (NF-κB), and promotes the expression of anti-inflammatory cytokines. In rodents, the consumption of a diet enriched in $n-3$ PUFAs prevents the dysregulation of cytokine production in hippocampal microglial cells in response to Lipopolysaccharide (LPS) [124], reduces hippocampal Aβ plaque density by modifying the fibrillar/prefibrillar Aβ oligomer ratio (the former are less toxic), and leads to mild improvements in the behavioral testing of the transgenic APP/PS1 rodent model of AD [125]. Moreover, dietary omega-3 and omega-6 PUFA and monounsaturated fatty acid (MUFA) intake may influence the membrane fluidity and enzyme activity in neurons, leading to the potential modulation of brain structures and functions [126].

5.4. Gut Microbiota Dysbiosis. The human gut microbiota includes approximately 10^{14} microbes belonging to hundreds different species and to five predominant phyla and is mainly composed of two phyla: *Firmicutes* (60-80%) and *Bacteroides* (20-30%), followed by *Actinobacteria*, *Verrucomicrobia*, and *Proteobacteria* [127, 128]. The gut microbiota plays a crucial role in human health but also in a variety of human diseases, at least in part through the production and release of numerous small molecules like vitamins (folate, vitamin B12), amino acids (tryptophan), and short chain fatty acids (SCFAs). Some of these bioactive substances (SCFAs, catecholamines, neurotransmitters, neuropeptides, and miRNAs) are transported into the blood and can cross the BBB thus affecting the central nervous system (CNS). Additionally, the CNS communicates with the gut through efferent autonomic pathways, thus modulating many gut functions like permeability, mucus secretion, motility, and immunity. This bidirectional communication between the gastrointestinal system and the CNS is called the gut-brain axis [129].

A healthy microbiota is fundamental for the metabolization of such dietary nutrients (like polyphenols) which require transformation to become active compounds having beneficial effects on the brain. Curcumin metabolites produced by the microbiota can exert anti-inflammatory and neuroprotective effects [130], including interesting positive effects on Tau pathology [131].

The composition of human gut microbiota is dynamic and can be shaped by various factors such as the type of childbirth and newborn feeding, diet, use of drugs, or pre/-probiotics, as well as age, sex, and geographical area. The Mediterranean diet, which is rich in plant-based foods, fibers, and monounsaturated fats, is considered the gold standard for gut health and promotes the diversification of the microbiota [132]. Conversely, the typical Western diet, which is made up of low dietary fiber and high animal protein and saturated fat, is associated with a negative change in the gut microbiota composition (dysbiosis), as documented by an increase in Firmicutes and Gram-negative bacteria [133]. As previously mentioned, age is another important modulating factor of human gut microbiota composition. Several reviews analyzed gut microbiota age-related changes and the potential relationships between gut dysbiosis and inflammaging [134–136].

During aging, the gut undergoes a continuous and profound remodeling, as a result of modification of lifestyle, nutrition, behavior, immunosenescence (a decline in an immune system functioning), and inflammaging (the chronic low-grade inflammatory status typical of the elderly). Moreover, aging-associated alterations in gut physiology (i.e., gastric motility disorders, hypochlorhydria, and degenerative changes in the enteric nervous system) have profound effects on the diversity, composition, and functional features of intestinal microbiome [137]. Several authors reported a reduction in the microbiota diversity and a greater interindividual variation in microbiota composition in elderly people (>65 years of age), with reduced numbers of *Bifidobacteria*, *Firmicutes*, *Faecalibacterium prausnitzii*, and *Clostridium* cluster XIV and increased numbers of *Bacteroidetes* and *Enterobacteriaceae* even if with some differences between various populations [138–140].

The inflammatory process can affect the gut environment by enhancing the level of aerobiosis and the production of ROS that inactivate the strict anaerobic *Firmicutes*, while allowing the growth of facultative aerobes (i.e., *Enterobacteriaceae*, *Enterococcaceae*, and *Staphylococcaceae*). These so-called "pathobionts" can survive in the presence of oxygen, so they can prosper in an inflamed gut, and in turn, they promote a proinflammatory profile (increase of Il-6 and Il-8 levels) and compromise the host immune homeostasis, in a sort of self-sustaining loop [141].

Gut dysbiosis has been linked to the development of several health problems, including psychiatric or neurodegenerative diseases. An increasing body of evidence suggests that alterations to the gut microbiota can play a role in the pathogenesis of AD. Dysbiosis can amplify neuroinflammation and accelerate neurodegeneration, and this brain-gut microbiota axis can actually be modulated by dietary factors. In aging mice, AD-like symptoms were associated with increased gut permeability, inflammation, and a microbiome profile similar to that of murine inflammatory bowel disease [142]. Experimental mice models of AD showed a decrease in microbiota diversity with age, an increased number of taxa with proinflammatory activity (e.g., Odoribacter, Helicobacter, and Sutterella), and impaired production of SCFAs [143, 144]. All these modifications can be worsened by feeding transgenic mice a high-fat diet [145]. Moreover, gut microbiota dysbiosis in mouse models of dementia may be involved in neuroinflammation, reduced expression of hippocampal brain-derived neurotrophic factor (BDNF) and other signaling molecules, and amyloid deposition [146–148]. Conversely, administering probiotics to rodents with artificially induced AD led to an improvement in cognitive functions, especially spatial working tasks, less neuron degeneration and lower levels of proinflammatory cytokines [149–151], microglial activation and oxidative stress, improved mitochondrial dysfunction, and restoration of hippocampal plasticity [152].

In conclusion, long-term dietary habits may influence gut microbiota biodiversity, its functions, and the secretion of metabolites that, once absorbed in the systemic circulation, can modulate neural function and possibly enhance neuroinflammation, neuronal apoptosis, and β-amyloid deposition [153]. The modulation of gut microbiota by adopting and maintaining a healthy diet can be an effective strategy in AD prevention. However, the transposition of these results into humans is still premature given the absence of clinical studies, especially in middle-aged or older patients at risk of developing AD or with MCI.

5.5. Adult Hippocampal Neurogenesis. The hippocampus is the key brain region for learning and memory. Incorporation of new neurons into the granular cell layer of the dentate gyrus of the hippocampus is substantial throughout life, and adult neurogenesis has important implications in maintaining cognitive functions [154]. This brain area is highly involved in the process of neurodegeneration that is typical of AD. Numerous studies have documented an impairment of neurogenesis and relative memory loss as well as cognitive dysfunction in mouse models of AD even in the very early stages of the disease [155]. Environmental factors such as exercise and calorie restriction (see the specific chapter below) have been shown to increase adult hippocampal neurogenesis, while low-grade inflammation and oxidative stress seem to decrease it [156]. Hippocampal neurogenesis is regulated by several signaling pathways such as presenilin-1, Notch 1, soluble amyloid precursor protein, CREB, and β-catenin and is also mediated by neurotrophins such as BDNF. A poor diet can have detrimental effects on hippocampal neurogenesis. Diets that are rich in saturated fats/trans fatty acids and refined sugars, like Western diets, reduce the levels of BDNF and contribute to an increased production of ROS and proinflammatory cytokines, leading to neurodegeneration and learning and memory impairment [157]. On the contrary, dietary patterns based on foods that are rich in omega-3 fatty acids, flavonoids, and other antioxidants stimulate neurogenesis, reduce oxidative activity, and downregulate proinflammatory processes [158, 159].

6. Intermittent Fasting: A New Dietary Pattern for AD?

Aging is one of the main risk factors for AD. The aging brain and the "AD-Brain" share many characteristics, both at an anatomical and at a cellular/molecular level [160]. Indeed, many of the principal hallmarks of aging (e.g., oxidative stress, mitochondrial dysfunction, accumulation of oxidatively damaged molecules, impaired autophagy, disruption of Ca^{2+} homeostasis, aberrant neuronal network excitability, and neuroinflammation) have also been documented in AD, and these changes may promote amyloidogenic APP processing and Tau pathology and vice versa [161, 162].

Over the last 30 years, emerging evidence has shown the beneficial effects of fasting and CR as alternative or complementary strategies to other lifestyle interventions (e.g., physical activity) and to pharmacological therapies in AD prevention and treatment.

During the first 10-14 hours of fasting, the main source of energy for neurons is made up of glucose derived from the degradation of the liver glycogen store. Then, a "metabolic switch" occurs, characterized by liver production of Ketone Bodies (KBs) like β-hydroxybutyrate (BHB) and acetoacetate (AcAc) from the fatty acids released by adipose tissue in response to fasting; in this second phase, KBs represent the main fuel for neurons [163]. Besides this "metabolic switch," fasting can enhance a complex series of adaptive responses to limited food availability, which are to some degree, the same endogenous stress-response systems activated by foods that are rich in polyphenols and other bioactive compounds (as described above) [164].

In prokaryotes, laboratory animals and humans, a daily 20-40% CR, can protect against aging, oxidative stress, and neurodegenerative disorders and can also extend longevity [165, 166], but the feasibility and long-term tolerability are low, especially in patients with AD. Other studies have shown the same positive results by alternating normal diets with more feasible regimens, such as intermittent fasting (IF) or periodic fasting (PF) that are short periods of fasting which differ from each other with regard to duration and frequency. The best-characterized form of fasting that has been studied both in animal models and in humans is Alternate-Day Fasting (ADF), which implies fasting every other day [167, 168] or a 70% CR every other day [169, 170] or even 2 consecutive days of Very Low-Calorie Diet (VLCD) per week [171]. Upcoming alternatives are the so-called Fasting-Mimicking Diets (FMDs), characterized by periodic cycles of plant-based dietary programs lasting from 3 to 7 days, that are low in protein and in overall caloric intake but contain all the necessary micronutrients [172, 173]. Regardless of how fasting has been applied in these studies, it should be emphasized that it is different from starvation, which leads to chronic insufficiency of nutrients, malnutrition, and ultimately degeneration and death.

6.1. Why Is Fasting Good for the Brain?

6.1.1. Looking Briefly at the Molecular Mechanisms. Fasting substantially modifies the neurochemistry and the activity of the neuronal network especially in several brain regions such as the hippocampus, the striatum, the hypothalamus, and the brainstem. At the molecular level, a variety of signaling pathways have been identified that mediate the structural (increased synaptic density, neurogenesis) and functional (Long-Term Potentiation, LTP) adaptive responses of neuronal circuits to nutrient restriction, in particular low glucose and amino acid levels [174] (see Figure 1). Both increased excitatory synaptic activity and neurotrophic factors (BDNF and fibroblast growth factor 2) lead to the activation of multiple kinases, including phosphatidylinositol 3-kinase (PI-3K), serine/threonine-protein kinase (AKT), Mitogen-Activated Protein Kinases (MAPKs) and Ca^{2+}/calmodulin-dependent kinase (CaMK), nitric oxide synthase (NOS), and calcineurin, which all converge on several transcription factors like cAMP-Responsive Element-Binding protein (CREB), nuclear regulatory factor 2 (Nrf2), and NF-κB [166, 175]. These transcription factors, which include BDNF

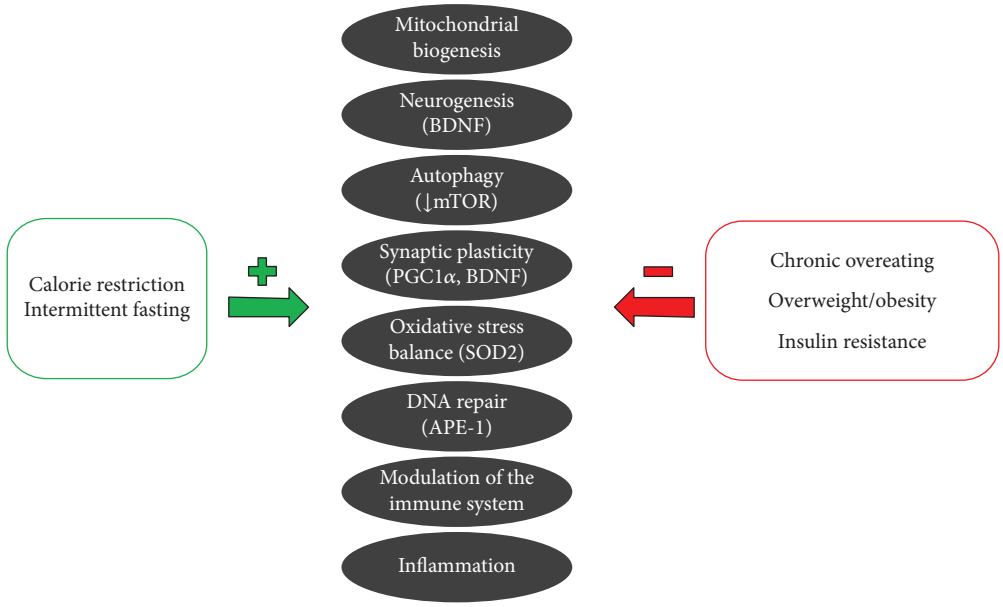

Figure 1: Signaling pathways involved in adaptive responses of neuronal circuits that contribute to maintain a healthy brain. Calorie restriction and intermittent fasting positively modulate these pathways, while chronic overfeeding and insulin resistance enhance neuroinflammation, neuronal damage, and apoptosis.

[166], sirtuin-3 (SIRT3) [176], peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) [177], and heat-shock protein 70 (HSP-70), induce the expression of genes and proteins involved in enhancing neuroplasticity and stress resistance [178]. Part of the benefits of CR/fasting seems to be related to protein restriction and reduced IGF1/insulin signaling. During protein restriction, mTOR, and in particular complex 1 (mTORC1), is repressed, thus allowing the cell to enter a "conservative" energy mode to inhibit protein and lipid synthesis and enhanced autophagy [179, 180].

6.2. Fasting and AD

6.2.1. Preclinical Studies. Most of the scientific evidence regarding IF or PF regimens and AD treatment derives from studies on laboratory animals. In rodents, several studies showed that fasting and FMDs are able to improve motor and cognitive functions, in particular hippocampal-dependent tasks like learning and memory [172, 181–183]. Moreover, IF has been associated with reduced oxidative stress and brain structural improvements such as increased thickness of the CA1 pyramidal cell layer and higher expression of the dendritic protein drebrin in the hippocampus [184].

IF and ketogenic diets can also modify neuronal network activity and synaptic plasticity. The neurons of rodents on an ADF regimen are more resistant to excitotoxin-induced degeneration of hippocampal neurons with kainic acid and perform better in water-maze learning and memory tasks [167, 185]. IF and/or regular physical activity can also prevent age-related deficits in LTP, a common cellular manifestation of learning and memory occurring in response to repetitive stimulation of synapses [186–189]. Other research groups also documented a role of CR/IF regimens in reducing Aβ deposition and Tau phosphorylation in the hippocampus and cerebral cortex of a transgenic mouse model of AD [190–194]. Recently, Zhang et al. highlighted the role of IF (and in particular BHB) in restoring the polarity of AQP4, a protein channel involved in Aβ clearance, that is often impaired in AD, thereby providing another possible explanation for the positive role of ADF in improving cognitive functions and protecting against Aβ pathology [195].

Lastly, IF, with or without exercise, stimulates the growth and differentiation of new neurons into granule neurons in the hippocampal dentate gyrus, i.e., adult hippocampal neurogenesis, and the creation of synaptic connections (dendritic spine growth and synaptogenesis) among themselves or with other neurons from other brain regions such as the entorhinal cortex, basal forebrain, and amygdala [172, 196, 197].

6.2.2. CR and IF in Humans. Several human studies have shown that the decrease in energy intake can reduce visceral fat (while preserving lean mass), improve glucose and lipid metabolism, and reduce blood pressure and blood biomarkers of inflammation (C-reactive protein and proinflammatory cytokines) [171, 198, 199]. All these beneficial effects may translate into a lower risk of cardiovascular disease, diabetes, and also neurodegenerative diseases such as AD.

Currently, no studies have been conducted on protein and/or calorie restriction in human subjects with established AD, but some authors have underlined the potential role of protein restriction against the aging process and aging-related chronic diseases [200]. However, it is important to properly time the application of protein restriction during life since the beneficial effects seem to be lost in people over 65 years of age [201].

Short periods of CR are able to improve cognitive function (verbal memory) in elderly subjects [202], and 30 days of a low glycemic diet in patients with MCI resulted in an improvement in delayed visual memory, cerebrospinal fluid biomarkers of Aβ metabolism, and brain bioenergetics [203], but it is hard to believe that severe restrictions could be tolerated for long periods, especially in elderly subjects affected by neurodegenerative diseases. IF regimens or FMD cycles every 1-2 months seem to be more feasible and tolerable in clinical practice. Recent pilot clinical trials applying such dietary regimens in healthy subjects and in patients with cancer, diabetes, and multiple sclerosis have been developed and have shown promising results [172, 173, 204], while several trials are still ongoing (https://clinicaltrials.gov/NCT03595540, https://clinicaltrials.gov/NCT03700437, and https://clinicaltrials.gov/NCT03811587).

The next step will be to demonstrate whether the various types of fasting (including ADF and FMD) have neuroprotective and regenerative effects in patients with early-stage AD. Our group will carry out a pilot study in an effort to assess the safety and feasibility of monthly cycles of an FMD diet in patients with a diagnosis of MCI or early AD.

In conclusion, emerging evidence on the effects of fasting on animal models of aging brain and neurodegenerative diseases is promising, but the applicability and potential efficacy of these dietary regimens in humans, and in particular in patients with MCI or AD, are yet to be established.

7. Conclusions

To date, it is not possible to establish with certainty a causal relationship between diet and the development of AD because there are still many confounding factors and biases:

(1) There may be confounding factors that contribute to increasing or reducing the risk of AD (physical activity, cardiovascular risk factors, and apoE4 status)

(2) There is broad heterogeneity among the characteristics of the studies, such as the mean age of the study subjects (50-85 years), diverse geographic settings (differences in the dietary patterns or in the environmental factors in Mediterranean or non-Mediterranean regions), sample size, study designs (cross-sectional, prospective, case-control, and RCTs), and length of follow-up

(3) Several different methods have been used for evaluating eating habits (different questionnaires, food diary, and 24-hour dietary recall)

(4) There is broad heterogeneity in the criteria used for the evaluation of cognitive performance and diagnosis of AD (single neuropsychological tests or neuropsychological batteries)

(5) None of the studies take into account the possible modifications in an individual's eating habits over the course of one's life as related to psychological (e.g., depression), physiological (e.g., difficulty in chewing and modification of appetite), or socioeconomic changes (e.g., social isolation, financial difficulties, and lack of family support)

(6) There is broad heterogeneity in the study outcomes (AD incidence or prevalence, worsening of the overall cognitive performance or even of some specific cognitive domains)

Currently available drugs (cholinesterase inhibitors and memantine) are able to partially control the symptoms but do not slow down the progression of AD. Therefore, there is an urgent need for new complementary therapeutic approaches, and in this context, the modulation of dietary habits and well-conducted nutritional interventions could be a useful and inexpensive tool.

Conflicts of Interest

The authors have no relevant disclosures of potential conflicts of interest.

References

[1] Alzheimer's Disease International, "World Alzheimer report 2015," *The Global Economic Impact of Dementia*, 2015.

[2] M. D. M. Haag, A. Hofman, P. J. Koudstaal, B. H. C. Stricker, and M. M. B. Breteler, "Statins are associated with a reduced risk of Alzheimer disease regardless of lipophilicity. The Rotterdam study," *Journal of Neurology, Neurosurgery & Psychiatry*, vol. 80, no. 1, pp. 13–17, 2009.

[3] E. M. van der Beek and P. J. G. H. Kamphuis, "The potential role of nutritional components in the management of Alzheimer's disease," *European Journal of Pharmacology*, vol. 585, no. 1, pp. 197–207, 2008.

[4] L. Nelson and N. Tabet, "Slowing the progression of Alzheimer's disease; what works?," *Ageing Research Reviews*, vol. 23, pp. 193–209, 2015.

[5] S. J. Shewale, R. M. Huebinger, M. S. Allen, and R. C. Barber, "The potential role of epigenetics in Alzheimer's disease etiology," *Biological Systems: Open Access*, vol. 2, no. 3, p. 114, 2013.

[6] S. S. M. Fernández and S. M. L. Ribeiro, "Nutrition and Alzheimer disease," *Clinics in Geriatric Medicine*, vol. 34, no. 4, pp. 677–697, 2018.

[7] D. Athanasopoulos, G. Karagiannis, and M. Tsolaki, "Recent findings in Alzheimer disease and nutrition focusing on epigenetics," *Advances in Nutrition*, vol. 7, no. 5, pp. 917–927, 2016.

[8] V. Nicolia, M. Lucarelli, and A. Fuso, "Environment, epigenetics and neurodegeneration: focus on nutrition in Alzheimer's disease," *Experimental Gerontology*, vol. 68, pp. 8–12, 2015.

[9] D. R. Gustafson, M. Clare Morris, N. Scarmeas et al., "New perspectives on Alzheimer's disease and nutrition," *Journal of Alzheimer's Disease*, vol. 46, no. 4, pp. 1111–1127, 2015.

[10] R. Shah, "The role of nutrition and diet in Alzheimer disease: a systematic review," *Journal of the American Medical Directors Association*, vol. 14, no. 6, pp. 398–402, 2013.

[11] C. Y. Santos, P. J. Snyder, W. C. Wu, M. Zhang, A. Echeverria, and J. Alber, "Pathophysiologic relationship between Alzheimer's disease, cerebrovascular disease, and cardiovascular

risk: a review and synthesis," *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, vol. 7, pp. 69–87, 2017.

[12] A. D. Dangour, P. J. Whitehouse, K. Rafferty et al., "B-vitamins and fatty acids in the prevention and treatment of Alzheimer's disease and dementia: a systematic review," *Journal of Alzheimer's Disease*, vol. 22, no. 1, pp. 205–224, 2010.

[13] M. W. Schelke, K. Hackett, J. L. Chen et al., "Nutritional interventions for Alzheimer's prevention: a clinical precision medicine approach," *Annals of the New York Academy of Sciences*, vol. 1367, no. 1, pp. 50–56, 2016.

[14] L. E. Middleton and K. Yaffe, "Promising strategies for the prevention of dementia," *Archives of Neurology*, vol. 66, no. 10, pp. 1210–1215, 2009.

[15] M. O. W. Grimm, D. M. Michaelson, and T. Hartmann, "Omega-3 fatty acids, lipids, and apoE lipidation in Alzheimer's disease: a rationale for multi-nutrient dementia prevention," *Journal of Lipid Research*, vol. 58, no. 11, pp. 2083–2101, 2017.

[16] K. Fischer, D. Melo van Lent, S. Wolfsgruber et al., "Prospective associations between single foods, Alzheimer's dementia and memory decline in the elderly," *Nutrients*, vol. 10, no. 7, p. 852, 2018.

[17] S. K. Ravi, R. B. Narasingappa, and B. Vincent, "Neuro-nutrients as anti-Alzheimer's disease agents: a critical review," *Critical Reviews in Food Science and Nutrition*, vol. 30, pp. 1–20, 2018.

[18] O. van de Rest, A. A. M. Berendsen, A. Haveman-Nies, and L. C. P. G. M. de Groot, "Dietary patterns, cognitive decline, and dementia: a systematic review," *Advances in Nutrition*, vol. 6, no. 2, pp. 154–168, 2015.

[19] A. Knight, J. Bryan, and K. Murphy, "Is the Mediterranean diet a feasible approach to preserving cognitive function and reducing risk of dementia for older adults in Western countries? New insights and future directions," *Ageing Research Reviews*, vol. 25, pp. 85–101, 2016.

[20] V. Solfrizzi, V. Frisardi, D. Seripa et al., "Mediterranean diet in predementia and dementia syndromes," *Current Alzheimer Research*, vol. 8, no. 5, pp. 520–542, 2011.

[21] V. Solfrizzi and F. Panza, "Mediterranean diet and cognitive decline. A lesson from the whole-diet approach: what challenges lie ahead?," *Journal of Alzheimer's Disease*, vol. 39, no. 2, pp. 283–286, 2014.

[22] N. Scarmeas, Y. Stern, R. Mayeux, and J. A. Luchsinger, "Mediterranean diet, Alzheimer disease, and vascular mediation," *Archives of Neurology*, vol. 63, no. 12, pp. 1709–1717, 2006.

[23] S. Gardener, Y. Gu, S. R. Rainey-Smith et al., "Adherence to a Mediterranean diet and Alzheimer's disease risk in an Australian population," *Translational Psychiatry*, vol. 2, no. 10, article e164, 2012.

[24] A. Trichopoulou, T. Costacou, C. Bamia, and D. Trichopoulos, "Adherence to a Mediterranean diet and survival in a Greek population," *New England Journal of Medicine*, vol. 348, no. 26, pp. 2599–2608, 2003.

[25] N. Scarmeas, Y. Stern, M. X. Tang, R. Mayeux, and J. A. Luchsinger, "Mediterranean diet and risk for Alzheimer's disease," *Annals of Neurology*, vol. 59, no. 6, pp. 912–921, 2006.

[26] N. Scarmeas, J. A. Luchsinger, N. Schupf et al., "Physical activity, diet, and risk of Alzheimer disease," *JAMA*, vol. 302, no. 6, pp. 627–637, 2009.

[27] Y. Gu, J. A. Luchsinger, Y. Stern, and N. Scarmeas, "Mediterranean diet, inflammatory and metabolic biomarkers, and risk of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 22, no. 2, pp. 483–492, 2010.

[28] M. C. Morris, C. C. Tangney, Y. Wang et al., "MIND diet slows cognitive decline with aging," *Alzheimer's & Dementia*, vol. 11, no. 9, pp. 1015–1022, 2015.

[29] N. Scarmeas, Y. Stern, R. Mayeux, J. J. Manly, N. Schupf, and J. A. Luchsinger, "Mediterranean diet and mild cognitive impairment," *Archives of Neurology*, vol. 66, no. 2, pp. 216–225, 2009.

[30] N. Scarmeas, J. A. Luchsinger, R. Mayeux, and Y. Stern, "Mediterranean diet and Alzheimer disease mortality," *Neurology*, vol. 69, no. 11, pp. 1084–1093, 2007.

[31] C. A. Anastasiou, M. Yannakoulia, M. H. Kosmidis et al., "Mediterranean diet and cognitive health: initial results from the Hellenic Longitudinal Investigation of Ageing and Diet," *PLoS One*, vol. 12, no. 8, article e0182048, 2017.

[32] D. B. Panagiotakos, C. Pitsavos, F. Arvaniti, and C. Stefanadis, "Adherence to the Mediterranean food pattern predicts the prevalence of hypertension, hypercholesterolemia, diabetes and obesity, among healthy adults; the accuracy of the MedDietScore," *Preventive Medicine*, vol. 44, no. 4, pp. 335–340, 2007.

[33] C. Féart, C. Samieri, V. Rondeau et al., "Adherence to a Mediterranean diet, cognitive decline, and risk of dementia," *JAMA*, vol. 302, no. 6, pp. 638–648, 2009.

[34] R. O. Roberts, Y. E. Geda, J. R. Cerhan et al., "Vegetables, unsaturated fats, moderate alcohol intake, and mild cognitive impairment," *Dementia and Geriatric Cognitive Disorders*, vol. 29, no. 5, pp. 413–423, 2010.

[35] E. Olsson, B. Karlström, L. Kilander, L. Byberg, T. Cederholm, and P. Sjögren, "Dietary patterns and cognitive dysfunction in a 12-year follow-up study of 70 year old men," *Journal of Alzheimer's Disease*, vol. 43, no. 1, pp. 109–119, 2015.

[36] T. Psaltopoulou, T. N. Sergentanis, D. B. Panagiotakos, I. N. Sergentanis, R. Kosti, and N. Scarmeas, "Mediterranean diet, stroke, cognitive impairment, and depression: a meta-analysis," *Annals of Neurology*, vol. 74, no. 4, pp. 580–591, 2013.

[37] F. Sofi, R. Abbate, G. F. Gensini, and A. Casini, "Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis," *The American Journal of Clinical Nutrition*, vol. 92, no. 5, pp. 1189–1196, 2010.

[38] B. Singh, A. K. Parsaik, M. M. Mielke et al., "Association of mediterranean diet with mild cognitive impairment and Alzheimer's disease: a systematic review and meta-analysis," *Journal of Alzheimer's Disease*, vol. 39, no. 2, pp. 271–282, 2014.

[39] S. D. Petersson and E. Philippou, "Mediterranean diet, cognitive function, and dementia: a systematic review of the evidence," *Advances in Nutrition*, vol. 7, no. 5, pp. 889–904, 2016.

[40] Y. S. Aridi, J. L. Walker, and O. R. L. Wright, "The association between the Mediterranean dietary pattern and cognitive health: a systematic review," *Nutrients*, vol. 9, no. 7, p. 674, 2017.

[41] M. Yusufov, L. L. Weyandt, and I. Piryatinsky, "Alzheimer's disease and diet: a systematic review," *International Journal of Neuroscience*, vol. 127, no. 2, pp. 161–175, 2017.

[42] R. Estruch, E. Ros, J. Salas-Salvadó et al., "Primary prevention of cardiovascular disease with a Mediterranean diet," *New England Journal of Medicine*, vol. 368, no. 14, pp. 1279–1290, 2013.

[43] E. H. Martínez-Lapiscina, P. Clavero, E. Toledo et al., "Mediterranean diet improves cognition: the PREDIMED-NAVARRA randomised trial," *Journal of Neurology, Neurosurgery & Psychiatry*, vol. 84, no. 12, pp. 1318–1325, 2013.

[44] C. Valls-Pedret, A. Sala-Vila, M. Serra-Mir et al., "Mediterranean diet and age-related cognitive decline: a randomized clinical trial," *JAMA Internal Medicine*, vol. 175, no. 7, pp. 1094–1103, 2015.

[45] A. Knight, J. Bryan, C. Wilson, J. Hodgson, C. Davis, and K. Murphy, "The Mediterranean diet and cognitive function among healthy older adults in a 6-month randomised controlled trial: the MedLey study," *Nutrients*, vol. 8, no. 9, p. 579, 2016.

[46] A. Marseglia, W. Xu, L. Fratiglioni et al., "Effect of the NU-AGE diet on cognitive functioning in older adults: a randomized controlled trial," *Frontiers in Physiology*, vol. 9, p. 349, 2018.

[47] B. C. Dickerson and H. Eichenbaum, "The episodic memory system: neurocircuitry and disorders," *Neuropsychopharmacology*, vol. 35, no. 1, pp. 86–104, 2010.

[48] S. Radd-Vagenas, S. L. Duffy, S. L. Naismith, B. J. Brew, V. M. Flood, and M. A. Fiatarone Singh, "Effect of the Mediterranean diet on cognition and brain morphology and function: a systematic review of randomized controlled trials," *The American Journal of Clinical Nutrition*, vol. 107, no. 3, pp. 389–404, 2018.

[49] L. Mosconi, J. Murray, W. H. Tsui et al., "Mediterranean diet and magnetic resonance imaging-assessed brain atrophy in cognitively normal individuals at risk for Alzheimer's disease," *The Journal of Prevention of Alzheimer's Disease*, vol. 1, no. 1, pp. 23–32, 2014.

[50] Y. Gu, A. M. Brickman, Y. Stern et al., "Mediterranean diet and brain structure in a multiethnic elderly cohort," *Neurology*, vol. 85, no. 20, pp. 1744–1751, 2015.

[51] S. C. Staubo, J. A. Aakre, P. Vemuri et al., "Mediterranean diet, micronutrients and macronutrients, and MRI measures of cortical thickness," *Alzheimer's & Dementia*, vol. 13, no. 2, pp. 168–177, 2017.

[52] A. J. Karstens, L. Tussing-Humphreys, L. Zhan et al., "Associations of the Mediterranean diet with cognitive and neuroimaging phenotypes of dementia in healthy older adults," *The American Journal of Clinical Nutrition*, vol. 109, no. 2, pp. 361–368, 2019.

[53] M. Luciano, J. Corley, S. R. Cox et al., "Mediterranean-type diet and brain structural change from 73 to 76 years in a Scottish cohort," *Neurology*, vol. 88, no. 5, pp. 449–455, 2017.

[54] F. N. Jacka, N. Cherbuin, K. J. Anstey, P. Sachdev, and P. Butterworth, "Western diet is associated with a smaller hippocampus: a longitudinal investigation," *BMC Medicine*, vol. 13, no. 1, p. 215, 2015.

[55] O. E. Titova, E. Ax, S. J. Brooks et al., "Mediterranean diet habits in older individuals: associations with cognitive functioning and brain volumes," *Experimental Gerontology*, vol. 48, no. 12, pp. 1443–1448, 2013.

[56] A. Pelletier, C. Barul, C. Feart et al., "Mediterranean diet and preserved brain structural connectivity in older subjects," *Alzheimer's & Dementia*, vol. 11, no. 9, pp. 1023–1031, 2015.

[57] R. Estruch, M. Á. Martínez-González, D. Corella et al., "Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial," *Annals of Internal Medicine*, vol. 145, no. 1, pp. 1–11, 2006.

[58] C. Chrysohoou, D. B. Panagiotakos, C. Pitsavos, U. N. Das, and C. Stefanadis, "Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: the Attica study," *Journal of the American College of Cardiology*, vol. 44, no. 1, pp. 152–158, 2004.

[59] M. P. Mena, E. Sacanella, M. Vazquez-Agell et al., "Inhibition of circulating immune cell activation: a molecular antiinflammatory effect of the Mediterranean diet," *The American Journal of Clinical Nutrition*, vol. 89, no. 1, pp. 248–256, 2009.

[60] R. Casas, E. Sacanella, M. Urpí-Sardà et al., "The effects of the Mediterranean diet on biomarkers of vascular wall inflammation and plaque vulnerability in subjects with high risk for cardiovascular disease. A randomized trial," *PLoS One*, vol. 9, no. 6, article e100084, 2014.

[61] D. C. Matthews, M. Davies, J. Murray et al., "Physical activity, Mediterranean diet and biomarkers-assessed risk of Alzheimer's: a multi-modality brain imaging study," *Advances in Molecular Imaging*, vol. 4, no. 4, pp. 43–57, 2014.

[62] M. Vassilaki, J. A. Aakre, J. A. Syrjanen et al., "Mediterranean diet, its components, and amyloid imaging biomarkers," *Journal of Alzheimer's Disease*, vol. 64, no. 1, pp. 281–290, 2018.

[63] S. R. Rainey-Smith, Y. Gu, S. L. Gardener et al., "Mediterranean diet adherence and rate of cerebral Aβ-amyloid accumulation: data from the Australian imaging, biomarkers and lifestyle study of ageing," *Translational Psychiatry*, vol. 8, no. 1, p. 238, 2018.

[64] M. J. Walters, J. Sterling, C. Quinn et al., "Associations of lifestyle and vascular risk factors with Alzheimer's brain biomarker changes during middle age: a 3-year longitudinal study in the broader New York City area," *BMJ Open*, vol. 8, no. 11, article e023664, 2018.

[65] F. M. Sacks, L. P. Svetkey, W. M. Vollmer et al., "Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet," *New England Journal of Medicine*, vol. 344, no. 1, pp. 3–10, 2001.

[66] A. M. Abbatecola, M. Russo, and M. Barbieri, "Dietary patterns and cognition in older persons," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 21, no. 1, pp. 10–13, 2018.

[67] C. C. Tangney, H. Li, Y. Wang et al., "Relation of DASH- and Mediterranean-like dietary patterns to cognitive decline in older persons," *Neurology*, vol. 83, no. 16, pp. 1410–1416, 2014.

[68] M. C. Morris, C. C. Tangney, Y. Wang, F. M. Sacks, D. A. Bennett, and N. T. Aggarwal, "MIND diet associated with reduced incidence of Alzheimer's disease," *Alzheimer's & Dementia*, vol. 11, no. 9, pp. 1007–1014, 2015.

[69] A. A. M. Berendsen, J. H. Kang, O. van de Rest, E. J. M. Feskens, L. C. P. G. M. de Groot, and F. Grodstein, "The dietary approaches to stop hypertension diet, cognitive function, and cognitive decline in American older women," *Journal of the American Medical Directors Association*, vol. 18, no. 5, pp. 427–432, 2017.

[70] B. Haring, C. Wu, L. H. Coker et al., "Hypertension, dietary sodium, and cognitive decline: results from the Women's Health Initiative Memory Study," *American Journal of Hypertension*, vol. 29, no. 2, pp. 202–216, 2016.

[71] P. J. Smith, J. A. Blumenthal, M. A. Babyak et al., "Effects of the dietary approaches to stop hypertension diet, exercise, and caloric restriction on neurocognition in overweight adults with high blood pressure," *Hypertension*, vol. 55, no. 6, pp. 1331–1338, 2010.

[72] N. Di Fiore, "Diet may help prevent Alzheimer's: MIND diet rich in vegetables, berries, whole grains, nutsRush University Medical Center websitehttps://www.rush.edu/new/diet-may-hel-prevent-alzheimers.

[73] M. Koch and M. K. Jensen, "Association of the MIND diet with cognition and risk of Alzheimer's disease," *Current Opinion in Lipidology*, vol. 27, no. 3, pp. 303-304, 2016.

[74] S. Larrieu, L. Letenneur, C. Helmer, J. F. Dartigues, and P. Barberger-Gateau, "Nutritional factors and risk of incident dementia in the PAQUID longitudinal cohort," *The Journal of Nutrition, Health & Aging*, vol. 8, no. 3, pp. 150–154, 2004.

[75] M. C. Morris, D. A. Evans, J. L. Bienias et al., "Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease," *Archives of Neurology*, vol. 60, no. 7, pp. 940–946, 2003.

[76] S. Kalmijn, L. J. Launer, A. Ott, J. C. M. Witteman, A. Hofman, and M. M. B. Breteler, "Dietary fat intake and the risk of incident dementia in the Rotterdam study," *Annals of Neurology*, vol. 42, no. 5, pp. 776–782, 1997.

[77] M. C. Morris, D. A. Evans, C. C. Tangney, J. L. Bienias, and R. S. Wilson, "Associations of vegetable and fruit consumption with age-related cognitive change," *Neurology*, vol. 67, no. 8, pp. 1370–1376, 2006.

[78] J. H. Kang, A. Ascherio, and F. Grodstein, "Fruit and vegetable consumption and cognitive decline in aging women," *Annals of Neurology*, vol. 57, no. 5, pp. 713–720, 2005.

[79] G. Casedas, F. Les, M. P. Gomez-Serranillos, C. Smith, and V. Lopez, "Anthocyanin profile, antioxidant activity and enzyme inhibiting properties of blueberry and cranberry juices: a comparative study," *Food & Function*, vol. 8, no. 11, pp. 4187–4193, 2017.

[80] L. Tavares, I. Figueira, G. J. McDougall et al., "Neuroprotective effects of digested polyphenols from wild blackberry species," *European Journal of Nutrition*, vol. 52, no. 1, pp. 225–236, 2013.

[81] B. Shukitt-Hale, V. Cheng, and J. A. Joseph, "Effects of blackberries on motor and cognitive function in aged rats," *Nutritional Neuroscience*, vol. 12, no. 3, pp. 135–140, 2009.

[82] D. H. Malin, D. R. Lee, P. Goyarzu et al., "Short-term blueberry-enriched diet prevents and reverses object recognition memory loss in aging rats," *Nutrition*, vol. 27, no. 3, pp. 338–342, 2011.

[83] G. Casadesus, B. Shukitt-Hale, H. M. Stellwagen et al., "Modulation of hippocampal plasticity and cognitive behavior by short-term blueberry supplementation in aged rats," *Nutritional Neuroscience*, vol. 7, no. 5-6, pp. 309–316, 2004.

[84] M. D. Pandareesh, R. B. Mythri, and M. M. Srinivas Bharath, "Bioavailability of dietary polyphenols: factors contributing to their clinical application in CNS diseases," *Neurochemistry International*, vol. 89, pp. 198–208, 2015.

[85] E. Kelly, P. Vyas, and J. Weber, "Biochemical properties and neuroprotective effects of compounds in various species of berries," *Molecules*, vol. 23, no. 1, p. 26, 2018.

[86] E. E. Devore, J. H. Kang, M. M. B. Breteler, and F. Grodstein, "Dietary intakes of berries and flavonoids in relation to cognitive decline," *Annals of Neurology*, vol. 72, no. 1, pp. 135–143, 2012.

[87] L. Fernández-Fernández, G. Comes, I. Bolea et al., "LMN diet, rich in polyphenols and polyunsaturated fatty acids, improves mouse cognitive decline associated with aging and Alzheimer's disease," *Behavioural Brain Research*, vol. 228, no. 2, pp. 261–271, 2012.

[88] C. Grossi, S. Rigacci, S. Ambrosini et al., "The polyphenol oleuropein aglycone protects TgCRND8 mice against Aß plaque pathology," *PLoS One*, vol. 8, no. 8, article e71702, 2013.

[89] C. Razquin, E. Toledo, C. B. Clish et al., "Plasma lipidomic profiling and risk of type 2 diabetes in the PREDIMED trial," *Diabetes Care*, vol. 41, no. 12, pp. 2617–2624, 2018.

[90] P. Saneei, A. Salehi-Abargouei, A. Esmaillzadeh, and L. Azadbakht, "Influence of Dietary Approaches to Stop Hypertension (DASH) diet on blood pressure: a systematic review and meta-analysis on randomized controlled trials," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 24, no. 12, pp. 1253–1261, 2014.

[91] Z. Arvanitakis, A. W. Capuano, S. E. Leurgans, D. A. Bennett, and J. A. Schneider, "Relation of cerebral vessel disease to Alzheimer's disease dementia and cognitive function in elderly people: a cross-sectional study," *The Lancet Neurology*, vol. 15, no. 9, pp. 934–943, 2016.

[92] K. Kisler, A. R. Nelson, A. Montagne, and B. V. Zlokovic, "Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer disease," *Nature Reviews Neuroscience*, vol. 18, no. 7, pp. 419–434, 2017.

[93] S. P. Didion, C. D. Sigmund, and F. M. Faraci, "Impaired endothelial function in transgenic mice expressing both human renin and human angiotensinogen," *Stroke*, vol. 31, no. 3, pp. 760–765, 2000.

[94] M. Wiesmann, C. Capone, V. Zerbi et al., "Hypertension impairs cerebral blood flow in a mouse model for Alzheimer's disease," *Current Alzheimer Research*, vol. 12, no. 10, pp. 914–922, 2015.

[95] N. D. L. Fisher, F. A. Sorond, and N. K. Hollenberg, "Cocoa flavanols and brain perfusion," *Journal of Cardiovascular Pharmacology*, vol. 47, Supplement 2, pp. S210–S214, 2006.

[96] F. di Domenico, G. Pupo, E. Giraldo et al., "Oxidative signature of cerebrospinal fluid from mild cognitive impairment and Alzheimer disease patients," *Free Radical Biology & Medicine*, vol. 91, pp. 1–9, 2016.

[97] A. Nehlig, "The neuroprotective effects of cocoa flavanol and its influence on cognitive performance," *British Journal of Clinical Pharmacology*, vol. 75, no. 3, pp. 716–727, 2013.

[98] M. Rossi, F. Caruso, L. Kwok et al., "Protection by extra virgin olive oil against oxidative stress *in vitro* and *in vivo*. Chemical and biological studies on the health benefits due to a major component of the Mediterranean diet," *PLoS One*, vol. 12, no. 12, article e0189341, 2017.

[99] S. Rigacci and M. Stefani, "Nutraceutical properties of olive oil polyphenols. An itinerary from cultured cells through animal models to humans," *International Journal of Molecular Sciences*, vol. 17, no. 6, p. 843, 2016.

[100] M. Martucci, R. Ostan, F. Biondi et al., "Mediterranean diet and inflammaging within the hormesis paradigm," *Nutrition Reviews*, vol. 75, no. 6, pp. 442–455, 2017.

[101] M. Martínez-Huélamo, J. Rodríguez-Morató, A. Boronat, and R. de la Torre, "Modulation of Nrf2 by olive oil and wine polyphenols and neuroprotection," *Antioxidants*, vol. 6, no. 4, p. 73, 2017.

[102] H. Zrelli, M. Matsuoka, S. Kitazaki et al., "Hydroxytyrosol induces proliferation and cytoprotection against oxidative injury in vascular endothelial cells: role of Nrf2 activation and HO-1 induction," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 9, pp. 4473–4482, 2011.

[103] M. H. Ahmad, M. Fatima, and A. C. Mondal, "Influence of microglia and astrocyte activation in the neuroinflammatory pathogenesis of Alzheimer's disease: rational insights for the therapeutic approaches," *Journal of Clinical Neuroscience*, vol. 59, pp. 6–11, 2019.

[104] R. Takamura, N. Watamura, M. Nikkuni, and T. Ohshima, "All-trans retinoic acid improved impaired proliferation of neural stem cells and suppressed microglial activation in the hippocampus in an Alzheimer's mouse model," *Journal of Neuroscience Research*, vol. 95, no. 3, pp. 897–906, 2017.

[105] S. Mishra, K. K. Kelly, N. L. Rumian, and J. A. Siegenthaler, "Retinoic acid is required for neural stem and progenitor cell proliferation in the adult hippocampus," *Stem Cell Reports*, vol. 10, no. 6, pp. 1705–1720, 2018.

[106] F. Regen, J. Hellmann-Regen, E. Costantini, and M. Reale, "Neuroinflammation and Alzheimer's disease: implications for microglial activation," *Current Alzheimer Research*, vol. 14, no. 11, pp. 1140–1148, 2017.

[107] M. T. Heneka, M. J. Carson, J. E. Khoury et al., "Neuroinflammation in Alzheimer's disease," *The Lancet Neurology*, vol. 14, no. 4, pp. 388–405, 2015.

[108] A. A. Raha, J. W. Henderson, S. R. Stott et al., "Neuroprotective effect of TREM-2 in aging and Alzheimer's disease model," *Journal of Alzheimer's Disease*, vol. 55, no. 1, pp. 199–217, 2017.

[109] S. Krasemann, C. Madore, R. Cialic et al., "The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases," *Immunity*, vol. 47, no. 3, pp. 566–581.e9, 2017.

[110] F. G. De Felice and S. T. Ferreira, "Inflammation, defective insulin signaling, and mitochondrial dysfunction as common molecular denominators connecting type 2 diabetes to Alzheimer disease," *Diabetes*, vol. 63, no. 7, pp. 2262–2272, 2014.

[111] J. P. Bastard, M. Maachi, C. Lagathu et al., "Recent advances in the relationship between obesity, inflammation, and insulin resistance," *European Cytokine Network*, vol. 17, no. 1, pp. 4–12, 2006.

[112] K. Ono, M. Hirohata, and M. Yamada, "Ferulic acid destabilizes preformed β-amyloid fibrils in vitro," *Biochemical and Biophysical Research Communications*, vol. 336, no. 2, pp. 444–449, 2005.

[113] T. Hamaguchi, K. Ono, A. Murase, and M. Yamada, "Phenolic compounds prevent Alzheimer's pathology through different effects on the amyloid-β aggregation pathway," *The American Journal of Pathology*, vol. 175, no. 6, pp. 2557–2565, 2009.

[114] J. J. Yan, J. Y. Cho, H. S. Kim et al., "Protection against β-amyloid peptide toxicity *in vivo* with long-term administration of ferulic acid," *British Journal of Pharmacology*, vol. 133, no. 1, pp. 89–96, 2001.

[115] R. Sultana, A. Ravagna, H. Mohmmad-Abdul, V. Calabrese, and D. A. Butterfield, "Ferulic acid ethyl ester protects neurons against amyloid β- peptide(1-42)-induced oxidative stress and neurotoxicity: relationship to antioxidant activity," *Journal of Neurochemistry*, vol. 92, no. 4, pp. 749–758, 2005.

[116] T. Shen, X. N. Wang, and H. X. Lou, "Natural stilbenes: an overview," *Natural Product Reports*, vol. 26, no. 7, pp. 916–935, 2009.

[117] S. P. Patil, N. Tran, H. Geekiyanage, L. Liu, and C. Chan, "Curcumin-induced upregulation of the anti-tau cochaperone BAG2 in primary rat cortical neurons," *Neuroscience Letters*, vol. 554, pp. 121–125, 2013.

[118] C. A. Ribeiro, M. J. Saraiva, and I. Cardoso, "Stability of the transthyretin molecule as a key factor in the interaction with a-beta peptide - relevance in Alzheimer's disease," *PLoS One*, vol. 7, no. 9, article e45368, 2012.

[119] K. J. Kwon, H. J. Kim, C. Y. Shin, and S. H. Han, "Melatonin potentiates the neuroprotective properties of resveratrol against beta-amyloid-induced neurodegeneration by modulating amp-activated protein kinase pathways," *Journal of Clinical Neurology*, vol. 6, no. 3, pp. 127–137, 2010.

[120] K. J. Kwon, J. N. Kim, M. K. Kim et al., "Melatonin synergistically increases resveratrol induced heme oxygenase-1 expression through the inhibition of ubiquitin-dependent proteasome pathway: a possible role in neuroprotection," *Journal of Pineal Research*, vol. 50, no. 2, pp. 110–123, 2011.

[121] X. Cheng, Q. Wang, N. Li, and H. Zhao, "Effects of resveratrol on hippocampal astrocytes and expression of TNF-α in Alzheimer's disease model rate," *Wei Sheng Yan Jiu*, vol. 44, no. 4, pp. 610–614, 2015.

[122] Y. Kim, S. Y. Lim, S. H. Rhee et al., "Resveratrol inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in β-amyloid-treated C6 glioma cells," *International Journal of Molecular Medicine*, vol. 17, no. 6, pp. 1069–1075, 2006.

[123] S. Layé, A. Nadjar, C. Joffre, and R. P. Bazinet, "Anti-inflammatory effects of omega-3 fatty acids in the brain: physiological mechanisms and relevance to pharmacology," *Pharmacological Reviews*, vol. 70, no. 1, pp. 12–38, 2018.

[124] T. Kavanagh, P. E. Lonergan, and M. A. Lynch, "Eicosapentaenoic acid and gamma-linolenic acid increase hippocampal concentrations of IL-4 and IL-10 and abrogate lipopolysaccharide-induced inhibition of long-term potentiation," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 70, no. 4, pp. 391–397, 2004.

[125] E. Teng, K. Taylor, T. Bilousova et al., "Dietary DHA supplementation in an APP/PS1 transgenic rat model of AD reduces behavioral and Aβ pathology and modulates Aβ oligomerization," *Neurobiology of Disease*, vol. 82, pp. 552–560, 2015.

[126] A. B. Segarra, J. I. Ruiz-Sanz, M. B. Ruiz-Larrea et al., "The profile of fatty acids in frontal cortex of rats depends on the type of fat used in the diet and correlates with neuropeptidase activities," *Hormone and Metabolic Research*, vol. 43, no. 2, pp. 86–91, 2011.

[127] The Human Microbiome Project Consortium, "Structure, function and diversity of the healthy human microbiome," *Nature*, vol. 486, no. 7402, pp. 207–214, 2012.

[128] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[129] L. Szablewski, "Human gut microbiota in health and Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 62, no. 2, pp. 549–560, 2018.

[130] L. Shen and H. F. Ji, "Bidirectional interactions between dietary curcumin and gut microbiota," *Critical Reviews in Food Science and Nutrition*, vol. 21, pp. 1–7, 2018.

[131] J. S. Rane, P. Bhaumik, and D. Panda, "Curcumin inhibits tau aggregation and disintegrates preformed tau filaments *in vitro*," *Journal of Alzheimer's Disease*, vol. 60, no. 3, pp. 999–1014, 2017.

[132] C. S. Oriach, R. C. Robertson, C. Stanton, J. F. Cryan, and T. G. Dinan, "Food for thought: the role of nutrition in the microbiota-gut–brain axis," *Clinical Nutrition Experimental*, vol. 6, pp. 25–38, 2016.

[133] M. K. Zinöcker and I. A. Lindseth, "The Western diet–microbiome-host interaction and its role in metabolic disease," *Nutrients*, vol. 10, no. 3, p. 365, 2018.

[134] T. W. Buford, "(Dis)trust your gut: the gut microbiome in age-related inflammation, health, and disease," *Microbiome*, vol. 5, no. 1, p. 80, 2017.

[135] A. M. Vaiserman, A. K. Koliada, and F. Marotta, "Gut microbiota: a player in aging and a target for anti-aging intervention," *Ageing Research Reviews*, vol. 35, pp. 36–45, 2017.

[136] S. C. Bischoff, "Microbiota and aging," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 19, no. 1, pp. 26–30, 2016.

[137] P. C. Konturek, D. Haziri, T. Brzozowski et al., "Emerging role of fecal microbiota therapy in the treatment of gastrointestinal and extra-gastrointestinal diseases," *Journal of Physiology and Pharmacology*, vol. 66, no. 4, pp. 483–491, 2015.

[138] E. Biagi, L. Nylund, M. Candela et al., "Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians," *PLoS One*, vol. 5, no. 5, article e10667, 2010.

[139] M. J. Claesson, I. B. Jeffery, S. Conde et al., "Gut microbiota composition correlates with diet and health in the elderly," *Nature*, vol. 488, no. 7410, pp. 178–184, 2012.

[140] S. Mueller, K. Saunier, C. Hanisch et al., "Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study," *Applied and Environmental Microbiology*, vol. 72, no. 2, pp. 1027–1033, 2006.

[141] A. Santoro, R. Ostan, M. Candela et al., "Gut microbiota changes in the extreme decades of human life: a focus on centenarians," *Cellular and Molecular Life Sciences*, vol. 75, no. 1, pp. 129–148, 2018.

[142] K. A. Scott, M. Ida, V. L. Peterson et al., "Revisiting Metchnikoff: age-related alterations in microbiota–gut–brain axis in the mouse," *Brain, Behavior, and Immunity*, vol. 65, pp. 20–32, 2017.

[143] C. Bäuerl, M. C. Collado, A. Diaz Cuevas, J. Viña, and G. Pérez Martínez, "Shifts in gut microbiota composition in an APP/PSS1 transgenic mouse model of Alzheimer's disease during lifespan," *Letters in Applied Microbiology*, vol. 66, no. 6, pp. 464–471, 2018.

[144] L. Zhang, Y. Wang, X. Xiayu et al., "Altered gut microbiota in a mouse model of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 60, no. 4, pp. 1241–1257, 2017.

[145] E. Sanguinetti, M. C. Collado, V. G. Marrachelli et al., "Microbiome–metabolome signatures in mice genetically prone to develop dementia, fed a normal or fatty diet," *Scientific Reports*, vol. 8, no. 1, p. 4907, 2018.

[146] T. Harach, N. Marungruang, N. Duthilleul et al., "Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota," *Scientific Reports*, vol. 7, no. 1, article 41802, 2017.

[147] M. R. Minter, C. Zhang, V. Leone et al., "Antibiotic-induced perturbations in gut microbial diversity influences neuroinflammation and amyloidosis in a murine model of Alzheimer's disease," *Scientific Reports*, vol. 6, no. 1, article 30028, 2016.

[148] E. E. Fröhlich, A. Farzi, R. Mayerhofer et al., "Cognitive impairment by antibiotic-induced gut dysbiosis: analysis of gut microbiota–brain communication," *Brain, Behavior, and Immunity*, vol. 56, pp. 140–155, 2016.

[149] L. Bonfili, V. Cecarini, S. Berardi et al., "Microbiota modulation counteracts Alzheimer's disease progression influencing neuronal proteolysis and gut hormones plasma levels," *Scientific Reports*, vol. 7, no. 1, article 2426, 2017.

[150] M. Nimgampalle and Y. Kuna, "Anti-Alzheimer properties of probiotic, *Lactobacillus plantarum* MTCC 1325 in Alzheimer's disease induced albino rats," *Journal of Clinical and Diagnostic Research*, vol. 11, no. 8, pp. KC01–KC05, 2017.

[151] Y. Kobayashi, H. Sugahara, K. Shimada et al., "Therapeutic potential of *Bifidobacterium breve* strain A1 for preventing cognitive impairment in Alzheimer's disease," *Scientific Reports*, vol. 7, no. 1, p. 13510, 2017.

[152] T. Chunchai, W. Thunapong, S. Yasom et al., "Decreased microglial activation through gut–brain axis by prebiotics, probiotics, or synbiotics effectively restored cognitive function in obese-insulin resistant rats," *Journal of Neuroinflammation*, vol. 15, no. 1, p. 11, 2018.

[153] C. Q. Li, Q. Zheng, Q. Wang, and Q. P. Zeng, "Biotic/abiotic stress-driven Alzheimer's disease," *Frontiers in Cellular Neuroscience*, vol. 10, p. 269, 2016.

[154] C. Hollands, N. Bartolotti, and O. Lazarov, "Alzheimer's disease and hippocampal adult neurogenesis; exploring shared mechanisms," *Frontiers in Neuroscience*, vol. 10, p. 178, 2016.

[155] O. Lazarov and R. A. Marr, "Neurogenesis and Alzheimer's disease: at the crossroads," *Experimental Neurology*, vol. 223, no. 2, pp. 267–281, 2010.

[156] S. Das and A. Basu, "Inflammation: a new candidate in modulating adult neurogenesis," *Journal of Neuroscience Research*, vol. 86, no. 6, pp. 1199–1208, 2008.

[157] R. Molteni, R. J. Barnard, Z. Ying, C. K. Roberts, and F. Gomez-Pinilla, "A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning," *Neuroscience*, vol. 112, no. 4, pp. 803–814, 2002.

[158] M. Fotuhi, P. Mohassel, and K. Yaffe, "Fish consumption, long-chain omega-3 fatty acids and risk of cognitive decline or Alzheimer disease: a complex association," *Nature Clinical Practice Neurology*, vol. 5, no. 3, pp. 140–152, 2009.

[159] A. Granzotto and P. Zatta, "Resveratrol and Alzheimer's disease: message in a bottle on red wine and cognition," *Frontiers in Aging Neuroscience*, vol. 6, p. 95, 2014.

[160] P. Scheltens, K. Blennow, M. M. B. Breteler et al., "Alzheimer's disease," *The Lancet*, vol. 388, no. 10043, pp. 505–517, 2016.

[161] M. P. Mattson and T. V. Arumugam, "Hallmarks of brain aging: adaptive and pathological modification by metabolic states," *Cell Metabolism*, vol. 27, no. 6, pp. 1176–1199, 2018.

[162] D. Wahl, V. C. Cogger, S. M. Solon-Biet et al., "Nutritional strategies to optimise cognitive function in the aging brain," *Ageing Research Reviews*, vol. 31, pp. 80–92, 2016.

[163] S. Camandola and M. P. Mattson, "Brain metabolism in health, aging and neurodegeneration," *The EMBO Journal*, vol. 36, no. 11, pp. 1474–1492, 2017.

[164] V. D. Longo and M. P. Mattson, "Fasting: molecular mechanisms and clinical applications," *Cell Metabolism*, vol. 19, no. 2, pp. 181–192, 2014.

[165] L. Fontana, L. Partridge, and V. D. Longo, "Extending healthy life span–from yeast to humans," *Science*, vol. 328, no. 5976, pp. 321–326, 2010.

[166] S. Golbidi, A. Daiber, B. Korac, H. Li, M. F. Essop, and I. Laher, "Health benefits of fasting and caloric restriction," *Current Diabetes Reports*, vol. 17, no. 12, p. 123, 2017.

[167] A. J. Bruce-Keller, G. Umberger, R. McFall, and M. P. Mattson, "Food restriction reduces brain damage and improves behavioral outcome following excitotoxic and metabolic insults," *Annals of Neurology*, vol. 45, no. 1, pp. 8–15, 1999.

[168] R. M. Anson, Z. Guo, R. de Cabo et al., "Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake," *Proceedings of the National Academy of Sciences of the United States America*, vol. 100, no. 10, pp. 6216–6220, 2003.

[169] J. B. Johnson, W. Summer, R. G. Cutler et al., "Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma," *Free Radical Biology & Medicine*, vol. 42, no. 5, pp. 665–674, 2007.

[170] K. A. Varady, V. T. Dam, M. C. Klempel et al., "Effects of weight loss via high fat vs. low fat alternate day fasting diets on free fatty acid profiles," *Scientific Reports*, vol. 5, no. 1, article 7561, 2015.

[171] M. N. Harvie, M. Pegington, M. P. Mattson et al., "The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: a randomized trial in young overweight women," *International Journal of Obesity*, vol. 35, no. 5, pp. 714–727, 2011.

[172] S. Brandhorst, I. Y. Choi, M. Wei et al., "A periodic diet that mimics fasting promotes multi-system regeneration, enhanced cognitive performance and healthspan," *Cell Metabolism*, vol. 22, no. 1, pp. 86–99, 2015.

[173] I. Y. Choi, L. Piccio, P. Childress et al., "A diet mimicking fasting promotes regeneration and reduces autoimmunity and multiple sclerosis symptoms," *Cell Reports*, vol. 15, no. 10, pp. 2136–2146, 2016.

[174] M. P. Mattson, K. Moehl, N. Ghena, M. Schmaedick, and A. Cheng, "Intermittent metabolic switching, neuroplasticity and brain health," *Nature Reviews Neuroscience*, vol. 19, no. 2, pp. 80–80, 2018.

[175] K. Marosi and M. P. Mattson, "BDNF mediates adaptive brain and body responses to energetic challenges," *Trends in Endocrinology & Metabolism*, vol. 25, no. 2, pp. 89–98, 2014.

[176] R. A. H. van de Ven, D. Santos, and M. C. Haigis, "Mitochondrial sirtuins and molecular mechanisms of aging," *Trends in Molecular Medicine*, vol. 23, no. 4, pp. 320–331, 2017.

[177] A. Cheng, R. Wan, J. L. Yang et al., "Involvement of PGC-1α in the formation and maintenance of neuronal dendritic spines," *Nature Communications*, vol. 3, no. 1, article 1250, 2012.

[178] T. V. Arumugam, T. M. Phillips, A. Cheng, C. H. Morrell, M. P. Mattson, and R. Wan, "Age and energy intake interact to modify cell stress pathways and stroke outcome," *Annals of Neurology*, vol. 67, no. 1, pp. 41–52, 2010.

[179] S. C. Johnson, P. S. Rabinovitch, and M. Kaeberlein, "mTOR is a key modulator of ageing and age-related disease," *Nature*, vol. 493, no. 7432, pp. 338–345, 2013.

[180] M. Laplante and D. M. Sabatini, "mTOR signaling in growth control and disease," *Cell*, vol. 149, no. 2, pp. 274–293, 2012.

[181] R. Singh, D. Lakhanpal, S. Kumar et al., "Late-onset intermittent fasting dietary restriction as a potential intervention to retard age-associated brain function impairments in male rats," *Age*, vol. 34, no. 4, pp. 917–933, 2012.

[182] R. Singh, S. Manchanda, T. Kaur et al., "Middle age onset short-term intermittent fasting dietary restriction prevents brain function impairments in male Wistar rats," *Biogerontology*, vol. 16, no. 6, pp. 775–788, 2015.

[183] Á. Fontán-Lozano, J. L. Saez-Cassanelli, M. C. Inda et al., "Caloric restriction increases learning consolidation and facilitates synaptic plasticity through mechanisms dependent on NR2B subunits of the NMDA receptor," *The Journal of Neuroscience*, vol. 27, no. 38, pp. 10185–10195, 2007.

[184] L. Li, Z. Wang, and Z. Zuo, "Chronic intermittent fasting improves cognitive functions and brain structures in mice," *PLoS One*, vol. 8, no. 6, article e66069, 2013.

[185] W. Duan, Z. H. Guo, and M. P. Mattson, "Brain-derived neurotrophic factor mediates an excitoprotective effect of dietary restriction in mice," *Journal of Neurochemistry*, vol. 76, no. 2, pp. 619–626, 2001.

[186] H. van Praag, B. R. Christie, T. J. Sejnowski, and F. H. Gage, "Running enhances neurogenesis, learning, and long-term potentiation in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13427–13431, 1999.

[187] R. M. O'Callaghan, R. Ohle, and Á. M. Kelly, "The effects of forced exercise on hippocampal plasticity in the rat: a comparison of LTP, spatial- and non-spatial learning," *Behavioural Brain Research*, vol. 176, no. 2, pp. 362–366, 2007.

[188] K. Eckles-Smith, D. Clayton, P. Bickford, and M. D. Browning, "Caloric restriction prevents age-related deficits in LTP and in NMDA receptor expression," *Molecular Brain Research*, vol. 78, no. 1-2, pp. 154–162, 2000.

[189] N. Hori, I. Hirotsu, P. J. Davis, and D. O. Carpenter, "Long-term potentiation is lost in aged rats but preserved by calorie restriction," *Neuroreport*, vol. 3, no. 12, pp. 1085–1088, 1992.

[190] V. K. M. Halagappa, Z. Guo, M. Pearson et al., "Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of Alzheimer's disease," *Neurobiology of Disease*, vol. 26, no. 1, pp. 212–220, 2007.

[191] N. V. Patel, M. N. Gordon, K. E. Connor et al., "Caloric restriction attenuates Aβ-deposition in Alzheimer transgenic models," *Neurobiology of Aging*, vol. 26, no. 7, pp. 995–1000, 2005.

[192] J. Wang, L. Ho, W. Qin et al., "Caloric restriction attenuates β-amyloid neuropathology in a mouse model of Alzheimer's disease," *The FASEB Journal*, vol. 19, no. 6, pp. 659–661, 2005.

[193] P. R. Mouton, M. E. Chachich, C. Quigley, E. Spangler, and D. K. Ingram, "Caloric restriction attenuates amyloid deposition in middle-aged dtg APP/PS1 mice," *Neuroscience Letters*, vol. 464, no. 3, pp. 184–187, 2009.

[194] E. Parrella, T. Maxim, F. Maialetti et al., "Protein restriction cycles reduce IGF-1 and phosphorylated tau and improve behavioral performance in an Alzheimer's disease mouse model," *Aging Cell*, vol. 12, no. 2, pp. 257–268, 2013.

[195] J. Zhang, Z. Zhan, X. Li et al., "Intermittent fasting protects against Alzheimer's disease possible through restoring aquaporin-4 polarity," *Frontiers in Molecular Neuroscience*, vol. 10, p. 395, 2017.

[196] J. Lee, K. B. Seroogy, and M. P. Mattson, "Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice," *Journal of Neurochemistry*, vol. 80, no. 3, pp. 539–547, 2002.

[197] C. Vivar, M. C. Potter, J. Choi et al., "Monosynaptic inputs to new neurons in the dentate gyrus," *Nature Communications*, vol. 3, no. 1, article 1107, 2012.

[198] J. F. Trepanowski, C. M. Kroeger, A. Barnosky et al., "Effect of alternate-day fasting on weight loss, weight maintenance, and cardioprotection among metabolically healthy obese adults," *JAMA Internal Medicine*, vol. 177, no. 7, pp. 930–938, 2017.

[199] M. Wei, S. Brandhorst, M. Shelehchi et al., "Fasting-mimicking diet and markers/risk factors for aging, diabetes cancer and cardiovascular disease," *Science Translational Medicine*, vol. 9, no. 377, article eeai8700, 2017.

[200] H. Mirzaei, J. A. Suarez, and V. D. Longo, "Protein and amino acid restriction, aging and disease: from yeast to humans," *Trends in Endocrinology & Metabolism*, vol. 25, no. 11, pp. 558–566, 2014.

[201] M. E. Levine, J. A. Suarez, S. Brandhorst et al., "Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population," *Cell Metabolism*, vol. 19, no. 3, pp. 407–417, 2014.

[202] A. V. Witte, M. Fobker, R. Gellner, S. Knecht, and A. Floel, "Caloric restriction improves memory in elderly humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 4, pp. 1255–1260, 2009.

[203] J. L. Bayer-Carter, P. S. Green, T. J. Montine et al., "Diet intervention and cerebrospinal fluid biomarkers in amnestic mild cognitive impairment," *Archives of Neurology*, vol. 68, no. 6, pp. 743–752, 2011.

[204] C. W. Cheng, V. Villani, R. Buono et al., "Fasting-mimicking diet promotes Ngn3-driven β-cell regeneration to reverse diabetes," *Cell*, vol. 168, no. 5, pp. 775–788.e12, 2017.

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Review Article

Oxidative Stress and Advanced Lipoxidation and Glycation End Products (ALEs and AGEs) in Aging and Age-Related Diseases

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Oxidative stress is a consequence of the use of oxygen in aerobic respiration by living organisms and is denoted as a persistent condition of an imbalance between the generation of reactive oxygen species (ROS) and the ability of the endogenous antioxidant system (AOS) to detoxify them. The oxidative stress theory has been confirmed in many animal studies, which demonstrated that the maintenance of cellular homeostasis and biomolecular stability and integrity is crucial for cellular longevity and successful aging. Mitochondrial dysfunction, impaired protein homeostasis (proteostasis) network, alteration in the activities of transcription factors such as Nrf2 and NF-κB, and disturbances in the protein quality control machinery that includes molecular chaperones, ubiquitin-proteasome system (UPS), and autophagy/lysosome pathway have been observed during aging and age-related chronic diseases. The accumulation of ROS under oxidative stress conditions results in the induction of lipid peroxidation and glycoxidation reactions, which leads to the elevated endogenous production of reactive aldehydes and their derivatives such as glyoxal, methylglyoxal (MG), malonic dialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) giving rise to advanced lipoxidation and glycation end products (ALEs and AGEs, respectively). Both ALEs and AGEs play key roles in cellular response to oxidative stress stimuli through the regulation of a variety of cell signaling pathways. However, elevated ALE and AGE production leads to protein cross-linking and aggregation resulting in an alteration in cell signaling and functioning which causes cell damage and death. This is implicated in aging and various age-related chronic pathologies such as inflammation, neurodegenerative diseases, atherosclerosis, and vascular complications of diabetes mellitus. In the present review, we discuss experimental data evidencing the impairment in cellular functions caused by AGE/ALE accumulation under oxidative stress conditions. We focused on the implications of ALEs/AGEs in aging and age-related diseases to demonstrate that the identification of cellular dysfunctions involved in disease initiation and progression can serve as a basis for the discovery of relevant therapeutic agents.

1. Introduction

Living cells produce various kinds of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) [1, 2]. The major endogenous sources of ROS include mitochondrial electron-transportation chain (ETC) complexes I and III and the NADPH oxidases of NOX family enzymes [3–5]. Additionally, ROS may be produced by xanthine oxidase, cyclooxygenases (COXs, prostaglandin G/H synthases), lipoxygenases, and the cytochrome P450- (CYP-) containing monooxygenase system [6–9]. ROS generation may be

induced by exogenous factors such as UV light, X-ray, and γ-ray irradiations, air pollutants, tobacco smoke, heavy metals, and certain drugs [10, 11].

Under physiological conditions, living cells maintain low intracellular concentrations of ROS due to the activity of the endogenous antioxidant system (AOS) composed of both enzymatic and nonenzymatic components capable of ROS scavenging and, thereby, protecting cells from the deleterious effects of high ROS concentrations (reviewed in [12–14]). The enzymatic components include superoxide dismutases (SODs), catalase, peroxiredoxins (Prxs), glutathione peroxidases (GPx), and glutathione reductase (GR), while nonenzymatic components include ascorbic acid, alpha-tocopherol, retinol, and various molecules with thiol groups such as glutathione, lipoic acid, small protein thioredoxin, as well as transition-metal ions such as Fe, Cu, Zn, and Mn [15–20].

At low concentrations, ROS exert regulatory effects on cellular functions including proliferation, differentiation, migration, and survival [21, 22]. This is provided by their involvement in reversible posttranslational modifications of key redox-sensitive amino acid residues in enzymes, intracellular effectors of signal transduction pathways (protein kinases and protein phosphatases), transcription factors, cytoskeletal proteins, and molecular chaperones [23–26]. Through this, oxidative protein modifications may be caused either directly by ROS themselves or indirectly by secondary products of ROS-induced oxidation reactions occurring on both protein backbone and amino acid side chains [27, 28].

However, insufficient AOS activity causes the accumulation of ROS, which leads to oxidative stress that is denoted as a persistent condition of an imbalance between ROS generation and the ability of a biological system to detoxify them leading to disruption in redox signaling/control and/or molecular damage [29]. Oxidative stress induces lipid peroxidation and glycoxidation reactions, which lead to the formation of highly reactive and electrophilic compounds that attack free amino groups in proteins causing their covalent modifications and resulting in the generation of advanced lipoxidation end products (ALEs) and advanced glycation end products (AGEs) [30].

ALE and AGE formation causes an impairment in the protein structure due to covalent cross-linking resulting in protein oligomerization and aggregation. This leads to alterations in cellular functions, cell damage, and death. For example, the impairment in mitochondrial, endoplasmic reticulum (ER), and extracellular matrix (ECM) proteins and those involved in cell cycle and control of gene expression has been observed in various studies [31, 32]. Oxidative stress and oxidative stress-induced ALE and AGE formation have been implicated in aging and in a variety of age-related chronic diseases [33–37].

The present review focuses on recent advancements in investigating the consequences of oxidative stress-induced ALE/AGE accumulation for cellular functions and the implication of ALE/AGE formation in aging and age-related human diseases such as chronic/acute inflammation, neurodegenerative disorders, atherosclerosis, and vascular complications of diabetes mellitus (DM).

2. Implication of ALEs and AGEs in Aging

2.1. Oxidative Stress and Aging. Aging is a progressive time-dependent functional decline in an organism's physiological integrity and adaptability followed by a consequent irreversible decrease in its fertility and an increase in morbidity and mortality risk [38]. In 1959, Denham Harman postulated a free radical theory of aging that points to ROS accumulation as the underlying reason for biomolecular oxidation and cellular damage and as the explanation for the alterations in cellular functions during aging [39]. Since that time, the oxidative stress theory of aging has gained considerable acceptance, despite numerous other proposed theories on biological aging and senescence [40].

The reduction of oxidative stress by ROS scavengers followed by the delay of the age-associated decline in physiological processes and marked prolongation in the mean lifespan can be considered as a confirmation of the oxidative stress theory of aging [41]. This theory has also been approved in many animal models including *S. cerevisiae*, transgenic mice, and long-lived species such as *C. elegans*, birds, and naked mole-rat [42–44]. Among them, the naked mole-rat (NMR, *Heterocephalus glaber*) is the longest-living rodent known with a maximum lifespan of more than 28.3 years, which is 9 times longer than that of similar-sized laboratory mice. Body composition, physiological functions including reproductive function, and tissue morphology of NMRs can be maintained from 2 to 24 years almost with no changes and showing negligible senescence and no spontaneous neoplasm [45].

Perez et al. [46] showed that the amount and activities of both ROS and the antioxidant system in NMRs are similar to those of shorter-living mice; however, NMRs exhibited higher levels of oxidative biomolecular damage (DNA damage, lipid peroxidation, and protein carbonyl formation) even at an early age. However, NMRs demonstrated a higher amount of free thiol groups and lower levels of urea-induced protein unfolding and protein ubiquitination as well as higher proteasome activity as compared to young C57BL/6 mice [46]. Interestingly, no one of these parameters was significantly altered during the two decades of the NMRs' lifespan. These data indicate that the existence of molecular mechanisms underlying the maintenance of cellular homeostasis and biomolecular stability and integrity are crucial for cellular longevity and successful aging.

Indeed, alterations in the structure, functions, and oxidation state of muscle proteins have been observed in aged F344BN mice [47]. The content of regulatory proteins was reduced by up to 75%, while the catalytic activity of enzymes decreased by up to 50% in mice with aging. Additionally, Duchenne muscular atrophy and loss of nerve supply along with increased expression of immunoproteasome subunits have been observed in aged animals [48]. The increased lifespan of Tq mice has been reported to associate with the stimulation of mitogen-activated protein kinase- (MAPK-) mediated redox signaling, the increased expression of stress-protective heat shock protein 25 (HSP25), and the activation of antioxidant enzymes, catalase, and SODs, suggesting that the oxidative stress-induced stimulation of

endogenous defense mechanisms plays key roles in providing health and longevity [49].

Proteostasis is an overall cellular protein homeostasis provided by integrated protein control quality pathways including biosynthesis, folding, trafficking, and elimination/-degradation of damaged proteins [50, 51]. Maintaining proteostasis is an important component of successful aging because, in most metazoans, aging has been shown to be accompanied by a decline in the activities of the protein quality control machinery that includes molecular chaperones, ubiquitin-proteasome system (UPS), and autophagy/-lysosome activity, which results in the accumulation of damaged and self-aggregating proteins [52]. During aging, oxidative protein damage and covalent cross-linking followed by the accumulation of the so-called "aggresomes" that are toxic for cells have been shown [53, 54]. Long-lived species have been observed to possess improved proteostasis in comparison with short-lived species as assessed by elevated HSP levels, enhanced macroautophagy, and the UPS activity [55]. Additionally, the reestablishment of proteostasis due to lysosome activation followed by a metabolic shift that mobilizes the degradation of protein aggregates has been observed in immortal *C. elegans* germ lineages [43].

One of the most studied features of aging is the manifestation of mitochondrial dysfunction [56, 57]. Mitochondria are considered as both a major site of ROS generation and the main target for ROS attack. The age-related increase in mitochondrial ROS production by complex I, oxidative stress-induced mutations in mitochondrial DNA (mtDNA), and accumulation of mtDNA fragments inside the nucleus have been observed in mouse liver [58]. These changes were accompanied by oxidative damage and lipoxidation of mitochondrial proteins including enzymatic components of ETC and accumulation of lipofuscin produced by covalently cross-linked and aggregated proteins; all these alterations were abolished by rapamycin treatment. Additionally, a lesser amount mitochondrial ROS production and higher cardiolipin content in erythrocytes of long-lived species as compared to short-lived ones have been reported [44]. Thus, age-dependent accumulation of oxidized proteins may be caused by both an increase in mitochondrial ROS production and a decline in the proteolytic capacity of either the ubiquitin/proteasomal or lysosomal pathway [59].

The Nrf2 transcription factor serves as a master regulator of cell response to oxidative stress, the Nrf2 dysfunction being observed in various cell types during aging. The overexpression of Nrf2 target genes, NADPH quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase (GCLM), and heme oxygenase 1 (HO1) have been shown in aged mouse retinal pigment epithelium (RPE) cells as compared to younger mice under oxidative stress conditions [60]. Old mice also exhibited higher $O_2^{\bullet -}$ and MDA levels than younger mice. The same genes were overexpressed under Nrf2 induction conditions in the bronchial epithelium cells of old humans as compared to young adult persons [61]. A disruption in Nrf2 signaling causes reduced cell migration and an impaired ability of the coronary artery endothelial cells to form capillary-like structures [62].

The antioxidant system, including the glutathione (GSH/GSSG) system and SODs, has been shown to be involved in successful aging through the maintenance of intracellular redox balance. Indeed, the altered ratio between reduced, GSH, and oxidized, GSSG, forms of glutathione in aging has been demonstrated by measurements of GSH concentration in red blood cells and levels of plasma oxidative stress biomarkers such as F2-isoprostanes in younger and elderly persons [63]. The elderly persons had markedly lower concentrations of glycine, cysteine, and GSH along with decreased GSH biosynthesis in erythrocytes as compared to those in younger persons. However, glycine and cysteine supplementation led to an increase in GSH concentration and rate of its biosynthesis along with a significant decrease in levels of oxidative stress biomarkers in the blood plasma.

A reduced ROS level due to the activation of another AOS component, Mn-superoxide dismutase (SOD2), through its deacetylation at the evolutionarily conserved Lys122 residue by the conserved family of NAD^+-dependent deacetylases, sirtuins, have been reported as a factor involved in lifespan control [64, 65]. Mammalian sirtuins 1 and 3, SIRT1 and SIRT3, have been shown to regulate the activity of SOD2 to protect muscle cells from oxidative stress [66]. They can promote mitochondrial biogenesis by activating PGC-1α that is a transcriptional coactivator upregulating antioxidant enzymes such as GPx, catalase, and SOD2 [67].

2.2. ALEs and AGEs in Aging. Oxidative stress induces endogenous formation and accumulation of both ALEs and AGEs, which can be produced from the same precursors such as glyoxal and methylglyoxal and through the same intermediates such as *N*-(carboxymethyl)-lysine (CML) and *N*-(carboxymethyl)-cysteine (CMC). ALEs are generated due to lipid peroxidation reactions, while AGEs result from glycoxidation reactions; both of the pathways give rise to an extraordinarily complex mixture of interrelated compounds [30]. These compounds include highly reactive electrophilic aldehydes and their derivatives such as 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE), 4-hydroxy-hexanal (HHE), acrolein (ACR), and malonic dialdehyde (MDA) [68, 69]. They interact with free amino groups in protein to cause their covalent modification, cross-linking, oligomerization, and aggregation. These processes cause intracellular damage, impaired cell functions, and, ultimately, cell death to be implicated in aging and various age-related chronic diseases [70, 71].

2.2.1. Roles of ALEs in Aging. Changes in the amount of lipid peroxidation products and activities of COX-2 and CYP2JA in human brain have been reported to occur in an age-dependent manner [72]. A significant increase in lipid peroxidation and oxidative protein modification levels accompanied by the loss of thiol groups, accumulation of dityrosine, and ALE formation has been observed in mitochondria and synaptosomes during brain aging in rats [73]. Interestingly, the higher membrane resistance to lipid peroxidation and the lower molecular damage caused by protein lipoxidation have been shown to associate with significantly reduced desaturase activity and peroxisomal betaoxidation

in the brain and spleen of exceptionally old (128 ± 4 weeks) and adult (28 ± 4 weeks) female mice as compared to old (76 ± 4 weeks) animals [74].

Aldehydes generated from polyunsaturated fatty acid (PUFA) peroxidation such as HNE, MDA, and ACR have been shown to form protein adducts that accumulate in the intima, media, and adventitia layers of the human aorta leading to progressive cellular dysfunction and contributing to the process of aging [75]. HNE, the most reactive and abundant endogenously generated α,β-unsaturated hydroxyl-aldehyde, has been shown to contribute to inhibiting elastin repair by antagonizing elastogenic signaling of transforming growth factor-β (TGF-β) through the inhibition of Smad2 translocation into the nucleus of human and murine skin fibroblasts [76].

Additionally, the accumulation of HNE-modified adducts, the decrease in elastin content, and the modification of the epidermal growth factor (EGF) receptor by NHE have been observed in the aorta of aged C57BL/6 mice. The content of elastin in connective tissue decreased, and the structure of elastin fibers was significantly altered with aging; however, the oxidative protein modification level was very poor indicating a complex role of ALEs in vascular wall remodeling during aging [76].

2.2.2. Ages and AGE-RAGE Axis in Aging. AGE manifestation, especially in connective tissue, which leads to age-dependent damage and covalent cross-linking in ECM adhesion proteins such as collagen, laminin, and elastin has been shown to contribute to the loss of skin and vessel elasticity and degeneration of cartilages, ligaments, and eye lens [77, 78]. The accumulation of AGEs and the fluorescent age pigment, lipofuscin, both of which are typically of brown color, has been shown to associate with aging and age-related chronic diseases contributed by age-dependent inhibition of both proteasomal and lysosomal protein degradation pathways [79, 80].

Various age-related diseases arise due to alterations in cell signaling pathways that proceed with the involvement of the receptors for AGEs (RAGEs) and the AGE-RAGE axis. For example, the colocalization of CML and RAGE along with the activation of nuclear factor-κB (NF-κB) has been observed in patients with age-related macular degeneration indicating the possible role of the AGE-RAGE axis and the NF-κB transcription factor in the pathogenesis of the disease [81]. The accumulation of both AGEs and RAGEs in RPE and photoreceptor cells has been accompanied by NF-κB nuclear translocation and cell apoptosis [82]. These data allowed suggesting that AGE accumulation induces RPE/-photoreceptor cell activation during normal aging and contributes to age-related pathologies in human retinas.

Additionally, diet-derived AGEs and lipofuscin have been reported to disrupt the overall protein homeostasis and to reduce the lifespan of *D. melanogaster* [83]. Oral administration of glucose-, fructose-, and ribose-modified albumin or artificial lipofuscin caused the accumulation of AGEs in fly somatic tissues and hemolymph, and this was accompanied by oxidative stress and the upregulation of lysosomal cathepsin B activity. Interestingly, the decreased glucose level observed under caloric restriction with no malnutrition conditions led to the inhibition of enzyme activities and the decrease in concentrations of metabolites of the polyol pathway, sorbitol and fructose. This contributed to the beneficial effects of caloric restriction including the increase in the NADPH level required for other reduction reactions such as GSH and other forms of AOS component regeneration, and counteracted age-related changes derived from the activities of the polyol pathway [84].

Thus, experimental data evidence key roles of both ALEs and AGEs in the process of aging, being considered as biomarkers of oxidative stress and mitochondrial dysfunction and as factors of aging and age-associated chronic pathologies [85].

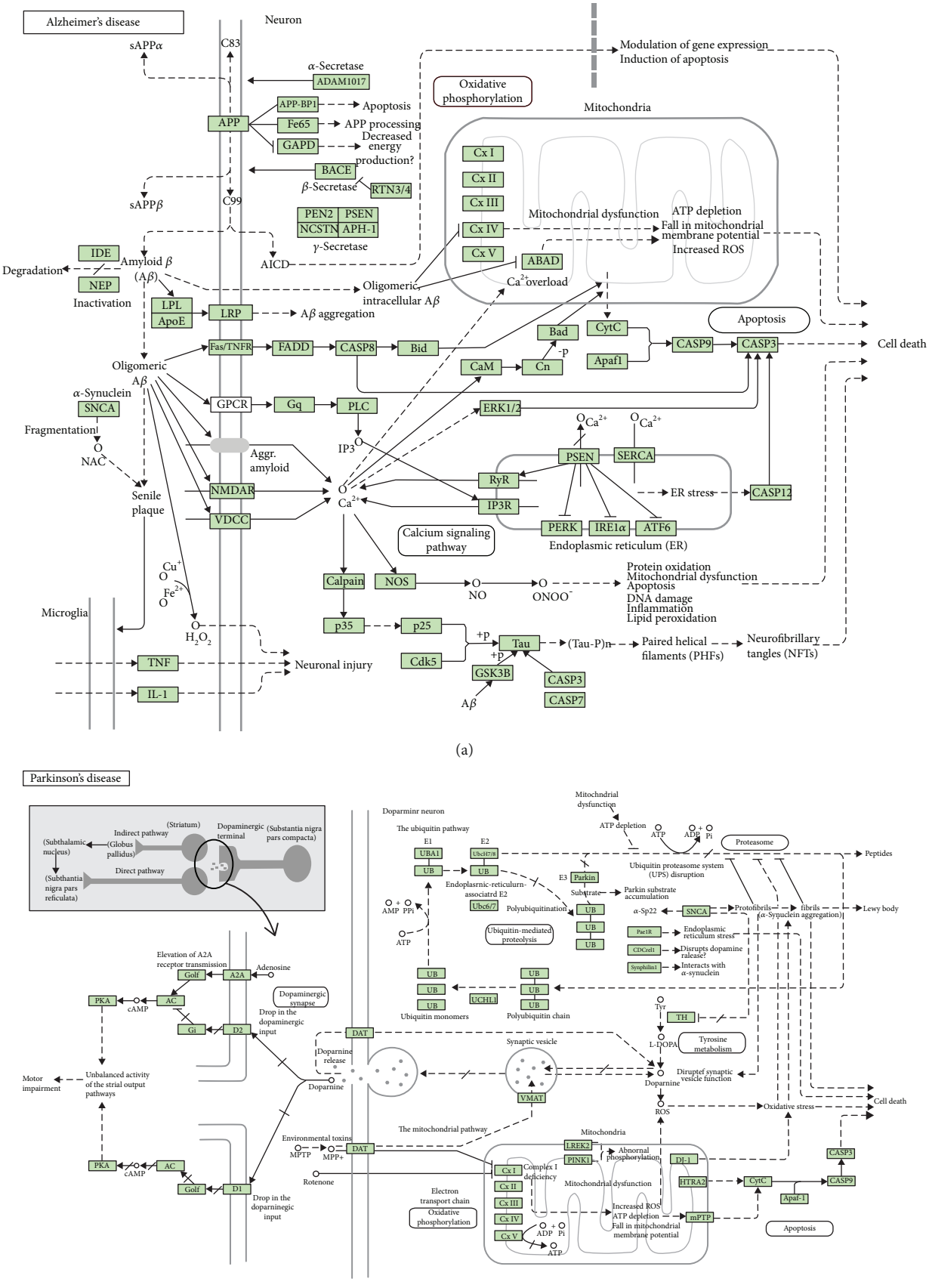
3. Roles of ALEs and AGEs in Age-Related Chronic Diseases

3.1. Neurodegenerative Diseases. Oxidative stress and oxidative protein damage can accelerate the formation of toxic protein oligomers and aggregates in the nucleus and cytoplasm of nerve cells, which contributes to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) [86–88]. Despite their distinct causative factors and clinical symptoms, these diseases have common pathogenetic features such as mitochondrial dysfunction and ER stress implicated in excessive ROS accumulation, impairment in proteostasis network, and neuroinflammation [89].

Normal aging and neurodegeneration can be distinguished by the measurement of AGE concentration in the brain tissue and cerebrospinal fluid. AGE/RAGE manifestation indicates neuropathological and biochemical alterations such as excessive protein cross-linking, inflammation, and neuronal cell death. For example, the accelerated accumulation of AGEs in pathological deposits such as amyloid fibrils and senile plaques has been observed in AD (Figure 1(a)), the most common age-related dementing disorder [90]. The measurement of various AGEs and ALEs in the brain cortex of AD patients demonstrated a significant, although heterogeneous increase in the concentrations of CML, *N*(epsilon)-malondialdehyde-lysine, *N*(epsilon)-carboxyethyl-lysine, and other protein oxidation adducts [91]. Methylglyoxal has been suggested to be one of the major carbonyl species responsible for AGE formation in AD [92].

AGEs can stimulate the expression of inducible nitric oxide synthase (iNOS), and colocalization of AGEs and iNOS has been demonstrated in astrocytes and microglia of AD patients (Figure 1(a)) as revealed by immunochemical analysis [93]. Additionally, an increase in traumatic brain injury-induced nitric oxide generation catalyzed by iNOS and persistent tyrosine nitration adjacent to the injury site have been reported [94]. These effects were accompanied by oxidative stress-induced cell death through apoptosis induction and receptor-mediated serine/threonine protein kinase-mediated necrosis.

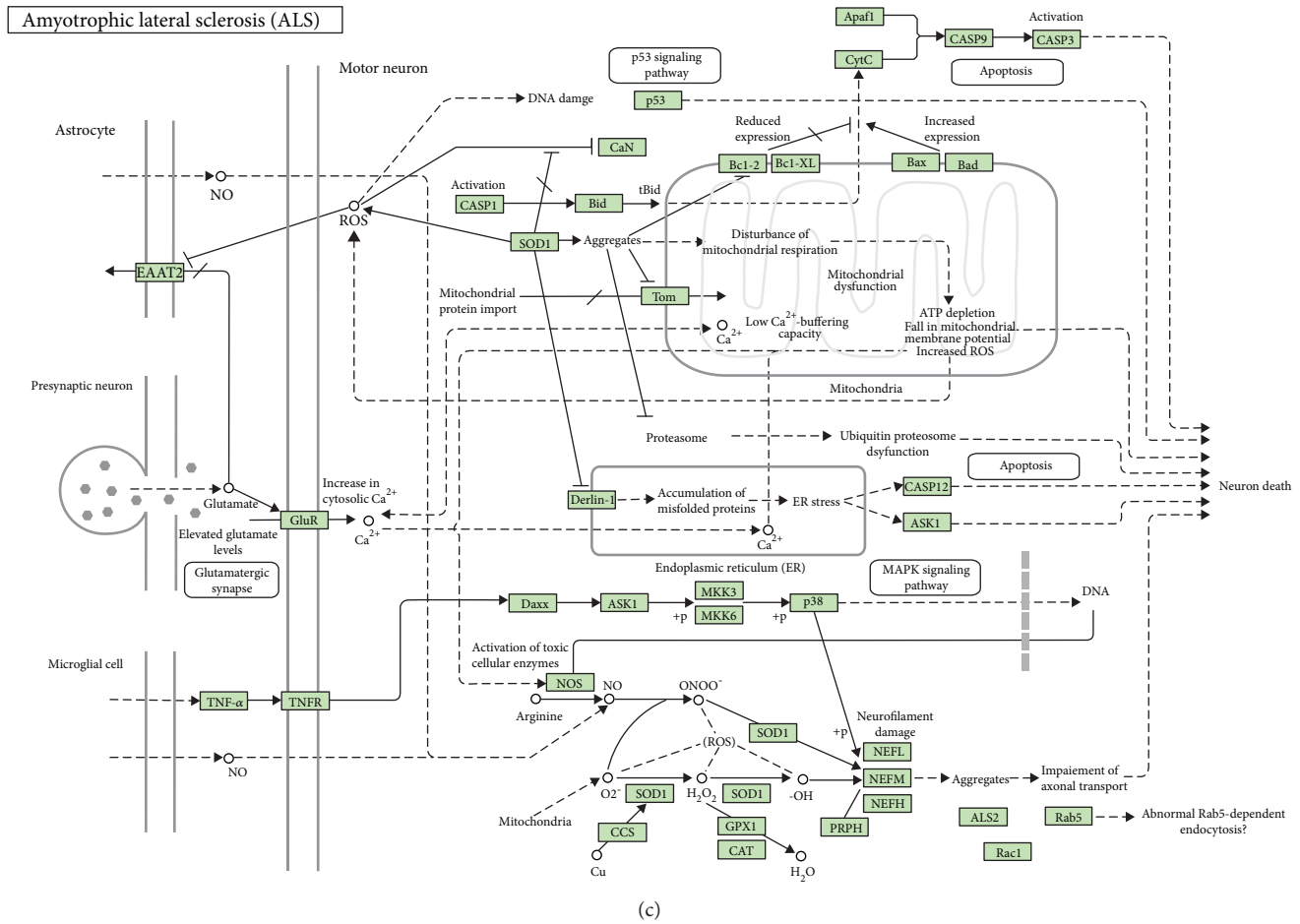
Mitochondrial dysfunction and mutations in mtDNA genes encoding ETC complex I subunits with the subsequent



(a)

(b)

FIGURE 1: Continued.



(c)

Figure 1: Schematic KEGG map representations of signaling pathways involved in Alzheimer's disease (a), Parkinson's disease (b), and amyotrophic lateral sclerosis (c). Oxidative stress-induced alterations in signaling pathways, which cause mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and dysregulation of the ubiquitin-proteasome system (UPS) and the autophagy/lysosomal protein quality control machinery, followed by neuronal death, are also shown. Here, → indicates stimulating effects and — indicates inhibitory effects. (a) AD is characterized by the formation of amyloid precursor protein-derived amyloid β-peptide (Aβ), a major component of senile plaques, which forms oligomers to induce pathways initiated by the following receptors: (i) LRP, an apoE receptor; (ii) amyloid precursor protein (APP), an integral membrane protein, mutations of which cause susceptibility to familial AD; (iii) TNF-α receptor (Fas/TNFR) to activate caspases; (iv) GNAQ (Gq)/G-protein-coupled receptor (GPCR) to stimulate phospholipid C (PLC) followed by the activation of inositol-3-phosphate receptor (IP_3R) and ER stress; (v) *N*-methyl-D-aspartate receptor (NMDAR) to cause hyperphosphorylation of tau receptors, and (vi) voltage-gated (dependent) calcium channels (VDCC) followed by neuronal damage through mitochondrial dysfunction and disruption of calcium release from ER. Presenilin 1 and 2 (PSEN1 and PSEN2) proteins belong to γ-secretases that generate Aβ. (b) PD results from the death of dopaminergic neurons in the substantia nigra pars compacta (SNs). Normally, dopamine active transporter (DAT) pumps dopamine out of the synaptic clefts into the cytoplasm. The early onset of PD is associated with mutations in synuclein-alpha (SNCA), ubiquitin carboxy-terminal hydrolase L1 (UCHL1), PTEN-induced kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), mitochondrial serine protease 2 (HTRA2), parkin, and parkin-associated protein DJ1 involved in oxidative stress. (c) ALS is a lethal disorder characterized by the death of motor neurons in the brain and spinal cord. Mutations in SOD1 may interfere with the neurofilament heavy polypeptide (NEFH) and the translocation machinery, the translocase of the inner/outer membrane (TIM/TOM) that is involved in familial ALS. Proapoptotic THFα acts through its receptor, TNFR, to induce inflammation and apoptotic cell death. The main glutamate transporter protein, excitatory amino acid transporter (EAAT2), is inhibited by ROS produced by mitochondria. Glutamate acts through its receptor (GluR) to increase calcium release from ER and to enhance oxidative stress and mitochondrial damage. Permission 190019 for usage of the following KEGG pathway images was kindly granted by Kanehisa Laboratories [141]: map05010—Alzheimer's disease; map05012—Parkinson's disease; map05014—amyotrophic lateral sclerosis (ALS).

impairment in ATP production and elevated ROS generation along with disruption in both UPS and autophagy-lysosome protein degradation pathways have been observed in all types of neurodegeneration [95]. Damaged mitochondria accumulate tensin homolog deleted from chromosome 10- (PTEN-) induced kinase 1 (PINK1) that recruits parkin, a protein of

the ubiquitin E3 ligase complex, as shown in PD patients (Figure 1(b)) [96, 97]. This causes the ubiquitination of mitochondrial proteins, which can further bind to the autophagic proteins, p62/SQSTM1 and lc3, resulting in the degradation of mitochondria through the autophagy pathway, the process denoted as mitophagy [98]. Significant increases in the expression of p62/SQSTM1 both at the mRNA and protein levels along with the activation of mitochondrial/lysosomal biogenesis following PINK1/parkin-mediated mitophagy have been observed in familial AD [99].

Nrf2 and transcription factor EB (TFEB), which play key roles in mitochondrial and lysosomal biogenesis, respectively, have been demonstrated to translocate into the nucleus following the mitophagy induction. Additionally, the multifaceted protective potential of Nrf2 signaling in patients with neurodegenerative diseases and in primary mouse HD and WT microglia and astrocytes has been reported [100, 101]. Oxidative stress-induced covalent modification of Cys151 in Kelch-like ECH-associated protein 1 (Keap1), the E3 ligase substrate adaptor protein and primary negative regulator of Nrf2, has been shown in HD [100]. Nrf2 expression is orchestrated and amplified by the coexpression of antioxidant and anti-inflammatory genes as shown, for example, in the primary monocytes from HD patients, in which the repressed expression of proinflammatory cytokines such as IL-1, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) was observed (Figure 1(a)).

The oxidative modification of Cys111 in Cu/Zn SOD (SOD1) has been implicated in the pathogenesis of various diseases, while mutation in SOD1 (Figure 1(c)) has been found in 20% of familial ALS [102]. Unlike native SOD1, cysteinylated SOD1 is not oxidized, suggesting that the cysteinylation protects this antioxidant enzyme from hydrogen peroxide-induced oxidation as shown in the culture of nerve cells. The existence of the cross-talk between the overexpression of SOD1 and regulation of mitochondrial unfolded protein response (UPR) has been postulated [103].

In the nervous system, proteasomes play key roles in maintaining the neuronal protein homeostasis, while an alteration in their activity contributes to pathogenesis of neurodegenerative diseases [104, 105]. The accumulation of large-ordered fibrils formed by β-sheet-enriched proteins denoted as amyloid fibrils in neuronal cells is characteristic for all types of neurodegenerative diseases, being a result of UPS dysfunction and, consequently, accumulation of polyubiquitinylated proteins in nervous tissue [106, 107]. A decreased capacity for the removal of oxidized proteins and the accumulation of damaged and misfolded proteins causes metabolic dysfunction and initiates cell death through apoptosis or necrosis. These disturbances lead to progressive amyloid plaque formation, loss of neurons, brain atrophy, cerebrovascular amyloid angiopathy, and vascular mineralization in an age-dependent manner [108].

ER stress has also been implicated in many chronic neurodegenerative diseases including AD and HD, while prolonging ER stress results in cell death. An important role in this process belongs to ER-localized stress-sensing and stress-triggering proteins such as IRE1α, ATF6, and PERK (Figure 1). During UPR, they activate the apoptotic signaling pathway, while fortilin, a prosurvival molecule, inhibits apoptosis by directly binding to IRE1α and reducing both its kinase and RNAse activities [109].

3.2. Atherosclerosis. The accumulation of both ALEs and AGEs progressively leads to cellular dysfunction and tissue damage involved in the progression of other oxidative stress-induced chronic diseases such as atherosclerosis and diabetes mellitus. Hyperglycemia can induce oxidative stress and tissue damage through either repeated acute changes in glucose metabolism or long-term biomolecular glycation and AGE formation [110]. This can further trigger inflammation and cell proliferation contributing to the development of atherosclerosis and vascular dysfunction through the initiation of oxidation of low-density lipoproteins (LDLs) and their interaction with mononuclear cells, endothelial cells, and smooth muscle cells [111–113]. Glycation of LDLs increases their atherogenicity, while high-density lipoproteins (HDLs) have been reported to impede the glycation of LDL apolipoprotein B (apoB) [114].

In an atherosclerotic lesion, macrophages express scavenger receptors on the surface of their cell membrane to bind oxidized LDLs from blood vessel walls and to develop into foam cells. The oxidation of LDLs causes the formation of HNE-apoB adducts that contribute to the atherogenicity of LDLs and their binding capacity to scavenger receptors [110]. Additionally, the transportation of oxidized lipids in lipoprotein complexes has been suggested to play a role in the pathogenesis of atherosclerosis, those transported by LDL being associated with high risk, while those transported by HDL being indicative for protection against disease progression [115].

The LDL receptor has a high affinity to apoE which in humans exists in three isoforms: apoE2, apoE3, and apoE4, the latter being a major risk factor for cardiovascular diseases and Alzheimer's disease (Figure 1(a)). The redox status of various serum apoE isoforms determined by oxidative modification in their redox-sensitive cysteine residues has been shown to be different. The quantitative ratios of nonreduced apoE to total serum apoE from patients with apoE4/E3 were higher than those from apoE3/E3 subjects; this may be used as the disease indicator [116].

3.3. Diabetes Mellitus. The key roles of oxidative stress in the onset of diabetes mellitus and in the development of its complications have been demonstrated in various animal models. For example, under impaired redox balance conditions, increased Nrf2 and nitrotyrosine levels along with decreased SOD2, GPx, HO1, and endothelial nitric oxide synthase (eNOS) levels have been demonstrated in diabetic skin in mice [117]. Impairment in lipid and glucose metabolism, oxidative phosphorylation, and phospho-5′-AMP-activated protein kinase-α- (AMPKα-) mediated signaling along with the downregulation of eNOS, HO1, and sarcoplasmic reticulum calcium-ATPases 1 and 2 (SERCA 1 and SERCA 2) has been observed in diabetic rat skeletal muscle [118].

Reactive aldehydes such as HNE, when excessively produced under oxidative stress conditions, exhibit cytotoxic effects and play key roles in the pathophysiology of diabetes

mellitus through the involvement in both development and progression of the disease [119]. For example, increased protein carbonyl content was observed in patients with type 2 DM associated with neuropathy [120]. Increased levels of oxidative stress biomarkers along with oxidized lipid accumulation and serum albumin glycoxidation have been reported in diabetic mice [121]. AGE-modified albumin causes diabetes-induced liver damage and impairment in the activities of proteolytic enzymes and ETC carriers. Both experimental and clinical diabetes mellitus are characterized by impaired wound healing and defect in vascular endothelial growth factor (VEGF) expression. Lipid peroxidation reactions have been shown to be involved in the pathogenesis of altered VEGF regulation and angiogenesis to stimulate wound healing in diabetic mice [122].

The high glucose concentration observed in diabetes mellitus activates the polyol (sorbitol-aldose reductase) pathway, which leads to intracellular sorbitol accumulation. The inability of sorbitol to pass through the cell membrane in insulin-independent tissues (the retina, kidney, and nervous system) causes an increase in intracellular osmotic pressure and, subsequently, cell damage. Under oxidative stress conditions, all intermediates of the polyol pathway (sorbitol, fructose, and fructose-1-phosphate) can glycate proteins leading to AGE formation, and this is implicated in microvascular complications of diabetes mellitus [123]. Interestingly, an increase in glucose and glycogen levels observed under caloric restriction conditions has been found to cause the significant decrease in the activities of the polyol pathway enzymes, along with the activation of hexokinase, glucose-6-phosphate-dehydrogenase (pentose phosphate pathway enzyme), and glucose-6-phosphatase (glycogen degradation enzyme) in both diabetic and nondiabetic rats [124]. Therefore, caloric restriction contributes to the attenuation of hyperglycemia observed in diabetes mellitus.

Also, glycoxidation of IgG by methylglyoxal generated by hydrogen peroxide has been shown to create novel epitopes and to alter IgG immunogenicity in patients with type 2 DM [125]. Through binding to their receptors, RAGEs, AGEs can greatly accelerate the progression of the disease and the development of its microvascular complications such as diabetic nephropathy, retinopathy, and neuropathy [126–128]. The AGE-RAGE axis has been implicated in cell capillary loss, capillary basement membrane thickening, increased vascular permeability, and disruption of the blood-tissue barrier, along with increased leukocyte-to-endothelial cell adhesion and neovascularization observed in experimental animal models with DM [129].

Type 2 diabetes has been characterized by the formation of glycated hemoglobin along with increased levels of serum AGEs and full-length RAGE [130, 131]. Furthermore, patients with vascular complications had a significantly higher level of the soluble form of RAGE (sRAGE), decoy AGE receptor, than those without complications, while the level of sRAGE was associated with the severity of nephropathy [132]. Patients with type 1 diabetes have been shown to demonstrate higher levels of sRAGE and endogenous secretory RAGE (esRAGE) as compared to healthy donors [133, 134].

The blockade of RAGE using the sRAGE extracellular ligand-binding domain has been demonstrated to cause wound healing and the suppression of cytokines TNF-α and IL-6 and matrix metalloproteinase-2, -3, and -9 expression in diabetic mice [135]. This was accompanied by increased levels of platelet-derived growth factor (PDGF) and VEGF along with the enhancement of well-vascularized granulation tissue. Impaired angiogenic response in diabetic mice was dependent on RAGE-mediated regulation, while sRAGE restored diabetes-associated impairment of angiogenic response *in vivo* [136].

The formation of AGEs has been reported to correlate with glycemic control. For example, AGE-modified serum albumin and apolipoprotein A-II levels are highest in patients with type 2 DM with poor glycemic control; in total, 19 modification sites corresponding to 11 proteins have been identified using a highly sensitive proteomic approach with the application of reverse-phase HPLC and mass spectrometry [137]. Additionally, fibrinogen and insulin-like growth factor- (IGF-) binding protein 1 are tightly connected to metabolic changes and vascular complications in patients with diabetes mellitus. The complexes of these two proteins have been shown to undergo glycoxidation, which reduces their stability and is possibly implicated in the hypercoagulation observed in type 2 DM [138].

All the abovementioned oxidative stress-induced metabolic and structural alterations may underlie the so-called "metabolic memory," the phenomenon that consists in the development of micro- and macrovascular complications of diabetes mellitus even after improved glucose levels [139]. Early intensive glycemic control can decrease the risk of diabetic vascular complications as shown in diabetic rats, in which oxidative stress and nitric oxide levels in urine and the renal cortex soon after the establishment of good glycemic control were not different from those observed in healthy animals [140]. However, when glycemic control was delayed to 6 months, diabetic nephropathy developed in diabetic rats. Hyperglycemia induces oxidative stress, which if prolonged, causes mitochondrial dysfunction, polyol pathway activation, ALE production, AGE-RAGE axis stimulation, and subsequent diabetic vascular complications.

4. Conclusion

Accumulated data evidence that oxidative stress-induced excessive generation of reactive aldehydes produced through lipid peroxidation and glycoxidation reactions with consequent protein cross-linking, oligomerization, and aggregation and formation of protein oxidation adducts are implicated in aging and various chronic age-related diseases. In the present review, we focused on neurodegenerative diseases and cardiovascular disorders, complications of diabetes mellitus, and atherosclerosis, the incidence and prevalence of which increase with age. These age-related chronic diseases are becoming a major challenge for medicine and public health worldwide, because the number of subjects suffering from these increases, causing demographic changes all over the world. This dictates more

investigations in the field to elucidate metabolic and structural changes that lead to alterations in cell signaling events with the involvement of ALEs and AGEs in the onset and progression of the age-associated diseases. The discovery of novel oxidative stress biomarkers and drug targets and new approaches in their clinical applications along with reconsidering health care policies are of crucial importance.

Abbreviations

ROS: Reactive oxygen species
AOS: Antioxidant system
ETC: Electron-transportation chain
NOS: Nitric oxide synthase
ALE: Advanced lipoxidation end product
AGE: Advanced glycation end product
GSH: Glutathione
RAGE: Receptor for AGEs
PUFA: Polyunsaturated fatty acid
PL: Phospholipid
RCS: Reactive carbonyl species
MDA: Malonic dialdehyde
ACR: Acrolein
HNE: 4-Hydroxy-2-nonenal
HHE: 4-Hydroxy-hexanal
ONE: 4-Oxo-2-nonenal
CML: *N*-(Carboxymethyl)-lysine
CMC: *N*-(Carboxymethyl)-cysteine
COX: Cyclooxygenase
NF-κB: Nuclear factor-κB
Nrf2: Nuclear factor-erythroid 2-related factor 2
TNF-α: Tumor necrosis factor-α
EGF: Epidermal growth factor
TGF-β: Transforming growth factor-β
VEGF: Vascular endothelial growth factor
IGF: Insulin-like growth factor
PDGF: Platelet-derived growth factor
MAPK: Mitogen-activated protein kinase
PTEN: Phosphatase tensin homolog deleted from chromosome 10
PINK1: PTEN-induced kinase 1
UPS: Ubiquitin-proteasome system
HSP: Heat shock protein
SOD: Superoxide dismutase
GPx: Glutathione peroxidase
GR: Glutathione reductase
ECM: Extracellular matrix
ER: Endoplasmic reticulum
UPR: Unfolded protein response
AD: Alzheimer's disease
PD: Parkinson's disease
HD: Huntington disease
ALS: Amyotrophic lateral sclerosis
LDL: Low-density lipoprotein
HDL: High-density lipoprotein
apoB: Apolipoprotein B
NMR: Naked mole-rat
RPE: Retinal pigment epithelium.

Conflicts of Interest

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

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References

[1] H. Sies, "Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress," *Redox Biology*, vol. 11, pp. 613–619, 2017.

[2] G. Bounocore, S. Perrone, and M. L. Tataranno, "Oxygen toxicity: chemistry and biology of reactive oxygen species," *Seminars in Fetal and Neonatal Medicine*, vol. 15, no. 4, pp. 186–190, 2010.

[3] M. P. Murphy, "How mitochondria produce reactive oxygen species," *Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.

[4] R. P. Brandes, V. Weissman, and K. Schröder, "Nox family NADPH oxidases: molecular mechanisms of activation," *Free Radical Biology & Medicine*, vol. 76, pp. 208–226, 2014.

[5] M. D. Brand, "Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling," *Free Radical Biology & Medicine*, vol. 100, pp. 14–31, 2016.

[6] I. M. Mokhosoev, G. P. Kuznetsova, M. A. Al'terman, G. I. Bachmanova, and A. I. Archakov, "Inactivation of sodium dithionite reduced cytochromes P-450 of different origins," *Biokhimia*, vol. 52, no. 10, pp. 1649–1658, 1987.

[7] S. Matsumoto, I. Koshiishi, T. Inoguchi, H. Nawata, and H. Utsumi, "Confirmation of superoxide generation via xanthine oxidase in streptozotocin-induced diabetic mice," *Free Radical Research*, vol. 37, no. 7, pp. 767–772, 2003.

[8] F. A. Fitzpatrick, "Cyclooxygenase enzymes: regulation and function," *Current Pharmaceutical Design*, vol. 10, no. 6, pp. 577–588, 2004.

[9] A. Andreou and I. Feussner, "Lipoxygenases – structure and reaction mechanism," *Phytochemistry*, vol. 70, no. 13–14, pp. 1504–1510, 2009.

[10] A. Phaniedra, D. B. Jestadi, and L. Periyasamy, "Free radicals: properties, sources, targets, and their implication in various diseases," *Indian Journal of Clinical Biochemistry*, vol. 30, no. 1, pp. 11–26, 2015.

[11] S. di Meo, T. T. Reed, P. Venditti, and V. M. Victor, "Role of ROS and RNS sources in physiological and pathological conditions," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 1245049, 44 pages, 2016.

[12] A. Kabel, "Free radicals and antioxidants: role of enzymes and nutrition," *World Journal of Nutrition*, vol. 2, no. 3, pp. 35–38, 2014.

[13] E. Niki, "Oxidative stress and antioxidants: *distress* or *eustress*?," *Archives of Biochemistry and Biophysics*, vol. 595, pp. 19–24, 2016.

[14] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *The International*

Journal of Biochemistry & Cell Biology, vol. 39, no. 1, pp. 44–84, 2007.

[15] T. Fukai and M. Ushio-Fukai, "Superoxide dismutases: role in redox signaling, vascular function, and diseases," *Antioxidants & Redox Signaling*, vol. 15, no. 6, pp. 1583–1606, 2011.

[16] R. Brigelius-Flohe and M. Maiorino, "Glutathione peroxidases," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1830, no. 5, pp. 3289–3303, 2013.

[17] L. Domınguez, A. Sosa-Peinado, and W. Hansberg, "Catalase evolved to concentrate H_2O_2 at its active site," *Archives of Biochemistry and Biophysics*, vol. 500, no. 1, pp. 82–91, 2010.

[18] M. G. Sharapov, V. K. Ravin, and V. I. Novoselov, "Peroxiredoxins as multifunctional enzymes," *Molecular Biology*, vol. 48, no. 4, pp. 520–545, 2014.

[19] L. Flohe, "The fairytale of the GSSG/GSH redox potential," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1830, no. 5, pp. 3139–3142, 2013.

[20] S. B. Nimse and D. Pal, "Free radicals, natural antioxidants, and their reaction mechanisms," *RSC Advances*, vol. 5, no. 35, pp. 27986–28006, 2015.

[21] R. H. Burdon, V. Gill, and D. Alliangana, "Hydrogen peroxide in relation to proliferation and apoptosis in BHK-21 hamster fibroblasts," *Free Radical Biology & Medicine*, vol. 24, no. 2, pp. 81–93, 1996.

[22] N. T. Moldogazieva, I. M. Mokhosoev, N. B. Feldman, and S. V. Lutsenko, "ROS and RNS signalling: adaptive redox switches through oxidative/nitrosative protein modifications," *Free Radical Research*, vol. 52, no. 5, pp. 507–543, 2018.

[23] N. Gould, P. T. Doulias, M. Tenopoulou, K. Raju, and H. Ischiropoulos, "Regulation of protein function and signaling by reversible cysteine S-nitrosylation," *Journal of Biological Chemistry*, vol. 288, no. 37, pp. 26473–26479, 2013.

[24] A. Wright, W. A. Bubb, C. L. Hawkins, and M. J. Davies, "Singlet oxygen–mediated protein oxidation: evidence for the formation of reactive side chain peroxides on tyrosine residues," *Photochemistry and Photobiology*, vol. 76, no. 1, pp. 35–46, 2002.

[25] A. Wright, C. L. Hawkins, and M. J. Davies, "Photo-oxidation of cells generates long-lived intracellular protein peroxides," *Free Radical Biology & Medicine*, vol. 34, no. 6, pp. 637–647, 2003.

[26] V. V. Agon, W. A. Bubb, A. Wright, C. L. Hawkins, and M. J. Davies, "Sensitizer-mediated photooxidation of histidine residues: evidence for the formation of reactive side-chain peroxides," *Free Radical Biology & Medicine*, vol. 40, no. 4, pp. 698–710, 2006.

[27] M. J. Davies, "Protein oxidation and peroxidation," *Biochemical Journal*, vol. 473, no. 7, pp. 805–825, 2016.

[28] H. A. Headlam and M. J. Davies, "Markers of protein oxidation: different oxidants give rise to variable yields of bound and released carbonyl products," *Free Radical Biology & Medicine*, vol. 36, no. 9, pp. 1175–1184, 2004.

[29] H. Sies, C. Berndt, and D. P. Jones, "Oxidative stress," *Annual Review of Biochemistry*, vol. 86, no. 1, pp. 715–748, 2017.

[30] G. Vistoli, D. de Maddis, A. Cipak, N. Zarkovic, M. Carini, and G. Aldini, "Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation," *Free Radical Research*, vol. 47, Supplement 1, pp. 3–27, 2013.

[31] D. Fuentealba, B. Friguet, and E. Silva, "Advanced glycation endproducts induce photocrosslinking and oxidation of bovine lens proteins through type-I mechanism," *Photochemistry and Photobiology*, vol. 85, no. 1, pp. 185–194, 2009.

[32] H. D. Yan, X. Z. Li, J. M. Xie, and M. Li, "Effects of advanced glycation end products on renal fibrosis and oxidative stress in cultured NRK-49F cells," *Chinese Medical Journal*, vol. 120, no. 9, pp. 787–793, 2007.

[33] N. Grossin, F. Auger, C. Niquet-Leridon et al., "Dietary CML-enriched protein induces functional arterial aging in a RAGE-dependent manner in mice," *Molecular Nutrition & Food Research*, vol. 59, no. 5, pp. 927–938, 2015.

[34] G. Waris and H. Ahsan, "Reactive oxygen species: role in the development of cancer and various chronic conditions," *Journal of Carcinogenesis*, vol. 5, no. 1, p. 14, 2006.

[35] N. Shanmugam, J. L. Figarola, Y. Li, P. M. Swiderski, S. Rahbar, and R. Natarajan, "Proinflammatory effects of advanced lipoxidation end products in monocytes," *Diabetes*, vol. 57, no. 4, pp. 879–888, 2008.

[36] J. Zhang, S. Shao, D. Han et al., "High mobility group box 1 promotes the epithelial-to-mesenchymal transition in prostate cancer PC3 cells via the RAGE/NF-κB signaling pathway," *International Journal of Oncology*, vol. 53, no. 2, pp. 659–671, 2018.

[37] S. Ahmad, Z. Siddiqui, S. Rehman et al., "A glycation angle to look into the diabetic vasculopathy: cause and cure," *Current Vascular Pharmacology*, vol. 15, no. 4, pp. 352–354, 2017.

[38] C. Lopez-Otin, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, pp. 1194–1217, 2013.

[39] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[40] T. Grune, K. Merker, T. Jung, N. Sitte, and K. J. A. Davies, "Protein oxidation and degradation during postmitotic senescence," *Free Radical Biology & Medicine*, vol. 39, no. 9, pp. 1208–1215, 2005.

[41] D. Canistro, C. Boccia, R. Falconi et al., "Redox-based flagging of the global network of oxidative stress greatly promotes longevity," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 70, no. 8, pp. 936–943, 2015.

[42] I. Shimokawa, T. Komatsu, N. Hayashi et al., "The life-extending effect of dietary restriction requires Foxo3 in mice," *Aging Cell*, vol. 14, no. 4, pp. 707–709, 2015.

[43] K. A. Bohnert and C. Keyon, "A lysosomal switch triggers proteostasis renewal in the immortal *C. elegans* germ lineage," *Nature*, vol. 551, no. 7682, pp. 629–633, 2017.

[44] J. Delhaye, N. Salamin, A. Roulin, F. Criscuolo, P. Bize, and P. Christe, "Interspecific correlation between red blood cell mitochondrial ROS production, cardiolipin content and longevity in birds," *Age*, vol. 38, no. 5-6, pp. 433–443, 2016.

[45] R. Buffenstein, "Negligible senescence in the longest living rodent, the naked mole-rat: insights from a successfully aging species," *Journal of Comparative Physiology B*, vol. 178, no. 4, pp. 439–445, 2008.

[46] V. I. Perez, R. Buffenstein, V. Masamsetti et al., "Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 9, pp. 3059–3064, 2009.

[47] D. A. Ferrington, A. D. Husom, and L. V. Thompson, "Altered proteasome structure, function, and oxidation in aged muscle," *The FASEB Journal*, vol. 19, no. 6, pp. 644–646, 2005.

[48] C.-N. J. Chen, T. G. Graber, W. M. Bratten, D. A. Ferrington, and L. V. Thompson, "Immunoproteasome in animal models of Duchenne muscular dystrophy," *Journal of Muscle Research and Cell Motility*, vol. 35, no. 2, pp. 191–201, 2014.

[49] S. Keipert, M. Ost, A. Chadt et al., "Skeletal muscle uncoupling-induced longevity in mice is linked to increased substrate metabolism and induction of the endogenous antioxidant defense system," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 304, no. 5, pp. E495–E506, 2013.

[50] D. M. Roth and W. E. Balch, "Modeling general proteostasis: proteome balance in health and disease," *Current Opinion in Cell Biology*, vol. 23, no. 2, pp. 126–134, 2011.

[51] J. Labbadia and R. I. Morimoto, "The biology of proteostasis in aging and disease," *Annual Review of Biochemistry*, vol. 84, no. 1, pp. 435–464, 2015.

[52] K. A. Rodriguez, J. M. Valentine, D. A. Kramer et al., "Determinants of rodent longevity in the chaperone-protein degradation network," *Cell Stress and Chaperones*, vol. 21, no. 3, pp. 453–466, 2016.

[53] T. C. Squier, "Oxidative stress and protein aggregation during biological aging," *Experimental Gerontology*, vol. 36, no. 9, pp. 1539–1550, 2001.

[54] T. Grune, T. Jung, K. Merker, and K. J. A. Davies, "Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease," *The International Journal of Biochemistry & Cell Biology*, vol. 36, no. 12, pp. 2519–2530, 2004.

[55] H. Pride, Z. Yu, B. Sunchu et al., "Long-lived species have improved proteostasis compared to phylogenetically-related shorter-lived species," *Biochemical and Biophysical Research Communications*, vol. 457, no. 4, pp. 669–675, 2015.

[56] A. Terman, T. Kurz, M. Navratil, E. A. Arriaga, and U. T. Brunk, "Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial–lysosomal axis theory of aging," *Antioxidants & Redox Signaling*, vol. 12, no. 4, pp. 503–535, 2010.

[57] A. V. Kudryavtseva, G. S. Krasnov, A. A. Dmitriev et al., "Mitochondrial dysfunction and oxidative stress in aging and cancer," *Oncotarget*, vol. 7, no. 29, pp. 44879–44905, 2016.

[58] V. Martínez-Cisuelo, J. Gómez, I. García-Junceda et al., "Rapamycin reverses age-related increases in mitochondrial ROS production at complex I, oxidative stress, accumulation of mtDNA fragments inside nuclear DNA, and lipofuscin level, and increases autophagy, in the liver of middle-aged mice," *Experimental Gerontology*, vol. 83, pp. 130–138, 2016.

[59] T. Grune, R. Shringarpure, N. Sitte, and K. Davies, "Age-related changes in protein oxidation and proteolysis in mammalian cells," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 56, no. 11, pp. B459–B467, 2001.

[60] M. M. Sachdeva, M. Cano, and J. T. Handa, "Nrf2 signaling is impaired in the aging RPE given an oxidative insult," *Experimental Eye Research*, vol. 119, pp. 111–114, 2014.

[61] M. N. Valcarcel-Ares, T. Gautam, J. P. Warrington et al., "Disruption of Nrf2 signaling impairs angiogenic capacity of endothelial cells: implications for microvascular aging," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 67, no. 8, pp. 821–829, 2012.

[62] L. Zhou, H. Zhang, K. J. A. Davies, and H. J. Forman, "Aging-related decline in the induction of Nrf2-regulated antioxidant genes in human bronchial epithelial cells," *Redox Biology*, vol. 14, pp. 35–40, 2018.

[63] R. V. Sekhar, S. G. Patel, A. P. Guthikonda et al., "Deficient synthesis of glutathione underlies oxidative stress in aging and can be corrected by dietary cysteine and glycine supplementation," *The American Journal of Clinical Nutrition*, vol. 94, no. 3, pp. 847–853, 2011.

[64] R. Tao, M. C. Coleman, J. D. Pennington et al., "Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress," *Molecular Cell*, vol. 40, no. 6, pp. 893–904, 2010.

[65] P. I. Merksamer, Y. Liu, W. He, M. D. Hirschey, D. Chen, and E. Verdin, "The sirtuins, oxidative stress and aging: an emerging link," *Aging*, vol. 5, no. 3, pp. 144–150, 2013.

[66] P. S. Pardo, J. S. Mohamed, M. A. Lopez, and A. M. Boriek, "Induction of *Sirt1* by mechanical stretch of skeletal muscle through the early response factor EGR1 triggers as antioxidative response," *Journal of Biological Chemistry*, vol. 286, no. 4, pp. 2559–2566, 2011.

[67] J. St-Pierre, S. Drori, M. Uldry et al., "Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators," *Cell*, vol. 127, no. 2, pp. 397–408, 2006.

[68] R. Aslebagh, B. A. Pfeffer, S. J. Fliesler, and C. C. Darie, "Mass spectrometry-based proteomics of oxidative stress: identification of 4-hydroxy-2-nonenal (HNE) adducts of amino acids using lysozyme and bovine serum albumin as model proteins," *Electrophoresis*, vol. 37, no. 20, pp. 2615–2623, 2016.

[69] C. E. McGrath, K. A. Tallman, N. A. Porter, and L. J. Marnett, "Structure-activity analysis of diffusible lipid electrophiles associated with phospholipid peroxidation: 4-hydroxynonenal and 4-oxononenal analogues," *Chemical Research in Toxicology*, vol. 24, no. 3, pp. 357–370, 2011.

[70] K. Uchida, "4-Hydroxy-2-nonenal: a product and mediator of oxidative stress," *Progress in Lipid Research*, vol. 42, no. 4, pp. 318–343, 2003.

[71] J. P. Castro, T. Jung, T. Grune, and W. Siems, "4-Hydroxynonenal (HNE) modified proteins in metabolic diseases," *Free Radical Biology & Medicine*, vol. 111, pp. 309–315, 2017.

[72] M. Dominguez-González, M. Puigpinós, M. Jové et al., "Regional vulnerability to lipoxidative damage and inflammation in normal human brain aging," *Experimental Gerontology*, vol. 111, pp. 218–228, 2018.

[73] E. Babusikova, J. Hatok, D. Dobrota, and P. Kaplan, "Age-related oxidative modifications of proteins and lipids in rat brain," *Neurochemical Research*, vol. 32, no. 8, pp. 1351–1356, 2007.

[74] L. Arranz, A. Naudi, M. De la Fuente, and R. Pamplona, "Exceptionally old mice are highly resistant to lipoxidation-derived molecular damage," *Age*, vol. 35, no. 3, pp. 621–635, 2013.

[75] K. Zarkovic, P. Larroque-Cardoso, M. Pucelle et al., "Elastin aging and lipid oxidation products in human aorta," *Redox Biology*, vol. 4, pp. 109–117, 2015.

[76] P. Larroque-Cardoso, E. Mucher, M. H. Grazide et al., "4-Hydroxynonenal impairs transforming growth factor-β1-induced elastin synthesis via epidermal growth factor receptor activation in human and murine fibroblasts," *Free Radical Biology & Medicine*, vol. 71, pp. 427–436, 2014.

[77] S. Viguet-Carrin, J. P. Roux, M. E. Arlot et al., "Contribution of the advanced glycation end product pentosidine and of maturation of type I collagen to compressive biomechanical properties of human lumbar vertebrae," *Bone*, vol. 39, no. 5, pp. 1073–1079, 2006.

[78] J. D. Furber, "Extracellular glycation crosslinks: prospects for removal," *Rejuvenation Research*, vol. 9, no. 2, pp. 274–278, 2006.

[79] S. Yamada, S. Kumazawa, T. Ishii et al., "Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine," *Journal of Lipid Research*, vol. 42, no. 8, pp. 1187–1196, 2001.

[80] K. Uchida, "Lipofuscin-like fluorophores originated from malondialdehyde," *Free Radical Research*, vol. 40, no. 12, pp. 1335–1338, 2006.

[81] H. P. Hammes, H. Hoerauf, A. Alt et al., "N^{ε} (carboxymethyl)lysine and the AGE receptor RAGE colocalize in age-related macular degeneration," *Investigative Ophthalmology & Visual Science*, vol. 40, no. 8, pp. 1855–1859, 1999.

[82] K. A. Howes, Y. Liu, J. L. Dunaief et al., "Receptor for advanced glycation end products and age-related macular degeneration," *Investigative Ophthalmology & Visual Science*, vol. 45, no. 10, pp. 3713–3720, 2004.

[83] E. N. Tsakiri, K. K. Iliaki, A. Hohn et al., "Diet-derived advanced glycation end products or lipofuscin disrupts proteostasis and reduces life span in *Drosophila melanogaster*," *Free Radical Biology & Medicine*, vol. 65, pp. 1155–1163, 2013.

[84] K. Hagopian, J. J. Ramsey, and R. Weindruch, "Caloric restriction counteracts age-related changes in the activities of sorbitol metabolizing enzymes from mouse liver," *Biogerontology*, vol. 10, no. 4, pp. 471–479, 2009.

[85] A. Naudi, M. Jove, D. Cacabelos et al., "Formation of *S*-(carboxymethyl)-cysteine in rat liver mitochondrial proteins: effects of caloric and methionine restriction," *Amino Acids*, vol. 44, no. 2, pp. 361–371, 2013.

[86] M. Kalousova, T. Zima, V. Tesar, S. Dusilova-Sulkova, and J. Skrha, "Advanced glycoxidation end products in chronic diseases—clinical chemistry and genetic background," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 579, no. 1-2, pp. 37–46, 2005.

[87] V. Srikanth, A. Maczurek, T. Phan et al., "Advanced glycation endproducts and their receptor RAGE in Alzheimer's disease," *Neurobiology of Aging*, vol. 32, no. 5, pp. 763–777, 2011.

[88] R. Thanan, S. Oikawa, Y. Hiraku et al., "Oxidative stress and its significant roles in neurodegenerative diseases and cancer," *International Journal of Molecular Sciences*, vol. 16, no. 1, pp. 193–217, 2015.

[89] E. Aso, S. Lomoio, I. Lopez-Gonzalez et al., "Amyloid generation and dysfunctional immunoproteasome activation with disease progression in animal model of familial Alzheimer's disease," *Brain Pathology*, vol. 22, no. 5, pp. 636–653, 2012.

[90] A. Rahmadi, N. Steiner, and G. Munch, "Advanced glycation endproducts as gerontotoxins and biomarkers for carbonyl-based degenerative processes in Alzheimer's disease," *Clinical Chemistry and Laboratory Medicine*, vol. 49, no. 3, pp. 385–391, 2011.

[91] R. Pamplona, E. Dalfo, V. Ayala et al., "Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets," *Journal of Biological Chemistry*, vol. 280, no. 22, pp. 21522–21530, 2005.

[92] M. Krautwald and G. Munch, "Advanced glycation end products as biomarkers and gerontotoxins – a basis to explore methylglyoxal-lowering agents for Alzheimer's disease?," *Experimental Gerontology*, vol. 45, no. 10, pp. 744–751, 2010.

[93] A. Wong, H. J. Luth, W. Deuther-Conrad et al., "Advanced glycation endproducts co-localize with inducible nitric oxide synthase in Alzheimer's disease," *Brain Research*, vol. 920, no. 1-2, pp. 32–40, 2001.

[94] M. Ucal, K. Kraitsy, A. Weidinger et al., "Comprehensive profiling of modulation of nitric oxide levels and mitochondrial activity in the injured brain: an experimental study based on the fluid percussion injury model in rats," *Journal of Neurotrauma*, vol. 34, no. 2, pp. 475–486, 2017.

[95] G. Lenaz, A. Baracca, R. Fato, M. L. Genova, and G. Solani, "Mitochondrial complex I: structure, function, and implications in neurodegeneration," *The Italian Journal of Biochemistry*, vol. 55, no. 3-4, pp. 232–253, 2006.

[96] T. M. Dawson and V. L. Dawson, "The role of parkin in familial and sporadic Parkinson's disease," *Movement Disorders*, vol. 25, Supplement 1, pp. S32–S39, 2010.

[97] A. M. Pickrell and R. J. Youle, "The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease," *Neuron*, vol. 85, no. 2, pp. 257–273, 2015.

[98] W.-X. Ding and X.-M. Yin, "Mitophagy: mechanisms, pathophysiological roles, and analysis," *Biological Chemistry*, vol. 393, no. 7, pp. 547–564, 2012.

[99] D. Ivankovic, K. Y. Chau, A. H. V. Schapira, and M. E. Gegg, "Mitochondrial and lysosomal biogenesis are activated following PINK1/parkin-mediated mitophagy," *Journal of Neurochemistry*, vol. 136, no. 2, pp. 388–402, 2016.

[100] L. Quinti, S. Dayalan Naidu, U. Träger et al., "KEAP1-modifying small molecule reveals muted NRF2 signaling responses in neural stem cells from Huntington's disease patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 23, pp. E4676–E4685, 2017.

[101] A. T. Dinkova-Kostova and A. G. Kazantsev, "Activation of Nrf2 signaling as a common treatment of neurodegenerative diseases," *Neurodegenerative Disease Management*, vol. 7, no. 2, pp. 97–100, 2017.

[102] D. A. Bosco, G. Morfini, N. M. Karabacak et al., "Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS," *Nature Neuroscience*, vol. 13, no. 11, pp. 1396–1403, 2010.

[103] L. Papa, G. Manfredi, and D. Germain, "SOD1, an unexpected novel target for cancer therapy," *Genes & Cancer*, vol. 5, no. 1-2, pp. 15–21, 2014.

[104] A. V. Sorokin, E. R. Kim, and L. P. Ovchinnikov, "Proteasome system of protein degradation and processing," *Biochemistry*, vol. 74, no. 13, pp. 1411–1442, 2009.

[105] S. Grimm, A. Hohn, and T. Grune, "Oxidative protein damage and the proteasome," *Amino Acids*, vol. 42, no. 1, pp. 23–38, 2012.

[106] J. N. Keller, K. B. Hanni, and W. R. Markesbery, "Impaired proteasome function in Alzheimer's disease," *Journal of Neurochemistry*, vol. 75, no. 1, pp. 436–439, 2000.

[107] K. S. P. McNaught and P. Jenner, "Proteasomal function is impaired in substantia nigra in Parkinson's disease," *Neuroscience Letters*, vol. 297, no. 3, pp. 191–194, 2001.

[108] S. A. Youssef, M. T. Capucchio, J. E. Rofina et al., "Pathology of the aging brain in domestic and laboratory animals, and animal models of human neurodegenerative diseases," *Veterinary Pathology*, vol. 53, no. 2, pp. 327–348, 2016.

[109] D. Pinkaew, A. Chattopadhyay, M. D. King et al., "Fortilin binds IRE1α and prevents ER stress from signaling apoptotic cell death," *Nature Communications*, vol. 8, no. 1, pp. 18–18, 2017.

[110] A. Negre-Salvayre, N. Auge, V. Ayala et al., "Pathological aspects of lipid peroxidation," *Free Radical Research*, vol. 44, no. 10, pp. 1125–1171, 2010.

[111] G. Basta, A. M. Schmidt, and R. De Caterina, "Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes," *Cardiovascular Research*, vol. 63, no. 4, pp. 582–592, 2004.

[112] A. Goldin, J. A. Beckman, A. M. Schmidt, and M. A. Creager, "Advanced glycation end products: sparking the development of diabetic vascular injury," *Circulation*, vol. 114, no. 6, pp. 597–605, 2006.

[113] H. K. Takahashi, S. Mori, H. Wake et al., "Advanced glycation end products subspecies-selectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 330, no. 1, pp. 89–98, 2009.

[114] N. N. Younis, H. Soran, V. Charlton-Menys et al., "High-density lipoprotein impedes glycation of low-density lipoprotein," *Diabetes and Vascular Disease Research*, vol. 10, no. 2, pp. 152–160, 2013.

[115] M. Ahotupa, "Oxidized lipoprotein lipids and atherosclerosis," *Free Radical Research*, vol. 51, no. 4, pp. 439–447, 2017.

[116] K. Yamauchi, Y. Ebihara, and Y. Kawakami, "Redox status of serum apolipoprotein E and its impact on HDL cholesterol levels," *Clinical Biochemistry*, vol. 50, no. 13-14, pp. 777–783, 2017.

[117] A. Jankovic, C. Ferreri, M. Filipovic et al., "Targeting the superoxide/nitric oxide ratio by L-arginine and SOD mimic in diabetic rat skin," *Free Radical Research*, vol. 50, Supplement 1, pp. S51–S63, 2016.

[118] A. Stancic, M. Filipovic, I. Ivanovic-Burmazovic et al., "Early energy metabolism-related molecular events in skeletal muscle of diabetic rats: The effects of L-arginine and SOD mimic," *Chemico-Biological Interactions*, vol. 272, pp. 188–196, 2017.

[119] M. Jaganjac, O. Tirosh, G. Cohen, S. Sasson, and N. Zarkovic, "Reactive aldehydes – second messengers of free radicals in diabetes mellitus," *Free Radical Research*, vol. 47, no. sup1, Supplement 1, pp. 39–48, 2013.

[120] E. Almogbel and N. Rasheed, "Protein mediated oxidative stress in patients with diabetes and its associated neuropathy: correlation with protein carbonylation and disease activity markers," *Journal of Clinical and Diagnostic Research*, vol. 11, no. 2, pp. BC21–BC25, 2017.

[121] J. Patche, D. Girard, A. Catan et al., "Diabetes-induced hepatic oxidative stress: a new pathogenic role for glycated albumin," *Free Radical Biology & Medicine*, vol. 102, pp. 133–148, 2017.

[122] D. Altavilla, A. Saitta, D. Cucinotta et al., "Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis in the genetically diabetic mouse," *Diabetes*, vol. 50, no. 3, pp. 667–674, 2001.

[123] M. Lorenzi, "The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient," *Experimental Diabetes Research*, vol. 2007, Article ID 61038, 10 pages, 2007.

[124] N. Ugochukwu and C. Figgers, "Modulation of the flux patterns in carbohydrate metabolism in the livers of streptozoticin-induced diabetic rats by dietary caloric restriction," *Pharmacological Research*, vol. 54, no. 3, pp. 172–180, 2006.

[125] S. Islam, A. R. Mir, A. Raghav et al., "Neo-epitopes generated on hydroxyl radical modified glycatedIgG have role in immunopathology of diabetes type 2," *PLoS One*, vol. 12, no. 1, article e0169099, 2017.

[126] S. Yamagishi, "Role of advanced glycation end products (AGEs) and receptor for AGEs (RAGE) in vascular damage in diabetes," *Experimental Gerontology*, vol. 46, no. 4, pp. 217–224, 2011.

[127] S. Yamagishi, H. Adachi, K. Nakamura et al., "Positive association between serum levels of advanced glycation end products and the soluble form of receptor for advanced glycation end products in nondiabetic subjects," *Metabolism*, vol. 55, no. 9, pp. 1227–1231, 2006.

[128] Y. Sharma, S. Saxena, A. Mishra, A. Saxena, and S. M. Natu, "Advanced glycation end products and diabetic retinopathy," *Journal of Ocular Biology, Diseases, and Informatics*, vol. 5, no. 3-4, pp. 63–69, 2012.

[129] A. M. Schmidt, O. Hori, J. X. Chen et al., "Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes," *The Journal of Clinical Investigation*, vol. 96, no. 3, pp. 1395–1403, 1995.

[130] K. C. B. Tan, S. W. M. Shiu, W. S. Chow, L. Leng, R. Bucala, and D. J. Betteridge, "Association between serum levels of soluble receptor for advanced glycation end products and circulating advanced glycation end products in type 2 diabetes," *Diabetologia*, vol. 49, no. 11, pp. 2756–2762, 2006.

[131] X. H. L. Tam, S. W. M. Shiu, L. Leng, R. Bucala, D. J. Betteridge, and K. C. B. Tan, "Enhanced expression of receptor for advanced glycation end-products is associated with low circulating soluble isoforms of the receptor in type 2 diabetes," *Clinical Science*, vol. 120, no. 2, pp. 81–89, 2011.

[132] K. Nakamura, S. I. Yamagishi, H. Adachi et al., "Elevation of soluble form of receptor for advanced glycation end products (sRAGE) in diabetic subjects with coronary artery disease," *Diabetes/Metabolism Research and Reviews*, vol. 23, no. 5, pp. 368–371, 2007.

[133] J. K. Y. Lam, Y. Wang, S. W. M. Shiu, Y. Wong, D. J. Betteridge, and K. C. B. Tan, "Effect of insulin on the soluble receptor for advanced glycation end products (RAGE)," *Diabetic Medicine*, vol. 30, no. 6, pp. 702–709, 2013.

[134] A. C. H. Lee, J. K. Y. Lam, S. W. M. Shiu, Y. Wong, D. J. Betteridge, and K. C. B. Tan, "Serum level of soluble receptor for advanced glycation end products is associated

with a disintegrin and metalloproteinase 10 in type 1 diabetes," *PLoS One*, vol. 10, no. 9, article e0137330, 2015.

[135] M. T. Goova, J. Li, T. Kislinger et al., "Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice," *The American Journal of Pathology*, vol. 159, no. 2, pp. 513–525, 2001.

[136] T. Shoji, H. Koyama, T. Morioka et al., "Receptor for advanced glycation end products is involved in impaired angiogenic response in diabetes," *Diabetes*, vol. 55, no. 8, pp. 2245–2255, 2006.

[137] U. Greifenhagen, A. Frolov, M. Blüher, and R. Hoffmann, "Plasma proteins modified by advanced glycation end products (AGEs) reveal site-specific susceptibilities to glycemic control in patients with type 2 diabetes," *Journal of Biological Chemistry*, vol. 291, no. 18, pp. 9610–9616, 2016.

[138] N. Gligorijevic, A. Penezic, and O. Nedic, "Influence of glyco-oxidation on complexes between fibrin(ogen) and insulin-like growth factor-binding protein-1 in patients with diabetes mellitus type 2," *Free Radical Research*, vol. 51, no. 1, pp. 64–72, 2017.

[139] R. Testa, A. R. Bonfigli, F. Prattichizzo, L. la Sala, V. de Nigris, and A. Ceriello, "The "metabolic memory" theory and the early treatment of hyperglycemia in prevention of diabetic complications," *Nutrients*, vol. 9, no. 5, p. 437, 2017.

[140] R. A. Kowluru, S. N. Abbas, and S. Odenbach, "Reversal of hyperglycemia and diabetic nephropathy: effect of reinstitution of good metabolic control on oxidative stress in the kidney of diabetic rats," *Journal of Diabetes and its Complications*, vol. 18, no. 5, pp. 282–288, 2004.

[141] M. Kanehisa, Y. Sato, M. Furumichi, K. Morishima, and M. Tanabe, "New approach for understanding genome variations in KEGG," *Nucleic Acids Research*, vol. 47, no. D1, pp. D590–D595, 2019.

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Research Article

Combined Exercise Training Performed by Elderly Women Reduces Redox Indexes and Proinflammatory Cytokines Related to Atherogenesis

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Cardiovascular benefits for the general population of combined aerobic-resistance exercise training are well-known, but the impact of this exercise training modality on the plasma lipid, inflammatory, and antioxidant status in elderly women that are exposed to a great risk of developing ischemic cardio- and cerebrovascular diseases has not been well investigated. So, we aimed to evaluate the plasma lipids, oxidative stress, and inflammatory cytokines in 27 elderly women (TRAINED group, 69.1 ± 8.1 yrs) that were performing moderate intensity combined aerobic-resistance exercise training (3 times/week for at least 18 months) and in 27 sedentary elderly women (SED group, 72.0 ± 6.4 yrs), not submitted to exercise training for at least 5 yrs. Our results showed that BMI was lower in the TRAINED group than in the SED group (25.1 ± 3.2 vs. 28.7 ± 5.1, $p < 0.05$). The TRAINED group had lower glycemia (92 ± 3 vs. 118 ± 12, $p < 0.05$), glycated hemoglobin (5.9 ± 0.1 vs. 6.4 ± 0.2, $p < 0.05$), and triglycerides (98 (75-122) vs. 139 (109-214), $p < 0.01$); equal total cholesterol (199 (175-230) vs. 194 (165-220)), LDL-cholesterol (108 (83-133) vs. 109 (98-136)), and non-HDL-cholesterol (54 (30-74) vs. 62 (26-80)); and also higher HDL-cholesterol (64 (52-77) vs. 52 (44-63), $p < 0.01$) and LDL-C/oxLDL ratio (13378 ± 2570 vs. 11639 ± 3113, $p < 0.05$) compared to the SED group. Proinflammatory cytokines as IL-1β (11.31 ± 2.4 vs. 28.01 ± 4.7, $p < 0.05$), IL-6 (26.25 ± 7.4 vs. 49.41 ± 17.8, $p < 0.05$), and TNF-α (25.72 ± 2.8 vs. 51.73 ± 4.2, $p < 0.05$) were lower in the TRAINED group than in the SED group. The TRAINED group had lower total peroxides (26.3 ± 7.4 vs. 49.0 ± 17.8, $p < 0.05$) and oxidized LDL (1551 ± 50.33 vs. 1773 ± 74, $p < 0.02$) and higher total antioxidant capacity (26.25 ± 7.4 vs. 49.41 ± 17.8, $p < 0.001$) compared to the SED group. In conclusion, in TRAINED women, BMI was lower, plasma lipid profile was better, plasma oxidative stress was diminished, and there was less expression of proinflammatory interleukins than in SED, suggesting that combined aerobic-resistance exercise training may promote the protection against the complications of ischemic cardio- and cerebrovascular disease in elderly women.

1. Introduction

The pace of population aging is being accelerated, and the WHO estimated that between 2015 and 2050 the proportion of the world population over 60 years will grow from 12% to 22% [1]. Thus, the widespread promotion of healthy aging is mandatory, inclusively, to keep the pace with the spending of the health systems.

During the aging process, it is common to observe increases of adipose tissue in the viscera and organs such as the liver and muscles [2]. Overweight and obesity are clearly associated with alterations in the lipid profile and raise in the markers of systemic inflammation, including C-reactive protein (CRP) and proinflammatory cytokines, such as interleukin- (IL-) 1 beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) [2, 3]. In the elderly, the increase in the systemic markers of inflammation has been pointed as risk factors for chronic diseases such as atherosclerosis, cancer, sarcopenic syndromes, and diabetes mellitus, among others [4].

Alterations in the plasma metabolism and concentration of lipoproteins [5], redox imbalance, and dysregulation of the inflammatory response [6] in the vessel are key factors in the development, progression, and clinical manifestations of atherosclerosis. In fact, lipid peroxidation begins with the previous accumulation of lipid peroxides (LOOH). Redox decomposition of LOOH molecules initiates chain reactions promoted by alkyl, peroxyl, and alkoxyl radicals ($L^{\cdot}$, $LOO^{\cdot}$, and $LO^{\cdot}$, respectively) that further oxidize other lipid and protein molecules through massive production of reactive carbonyl species [7]. Moreover, bioactive "oxylipids" or aldehyde derivatives are involved in the activation of immune responses and accumulation of neutrophil/macrophage in atherosclerotic lesions culminating in an aggravated vascular condition [8].

The beneficial effects in the elderly population of aerobic and of resistance exercise training on the plasma lipid metabolism, oxidative, and inflammatory processes have been documented in the literature [9]. Whereas the aerobic training preferentially improves cardiovascular fitness, resistance training increases the muscle mass and both training modalities promote loss of body fat mass [9, 10]. It is widely accepted that the regular practice of exercise training, both aerobic (endurance) and resistance (anaerobic/strength) sets, is one of the most effective nonpharmacological interventions that can partially reverse the effects of vascular dysfunction, thereby decreasing the risk of death and consequently increase longevity [11–13]. Although it has been generally agreed that combined aerobic-resistance exercise training may equally attain both cardiovascular and muscle targets [14], the effects of combined aerobic-resistance exercise training on the lipid profile, oxidative stress, and inflammatory markers of atherosclerosis have been scarcely explored in aged subjects. These data are particularly important in women because the female life expectancy is longer than that of men. Those considerations lead us to investigate the status of plasma lipids and oxidized LDL (oxLDL), redox indexes, and inflammatory markers in elderly women under a combined aerobic-resistance exercise training program as compared to sedentary women in the equivalent age range.

2. Methods

2.1. Study Subjects. Fifty-four volunteer elderly women, aged 60-80 years, were selected for the study. A flow diagram is shown in Figure 1. Twenty-seven were participating in a combined aerobic-resistance exercise program (TRAINED group) sponsored by the municipality of the city of São Paulo, and twenty-seven age-paired women were sedentary (SED group) participants of a primary health care program of the Department of Preventive Medicine of the Federal University of São Paulo Medical School. Both groups of women were residents in the same neighborhood.

None of the subjects presented asthma, type-1 diabetes mellitus; neoplastic, renal, or liver diseases; dementia; thrombosis; or manifested cardiovascular disease. None was under statin or other lipid-lowering drugs. The participants responded to a Food Frequency Questionnaire in which the consumption of antioxidants was assessed. The study was in agreement with the Declaration of Helsinki and with the Ethical Standards defined by Harriss and Atkinson [15] and was approved by the Ethics Committee of the Federal University of São Paulo (UNIFESP, protocol number 0788/10). The volunteers signed an informed written consent form.

2.2. Combined Aerobic-Resistance Exercise Training Program. The physical exercise protocol is a combination of aerobic and resistance exercises performed in moderate intensity (Table 1). The combined aerobic-resistance exercise training followed the guidelines for exercise prescription recommended by the American College of Sports Medicine [9, 16].

Volunteers from the TRAINED group performed their prescribed exercises during 60-75 minutes per session, 3 times a week, on nonconsecutive days, for at least 18 months. The same experienced instructor supervised all the volunteers.

A description of combined physical exercise regime performed by the TRAINED group is described in Table 1.

Volunteers of the SED group, although independent and active, were not involved in any regular exercise program for at least five years, and they were oriented to maintain their normal routine during the study.

2.3. Sample Collection. Blood sampling occurred at 8:00 AM after a 12 h fast. The TRAINED group performed its last exercise training session at least 24 h beforehand. Serum/plasma aliquots of 500 μL were obtained after centrifugation (10 min, 400xg) of blood samples and stored at -80°C.

2.4. Laboratorial Analysis. Plasma total cholesterol concentration was measured by the CHOD-PAP method using commercial kits (Kovalente, São Gonçalo, Brazil), and the results were analyzed with an automated system (Dimension® RxL Max® Integrated Chemistry System, Siemens, Deerfield, IL, USA). HDL-C and triglyceride concentrations were determined using commercial kits and an automated analysis system (ADVIA® 2400, Siemens, Deerfield, IL, USA). LDL-C was estimated by the Friedewald formula [17]. Plasma glucose concentration and glycated hemoglobin

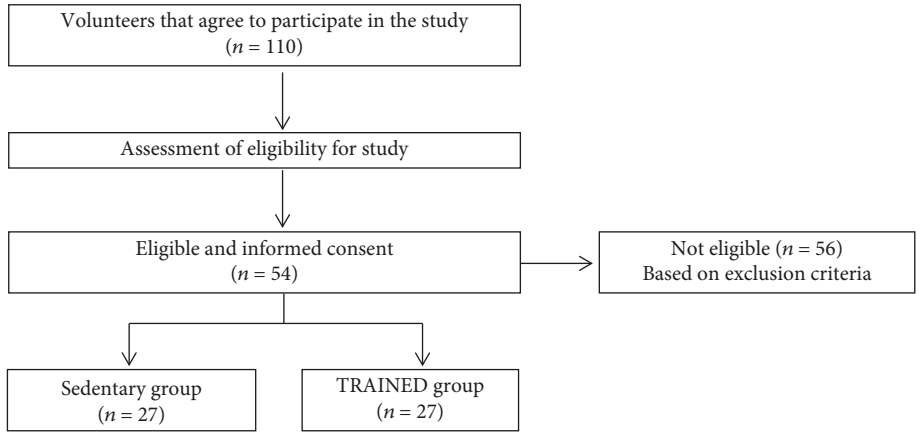

Figure 1: Flow diagram of the study.

Table 1: Description of combined physical exercise regime performed by the TRAINED group.

Aerobics	Exercise frequency: 60 to 70% of the maximal heart rate reserve (MHHR), calculated by the equation (208-0.7 × age) proposed by Tanaka et al. [51]
	Exercise type: physical exercises in step platform, jump, coordination, and rhythmic movements (dance sometimes)
	Impact: low impact
	Monthly cardiac control: Polar, FT1, Finland
Resistance	At least 5 different exercises for different muscle groups (upper and lower limb muscles, abdomen, gluteus, and muscles related to core/postural stabilization, including dorsal and lumbar muscles)
	Performed slowly in two series with 10-20 repetitions, between 50 and 60% of 1 RM (repetition maximum)
	Different combinations of two muscle groups (described above) performed in four consecutive sessions
	Borg scale to adjust the weight load monthly

percentage were measured by commercial kits and an automated analysis system (ADVIA® 2400, Siemens, Deerfield, IL, USA).

Serum concentration of oxLDL was evaluated by ELISA, using the Indirect Enzyme Immunoassay kit (USCN® Life Science Inc., Wuhan, China), total antioxidant capacity (TAC) by a colorimetric commercial kit (Cayman Chemical Corporation, Ann Arbor, MI, USA), total lipid peroxide content (LOOH) using the QuantiChrom™ Peroxide Assay Kit, a colorimetric commercial kit (BioAssay Systems, Hayward, CA, USA), and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) using Millipore® Multiplex Assays Using Luminex® (EMD Millipore Corporation, Billerica, MA, USA). All analyses were performed in accordance with the manufacturer's instructions.

2.5. Statistical Analysis. All data were previously analyzed by the Shapiro-Wilk test used to evaluate the normal distribution and after by the Levene test used to evaluate the variance homogeneity. Student's t-test was used to analyze the differences in age, height, weight, and body mass index (BMI). The Mann-Whitney test was used to determine the differences in plasma lipids and glucose, glycated hemoglobin, oxLDL, total peroxides, TAC, proinflammatory cytokines (IL-1β, IL-6, and TNF-α), and the relation between plasma concentrations of LDL-C and oxLDL ((LDL − C/oxLDL) × 100). Spearman's rank correlation coefficient was employed to identify any correlation between oxLDL and BMI or LDL-C or TAC or total peroxides. The significance level was set to 5% ($p < 0.05$).

3. Results

Physical and clinical characteristics of the volunteers are presented in Table 2. No differences were observed between the two groups regarding the antioxidant or vitamin intake of the participant subjects.

As shown in Table 2, the TRAINED group had lower BMI than the SED group ($p = 0.002$). Table 3 shows that both plasma glucose ($p = 0.02$) and glycated hemoglobin ($p = 0.04$) were lower in the TRAINED group than in the SED group. LDL-C and non-HDL-C were similar but HDL-C was higher ($p = 0.001$) and triglycerides were lower ($p = 0.008$) in the TRAINED group compared to the SED group.

As shown in Figure 2, the TAC serum concentration was higher ($p < 0.001$), whereas LOOH ($p < 0.001$) and oxLDL ($p = 0.02$) were lower in the TRAINED group than in the SED group. In addition, as shown in Table 3, the relationship between LDL-C and oxLDL ((LDL − C/oxLDL) × 100) was higher in the TRAINED group than in the SED group ($p = 0.04$).

Figure 3 shows that the serum concentration of proinflammatory cytokines IL-1β ($p = 0.001$), IL-6 ($p = 0.02$),

Table 2: Physical (means ± SD) and clinical characteristics of the TRAINED and SED groups. Significance level of $^*p < 0.05$.

Characteristics	Volunteers ($n = 54$) SED ($n = 27$)	TRAINED ($n = 27$)	p value
Physical			
Age (year)	72.0 ± 6.4	69.1 ± 8.1	0.257
Height (m)	1.55 ± 0.05	1.54 ± 0.07	0.543
Weight (kg)	71.2 ± 13.3*	59.8 ± 9.3	0.005
Body mass index (kg/m^2)	28.7 ± 5.1*	25.1 ± 3.2	0.002
Clinical (n)#			
Type 2 diabetes mellitus	6	4	>0.05
Dyslipidemia	13	12	>0.05
Obesity	4	3	>0.05
Arterial hypertension	15	15	>0.05
Depression	2	1	>0.05
Lifestyle			
Physical exercise training			
≤2 times/week	0	0	>0.05
≥2 times/week	0*	27	0.0001
Current alcohol use	0	0	
Smoking			
Current smoker	1	1	>0.05

#n = number of individuals.

Table 3: Values of the lipid profile and glucose are expressed as the median and interquartile range (mg/dL); glycated hemoglobin (HbA1c) is expressed in percentage (%) and oxLDL is expressed in means ± SD (pg/mL) in the TRAINED and SED groups. Significance level of $^*p < 0.05$.

Variables	Volunteers ($n = 54$) SED ($n = 27$)	TRAINED ($n = 27$)	p value
Glucose (mg/dL)	117.8 ± 11.6*	91.9 ± 2.5	0.02
HbA1c (%)	6.4 ± 0.2*	5.9 ± 0.1	0.04
Cholesterol (mg/dL)			
Total	194 (165-220)	199 (175-230)	0.709
LDL	108 (83-133)	109 (98-136)	0.721
Non-HDL	62 (26-80)	54 (30-74)	0.467
HDL	52 (44-63)*	64 (52-77)	0.001
Triglycerides (mg/dL)	139 (109-214)*	98 (75-122)	0.008
oxLDL (pg/mL)	1773 ± 384.8*	1551 ± 261.5	0.02
LDL/oxLDL ratio	11639 ± 3113*	13378 ± 2570	0.04

and TNF-α ($p = 0.01$) was higher in the SED group than in the TRAINED group.

Spearman's coefficient analysis (Figure 4) showed a positive correlation between BMI and oxLDL concentration in the SED group (Figure 4(a)), which was not observed in the TRAINED group (Figure 4(e)). LDL-C and oxLDL were linearly correlated in the TRAINED group. However, this correlation was not significant in the SED group (Figure 4(f)). Moreover, LOOH values were correlated with those of oxLDL in the TRAINED group (Figure 4(g)). No other significant correlations were found.

4. Discussion

In this transversal study enrolling elderly women, it was shown that the regular practice of combined aerobic-resistance exercise leads to a clear-cut improvement of markers of lipid and inflammatory status and of the oxidative stress that are related to the prevention of the manifestations of atherosclerosis.

LDL-C was not lower in the TRAINED group than in the SED group, which is in accordance with studies showing that several modalities of exercise training do not alter LDL-C plasma levels [10, 18]. However, regardless of the lack of effect on LDL-C levels, exercise training can improve the LDL metabolism by increasing the LDL removal from the plasma and the lipoprotein turnover, as shown in the study by Vinagre et al. [19]. In that study, the plasma kinetics of LDL-like particles measured in cycling practitioners was shown to increase severalfold in comparison with sedentary subjects, possibly due to an exercise-training induction of LDL receptor overexpression. In addition, the shorter residence time of LDL in the bloodstream can conceivably decrease the exposure of the lipoprotein to oxidation, and in fact, those authors found a correlation between the LDL-like particle clearance and the plasma levels of oxLDL [19]. Resistance exercise training program administered to healthy sedentary men had also the ability to increase LDL clearance with lowering of the oxLDL levels [20]. Sedentary hypercholesterolemic subjects had the LDL clearance increased after an aerobic exercise training program, with a concomitant decrease of the LDL susceptibility to oxidation [21]. In the current study, the lower oxLDL levels achieved by combined aerobic-resistance exercise training can presumptively be ascribed to increased LDL clearance, in view of the above-mentioned previous studies.

Besides the decrease in the plasma levels of oxLDL, an established risk factor for ischemic cardio- and cerebrovascular diseases [22–26], we also documented a decrease in the plasma LOOH levels in the TRAINED group. Higher LOOH plasma levels are also a risk factor for ischemic cardio- and cerebrovascular diseases [27–30]. According to Wonisch et al. [31], the oxidative stress increases with increasing BMI and age and, as a consequence, occurs an increase of peroxide molecules that can lead to an unbalanced lipid profile. So, the BMI values found in the TRAINED group and also the negative correlation found in our study between LOOH and the oxLDL levels reinforce the consistence of the favorable effects of the combined aerobic-resistance exercise training on the redox indexes. Moreover, we also found that in the TRAINED group, TAC levels were higher than in the SED group, which could buffer oxidative stress, thereby preventing the LDL oxidation [32]. Previous studies have also shown beneficial actions of exercise training protocols, including combined aerobic-resistance exercise training on the antioxidant defenses [33–35]. Taken together, these

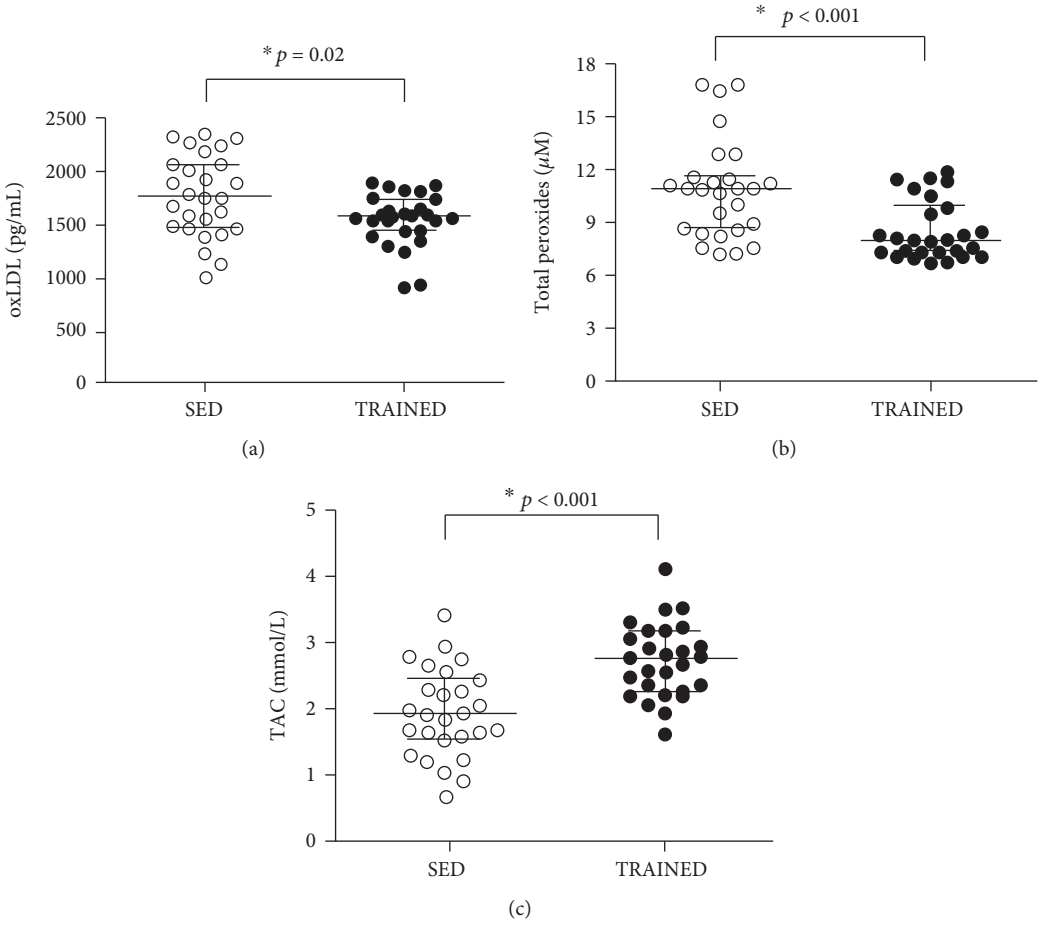

Figure 2: Serum concentrations of oxidized LDL (oxLDL, pg/mL (a)), lipid peroxide (LOOH, μM (b)), and plasma total antioxidant capacity (TAC, mmol/L (c)) in the SED and TRAINED groups were statistically analyzed using the Mann-Whitney test. These data were presented as the median (interquartile range) with a significance level of $^*p < 0.05$.

results support the assumption that combined aerobic-resistance exercise training can be a valid tool to improve the body antioxidation mechanisms and thereby atherosclerosis prevention.

Here, the TRAINED group exhibited higher levels of HDL-C as compared to the SED group. Exercise training is a classical factor to promote an increase in HDL-C [9, 10, 36–38], and some studies had already reported that combined aerobic-resistance exercise training also has the capability of HDL-C increase [10, 18]. It is noteworthy that the effects on HDL-C are largely related to exercise training intensity [9, 10]. HDL-C levels are inversely correlated with the risk of ischemic cardio- and cerebrovascular diseases [39, 40]. However, this lipoprotein has several functions that are protective against atherosclerosis and other diseases that are not necessarily dependent of the HDL-C levels. Among these functions, HDL is the main lipoprotein to exert cholesterol esterification and reverse cholesterol transport and has also many other protective functions such as antioxidant, promoter of vasodilation, antiapoptotic, and anti-inflammatory [40]. Previously, we have shown that combined aerobic-resistance exercise training performed in aged women increased the transfer of cholesterol to HDL [41]. This effect is presumably beneficial, since patients with cardiovascular disease exhibit low cholesterol transfer values compared to controls without the disease [42, 43].

As judged from the comparison of the TRAINED and SED groups, the practice of combined aerobic-resistance exercise training also had anti-inflammatory actions, since the TRAINED group presented lower serum levels of proinflammatory cytokines than the SED group. Similar to our results, some authors have reported that combined aerobic-resistance exercise training improves the inflammatory status [44–46]. It is reasonable to hypothesize that these anti-inflammatory effects may be associated with the antioxidant effects of exercise training observed here, since the relationship between chronic inflammatory processes and oxidative stress, especially in aging, is well-known [47, 48].

It is also widely accepted that exercise training is a chief factor to attain the control of glycemic levels, which was also documented here and in other studies [9, 49, 50].

As a limitation of this study, it should be mentioned that a longitudinal interventional study would be rigorously more appropriate to establish the biohumoral changes elicited by exercise training. However, in this cross-sectional protocol,

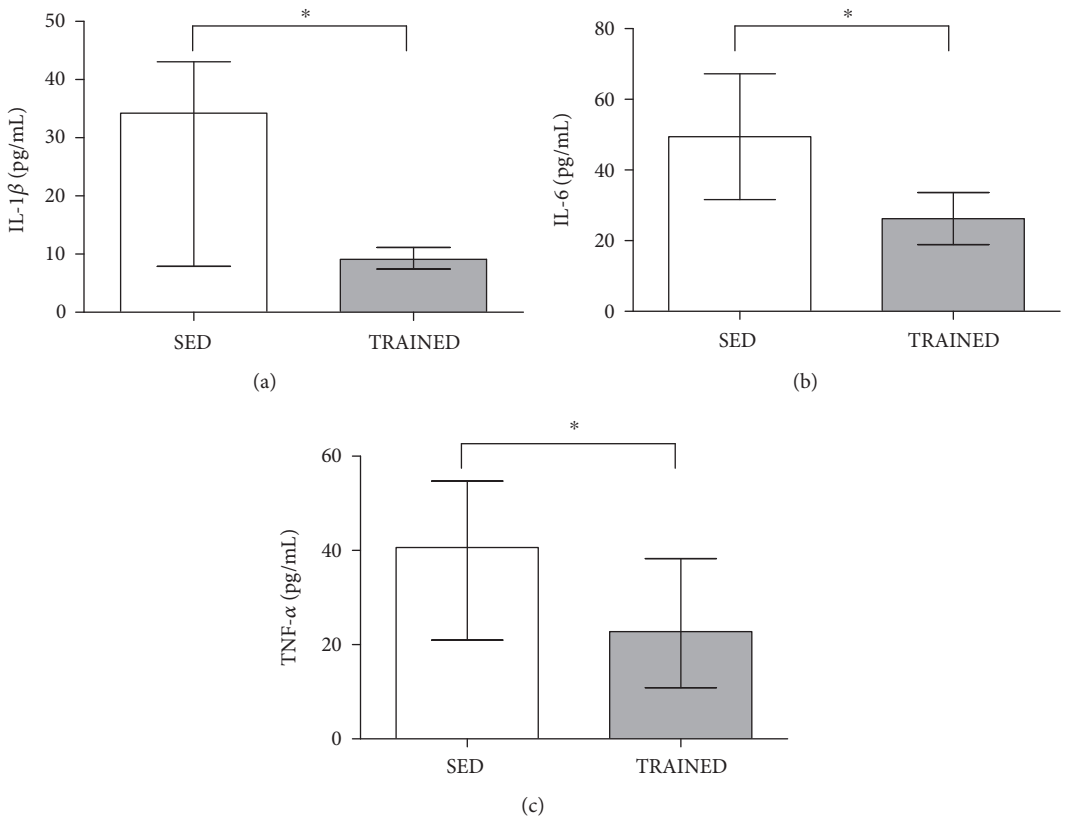

Figure 3: Serum concentrations (pg/mL) of IL-1β (a), IL-6 (b), and TNF-α (c) in the SED and TRAINED groups were statistically analyzed using the Mann-Whitney test. These data were presented as the median (interquartile range) with a significance level of $^*p < 0.05$.

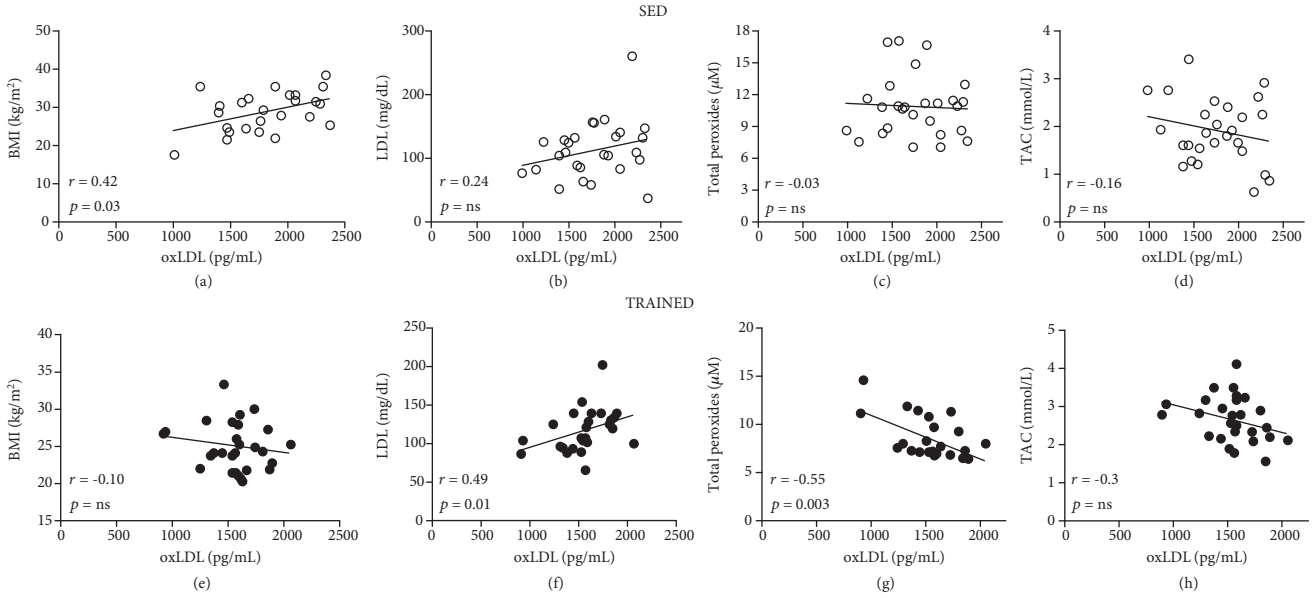

Figure 4: Spearman's rank correlation coefficient analysis was used to identify the correlation between oxidized LDL (oxLDL, pg/mL) and BMI (kg/m^2 (a, e)) or LDL-C (mg/dL (b, f)) or lipid peroxide (LOOH, μM (c, g)) or total antioxidant capacity (TAC, mmol/L (d, h)) in the SED group (a–d) and the TRAINED group (e–h). Significance level of $^*p < 0.05$.

the results of long-standing (over 18 months) regular practice of training, as compared to nonpractitioners of the same community, offer a fair evaluation of individuals in their real-life conditions.

5. Conclusion

The results of the current cross-sectional study suggest that elderly women may benefit from the regular practice of

combined aerobic-resistance exercise training in many metabolic aspects that are related to protection against the complications of ischemic cardio- and cerebrovascular disease.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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References

[1] World Health Organization, "The top 10 causes of death, fact sheet, updated January 2017. Ageing and health, fact sheet N°404," 2015, http://www.who.int/.

[2] D. Baylis, D. B. Bartlett, H. P. Patel, and H. C. Roberts, "Understanding how we age: insights into inflammaging," *Longevity & Healthspan*, vol. 2, no. 1, p. 8, 2013.

[3] G. Pawelec, D. Goldeck, and E. Derhovanessian, "Inflammation, ageing and chronic disease," *Current Opinion in Immunology*, vol. 29, pp. 23–28, 2014.

[4] M. Michaud, L. Balardy, G. Moulis et al., "Proinflammatory cytokines, aging, and age-related diseases," *Journal of the American Medical Directors Association*, vol. 14, no. 12, pp. 877–882, 2013.

[5] E. Orsó and G. Schmitz, "Lipoprotein(a) and its role in inflammation, atherosclerosis and malignancies," *Clinical Research in Cardiology Supplements*, vol. 12, Supplement 1, pp. 31–37, 2017.

[6] P. Mury, E. N. Chirico, M. Mura, A. Millon, E. Canet-Soulas, and V. Pialoux, "Oxidative stress and inflammation, key targets of atherosclerotic plaque progression and vulnerability: potential impact of physical activity," *Sports Medicine*, vol. 48, no. 12, pp. 2725–2741, 2018.

[7] J. R. Requena, M. X. Fu, M. U. Ahmed, A. J. Jenkins, T. J. Lyons, and S. R. Thorpe, "Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions," *Nephrology Dialysis Transplantation*, vol. 11, Supplement 5, pp. 48–53, 1996.

[8] A. D. Watson, N. Leitinger, M. Navab et al., "Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence *in vivo*," *The Journal of Biological Chemistry*, vol. 272, no. 21, pp. 13597–13607, 1997.

[9] W. J. Chodzko-Zajko, D. N. Proctor, M. A. Fiatarone Singh et al., "Exercise and physical activity for older adults," *Medicine & Science in Sports & Exercise*, vol. 41, no. 7, pp. 1510–1530, 2009.

[10] S. Mann, C. Beedie, and A. Jimenez, "Differential effects of aerobic exercise, resistance training and combined exercise modalities on cholesterol and the lipid profile: review, synthesis and recommendations," *Sports Medicine*, vol. 44, no. 2, pp. 211–221, 2014.

[11] S. A. Phillips, A. M. Mahmoud, M. D. Brown, and J. M. Haus, "Exercise interventions and peripheral arterial function: implications for cardio-metabolic disease," *Progress in Cardiovascular Diseases*, vol. 57, no. 5, pp. 521–534, 2015.

[12] M. El Assar, J. Angulo, and L. Rodríguez-Mañas, "Oxidative stress and vascular inflammation in aging," *Free Radical Biology and Medicine*, vol. 65, pp. 380–401, 2013.

[13] V. Gremeaux, M. Gayda, R. Lepers, P. Sosner, M. Juneau, and A. Nigam, "Exercise and longevity," *Maturitas*, vol. 73, no. 4, pp. 312–317, 2012.

[14] L. Schwingshackl, S. Dias, B. Strasser, and G. Hoffmann, "Impact of different training modalities on anthropometric and metabolic characteristics in overweight/obese subjects: a systematic review and network meta-analysis," *PLoS One*, vol. 8, no. 12, article e82853, 2013.

[15] D. J. Harriss and G. Atkinson, "Ethical standards in sport and exercise science research: 2016 update," *International Journal of Sports Medicine*, vol. 36, no. 14, pp. 1121–1124, 2015.

[16] C. E. Garber, B. Blissmer, M. R. Deschenes et al., "Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise," *Medicine & Science in Sports & Exercise*, vol. 43, no. 7, pp. 1334–1359, 2011.

[17] W. T. Friedewald, R. I. Levy, and D. S. Fredrickson, "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clinical Chemistry*, vol. 18, no. 6, pp. 499–502, 1972.

[18] K. Tambalis, D. B. Panagiotakos, S. A. Kavouras, and L. S. Sidossis, "Responses of blood lipids to aerobic, resistance, and combined aerobic with resistance exercise training: a systematic review of current evidence," *Angiology*, vol. 60, no. 5, pp. 614–632, 2009.

[19] C. G. C. Vinagre, E. S. Ficker, C. Finazzo et al., "Enhanced removal from the plasma of LDL-like nanoemulsion cholesteryl ester in trained men compared with sedentary healthy men," *Journal of Applied Physiology*, vol. 103, no. 4, pp. 1166–1171, 2007.

[20] J. L. da Silva, C. G. C. M. Vinagre, A. T. Morikawa, M. J. N. N. Alves, C. H. Mesquita, and R. C. Maranhão, "Resistance training changes LDL metabolism in normolipidemic subjects: a study with a nanoemulsion mimetic of LDL," *Atherosclerosis*, vol. 219, no. 2, pp. 532–537, 2011.

[21] E. S. Ficker, R. C. Maranhão, A. P. M. Chacra et al., "Exercise training accelerates the removal from plasma of LDL-like nanoemulsion in moderately hypercholesterolemic subjects," *Atherosclerosis*, vol. 212, no. 1, pp. 230–236, 2010.

[22] S. Mitra, A. Deshmukh, R. Sachdeva, J. Lu, and J. L. Mehta, "Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy," *The American Journal of the Medical Sciences*, vol. 342, no. 2, pp. 135–142, 2011.

[23] S. Gao and J. Liu, "Association between circulating oxidized low-density lipoprotein and atherosclerotic cardiovascular disease," *Chronic Diseases and Translational Medicine*, vol. 3, no. 2, pp. 89–94, 2017.

[24] S. Gao, D. Zhao, M. Wang et al., "Association between circulating oxidized LDL and atherosclerotic cardiovascular disease: a meta-analysis of observational studies," *The Canadian Journal of Cardiology*, vol. 33, no. 12, pp. 1624–1632, 2017.

[25] A. Trpkovic, I. Resanovic, J. Stanimirovic et al., "Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases," *Critical Reviews in Clinical Laboratory Sciences*, vol. 52, no. 2, pp. 70–85, 2015.

[26] E. Verhoye, M. R. Langlois, and Asklepios Investigators, "Circulating oxidized low-density lipoprotein: a biomarker of atherosclerosis and cardiovascular risk?," *Clinical Chemistry and Laboratory Medicine*, vol. 47, no. 2, pp. 128–137, 2009.

[27] S. Riahi, M. T. Mohammadi, and V. Sobhani, "Role of oxygen and nitrogen free radicals in diabetes-induced atherosclerosis and effects of exercise on it," *Physiology and Pharmacology*, vol. 18, no. 1, pp. 1–15, 2014.

[28] Y. C. Cheng, J. M. Sheen, W. L. Hu, and Y. C. Hung, "Polyphenols and oxidative stress in atherosclerosis-related ischemic heart disease and stroke," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 8526438, 16 pages, 2017.

[29] H. N. Siti, Y. Kamisah, and J. Kamsiah, "The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review)," *Vascular Pharmacology*, vol. 71, pp. 40–56, 2015.

[30] P. Pignatelli, D. Menichelli, D. Pastori, and F. Violi, "Oxidative stress and cardiovascular disease: new insights," *Kardiologia Polska*, vol. 76, no. 4, pp. 713–722, 2018.

[31] W. Wonisch, A. Falk, I. Sundl, B. M. Winklhofer-Roob, and M. Lindschinger, "Oxidative stress increases continuously with BMI and age with unfavourable profiles in males," *The Aging Male*, vol. 15, no. 3, pp. 159–165, 2012.

[32] A. L. L. Bachi, A. P. R. Sierra, F. J. O. Rios et al., "Athletes with higher VO_2max present reduced oxLDL after a marathon race," *BMJ Open Sport & Exercise Medicine*, vol. 1, no. 1, 2015.

[33] G. Vinetti, C. Mozzini, P. Desenzani et al., "Supervised exercise training reduces oxidative stress and cardiometabolic risk in adults with type 2 diabetes: a randomized controlled trial," *Scientific Reports*, vol. 5, no. 1, article 9238, 2015.

[34] K. Fisher-Wellman, H. K. Bell, and R. J. Bloomer, "Oxidative stress and antioxidant defense mechanisms linked to exercise during cardiopulmonary and metabolic disorders," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 1, pp. 43–51, 2009.

[35] A. Cebula, A. Tyka, W. Pilch et al., "Effects of 6-week Nordic walking training on body composition and antioxidant status for women > 55 years of age," *International Journal of Occupational Medicine and Environmental Health*, vol. 30, no. 3, pp. 445–454, 2017.

[36] Y. Kesaniemi, E. Danforth Jr., M. D. Jensen, P. G. Kopelman, P. Lefèbvre, and B. A. Reeder, "Dose–response issues concerning physical activity and health: an evidence-based symposium," *Medicine and Science in Sports and Exercise*, vol. 33, 6 Supplement, pp. S351–S358, 2001.

[37] A. S. Leon and O. A. Sanchez, "Response of blood lipids to exercise training alone or combined with dietary intervention," *Medicine and Science in Sports and Exercise*, vol. 33, 6 Supplement, pp. S502–S515, 2001.

[38] D. Sheikholeslami Vatani, S. Ahmadi, K. Ahmadi Dehrashid, and F. Gharibi, "Changes in cardiovascular risk factors and inflammatory markers of young, healthy, men after six weeks of moderate or high intensity resistance training," *The Journal of Sports Medicine and Physical Fitness*, vol. 51, no. 4, pp. 695–700, 2001.

[39] D. J. Rader and G. K. Hovingh, "HDL and cardiovascular disease," *The Lancet*, vol. 384, no. 9943, pp. 618–625, 2014.

[40] R. C. Maranhão and F. R. Freitas, "Chapter One - HDL Metabolism and Atheroprotection: Predictive Value of Lipid Transfers," *Advances in Clinical Chemistry*, vol. 65, pp. 1–41, 2014.

[41] A. L. L. Bachi, G. A. Rocha, M. C. O. Sprandel et al., "Exercise training improves plasma lipid and inflammatory profiles and increases cholesterol transfer to high-density lipoprotein in elderly women," *Journal of the American Geriatrics Society*, vol. 63, no. 6, pp. 1247–1249, 2015.

[42] M. C. O. Sprandel, W. A. Hueb, A. Segre, J. A. F. Ramires, R. Kalil-Filho, and R. C. Maranhão, "Alterations in lipid transfers to HDL associated with the presence of coronary artery disease in patients with type 2 diabetes mellitus," *Cardiovascular Diabetology*, vol. 14, no. 1, p. 107, 2015.

[43] C. H. M. Azevedo, M. Wajngarten, A. C. L. Prete, J. Diament, and R. C. Maranhão, "Simultaneous transfer of cholesterol, triglycerides, and phospholipids to high-density lipoprotein in aging subjects with or without coronary artery disease," *Clinics*, vol. 66, no. 9, pp. 1543–1548, 2011.

[44] F. F. Conti, J. . O. Brito, N. Bernardes et al., "Positive effect of combined exercise training in a model of metabolic syndrome and menopause: autonomic, inflammatory, and oxidative stress evaluations," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 309, no. 12, pp. R1532–R1539, 2015.

[45] I. C. Sanches, M. Buzin, F. F. Conti et al., "Combined aerobic and resistance exercise training attenuates cardiac dysfunctions in a model of diabetes and menopause," *PLoS One*, vol. 13, no. 9, article e0202731, 2018.

[46] G. L. Shimojo, D. . Silva Dias, C. Malfitano et al., "Combined aerobic and resistance exercise training improve hypertension associated with menopause," *Frontiers in Physiology*, vol. 9, article 1471, 2018.

[47] P. J. Barnes, "Mechanisms of development of multimorbidity in the elderly," *European Respiratory Journal*, vol. 45, no. 3, pp. 790–806, 2015.

[48] L. Ferrucci and E. Fabbri, "Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty," *Nature Reviews Cardiology*, vol. 15, no. 9, pp. 505–522, 2018.

[49] C. P. Rowan, M. C. Riddell, N. Gledhill, and V. K. Jamnik, "Aerobic exercise training modalities and prediabetes risk reduction," *Medicine and Science in Sports and Exercise*, vol. 49, no. 3, pp. 403–412, 2017.

[50] B. Strasser, U. Siebert, and W. Schobersberger, "Resistance training in the treatment of the metabolic syndrome: a systematic review and meta-analysis of the effect of resistance training on metabolic clustering in patients with abnormal glucose metabolism," *Sports Medicine*, vol. 40, no. 5, pp. 397–415, 2010.

[51] H. Tanaka, K. D. Monahan, and D. R. Seals, "Age-predicted maximal heart rate revisited," *Journal of the American College of Cardiology*, vol. 37, no. 1, pp. 153–156, 2001.

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Research Article

Antioxidant and Anti-Inflammatory Properties of Anthocyanins Extracted from *Oryza sativa* L. in Primary Dermal Fibroblasts

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Flavonoids are naturally active substances that form a large class of phenolic compounds abundant in certain foods. Black rice (*Oryza sativa* L.) contains high levels of anthocyanin polyphenols, which have beneficial effects on health owing to their antioxidant properties. The breakdown of collagenous networks with aging or skin deterioration results in the impairment of wound healing in the skin. Accordingly, reviving stagnant collagen synthesis can help maintain dermal homeostasis during wound healing. This study presents an assessment of the cellular activity of anthocyanins (ANT) extracted from *Oryza sativa* L., providing information necessary for the development of new products that support natural healing processes. The relative composition of ANT from *Oryza sativa* L. was determined by high-performance liquid chromatography/diode array detection. ANT promoted the migration of rat dermal fibroblasts (RDFs) and demonstrated antioxidant properties. ANT increased the mRNA expression of *collagen type I alpha 2* (*COL1A2*) and upregulated type I collagen protein levels in H_2O_2-stimulated RDFs without cytotoxicity. Compared with the untreated group, treatment of RDFs with ANT in the presence of H_2O_2 led to the activation of signaling pathways, including the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and Akt, whereas it significantly ($p < 0.001$) inhibited the phosphorylation of IκBα and suppressed the activation of the nuclear factor-kappa B (NF-κB) subunits, p50 and p65, which are transcription factors responsible for inflammation. Taken together, our findings suggest that ANT from *Oryza sativa* L. have anti-inflammatory properties and antiaging potential by modulating type I collagen gene expression and suppressing H_2O_2-induced NF-κB activation in skin fibroblasts.

1. Introduction

Black rice (*Oryza sativa* L.) has been widely consumed since ancient times in parts of Asia including present-day Thailand. In an attempt to know its effective beneficial health components, many investigators have aimed on the characteristics of flavonoids, a large class of phenolic compounds that is found plenty in foods including black rice. The most prominent flavonoids in *O. sativa* L. are the anthocyanins (ANT), which are ubiquitous coloring agents in plants accountable for the purple, red, and blue hues of many types of fruit, vegetables, cereal grains, and flowers [1]. *O. sativa* L. has been considered as a health-supporting food given its abundance of ANT [2]. Many studies have revealed that ANT have positive effects on a variety of health conditions [3]. One reason for this is

their anti-inflammatory properties, which occur via effects on collagen synthesis [4, 5].

Proanthocyanidins are a class of biologically active polyphenolic bioflavonoids known to be synthesized by many plants. ANT-demonstrated benefits include protection against diabetes [6, 7], impressive reduction of blood pressure, enhancement of vision [8, 9], and strong anti-inflammatory effects [10, 11]. These phenolic compounds also facilitate dermal and oral wound healing [11, 12]. Fibroblast proliferation influences the formation of granulation tissue, collagen synthesis, and wound contraction. The granulation tissue generated by fibroblasts immersed in a loose matrix of collagen fibers consisting primarily of type I collagen is accumulated in the wound base [13]. In addition, ANT stimulate wound healing while suppressing superfluous inflammation by inducing vascular endothelial growth factor (VEGF) production in fibroblasts and keratinocytes [11].

Oxidative stress has been implicated to damage various cellular portions involving lipids, proteins, and nucleic acids through oxidation by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and superoxide anion radical (O_2^-) [14]. ROS are very harmful to the wound healing process owing to their harmful effects on extracellular matrix components, including collagens, and on keratinocyte functions and the damage they induce in dermal fibroblasts [15]. Oxidative process involved in the pathogenesis of many diseases suggests that antioxidants may play an important role for the treatment in these conditions. Topical treatment with compounds that have antioxidant properties has been shown to significantly enhance the wound healing process and protect tissues against oxidative damage [16]. The free-radical-scavenging and antioxidant capacities of ANT pigments are introduced as the most highly recognized modes of action for interposing with numerous human therapeutic targets [17]. Moreover, with their anabolic effects *in vivo*, there is substantial verification suggesting that these phytochemicals have positive effects on wound healing [18]. In a previous study, ANT were found to promote wound healing by preventing oxidative damage [19]; however, to the best of our knowledge, the mechanisms by which ANT extracted from *O. sativa* L. act in modulating collagen formation and inflammation in skin fibroblasts have not been investigated.

The transcription factor, nuclear factor-κB (NF-κB), regulates a large number of genes that are involved in inflammation and immune responses. This stress-regulated transcription factor has multiple effects on the immune system [20] and can modulate the expression of proinflammatory genes encoding nitric oxide synthase, cytokines, and chemokines [21]. NF-κB overstimulation has been shown to result in cellular and DNA damage by ROS production [22, 23]. In addition, one of the key elements in the inflammatory process is the activation and translocation of NF-κB from the cytosol to the nucleus. Subunits p50 and p65 which are the classical NF-κB heterodimer exist as the potent activator of gene expression [24]. The activation of variable extracellular stimuli such as environmental stress including UV light and stimuli such as interleukin- (IL-) 1, tumor necrosis factor- (TNF-) α, viruses and endotoxins, and IκB will be phosphorylated and degraded by the *26S* proteasome. This gives rise to the translocation of p50/p65 heterodimer to the nucleus, where it links to its responsive elements present in a variety of genes [25]. In consequence, the various combinations of NF-κB subunits may organize the complicated regulatory pathway of NF-κB like orchestral performance. We hypothesized that ANT extracted from black rice may have anti-inflammatory and antioxidant effects associated with collagen production in skin fibroblasts. In this study, we showed that ANT extracted from *O. sativa* L. have strong antioxidant and anti-inflammatory activities in rat primary dermal fibroblasts. ANT protect against the effects of H_2O_2-induced collagen degradation, by suppressing IκB phosphorylation and the activation of NF-κB p50/p65.

2. Materials and Methods

2.1. Chemicals, Reagents, and Antibodies. ERK1/2 (pT202/Y204+Total) ELISA Kit, Akt (pS473+Total) ELISA kit, Rat IL-6 ELISA Kit, Abs for isotype control IgG, rabbit polyclonal Abs for NF-κB p50, p65, phosphor-Y42 IκBα, and Histone H3, mouse monoclonal Abs for β-actin, anti-rabbit IgG (HRP), and anti-mouse IgG (HRP), and anti-rabbit fluorescein isothiocyanate (FITC) secondary Abs were obtained from Abcam (Cambridge, MA, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was obtained from Promega (Madison, WI, USA). A Total Collagen Assay (Colorimetric) Kit was purchased from BioVision (Milpitas, CA, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). A Cell Fractionation Kit was purchased from Abcam. Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich Inc.

2.2. Plant Material and Extraction. Black rice was obtained from Chachoengsao Province, Thailand, and subjected to ANT extraction as previously reported [26]. ANT were extracted in ethanol (60/40, *v*/*v*%), concentrated using a Büchi B-490 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) and lyophilized with a freeze dryer (Labconco Corp., Kansas City, MO, USA). The crude extract was stored at room temperature (RT).

2.3. Determination of Flavonoids and ANT Composition by High-Performance Liquid Chromatography (HPLC). The composition of ANT extract was identified using HPLC/diode array detection (DAD) as previously reported [27]. A Waters LC-MSD 1525 series equipped with a UV detector and Agilent Zorbax SB-C18 column was used. The solvents were aqueous 2% formic acid and acetonitrile : water (1 : 1 *v*/*v*) containing 2% formic acid. Injection volumes were 15 μL. The separated ANT components were measured at 516 nm and were identified based on their retention times. ANT were quantified by UV-visible (Vis) spectroscopy as previously described [28]. The model reaction solution was diluted with 0.01% HCl in distilled water. Absorbance was measured using a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific, Grand Island, NY, USA).

2.4. Estimation of Total Phenolic Content. The total phenolic content was determined using Folin-Ciocalteu reagent (FRC) as previously described [29], with minor modifications. The absorbance of the mixture was measured at 765 nm using a UV-Vis Genesys 10 UV spectrophotometer. A standard curve was plotted using gallic acid (0.07–10 mg/mL in methanol; Sigma-Aldrich) as a standard. The total phenolic content was expressed as gallic acid equivalents (mM GAE/gFW).

2.5. Cell Culture and Treatment. Rat primary dermal fibroblasts (RDFs), growth media, and passaging solutions were purchased from Cell Applications (San Diego, CA, USA). Cells were maintained in Culture Complete Growth Medium and penicillin/streptomycin solution in a humidified incubator with 5% CO_2 at 37°C. To eliminate any possible side effects of growth factors, cells were cultured in serum-free medium for 24 h before treatment with ANT extract.

2.6. Cell Viability Test. Cell viability was measured using the MTT assay in accordance with a previously described method [30]. Briefly, after incubation of the cells (1×10^5 cells/mL) in 96-well plates with various concentrations of ANT (5, 10, 25, and 50 μg/mL) for 24 h, they were incubated with MTT (0.5 mg/mL). Three hours later, dimethyl sulfoxide (DMSO) was added and the absorbance of the solution was measured at 570 nm with an automatic microplate reader (Immuno-Mini NJ-2300; InterMed, Tokyo, Japan). The cell survival percentage was calculated using DMSO-treated cells as a standard. The experiments were repeated three times with five samples for each group.

2.7. Determination of Total Antioxidant Capacity and Copper Ion Reduction Activity. The Total Antioxidant Capacity (TAC) Assay Kit (Cell Biolabs OxiSelect™, San Diego, CA, USA) was used to measure the antioxidant capacity of ANT from black rice. The TAC Assay is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid. Briefly, RDFs were treated with 5–50 μg/mL ANT and stimulated with 0.3 mM H_2O_2 for 24 h. The supernatant was dispensed in a 96-well microtiter plate, after which 180 μL of 1× reaction buffer was added to each well and mixed. An initial reading at 490 nm was taken for each sample. Then, 50 μL of 1× copper ion reagent was added and incubated for 5 min on an orbital shaker. Next, 50 μL of stop solution was added to terminate the reaction, and the plate was subjected to additional measurement of absorption at 490 nm using a spectrophotometer (Epoch, BioTek, Winooski, VT, USA) with the Gen 5 data analysis software interface. All determinations were performed in triplicate, and results were averaged. The copper ion reduction activity of each sample with H_2O_2 was then calculated as the percent increase in ion reduction.

2.8. Monitoring Cell Migration by Real-Time Cell Analysis (RTCA). The rate of cell migration was monitored in real time with the xCELLigence system using CIM plates (ACEA Biosciences Inc., San Diego, CA, USA). Video clips of several experimental tests were observed to determine the optimal seeding density of the RDFs (Supplementary Movie 1). The optimal cell density at each time point for these migration profiles was 5000 cells/well, and hence, this number of RDFs was seeded in each well. Cells were serum-starved for approximately 24 h. The upper chamber (UC) of the CIM plates was coated with 1 μg/μL fibronectin. A total of 5000 cells were seeded in the UC of each well in a serum-free medium. Fresh medium was added to the lower chamber (LC) of each well, and cells were stimulated with 5–25 μg/mL ANT. The CIM plates were left in an incubator for 1 h to allow cells to attach. The impedance value of each well was automatically monitored by the xCELLigence system at 6, 12, and 24 h and expressed as CI (cell index). Data of cell migration were normalized at 30 min. The CI was calculated automatically by the RTCA Software Package 2.1.0. The CI calculation is based on the following formula:

$$\mathrm{CI} = \frac{(Z_i - Z_0)}{15\varsigma}, \tag{1}$$

CI calculation used to measure the relative change in electrical impedance, representing the cell status (equation (1)). The unit of the impedance is ohm (Ω):

(i) Z_i = impedance at an individual point of time during the experiment

(ii) Z_0 = impedance at the start of the experiment

Normalized CI was calculated as CI at a given time point divided by the CI at the normalization time point. The rate of cell migration was determined by calculating the slope of the line between two given time points.

2.9. Cell Migration Assay Using a Boyden Chamber. To analyze the migration of RDFs, a modified Boyden chamber with 24-well Nunc™ cell culture inserts containing a polycarbonate filter with 8 μm pores was used. The inner layer of Transwell inserts was coated with 1 mg/mL Matrigel (BD Biosciences, Palo Alto, CA, USA) overnight at 4°C. Fibroblasts were placed in the inner well of the Transwell at 5×10^4 cells/well in 200 μL of Dulbecco's modified Eagle's media containing 1% FBS. Various concentrations of ANT (5, 10, and 25 μg/mL) in 750 μL of culture medium were added to the bottom chamber. After incubation at 37°C for 24 h, the inner well was wiped with a cotton swab to remove nonmigrating cells. The cells were fixed using 100% (w/v) methanol at RT for 10 min, after which the membrane was transferred onto a cover slide. Cells were stained with 10% Giemsa stain and assessed by light microscopy. Fifteen high-power fields (hpf) per membrane were counted, and migration was expressed as the mean number of cells/hpf ± SEM. To ensure reproducibility, each condition was tested in three separate wells. For each experiment, cell viability was assessed by trypan blue exclusion before using cells in the assay. In all cases, cell viability exceeded 90%.

2.10. RNA Extraction, cDNA Synthesis, and Quantitative Reverse Transcription-PCR (RT-qPCR). RDFs were treated with 5, 10, and 25 μg/mL ANT or PMA with or without 0.3 mM H_2O_2 for 4 and 10 h. After removing the supernatant

Table 1: Primer sequences used for RT-qPCR.

Target gene	Forward primer	Reverse primer	Amplicon size (bp)	Accession number
COL1A2	5′-ACCTCAGGGTGTTCAAGGTG-3′	5′-CGGATTCCAATAGGACCAGA-3′	222	NM_053356.1:1
NF-κB p50	5′-AGAGGATGTGGGGTTTCAGG-3′	5′-GCTGAGCATGAAGGTGGATG-3′	200	NM_001276711.1
NF-κB p65	5′-CGCCACCGGATTGAAGAAA-3′	5′-TTGATGGTGCTGAGGGATGT-3′	194	LC 369719.1
IL-6	5′-GCCCTTCAGGAACAGCTATGA-3′	5′-TGTCAACAACATCAGTCCCAAGA-3′	80	NM_012589.2
GAPDH	5′-CCCCCAATGTATCCGTTGTG-3′	5′-TAGCCCAGGATGCCCTTTAGT-3′	118	NM_017008.4

NF-κB: nuclear factor kappa-B; COL1A2: collagen type I alpha 2; IL-6: interleukin-6; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

and collecting the cell pellets, total RNA was isolated with FavorPrep Tissue Total RNA Mini Kit (Favorgen Biotech, Pingtung, Taiwan) in accordance with the manufacturer's instructions. The absorbance of nucleic acids was measured using a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific). One microgram of RNA was subjected to DNase I digestion, followed by reverse transcription using a DNase I, RNase-free kit (Thermo Fisher Scientific). Total RNA (1 μg) was reverse-transcribed into cDNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μL using oligo-dT primers. cDNA (<20 ng/well) was used as a template in qPCR reactions with oligonucleotides specific for the genes of interest (Table 1). PCR amplification was conducted in a total volume of 20 μL including 20 ng of the template cDNA, 7.2 μL of PCR-grade water (Welgene, Daegu, South Korea), 10 μM of each primer, and 10 μL of 2× KAPA SYBR® FAST qPCR Master Mix Universal from KAPA SYBR® FAST qPCR Kit Master Mix (2×) Universal (KAPA Biosystems, Selangor, Malaysia). A nontemplate control and an RNA sample without reverse transcription for each sample were used as control for potential DNA contamination. Unless stated otherwise, the temperature protocol for PCR amplification was as follows: enzyme deactivation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 3 s plus combined annealing/extension at 60°C for 30 s. Melting curve analysis was then performed for 10 s at 95°C with 0.5°C increments between 65°C and 95°C. All qPCR reactions were performed in triplicate. Relative quantification was determined using the CFX Manager Software (version 2.0; Bio-Rad) measuring SYBR green fluorescence.

2.11. Total Collagen Estimation by Colorimetric Assay. RDFs cultured in 96-well plates at a density of 3×10^4 cells/well in 200 μL were pretreated with 5, 10, and 25 μg/mL ANT for 2 h with or without 0.3 mM H_2O_2, followed by incubation for 24 h. Estimation of the collagen level was carried out using a Total Collagen Assay Kit, in accordance with the manufacturer's instructions. Each sample was homogenized in 100 μL of ddH_2O. Briefly, each cell lysate was hydrolyzed with concentrated HCl at 120°C for 3 h, followed by vortexing and centrifugation at 10,000 × g for 3 min to remove the precipitate. A total of 10–30 μL of each hydrolyzed sample was transferred to a 96-well plate and dried by evaporation at 70°C. Next, 100 μL of Chloramine T reagent was added to each sample and incubated at RT for 5 min, after which 4-(dimethylamino)benzaldehyde (DMAB) reagent was added and incubated for 90 min at 60°C. Finally, hydroxyproline was oxidized to form a reaction intermediate and absorbance at 560 nm was determined in a microtiter plate using a microplate reader with an Epoch Microplate Spectrophotometer (Epoch; BioTek). The concentration of collagen was calculated from the collagen I standard curve provided with the kit.

2.12. Western Blot Analysis. The cells were plated in 10 cm dishes and pretreated with 10 or 25 μg/mL ANT for 2 h before adding 0.3 mM H_2O_2, followed by incubation for 10 h. The cytoplasmic and nuclear extracts for immunoblotting were prepared using a Cell Fractionation Kit. Protein concentrations were measured and normalized with the BCA protein assay kit (Thermo Fisher Scientific). Each sample was subjected to electrophoresis on 12% SDS-polyacrylamide gels. Then, the protein was blotted onto nitrocellulose membranes. Membranes were incubated with primary antibodies against NF-κB p50 (1 : 1000), NF-κB p65 (1 : 1000), p-IκBα Y42 (1 : 1000), β-actin (1 : 1000), and Histone H3 (1 : 500) overnight at 4°C. After washing, the membranes were incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG) for 1 h. Immunoreactive proteins were detected with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). The intensity of the protein bands was quantified using ImageJ software.

2.13. Immunofluorescence Staining. RDFs were seeded on a four-well chamber slide (Nunc™ Lab-Tek™ II Chamber Slide™ System; Thermo Fisher Scientific) at a density of 2×10^4 cells per well for 24 h. Cells were preincubated with ANT at a final concentration of 25 μg/mL for 2 h, and NF-κB activity was induced by 0.3 mM H_2O_2 for 24 h, after which cells were washed with PBS and then fixed with 100% methanol at −20°C for 20 min. Cells were blocked with 1% BSA in PBST (PBS+0.1% Tween 20) for 30 min at RT. After washing with PBS, the cells were incubated for 1 h at RT with anti-NF-κB/p50 or anti-NF-κB/p65 Ab (1 : 1000 dilution). Cells were also incubated with an isotype control rabbit monoclonal Ab as a negative control. After three washes with PBS, the cells were incubated for 1 h at RT with goat anti-rabbit IgG H&L conjugated with FITC (1 : 2500 dilution). Following three washes with PBS, the nuclei were counterstained with DAPI (Miltenyi Biotec,

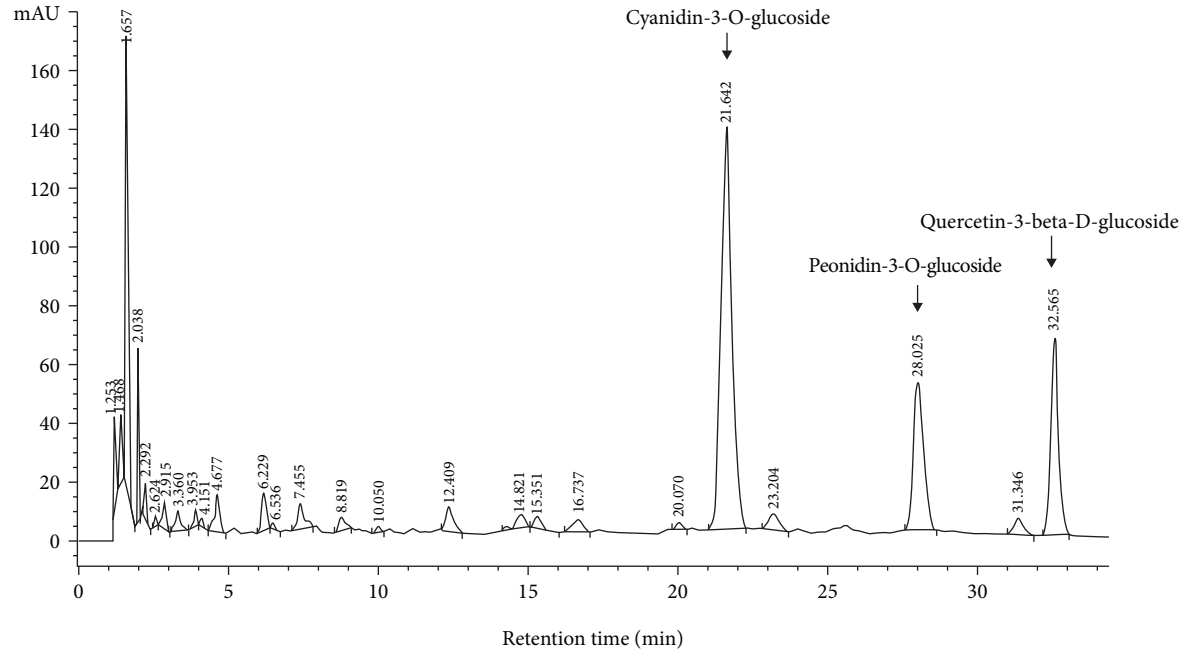


Figure 1: HPLC analysis of anthocyanins (ANT) in *Oryza sativa* L. Representative HPLC/DAD chromatograms of cyanidin-3-O-glucoside, peonidin-3-O-glucoside, and quercetin-3-beta-D-glucoside in the extracts at 516 nm. Peaks were detected with a retention time of 21.6, 28.0, and 32.5 min, respectively. Duplicate experiments were performed.

Bergisch Gladbach, Germany). The cells on the glass coverslips were embedded in a mounting medium (Aquamount; Lerner Laboratories, Pittsburgh, PA, USA) and then photographed with a fluorescence microscope (BX53 Digital Upright Microscope; Olympus, Tokyo, Japan).

2.14. Quantitative Estimation of Phosphorylated Akt and ERK1/2 and of IL-6 by Enzyme-Linked Immunosorbent Assay (ELISA). RDFs were treated with 25 μg/mL ANT or 0.3 mM H_2O_2 at various time intervals. After the treatment, the ANT and H_2O_2 effect on Akt and ERK1/2 phosphorylation was determined. Cells were then pretreated with 25 μg/mL ANT for 2 h followed by a 30 min incubation with 0.3 mM H_2O_2 for Akt and 2 h for ERK1/2. The amounts of total and phosphorylated Akt and ERK1/2 in cell lysates were determined using Akt (pS473+Total) and ERK1/2 (pT202/Y204+Total) ELISA kits in accordance with the manufacturer's protocol. Briefly, cells were lysed in lysis buffer and then transferred to the ELISA plate coated with immobilized antibodies. The wells were washed, and antibodies from the kits were used to detect the phosphorylated and total forms of Akt and ERK1/2. For IL-6 determination, the protein release of IL-6 in RDF supernatants was evaluated with a commercial Rat IL-6 ELISA Kit in accordance with the manufacturer's instructions. The optical densities were measured at 450 nm with an Epoch Microplate Spectrophotometer (Epoch; BioTek).

2.15. Statistical Analysis. SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA) was used for statistical analyses. All results are expressed as mean ± SD. The significance of differences between two groups was assessed using Student's *t*-test, and differences among multiple groups were assessed by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer method. The level of significance was set at $p < 0.05$.

3. Results

3.1. Identification of Anthocyanins and Total Phenolic Content in Oryza sativa L. The ANT composition in *O. sativa* L. was determined by HPLC/DAD. The resulting chromatograms at 516 nm are shown (Figure 1). The chromatogram within the retention time of 21.6 min indicated the presence of cyanidin-3-O-glucoside (C3G), and the chromatogram within the retention time of 28.0 min was identified as peonidin-3-O-glucoside. Additionally, the retention time of 32.5 min indicated the presence of quercetin-3-beta-D-glucoside. The UV-Vis wavelength spectra quantification of total ANT showed that the phenolic-rich extract contained ~1704 mg/kg of total ANT (calculated as C3G equivalents), ~572.89 mg/kg of peonidin-3-O-glucoside, and ~728.37 mg/kg of quercetin-3-beta-D-glucoside. The total phenolic content was ~30 ± 1.5 mM gallic acid equivalent (gFW).

3.2. ANT Treatment Affects RDF Migration in a Dose-Dependent Manner. To examine cell viability in ANT-exposed RDFs, cells were treated for 24 h with various concentrations of ANT extracted from black rice. Cell viability was determined using the MTT assay. Our results demonstrated that 5, 10, and 25 μg/mL ANT had no cytotoxic effects (Figure 2(a)). However, 50 μg/mL ANT reduced cell viability to 90%. To minimize confounding effects due to reduced cell viability, ANT were used at the maximal noncytotoxic concentration, namely, 25 μg/mL, in subsequent experiments. Based on the above results, we next investigated

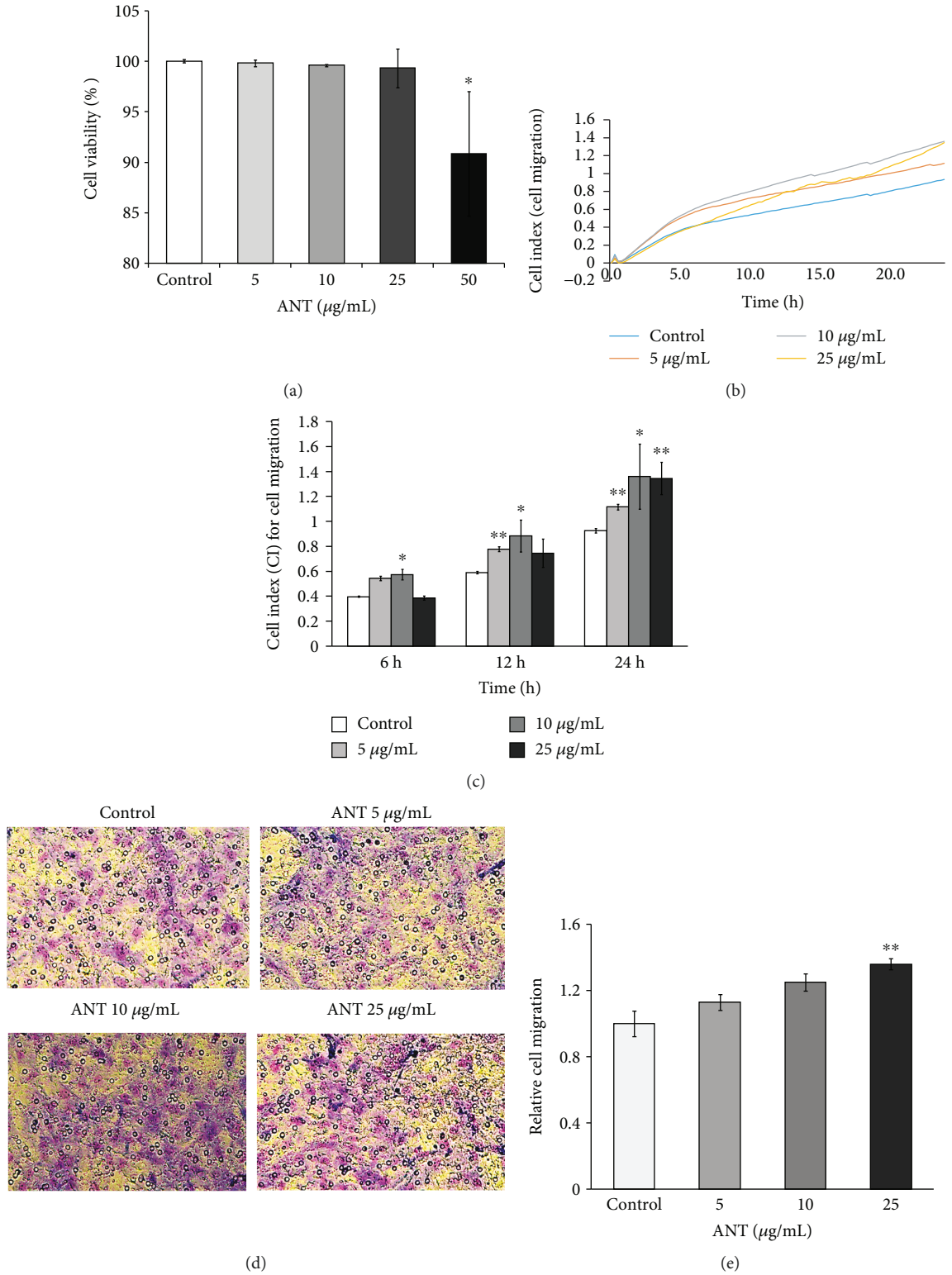


Figure 2: Effects of ANT on cell viability, migration, and chemotaxis of RDFs. RDFs were treated with or without ANT at 5–25 μg/mL. (a) Cell viability was measured by the MTT assay. (b) Representative figure of CI values for cell migration over 24 h. (c) CI values for ANT at different time points following treatment with varying concentrations of ANT. (d) Boyden chamber assay demonstrating increased migration of RDFs incubated with ANT. Cells were allowed to migrate for 24 h, and then, they were fixed and stained with 10% Giemsa stain. The numbers of migrating cells were averaged from three ×10 field-of-view images. Original magnification ×100. (e) Results were quantified by counting the stained cells and are expressed relative to the mean number of cells randomly migrating in the control wells ± SEM. The results presented are from three independent experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

the effects of ANT on RDF migration, using the RTCA-DP xCELLigence system.

The migration profile of RDFs was indicated by CI values obtained within 24 h of testing. A marked increase in CI values was observed when cells were incubated with ANT at 5, 10, and 25 μg/mL (Figure 2(b)). The results showed a significant increase in cell migration following stimulation with 5 μg/mL ($p < 0.05$) and 10 μg/mL ($p < 0.01$) ANT for 12 and 24 h (Figure 2(c)). However, a significant increase in cell migration ($p < 0.01$) was observed following stimulation with 25 μg/mL ANT for 24 h. To confirm the effect of ANT on RDF chemotaxis, we determined the optimal concentration of ANT required to stimulate the migration of these cells using a Boyden chamber (Figure 2(d)). We found that 25 μg/mL ANT induced a significant increase in chemotactic capacity ($p < 0.01$), which is consistent with the RTCA results (Figure 2(e)).

3.3. The Copper Ion-Reducing Capacity of ANT Extracts. To confirm the antioxidant capacity of ANT against cellular damage, we examined their total antioxidant capacity and their copper ion reduction effect against H_2O_2 using the TAC Assay. We found that the copper ion-reducing capacity improved with the increase in ANT concentration in a dose-dependent manner (Figure 3). An extract concentration as low as 10 μg/mL showed a significant increase in total antioxidant capacity compared with the control (32.96% ± 0.33, $p < 0.001$). Furthermore, 25 and 50 μg/mL ANT showed an increase in the percent of copper ion reduction with the increase in ANT concentration (78.71% ± 2.79 and 84.56% ± 7.71, respectively, $p < 0.001$). Our results strongly indicated that ANT from *O. sativa* L. extract exhibit antioxidant capacity.

3.4. ANT Induce Collagen Type I Alpha 2 mRNA Expression and Suppress NF-κB p50 and p65 mRNA Expression in H_2O_2-Stimulated RDFs. ANT affected the mRNA expression of *collagen type I alpha 2* (*COL1A2*), which has been determined to be upregulated when the skin is capable of regeneration. RDFs were incubated with 5, 10, or 25 μg/mL ANT. RT-qPCR analysis showed that ANT significantly upregulated *COL1A2* mRNA at 25 μg/mL ($p < 0.05$; Figure 4(a)). To further examine the effects of ANT on collagen synthesis under oxidative stress conditions, their action on cells stimulated with H_2O_2 was evaluated. The viability of H_2O_2-exposed RDFs was determined by the MTT assays. At 24 h, while 0.3 mM H_2O_2 did not alter the number of viable RDFs, an H_2O_2 concentration above 0.6 mM significantly decreased the population of viable RDFs (Supplementary Figure S1). Based on this assay, 0.3 mM H_2O_2 was used in further experiments. The presence of a low concentration of H_2O_2 suppressed the *COL1A2* mRNA level (Figure 4(b)). However, following 25 μg/mL ANT pretreatment, the *COL1A2* mRNA level significantly increased ($p < 0.05$) compared with H_2O_2 stimulation alone (Figure 4(b)).

NF-κB signaling plays an important role in inflammation and oxidative stress [31]. To investigate whether ANT reduce the upregulation of NF-κB p50 and p65 subunits, RDF cultures were treated with H_2O_2 to induce transcriptional activation under oxidative stress. We assessed the mRNA expression of genes encoding these NF-κB subunits using RT-qPCR. Compared with the control levels, H_2O_2 treatment for 10 h resulted in significant increases in the mRNA level of *NF-κB p50* (Figure 4(c)) and *p65* (Figure 4(d)), while treatment with H_2O_2 for 4 h had no effect on NF-κB activation (data not shown). The addition of ANT prior to H_2O_2 treatment reduced the *NF-κB p50* and *p65* mRNA level induced by H_2O_2.

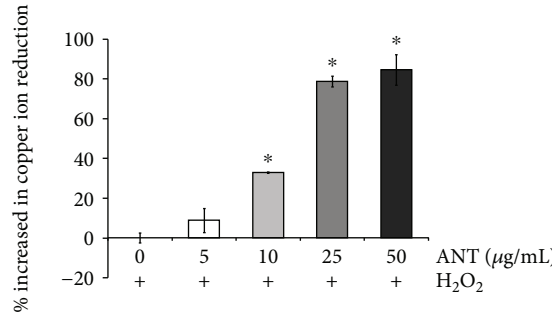


Figure 3: The Total Antioxidant Capacity (TAC) Assay for estimating copper ion reduction activity of the extracts under H_2O_2 stimulation. RDFs were treated with 5–50 μg/mL ANT and stimulated with H_2O_2 for 24 h. The supernatant was used for TAC analysis. The mean values ± SD from three independent experiments are presented. $^*p < 0.001$ vs. H_2O_2 alone.

We also evaluated whether ANT influence the activation of NF-κB directly or via ROS modification using PMA, which is an NF-κB transcription inducer [32]. In this experiment, treatment with 40 ng/mL PMA induced almost 4-fold activation of NF-κB expression (Figures 4(c) and 4(d)). In contrast to H_2O_2, a combination of ANT and PMA did not influence the inducing effect of PMA on both *NF-κB p50* and *p65* genes. In addition, ANT displayed a concentration-dependent inhibition of H_2O_2 stimulation of both NF-κB subunits. These findings support the notion that cotreatment with ANT suppressed the H_2O_2-induced *NF-κB* mRNA level via the antioxidant effect rather than via direct inhibition of the NF-κB pathway. Interestingly, the mRNA level of the NF-κB subunits significantly and negatively correlated with the increased level of *COL1A2* in RDFs, suggesting a relationship between the collagen synthesis marker and the synthesis of transcription factors.

3.5. ANT Extract Protects Collagen Synthesis in RDFs from the Effects of H_2O_2. Collagen biosynthesis-associated hydroxyproline was measured in RDFs that were treated with 5, 10, or 25 μg/mL ANT and evaluated using a Total Collagen Assay Kit. ANT induced an increase in collagen biosynthesis in a dose-dependent manner (Figure 5(a)). At 25 μg/mL, ANT caused approximately 2.5-fold induction of collagen formation compared with that in the control. Notably, the H_2O_2 group exhibited significantly decreased levels of collagen synthesis compared with the control (Figure 5(b), $p < 0.01$). The levels of collagen synthesis were significantly increased when RDFs were cocultured with H_2O_2 and 5 μg/mL, 10 μg/mL ($p < 0.05$), or 25 μg/mL ANT ($p < 0.001$). In addition, pretreatment with ANT (25 μg/mL) before

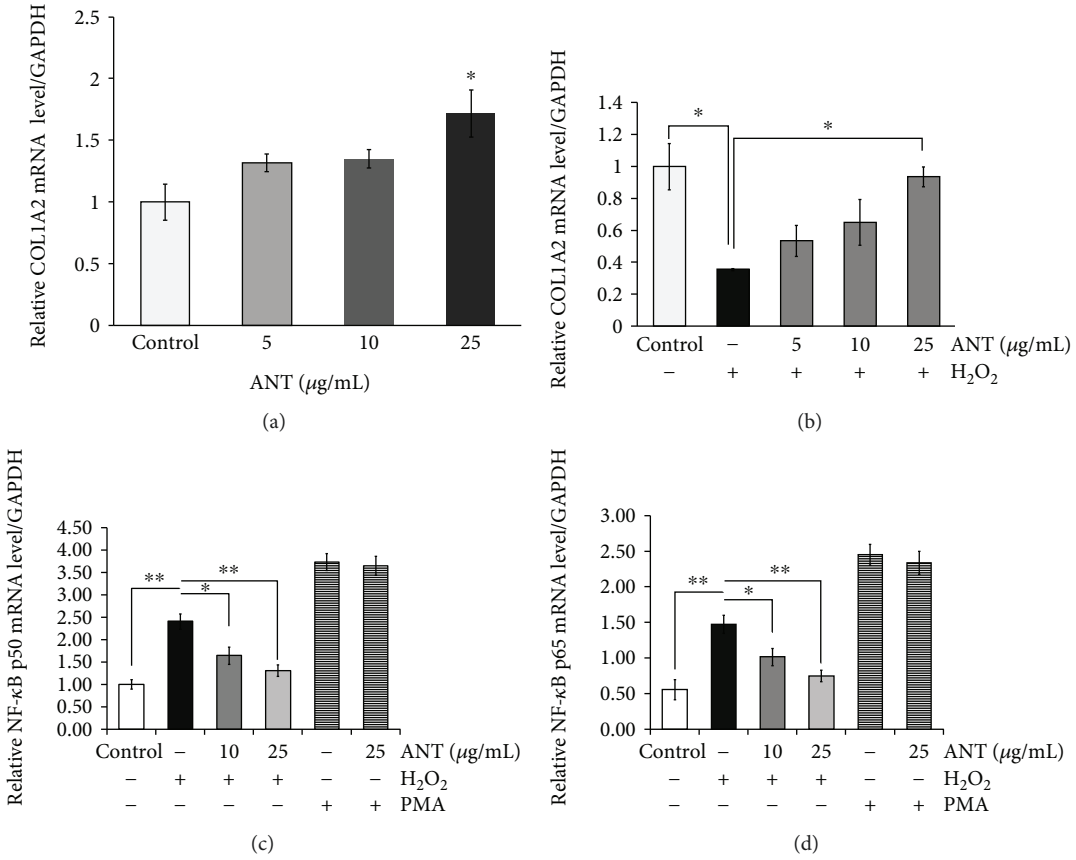


Figure 4: RT-qPCR analysis of the effects of ANT on H_2O_2-induced mRNA expression in RDFs at 10 h. (a) RDFs were treated with the indicated concentrations of ANT for 2 h, and then, the *COL1A2* mRNA levels were quantified. (b) RDFs were treated with the indicated concentrations of ANT for 2 h before the addition of 0.3 mM H_2O_2. *COL1A2* mRNA levels were quantified. Effects of ANT pretreatment on H_2O_2- or PMA-induced *NF-κB p50* (c) and (d) *p65* mRNA expression. The relative levels of mRNA were normalized against *GAPDH* from the same cDNA preparation. Values are presented as the mean ± SEM of three independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

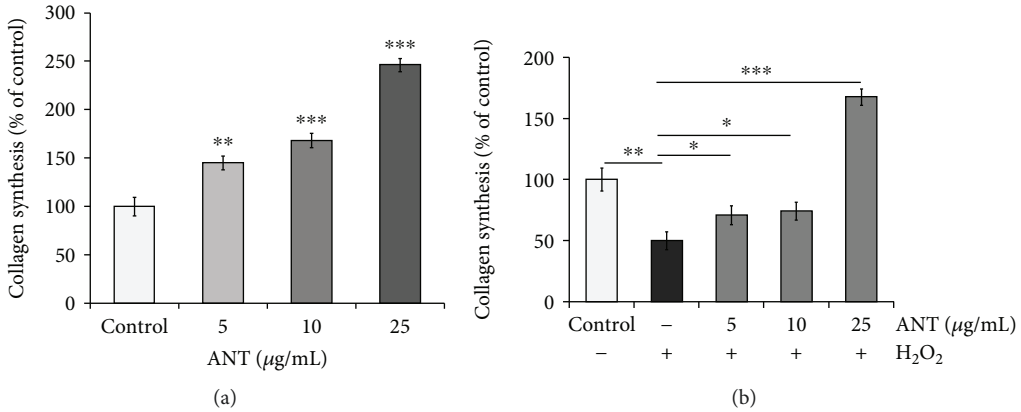


Figure 5: Effect of ANT on H_2O_2-stimulated RDF collagen synthesis. (a) RDFs were incubated with different concentrations of ANT for 24 h, and the effect on collagen synthesis was analyzed in terms of percentage change over the control. (b) Upon coculturing with various concentrations of ANT for 2 h with H_2O_2, collagen formation was measured in terms of percentage change compared with the control. Data are presented as the mean ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

H_2O_2 exposure induced ~3.4-fold induction of collagen formation compared with that in the H_2O_2-treated cells. These results suggest that ANT reverse the suppression of H_2O_2-induced collagen synthesis in RDFs.

3.6. ANT Effect on the Akt and ERK1/2 Signaling Pathways under Oxidative Stress. Protein kinase B (Akt) controls a wide variety of cellular processes including complex cellular programs such as differentiation, proliferation, and apoptosis

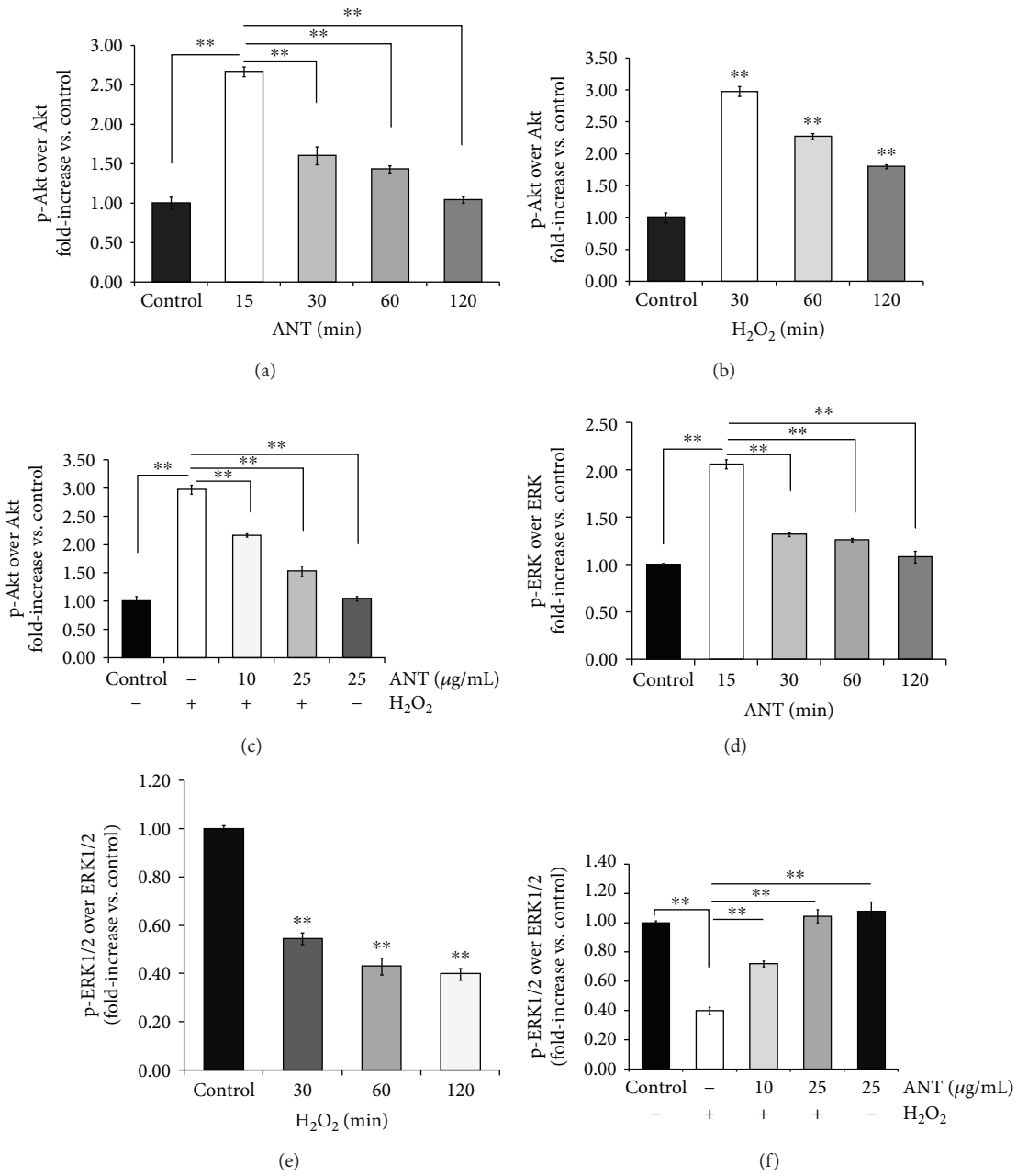


Figure 6: Involvement of the Akt and ERK1/2 signaling pathways in the antioxidative effect of ANT in RDFs determined by ELISA. Total and phosphorylated Akt expression after treatment with 25 μg/mL ANT (a) or 0.3 mM H_2O_2 (b) for the indicated periods of time. RDFs were then pretreated with 10 or 25 μg/mL ANT for 2 h and then incubated with 0.3 mM H_2O_2 for 30 min (c). Total and phosphorylated ERK1/2 expression after treatment with ANT (d) or H_2O_2 (e) under the same conditions as described for Akt. RDFs were then pretreated with 10 or 25 μg/mL ANT for 2 h, followed by incubation with 0.3 mM H_2O_2 for 2 h (f). Results were analyzed by calculation of the phosphorylated protein relative to the total protein. Data are presented as the mean ± SD ($n = 3$). $^{*}p < 0.01$ and $^{**}p < 0.001$.

and processes involved in immune responses [33]. Akt prompts NF-κB activation through IκB degradation [34, 35]. Herein, we revealed that ANT induced Akt phosphorylation as early as 15 min after the addition of ANT compared with the control (2.67 ± 0.06 vs. 1.00 ± 0.01, $p < 0.001$; Figure 6(a)). Figure 6(b) shows that treatment of RDFs with 0.3 mM H_2O_2 induced ~3-fold Akt phosphorylation in 30 min (2.98 ± 0.08, $p < 0.001$). However, pretreatment with 10 or 25 μg/mL ANT for 2 h was found to significantly decrease the level of H_2O_2-induced Akt phosphorylation in dermal fibroblasts at 30 min (2.17 ± 0.03 and 1.53 ± 0.09, respectively, $p < 0.001$; Figure 6(c)).

ERK1/2 signaling also has a prosurvival effect [36], and its activation is involved in the migration of skin fibroblasts [37]. Here, we showed the positive effect of ANT on fibroblast migration and then further explored the upstream regulator that protects dermal fibroblasts from oxidative stress. Thus, we investigated the effects of ANT on ERK1/2

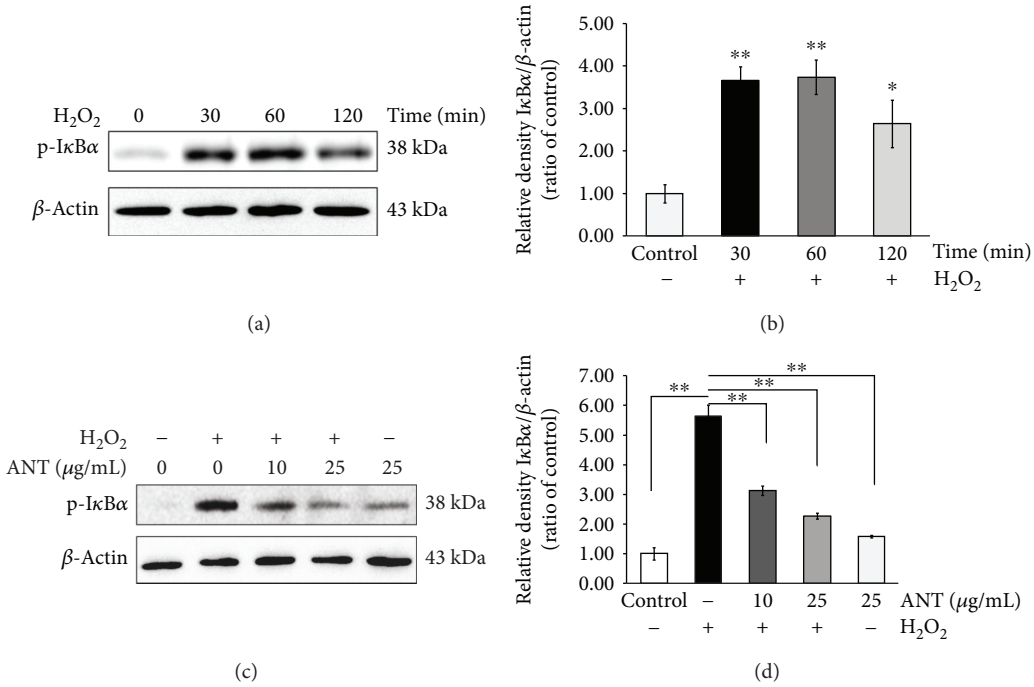


Figure 7: ANT inhibit the phosphorylation of IκBα in H_2O_2-stimulated RDFs. Whole cell extracts were taken and assayed using western blotting. (a) RDFs were stimulated with 0.3 mM H_2O_2 for the indicated time points to evaluate the IκBα phosphorylation status. (b) Data are expressed relative to untreated cells. (c) RDFs were pretreated with 10 or 25 μg/mL ANT for 2 h, followed by the absence or presence of 0.3 mM H_2O_2 for 1 h. (d) Data are expressed relative to untreated cells. β-Actin was used as a loading control. Results are expressed as means ± SD of three independent experiments. $^*p < 0.01$ and $^{**}p < 0.001$.

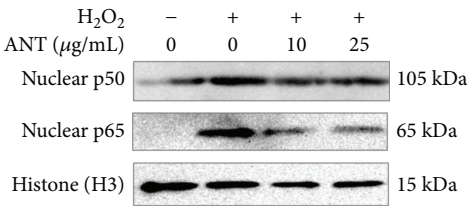
phosphorylation under H_2O_2 exposure. ANT significantly induced ERK1/2 phosphorylation compared with the control (2.06 ± 0.05 vs. 1.00 ± 0.01, $p < 0.001$; Figure 6(d)). In contrast, 0.3 mM H_2O_2 incubation for up to 2 h suppressed ERK1/2 phosphorylation in a time-dependent manner, with the maximum effect observed at 2 h (0.40 ± 0.03, $p < 0.001$, Figure 6(e)). ANT pretreatment for 2 h at 10 or 25 μg/mL effectively rescued the H_2O_2-induced ERK1/2 suppression (0.72 ± 0.02 and 1.05 ± 0.04, respectively, $p < 0.001$, Figure 6(f)).

3.7. Inhibitory Effects of ANT on the H_2O_2-Induced IκBα Signaling. As our previous experiment showed that the H_2O_2-induced oxidative response may depend on NF-κB activity, we investigated the effect of ANT on this pathway. In the canonical NF-κB pathway, the degradation of IκBα protein occurs after a signal-induced phosphorylation by IκB kinase (IKK) [38]. This enables the translocation of the NF-κB p50 and p65 subunits into the nucleus, resulting in the production and secretion of inflammatory cytokines [39]. Western blot analysis revealed markedly increased phosphorylation of IκBα at Tyr42 within 30 min after H_2O_2 stimulation (Figure 7(a)). Quantification of these results showed a significantly increased expression of phosphor-IκBα compared with the untreated cells (3.66 ± 0.325 vs. 1.00 ± 0.21, $p < 0.001$, Figure 7(b)), which decreased at the 2 h time point.

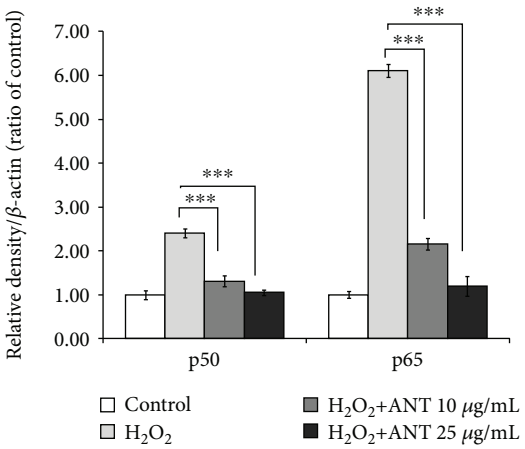
To determine whether ANT inhibit H_2O_2-induced IκBα phosphorylation, we pretreated cells with ANT for 2 h and then exposed them to H_2O_2 for 1 h (Figure 7(c)). ANT-treated RDFs at 10 or 25 μg/mL showed significantly reduced phosphorylation of IκBα in response to H_2O_2 compared with H_2O_2 stimulation alone (5.63 ± 0.38 vs. 2.27 ± 0.10; Figure 7(d)). These results indicated that ANT inhibited H_2O_2-induced phosphorylation and degradation of IκBα and thus the subsequent nuclear translocation of NF-κB.

3.8. The Effects of ANT on the H_2O_2-Induced NF-κB Nuclear Expression and Translocation. To confirm the effect of ANT on NF-κB subunit-mediated transcriptional activation, further analysis of H_2O_2-induced NF-κB expression in RDFs was performed. We examined the changes in NF-κB p50 and p65 levels in the cytoplasmic and nuclear fractions. H_2O_2-induced RDFs showed an increase in NF-κB protein in the nuclear fraction compared with the unstimulated control cells (Figure 8(a)). The NF-κB expression upon H_2O_2 stimulation was significantly suppressed by 10 μg/mL ANT ($p < 0.001$) and 25 μg/mL ANT ($p < 0.001$) pretreatment in a dose-dependent manner (Figure 8(b)). Western blot analysis also showed that in normal conditions, the NF-κB subunits localized in the cytosolic region of quiescent fibroblasts. However, after oxidative stimulation, the protein translocated into the nuclear region, resulting in a decrease in the expression in the cytosol (Figure 8(c)). ANT treatment resulted in significant inhibition of both NF-κB p50 ($p < 0.05$) and p65 ($p < 0.01$) activation and translocation to the nucleus (Figures 8(a)–8(d)).

To confirm the cellular distribution of NF-κB, immunofluorescence analysis was performed to investigate the effects



(a)



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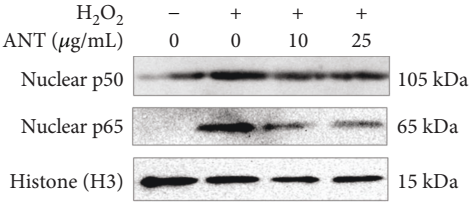

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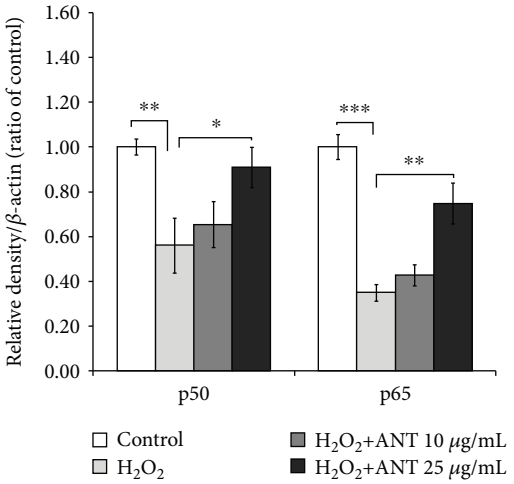

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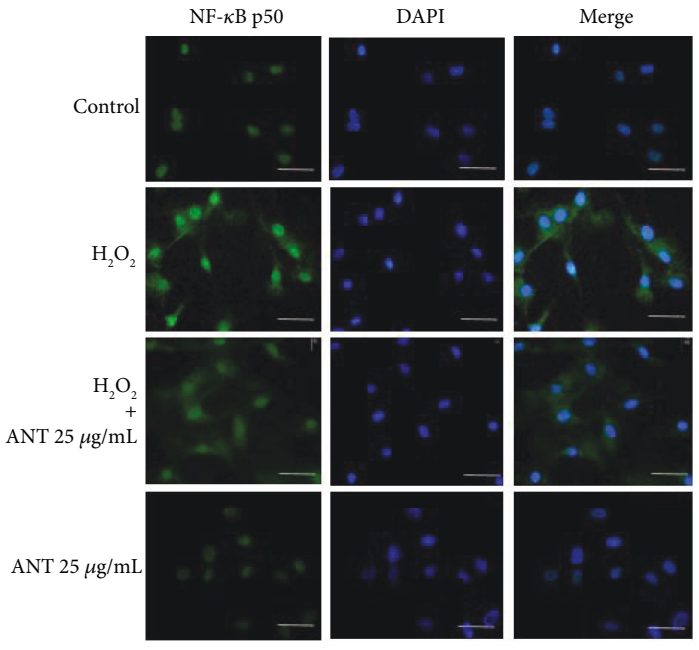

(e)

Figure 8: Continued.

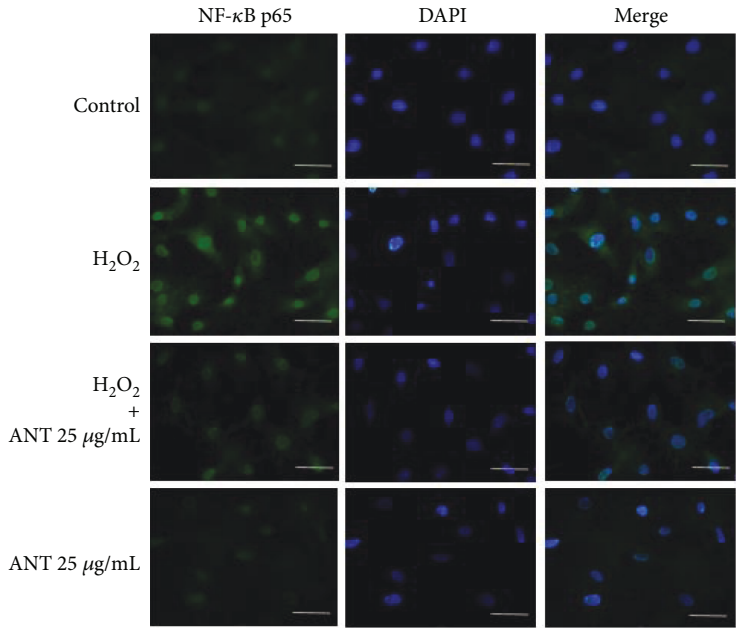

Figure 8: Effects of ANT on the activation of NF-κB transcription factors in RDFs stimulated with H_2O_2. RDFs were treated with different doses of ANT for 2 h and then exposed to 0.3 mM H_2O_2. Ten hours after H_2O_2 exposure, cytoplasmic and nuclear extracts were subjected to western blot analysis. (a) Nuclear extracts were immunoblotted for NF-κB p50 and NF-κB p65. (b) Data are expressed relative to the control. (c) Cytoplasmic extracts from the same RDF preparations were immunoblotted for NF-κB p50 and NF-κB p65. (d) Data are expressed relative to the control. Histone H3 and β-actin were used as a loading control for the nuclear and cytosol fraction, respectively. Representative images of immunofluorescence staining showing NF-κB p50 (e) and NF-κB p65 (f) localization conjugated to FITC (green). Nuclei were stained with DAPI (blue). Scale bars: 50 μm. Results are expressed as means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

of ANT on H_2O_2-induced NF-κB translocation. Untreated RDFs showed a low signal of the cytoplasmic and nuclear staining of NF-κB p50 Ab (Figure 8(e)). After H_2O_2 treatment, a marked nuclear staining was observed. However, ANT treatment prior to H_2O_2 stimulation decreased the level of nuclear translocation of NF-κB p50 compared with that of cells treated with H_2O_2 alone. Similarly, there was an increase in the nuclear localization of NF-κB p65 after cells were exposed to H_2O_2 compared with the control cells, and ANT treatment suppressed the localization of NF-κB p65 in the nucleus (Figure 8(f)). There was no positive staining of the cells upon staining RDFs with the isotype-matched control IgG (Supplementary Figure S2). These findings further confirmed the results of the protein transcriptional activity in RDFs following free radical exposure and ANT pretreatment.

3.9. ANT Reduce IL-6 Production under Hydrogen Peroxide Stimulation in RDFs. Hydrogen peroxide activates the transcription process of various inflammatory mediators and cytokines [40]. To determine the consequences of NF-κB activation, the mRNA transcription levels of *IL-6* were examined in H_2O_2-treated RDFs. H_2O_2 treatment induced the transcription of *IL-6* mRNA (6.92 ± 1.39 vs. 1.00 ± 0.12, $p < 0.001$, Figure 9(a)). However, when RDFs were pretreated with 10 or 25 μg/mL ANT, the *IL-6* mRNA level was significantly decreased to 3.08 ± 0.67 ($p < 0.05$) and 1.86 ± 0.29 ($p < 0.01$), respectively. The protein released into the cell culture supernatant also increased after H_2O_2 treatment compared with the nontreated cells ($236.37\% \pm 20.33$ vs. $100\% \pm 13.01$, $p < 0.001$, Figure 9(b)). Furthermore, a significant decrease in IL-6 protein release was found after pretreatment with ANT at 10 μg/mL ($193.36\% \pm 15.52$, $p < 0.05$) and 25 μg/mL ($142.84\% \pm 17.63$, $p < 0.01$) compared with the untreated control cells.

4. Discussion

ANT are members of the flavonoid group of phytochemicals, which have been shown to have substantial antioxidant effects, and are able to reduce lipid peroxidation and the deleterious effects of ROS [41]. Despite these findings, the preventive effects of black rice-derived ANT on wound healing and antiaging of skin have not been comprehensively examined. In this study, we initially performed a molecular target-based screening assay to identify the substance causing the induction of skin regeneration in rat primary dermal fibroblasts. We focused on the regulation of type I collagen expression by evaluating the level of *COL1A2* mRNA expression and determining the excreted proteins as well as the transcriptional regulation of genes involved in inflammation in RDFs. Herein, we report that ANT extracted from *O. sativa* L. induced collagen formation and prevented collagen degradation following H_2O_2 stimulation. The protective effects of ANT against cellular inflammation were investigated focusing on transcriptional activation of NF-κB, which

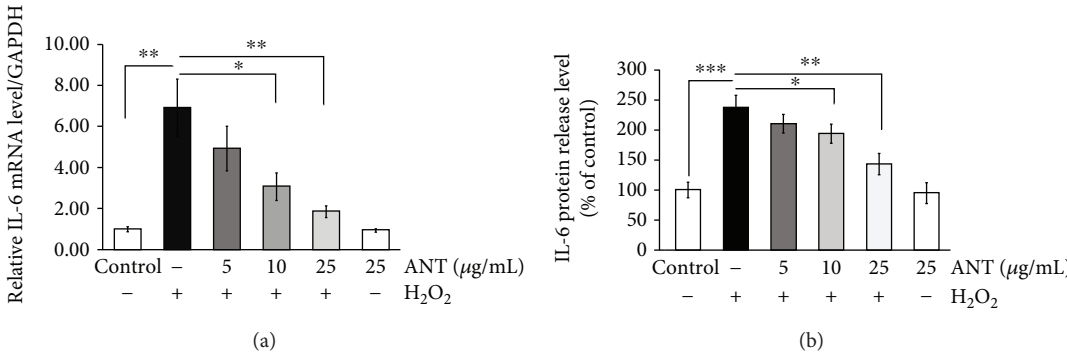

FIGURE 9: ANT reduce H_2O_2-induced interleukin-6 (IL-6) expression in RDFs. (a) RDFs were treated with different doses of ANT for 2 h and then exposed to 0.3 mM H_2O_2 for 10 h. *IL-6* mRNA levels were quantified by RT-qPCR. The relative levels of mRNA were normalized against *GAPDH* from the same cDNA preparation. (b) RDFs were exposed to 0.3 mM H_2O_2 for 24 h after a 2 h ANT pretreatment. The amount of IL-6 protein released into the cell culture supernatant following H_2O_2 treatment was measured by ELISA. The control values were arbitrarily set to 100%; all other samples are presented as percentage of control. Values are presented as the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

results from the inhibition of IκBα phosphorylation [24]. Our study demonstrated that ANT inhibited the H_2O_2-induced nuclear translocation of NF-κB p50/p65 heterodimers in primary dermal fibroblasts. Furthermore, ANT induced Akt/ERK1/2 phosphorylation and inhibited IL-6 expression in response to H_2O_2 stimulation. RDFs also exhibited cell migration capacities following ANT treatment.

The effect of exogenous oxidants highlights the importance of the balance among the proinflammatory signals that control skin repair. ROS generated by disturbance of the oxidation/reduction state of the cell have been implicated in the pathogenesis of various inflammatory diseases including skin damage. Our results demonstrated a protective effect of ANT extract by ameliorating the H_2O_2-induced cytotoxicity in primary dermal fibroblasts, which is consistent with previous studies indicating that the antioxidant activities of polyphenols can decrease oxidative stress by inhibiting ROS production and lipid peroxidation [42, 43]. Previous studies have shown that cell injury induced by H_2O_2 can be reversed by decreasing ROS generation; ANT extracted from red cabbage protected animals against oxidative stress by suppressing the NADPH-cytochrome-P450-reductase activity [44]. Cell injury causes inflammatory cells to migrate to the wound site and generate free radicals via a nonphagocytic NAD(P)H oxidase mechanism [15]. This causes an abundance of free radicals at the wound site. A measurement of NADPH-P450 reductase by its NADPH-cytochrome c reduction activity should be further determined in the black rice extract used in this study.

In our study, ANT were extracted in ethanol and contained molecules such as C3G, peonidin-3-O-glucoside, and quercetin-3-beta-D-glucoside. The total phenolic content was ~30 ± 1.5 mM gallic acid equivalent (gFW). Our results of the levels of C3G and the antioxidant properties of ANT extracted from *O. sativa* L. were lower than the levels in a previous study [26]. The differences in the C3G content and antioxidant capacity between the previous study and our findings probably stem from the diversity of rice cultivars, as well as the variety of extraction methods and analyses.

Numerous studies on the effects of natural or synthetic antioxidants on collagen deposition and on antioxidant defense have generated highly conflicting data, depending on the experimental system used. However, with various wound healing models, it has been repeatedly demonstrated that complex plant extracts containing active secondary metabolites (polyphenols, flavonoids, and alkaloids) [42, 43] and components of collagen-inducing polysaccharides, such as chitosan, and antioxidants such as curcumin [45] and resveratrol [46] ameliorated wound healing and increased skin collagen deposition, while suppressing proinflammatory markers. ANT extract from black soybean seed coats has been shown to decrease inflammation by suppressing the translocation of NF-κB p65 into the nucleus of human dermal fibroblasts and keratinocytes [11]. Consistently, analysis of the effects of berries, including extracts and purified ANT, has revealed that ANT suppressed NF-κB activation in monocytes and reduced the plasma concentration of proinflammatory mediators [47, 48]. In the wound healing mechanism, collagen type I plays a crucial role in maintaining the tensile strength and elasticity of the skin. Therefore, an approach that enhances type I collagen is an attractive strategy for dermal wound healing. ANT are the major active components in *O. sativa* L. extract, which may stimulate type I collagen biosynthesis. In this context, further clinical studies on the wound healing or antiaging activities of black rice extract should be performed *in vivo*.

Previous studies have reported that the main factor in UVA-induced damage to skin cells is the presence of ROS, including H_2O_2 and hydroxyl radicals, which affect the activation of the downstream cellular signaling pathways and cause damage to the skin [49]. The addition of H_2O_2 to a mammalian cell culture at concentrations of typically 50–1000 μM induces growth arrest, cell death, and survival signaling pathways, including Akt [50, 51] and ERK1/2 signaling [52, 53]. Akt is a downstream target of NF-κB and is involved in oxidative stress and inflammation [54]. ERK1/2 activation is associated with cell death induced by ROS [36]. However, signaling through ERK1/2

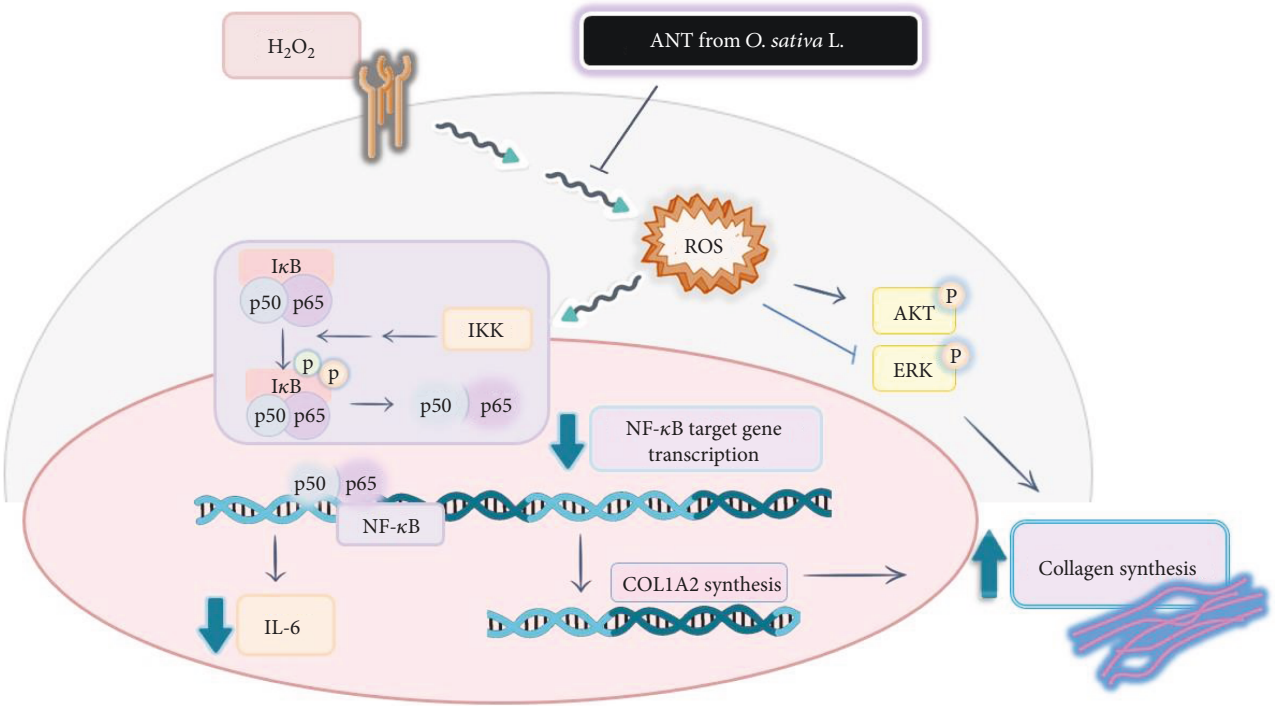

Figure 10: A schematic of the antioxidant and anti-inflammatory properties of anthocyanins (ANT) extracted from *Oryza sativa* L. in primary dermal fibroblasts. Black rice-derived ANT suppressed reactive oxygen species (ROS) signaling, which regulates phosphor-Akt/extracellular signal-regulated kinases (ERK) and NF-κB p50/p65 signaling in inflammation, and modulated *collagen type I alpha 2* (*COL1A2*) upregulation. In addition, ANT suppressed hydrogen peroxide- (H_2O_2-) induced interleukin- (IL-) 6 production. The present study demonstrated that ANT may promote collagen synthesis in the skin and function as antioxidants and anti-inflammatory substances.

also has a prosurvival effect [55]. We demonstrated that H_2O_2 stimulated strong phosphorylation of Akt; however, ANT pretreatment suppressed this Akt signaling in a dose-dependent manner in RDFs. Under similar cell conditions, ANT prevented H_2O_2-induced suppression of ERK1/2 phosphorylation. The ERK/Akt pathways are involved in the differentiation of cells and the formation of collagen during tissue regeneration [56]. In addition, our study indicated that ANT induced collagen formation. Our results suggest that ANT can protect against ROS via modulations of the Akt/ERK1/2 signaling cascades, upstream of NF-κB activation. Our investigations imply that ANT are a necessary requirement for collagen formation, which may occur via the ERK/Akt/NF-κB signaling pathways. However, the molecular mechanisms that define the conditions for ANT-mediated ERK1/2 phosphorylation should be further investigated.

In aging skin, the NF-κB signaling pathway may be attributable to the transcriptional control of collagen I gene expression in human skin fibroblasts [57]. NF-κB is an early nuclear transcription factor that is triggered by many pathogenic stimuli such as oxidative stress. It is composed of various subunits, of which the p65/p50 heterodimer is the best known combination, and it has been shown to play a critical role in binding to a DNA consensus sequence to initiate the expression of proinflammatory genes [31, 58]. Several studies have demonstrated the role of NF-κB in matrix metalloproteinases [59] and IL expression [60], which have been shown to be responsible for collagen degradation. In human dermal fibroblasts treated with H_2O_2, NF-κB activity was induced; however, peptides derived from seaweed pipefish can suppress the NF-κB activation thus decreasing the oxidative stress-mediated damage of the cells [61]. We observed that the H_2O_2-induced expression of p65 or p50 triggered an increase in NF-κB activity accompanied by the downregulation of type I collagen expression in RDFs. Our results showing the declined transcriptional activity of *COL1A2* and type I collagen protein secretion in H_2O_2-stimulated cells suggest that NF-κB affects *COL1A2* transcriptional activity. The inhibition of NF-κB activation and induced collagen formation by ANT, which are among the best-described flavonoids, indicate an important role of ANT extract from the black rice in aging skin protection. Our investigation is in agreement with an *in vivo* study that demonstrated the anti-inflammatory role of ANT from Thai black rice using a 5-FU-induced oral mucositis model. This study indicated that ANT reduced the lesions induced by 5-FU in the oral mucosa via NF-κB-mediated biological manipulations [26]. Furthermore, ANT suppressed the H_2O_2-induced IL-6 production and NF-κB activation, which is in agreement with a previous study on normal human dermal fibroblasts exposed to UVB [62]. Our findings are consistent with previous observations in animal and clinical studies, where ANT demonstrated anti-inflammatory effects by inhibiting NF-κB transactivation and suppressing proinflammatory mediators [48].

Several studies have reported that the dissociation of the p65-p50 NF-κB heterodimer from phosphorylated IκBα is

accompanied by proteolytic degradation of IκB. IκBα degradation precedes NF-κB activation, which in turn promotes IκBα synthesis, restoring the unstimulated inhibited state [63]. To confirm the effect of ANT on oxidative stress-induced NF-κB activation, the experiment with different time intervals of H_2O_2-induced NF-κB activation and ANT pretreatment was conducted. Our results indicated that H_2O_2 induced tyrosine phosphorylation of IκBα, which is in agreement with reports by Takada et al. [64] and Schoonbroodt et al. [65]. This favors our hypothesis that the intracellular levels of ROS control the level of phosphor-IκBα by activating a kinase or inactivating a phosphatase that is specific to this protein. Therefore, the ANT-mediated low levels of ROS, by suppressing IκBα phosphorylation, may abolish the specific proteolysis of phosphorylated IκBα that induces NF-κB activation. NF-κB p50 phosphorylation at S337 is mediated by protein kinase A (PKA) [66] and promotes DNA damage-induced cytotoxicity [67]. In addition to PKA, NF-κB p65 S276 phosphorylation is mediated by a number of other kinases and inhibits inflammation by regulating cytokine expression [68]. Our results showed that at 25 μg/mL, ANT caused ~50% reduction in both NF-κB p50 and p65 mRNA and protein expression in the nucleus, compared with cells exposed to H_2O_2 alone, suggesting that a combination therapy with other medicines may be required to fully affect NF-κB transcriptional activity.

5. Conclusions

In conclusion, our data indicated that ANT extracted from black rice protected RDFs against H_2O_2-induced cytotoxicity by suppressing oxidative stress, confirming their antioxidative activity. ANT extract effectively upregulated the mRNA and protein expression levels of type I collagen in a dose-dependent manner. They also protected RDFs from H_2O_2-mediated inflammatory damage by inhibiting IκBα phosphorylation, NF-κB activation, and IL-6 production (Figure 10). We propose that ANT may be suitable as adjuncts in treatment promoting wound healing and antiaging. Further detailed validation studies are needed to investigate ANT as possible wound dressing materials, not only stimulating collagen synthesis but also accelerating wound healing by reducing oxidative stress.

Abbreviations

ANT: Anthocyanins
NF-κB: Nuclear factor-kappa B
RDF: Rat dermal fibroblast
ERK: Extracellular signal-regulated kinase
Akt: Protein kinase B
MAPKs: Mitogen-activated protein kinases.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

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Supplementary Materials

Supplementary materials can be found at Supplementary Movie 1.gif and Supplementary Figures. *(Supplementary Materials)*

References

[1] X. Q. Chen, N. Nagao, T. Itani, and K. Irifune, "Anti-oxidative analysis, and identification and quantification of anthocyanin pigments in different coloured rice," *Food Chemistry*, vol. 135, no. 4, pp. 2783–2788, 2012.

[2] Y. Shao, Z. Hu, Y. Yu, R. Mou, Z. Zhu, and T. Beta, "Phenolic acids, anthocyanins, proanthocyanidins, antioxidant activity, minerals and their correlations in non-pigmented, red, and black rice," *Food Chemistry*, vol. 239, pp. 733–741, 2018.

[3] S. B. Lotito and B. Frei, "Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon?," *Free Radical Biology and Medicine*, vol. 41, no. 12, pp. 1727–1746, 2006.

[4] H. J. Kim, I. Tsoy, J. M. Park, J. I. Chung, S. C. Shin, and K. C. Chang, "Anthocyanins from soybean seed coat inhibit the expression of TNF-α-induced genes associated with ischemia/reperfusion in endothelial cell by NF-κB-dependent pathway and reduce rat myocardial damages incurred by ischemia and reperfusion in vivo," *FEBS Letters*, vol. 580, no. 5, pp. 1391–1397, 2006.

[5] Y. H. He, J. Zhou, Y. S. Wang et al., "Anti-inflammatory and anti-oxidative effects of cherries on Freund's adjuvant-induced arthritis in rats," *Scandinavian Journal of Rheumatology*, vol. 35, no. 5, pp. 356–358, 2006.

[6] R. Boniface and A. M. Robert, "Einfluß von Anthocyanen auf den Bindegewebsmetabolismus beim Menschen," *Klinische Monatsblätter für Augenheilkunde*, vol. 209, no. 12, pp. 368–372, 1996.

[7] F. Cohen-Boulakia, P. E. Valensi, H. Boulahdour et al., "In vivo sequential study of skeletal muscle capillary permeability in diabetic rats: effect of anthocyanosides," *Metabolism: Clinical and Experimental*, vol. 49, no. 7, pp. 880–885, 2000.

[8] D. Zadok, Y. Levy, and Y. Glovinsky, "The effect of anthocyanosides in a multiple oral dose on night vision," *Eye*, vol. 13, no. 6, pp. 734–736, 1999.

[9] H. Nakaishi, H. Matsumoto, S. Tominaga, and M. Hirayama, "Effects of black current anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alteration

in healthy humans," *Alternative Medicine Review*, vol. 5, no. 6, pp. 553–562, 2000.

[10] P. Borissova, S. Valcheva, and A. Belcheva, "Antiinflammatory effect of flavonoids in the natural juice from Aronia melanocarpa, rutin and rutin-magnesium complex on an experimental model of inflammation induced by histamine and serotonin," *Acta physiologica et pharmacologica Bulgarica*, vol. 20, no. 1, pp. 25–30, 1994.

[11] I. T. Nizamutdinova, Y. M. Kim, J. I. Chung et al., "Anthocyanins from black soybean seed coats stimulate wound healing in fibroblasts and keratinocytes and prevent inflammation in endothelial cells," *Food and Chemical Toxicology*, vol. 47, no. 11, pp. 2806–2812, 2009.

[12] S. Limsitthichaikoon, B. Khampaenjiraroch, T. Damrongrungruang, W. Limphirat, S. Thapphasaraphong, and A. Priprem, "Topical oral wound healing potential of anthocyanin complex: animal and clinical studies," *Therapeutic Delivery*, vol. 9, no. 5, pp. 359–374, 2018.

[13] R. A. Clark, "Cutaneous tissue repair: basic biologic considerations. I," *Journal of the American Academy of Dermatology*, vol. 13, no. 5, pp. 701–725, 1985.

[14] M. Nita and A. Grzybowski, "The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 3164734, 23 pages, 2016.

[15] T. Tang, L. Yin, J. Yang, and G. Shan, "Emodin, an anthraquinone derivative from Rheum officinale Baill, enhances cutaneous wound healing in rats," *European Journal of Pharmacology*, vol. 567, no. 3, pp. 177–185, 2007.

[16] B. Thiem and O. Goslinska, "Antimicrobial activity of Rubus chamaemorus leaves," *Fitoterapia*, vol. 75, no. 1, pp. 93–95, 2004.

[17] S. Khanna, M. Venojarvi, S. Roy et al., "Dermal wound healing properties of redox-active grape seed proanthocyanidins," *Free Radical Biology and Medicine*, vol. 33, no. 8, pp. 1089–1096, 2002.

[18] A. Priprem, T. Damrongrungruang, S. Limsitthichaikoon et al., "Topical niosome gel containing an anthocyanin complex: a potential oral wound healing in rats," *AAPS PharmSciTech*, vol. 19, no. 4, pp. 1681–1692, 2018.

[19] L. Xu, T. H. Choi, S. Kim et al., "Anthocyanins from black soybean seed coat enhance wound healing," *Annals of Plastic Surgery*, vol. 71, no. 4, pp. 415–420, 2013.

[20] P. A. Baeuerle and T. Henkel, "Function and activation of NF-kappaB in the immune system," *Annual Review of Immunology*, vol. 12, no. 1, pp. 141–179, 1994.

[21] R. Piva, G. Belardo, and M. G. Santoro, "NF-κB: a stress-regulated switch for cell survival," *Antioxidants & Redox Signaling*, vol. 8, no. 3-4, pp. 478–486, 2006.

[22] S. Cuzzocrea, D. P. Riley, A. P. Caputi, and D. Salvemini, "Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury," *Pharmacological Reviews*, vol. 53, no. 1, pp. 135–159, 2001.

[23] M. Conti and J. Beavo, "Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling," *Annual Review of Biochemistry*, vol. 76, no. 1, pp. 481–511, 2007.

[24] T. S. Finco and A. S. Baldwin, "Mechanistic aspects of NF-kappa B regulation: the emerging role of phosphorylation and proteolysis," *Immunity*, vol. 3, no. 3, pp. 263–272, 1995.

[25] T. S. Finco, A. A. Beg, and A. S. Baldwin Jr., "Inducible phosphorylation of I kappa B alpha is not sufficient for its dissociation from NF-kappa B and is inhibited by protease inhibitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 25, pp. 11884–11888, 1994.

[26] S. Tancharoen, P. Shakya, S. Narkpinit, P. Dararat, and K. Kikuchi, "Anthocyanins extracted from *Oryza sativa* L. prevent fluorouracil-induced nuclear factor-κB activation in oral mucositis: in vitro and in vivo studies," *International journal of molecular sciences*, vol. 19, no. 10, article 2981, 2018.

[27] W.-J. Huang, "Isolation and determination of major anthocyanin pigments in the pericarp of P. communis L. cv. 'Red Du Comices' and their association with antioxidant activity," *African Journal of Agricultural Reseearch*, vol. 7, no. 26, 2012.

[28] A. Smeriglio, D. Barreca, E. Bellocco, and D. Trombetta, "Chemistry, pharmacology and health benefits of anthocyanins," *Phytotherapy Research*, vol. 30, no. 8, pp. 1265–1286, 2016.

[29] A. Sarikaphuti, T. Nararatwanchai, T. Hashiguchi et al., "Preventive effects of Morus alba L. anthocyanins on diabetes in Zucker diabetic fatty rats," *Experimental and Therapeutic Medicine*, vol. 6, no. 3, pp. 689–695, 2013.

[30] S. Tancharoen, T. Matsuyama, K. Kawahara et al., "Cleavage of host cytokeratin-6 by lysine-specific gingipain induces gingival inflammation in periodontitis patients," *PLoS One*, vol. 10, no. 2, article e0117775, 2015.

[31] T. Liu, L. Zhang, D. Joo, and S.-C. Sun, "NF-κB signaling in inflammation," *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, article 17023, 2017.

[32] K. N. Islam and W. J. Koch, "Involvement of nuclear factor κB (NF-κB) signaling pathway in regulation of cardiac G protein-coupled receptor kinase 5 (GRK5) expression," *Journal of Biological Chemistry*, vol. 287, no. 16, pp. 12771–12778, 2012.

[33] V. J. Hernandez, J. Weng, P. Ly et al., "Cavin-3 dictates the balance between ERK and Akt signaling," *eLife*, vol. 2, article e00905, 2013.

[34] M. Etscheid, N. Beer, and J. Dodt, "The hyaluronan-binding protease upregulates ERK1/2 and PI3K/Akt signalling pathways in fibroblasts and stimulates cell proliferation and migration," *Cellular Signalling*, vol. 17, no. 12, pp. 1486–1494, 2005.

[35] J. Wei and J. Feng, "Signaling pathways associated with inflammatory bowel disease," *Recent Patents on Inflammation & Allergy Drug Discovery*, vol. 4, no. 2, pp. 105–117, 2010.

[36] J. Dong, S. Ramachandiran, K. Tikoo, Z. Jia, S. S. Lau, and T. J. Monks, "EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death," *American Journal of Physiology Renal Physiology*, vol. 287, no. 5, pp. F1049–F1058, 2004.

[37] T. H. Lee, G. W. Lee, K. H. Park et al., "The stimulatory effects of Stewartia koreana extract on the proliferation and migration of fibroblasts and the wound healing activity of the extract in mice," *International Journal of Molecular Medicine*, vol. 34, no. 1, pp. 145–152, 2014.

[38] M. S. Byun, K. I. Jeon, J. W. Choi, J. Y. Shim, and D. M. Jue, "Dual effect of oxidative stress on NF-κB activation in HeLa cells," *Experimental & Molecular Medicine*, vol. 34, no. 5, pp. 332–339, 2002.

[39] L. J. Simmons, M. C. Surles-Zeigler, Y. Li, G. D. Ford, G. D. Newman, and B. D. Ford, "Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial

cells in vitro involves the NF-kappa B pathway," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 237, 2016.

[40] N. Miyoshi, H. Oubrahim, P. B. Chock, and E. R. Stadtman, "Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 6, pp. 1727–1731, 2006.

[41] C. S. Bowen-Forbes, Y. Zhang, and M. G. Nair, "Anthocyanin content, antioxidant, anti-inflammatory and anticancer properties of blackberry and raspberry fruits," *Journal of Food Composition and Analysis*, vol. 23, no. 6, pp. 554–560, 2010.

[42] M. Gangwar, M. K. Gautam, S. Ghildiyal, G. Nath, and R. K. Goel, "*Mallotus philippinensis* Muell. Arg fruit glandular hairs extract promotes wound healing on different wound model in rats," *BMC Complementary and Alternative Medicine*, vol. 15, no. 1, p. 123, 2015.

[43] S. Murthy, M. K. Gautam, S. Goel, V. Purohit, H. Sharma, and R. K. Goel, "Evaluation of *in vivo* wound healing activity of *Bacopa monniera* on different wound model in rats," *BioMed Research International*, vol. 2013, Article ID 972028, 9 pages, 2013.

[44] K. Igarashi, Y. Kimura, and A. Takenaka, "Preventive effects of dietary cabbage acylated anthocyanins on paraquat-induced oxidative stress in rats," *Bioscience, Biotechnology, and Biochemistry*, vol. 64, no. 8, pp. 1600–1607, 2000.

[45] S. Jebahi, M. Saoudi, L. Farhat et al., "Effect of novel curcumin-encapsulated chitosan-bioglass drug on bone and skin repair after gamma radiation: experimental study on a Wistar rat model," *Cell Biochemistry and Function*, vol. 33, no. 3, pp. 150–159, 2015.

[46] I. Yaman, H. Derici, C. Kara et al., "Effects of resveratrol on incisional wound healing in rats," *Surgery Today*, vol. 43, no. 12, pp. 1433–1438, 2013.

[47] S. V. Joseph, I. Edirisinghe, and B. M. Burton-Freeman, "Berries: anti-inflammatory effects in humans," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 18, pp. 3886–3903, 2014.

[48] A. Karlsen, L. Retterstøl, P. Laake et al., "Anthocyanins inhibit nuclear factor-κB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults," *The Journal of Nutrition*, vol. 137, no. 8, pp. 1951–1954, 2007.

[49] S. Wu, Y. Hu, Z. Li et al., "The effect of cyanidin-3-o-glucoside on UVA-induced damage in human dermal fibroblasts," *Photodermatology, photoimmunology & photomedicine*, vol. 34, no. 4, pp. 224–231, 2017.

[50] M. Ushio-Fukai, R. W. Alexander, M. Akers et al., "Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells," *Journal of Biological Chemistry*, vol. 274, no. 32, pp. 22699–22704, 1999.

[51] Y. Sonoda, S. Watanabe, Y. Matsumoto, E. Aizu-Yokota, and T. Kasahara, "FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line," *Journal of Biological Chemistry*, vol. 274, no. 15, pp. 10566–10570, 1999.

[52] A. J. Crossthwaite, S. Hasan, and R. J. Williams, "Hydrogen peroxide-mediated phosphorylation of ERK1/2, Akt/PKB and JNK in cortical neurones: dependence on Ca(2+) and PI3-kinase," *Journal of Neurochemistry*, vol. 80, no. 1, pp. 24–35, 2002.

[53] F. Esposito, G. Chirico, N. Montesano Gesualdi et al., "Protein kinase B activation by reactive oxygen species is independent of tyrosine kinase receptor phosphorylation and requires SRC activity," *Journal of Biological Chemistry*, vol. 278, no. 23, pp. 20828–20834, 2003.

[54] J. Zhang, X. Wang, V. Vikash et al., "ROS and ROS-mediated cellular signaling," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 4350965, 18 pages, 2016.

[55] W. B. Jang, J. H. Park, S. T. Ji et al., "Cytoprotective roles of a novel compound, MHY-1684, against hyperglycemia-induced oxidative stress and mitochondrial dysfunction in human cardiac progenitor cells," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 4528184, 10 pages, 2018.

[56] I. J. Lim, T. T. Phan, E. K. Tan et al., "Synchronous activation of ERK and phosphatidylinositol 3-kinase pathways is required for collagen and extracellular matrix production in keloids," *Journal of Biological Chemistry*, vol. 278, no. 42, pp. 40851–40858, 2003.

[57] N. Bigot, G. Beauchef, M. Hervieu et al., "NF-κB accumulation associated with *COL1A1 Transactivators* defects during chronological aging represses Type I collagen expression through a –112/–61-bp region of the *COL1A1* promoter in human skin fibroblasts," *Journal of Investigative Dermatology*, vol. 132, no. 10, pp. 2360–2367, 2012.

[58] T. Lawrence, "The nuclear factor NF-kappaB pathway in inflammation," *Cold Spring Harbor perspectives in biology*, vol. 1, no. 6, article a001651, 2009.

[59] S. Mine, N. O. Fortunel, H. Pageon, and D. Asselineau, "Aging alters functionally human dermal papillary fibroblasts but not reticular fibroblasts: a new view of skin morphogenesis and aging," *PLoS One*, vol. 3, no. 12, article e4066, 2008.

[60] H. J. Hahn, K. B. Kim, I. S. An, K. J. Ahn, and H. J. Han, "Protective effects of rosmarinic acid against hydrogen peroxide-induced cellular senescence and the inflammatory response in normal human dermal fibroblasts," *Molecular Medicine Reports*, vol. 16, no. 6, pp. 9763–9769, 2017.

[61] B. Ryu, S. W. Himaya, Z. J. Qian, S. H. Lee, and S. K. Kim, "Prevention of hydrogen peroxide-induced oxidative stress in HDF cells by peptides derived from seaweed pipefish, *Syngnathus schlegeli*," *Peptides*, vol. 32, no. 4, pp. 639–647, 2011.

[62] K. Shirato, T. Koda, J. Takanari et al., "ETAS®50 attenuates ultraviolet-B-induced interleukin-6 expression by suppressing Akt phosphorylation in normal human dermal fibroblasts," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 1547120, 8 pages, 2018.

[63] P. J. Chiao, S. Miyamoto, and I. M. Verma, "Autoregulation of I kappa B alpha activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 1, pp. 28–32, 1994.

[64] Y. Takada, A. Mukhopadhyay, G. C. Kundu, G. H. Mahabeleshwar, S. Singh, and B. B. Aggarwal, "Hydrogen peroxide activates NF-κB through tyrosine phosphorylation of IκBα and serine phosphorylation of p65: evidence for the involvement of IκBα kinase and Syk protein-tyrosine kinase," *Journal of Biological Chemistry*, vol. 278, no. 26, pp. 24233–24241, 2003.

[65] S. Schoonbroodt, V. Ferreira, M. Best-Belpomme et al., "Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of I κBα in NF-κB activation by an oxidative stress," *The Journal of immunology*, vol. 164, no. 8, pp. 4292–4300, 2000.

[66] H. Guan, S. Hou, and R. P. Ricciardi, "DNA binding of repressor nuclear factor-kappaB p50/p50 depends on phosphorylation of Ser337 by the protein kinase A catalytic subunit," *Journal of Biological Chemistry*, vol. 280, no. 11, pp. 9957–9962, 2005.

[67] C. D. Crawley, D. R. Raleigh, S. Kang et al., "DNA damage-induced cytotoxicity is mediated by the cooperative interaction of phospho-NF-κB p50 and a single nucleotide in the κB-site," *Nucleic Acids Research*, vol. 41, no. 2, pp. 764–774, 2013.

[68] C. C. Ghosh, S. Ramaswami, A. Juvekar et al., "Gene-specific repression of proinflammatory cytokines in stimulated human macrophages by nuclear IκBα," *The Journal of Immunology*, vol. 185, no. 6, pp. 3685–3693, 2010.

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Review Article

Nutrient Sensing and Redox Balance: GCN2 as a New Integrator in Aging

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Aging is a complex process in which the accumulation of molecular, cellular, and organism dysfunction increases the probability of death. Several pieces of evidence have revealed a contribution of stress responses in aging and in aging-related diseases, in particular, the key role of signaling pathways associated to nutritional stress. Here, we review the possible interplay between amino acid sensing and redox balance maintenance mediated by the nutritional sensor general control nonderepressive 2 (GCN2). We discuss this new dimension of nutritional stress sensing consequences, standing out GCN2 as a central coordinator of key cellular processes that assure healthy homeostasis in the cell, raising GCN2 as a novel interesting target, that when activated, could imply pleiotropic benefits, particularly GCN2 intervention and its new unexplored therapeutic role as a player in the aging process.

1. Introduction

Aging is a time-dependent physiological process characterized for being dynamic and multifactorial, and contrary to the common conception, it has been proposed that aging does not start in adulthood but begins with the birth of an organism [1]. In aging, organic changes occur limiting the adaptability of the organism to the environment, leading to an increased risk of weakness, disease, and death [2]. Through the course of time, the biological functions progressively decay, accompanied by a deterioration of the ability to adapt to the metabolic stress [3].

The aging research field has recently born in response to the impact that it exhibits in the healthspan of a worldwide population that is getting older demographically [4]. Given the complexity of the biological phenomenon, in 2013, Lopez-Otin and colleagues [5] made the first effort in categorizing the main cellular features of aging; each one considers fulfilling three aspect criteria: (1) it manifests during normal aging, (2) its experimental aggravation should accelerate aging, and (3) its experimental amelioration should retard the normal aging process. These hallmarks, which are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [5], contribute to the aging process, and together, they establish an aging phenotype. This first attempt for shaping conceptually the aging process was fundamental in the field; however, nowadays, other authors disagree [2] regarding the applicability of the criteria of features that have only been demonstrated, so far, in proliferative peripheral tissue-associated aging such as cellular senescence and telomere attrition, in which utility in another context, namely, of a nonproliferative tissue like the brain, should be established [2]. Despite of these discrepancies, even some authors may include new mediators [6, 7]; there is a general accordance in the field about the relevance of the deregulated nutrient sensing and energy metabolism dysregulation as a key hallmark of aging [5, 8–10] (Figure 1). In general terms, the main aging hallmarks may be grouped in four sets: DNA alterations, mitochondrial dysfunction, impaired adaptive/stress response, and cell

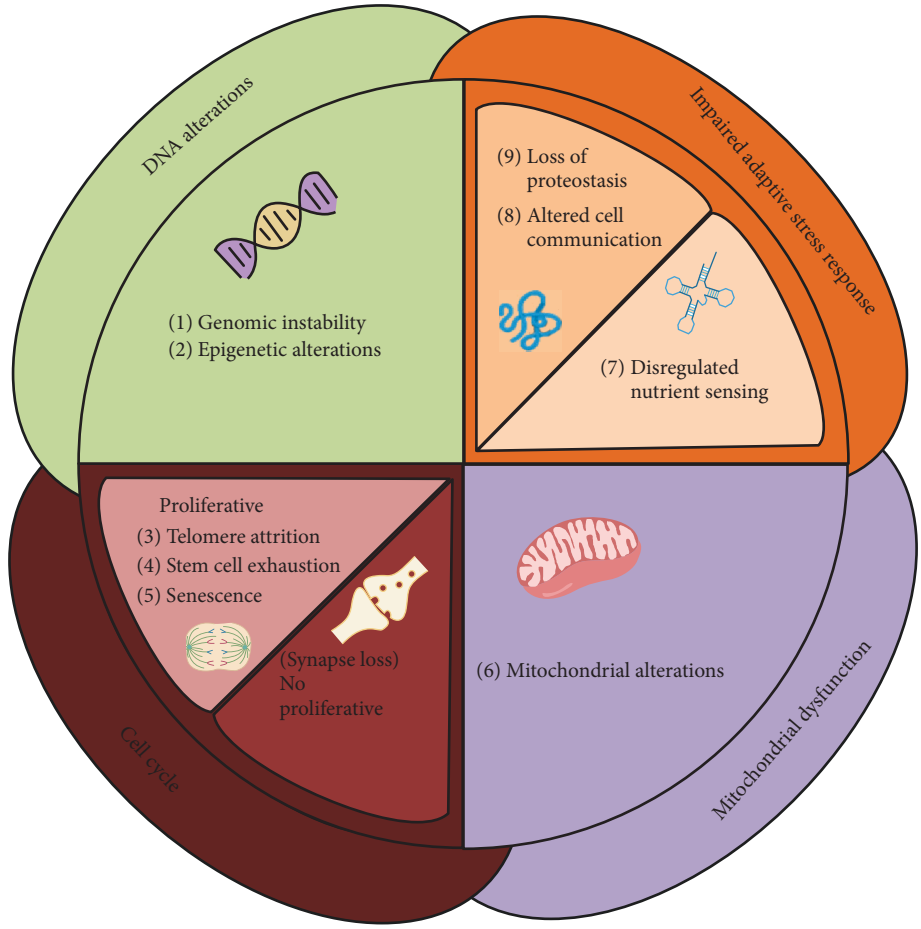

Figure 1: Main aging hallmarks studied in chordates. In the diagram, the nine-group aging hallmarks were grouped into four: mitochondrial dysfunction, DNA alterations (containing epigenetic alterations and genomic stability), impaired adaptive/stress response (containing loss of proteostasis and nutrient sensing deregulation), and cell cycle state dependent in function if they are differentiated or not, proliferative tissue alterations (telomere attrition, senesce), or nonproliferative cell disturbances such as synaptic loss.

cycle-related perturbations exhibited in proliferative tissue (such as telomere attrition, stem cell exhaustion, and senesce) or nonproliferative cell disturbances such as synaptic loss (Figure 1). In this review, we will focus on stress responses evoked by nutrient scarcity and how nutrient sensing pathways could be involved in aging.

2. Nutrient Sensing in Aging

The importance of nutrient sensing (dys)regulation along the aging process was first demonstrated more than 80 years ago by McCay et al. [11], with the seminal observation that reduced food intake in rats, without malnutrition, extends both mean and maximal lifespan as compared to *ad libitum* fed controls. This nutritional strategy, named calorie restriction (CR), has been successfully tested in diverse eukaryotic species [12]. Thus, many efforts have been focused on delineating the molecular components linking metabolic balance induced by CR and the biology of aging, and the research has revealed a major importance of nutrient sensing in aging [13].

Nonetheless, besides the CR, lifespan extension can also be achieved by altering the diet composition; hence, nutrient and amino acid sensing mechanisms have emerged as attractive lifespan determinants. In the nutrient metabolism scene, current available evidence strongly supports the idea that amino acid (AA) sensing signaling can modify longevity [14]. The mammalian target of rapamycin (mTOR), a master growth regulator kinase, when part of mTOR complex 1 (mTORC1), can be activated by the absence of certain AAs (reviewed in [15]) and has been widely described as a key signaling pathway involved in aging [16–18]. Decreased activation of mTORC1 leads to lifespan extension in yeast, worms, flies, and mice [19], being the role of mTOR in aging extensively reviewed elsewhere [16–18]. We will focus on another, less explored in the field on aging, AA deficiency sensor, the kinase general control nonderepressive 2 (GCN2). The kinase GCN2 is a highly conserved nutrient sensor in eukaryotes at structural and functional levels [20, 21] and was identified as a critical regulator of cellular responses under AA deficiency [20, 22]. At molecular level, the mechanism of activation of GCN2 requires accumulation of uncharged tRNAs present near to the ribosome [23]. Once activated, GCN2 phosphorylates the alpha subunit of the eukaryotic initiation factor 2 alpha (eIF2α), causing general protein synthesis inhibition [20], an effect that promotes energy saving.

At the same time, the translation of specific genes is induced, being the one coding for the activating transcription factor 4 (ATF4), the best characterized [24, 25]. ATF4 is translated using an alternative and functional open reading frame of the *ATF4* gene [26, 27] and functions to promote cell survival and adaptation during stress and AA insufficiency [25, 28]. GCN2 and its downstream activation consequences are part of the signaling pathway called the Integrated Stress Response (ISR), because it integrates cellular responses to diverse stress stimuli such as nutrient deficiency (through GCN2), viral infection (through the double-stranded RNA-dependent protein kinase (PKR)), endoplasmic reticulum stress (through the PKR-like ER kinase (PERK)), and heme deprivation (through heme-regulated eIF2α kinase (HRI)) [29]. The activation of the ISR in response to nutrient starvation engages adaptive changes mediated by the induction of genes necessary to produce all the amino acids [30, 31], known as the amino acid stress response (AAR). The capacity of synthesis of AA is not only a crucial step for new protein synthesis. Amino acids serve a wide variety of cellular functions. For example, amino acids supply substrates to keep the Krebs cycle activity for ATP generation and also provide reducing equivalents for maintaining redox homeostasis [25]. Thus, the consequences of AAR can be considered a strategy to cope with metabolic stress and challenges after amino acid scarcity.

The contribution of AA deficiency signaling pathways to aging, as mentioned, has been widely described based on mTOR. However, the role of GCN2 has not been extensively approached, and how GCN2 impacts the cell biology during the aging course is a question that still has no answer.

3. GCN2: A Nutrient Sensor That Plays a Role in Lifespan Extension

The yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) has enormously contributed to the identification of mammalian genes that affect aging [32]. It was precisely in this model where there are first clues about the possible role of GCN2 in longevity [33], using the chronological aging model. In the chronological aging model, the length of time that yeast cells remain viable in a nondividing state is measured [32]. In the study by Wu et al., they showed that the extended survival induced by amino acid restriction observed in yeast was dependent on GNC2 [33]. Using another aging model in the same organism, the replicative aging, which tests the number of times a mother cell can divide and produce daughters [32], Tyler's group showed that Gcn2 (the yeast homolog of the mammalian GCN2) activation suppresses global translation efficiency, extending lifespan [34]. Considering that translation is a process that implies high energy expenditure (more than the 50% of the overall energy budget), they suggested that homeostasis is better preserved when transcript translation is reduced. Hence, reducing the translation entails a significant energy saving, which in turn could be used in restoring and maintaining cell requirements [34].

The other piece of evidence was obtained in mammals, which demonstrates that GCN2-deficient mice exhibit two main age-related effects [35] related to nutrient preference described in aging. First, GCN2 deficiency exacerbates aged mice' fat consumption at the expense of carbohydrate intake, and second, it prevented the increase in protein consumption. In this study, they suggested that GCN2 signaling might be an ancient pathway that contributes to the macronutrient selection and food preference [35]. Moreover, Kang et al. recently showed that, in response to dietary protein restriction, the lifespan of *Drosophila melanogaster* is extended, in a GCN2-ATF4 signaling axis-dependent manner [36]. The role of GCN2 as amino acid deficiency sensor has also been described in nematode *Caenorhabditis elegans* (*C. elegans*) [37], and the loss of GCN2 function is necessary for lifespan extension under nutritional stress [37]. Interestingly, for the protective role of CR on extension survival in *C. elegans*, the transcription factor PHA-4/FoxA is required [38]. PHA-4/FoxA (the nematode homolog of the mammal FoxA2) induces the expression of genes mostly involved in metabolic processes and defenses response [39]. In the context of amino AA deprivation, the extension of survival that depends on GCN2 involves the modulation of PHA-4/FoxA [37]. The other evidence is related to the protein IMPACT, which in mammals is a negative regulator of GCN2 [40]. Interestingly, the partial loss of function of IMPT-1 (yeast IMPACT Homolog 1) in *C. elegans* induces eIF2α phosphorylation even in a fed state. Moreover, *impt-1* knockdown exacerbates CR-induced extended lifespan and confers stress resistance [41]. This effect was dependent on other genes required for CR life extension, including *daf-16* (the yeast homolog of FOXO) and *skn-1* (the yeast homolog of Nrf 2). Thus, the discoveries found associating GCN2-dependent nutrient sensing and longevity suggest that under reduced amino acid availability, mRNA translation is inhibited and the expression of stress responses is activated, extending lifespan and improving healthspan, so far, in invertebrates. The beneficial effects of CR are also associated to the ISR activation. Still, whether GCN2 signaling or activation is impaired throughout aging in mammals is not known. Moreover, whether its functional modulation could exacerbate or attenuate the aging process is an issue that remains unclear.

Dietary methionine restriction (MR) is a proved approach to increase life span that has been shown in a variety of species (reviewed in [42]) and induce several physiological responses that confer resistance to metabolic disease [43–47]. The physiological responses to MR encompass adiposity decrease, energy expenditure increase, and thermogenic gene expression induction in the liver [48, 49]. The restriction of essential amino acids (EAAs), including methionine or leucine, limits aminoacylation of tRNAs by their cognate EAAs and induces the activation of GCN2 [50, 51]. Thus, it is possible that under MR, activation of GCN2 occurs, triggering the AAR that could be contributing to the beneficial effects observed under this specific AA diet restriction. Some of the metabolic effects of MR, including body weight reduction or elevated energy expenditure, are still present in GCN2-deficient mice [52]. GCN2 has been shown to play a pivotal role in the acute response to the essential amino acid deprivation induced by methionine restriction, while the long-term metabolic changes seem to be mediated

by a GCN2-independent eIF2α phosphorylation [52]. These results demonstrate the complexity of the response under AA deficiency and the presence of other sensing mechanisms of the MR phenotype. However, other responses associated to GCN2 activation, described in young or old, could be driven by this amino acid deficiency during aging. For instance, GCN2 protects against hepatotoxicity after AA depletion. GCN2-deficient animals lost the capacity to engage the AAR, which is associated with hepatic triglyceride accumulation, DNA damage, oxidative stress, and inflammation [53]. In the context of ischemia reperfusion (IRI) injury, GCN2 is required for protection from renal and hepatic IRI [54]. The work from Anthony et al.'s group has revealed the protective effects of GCN2 in the central nervous system. In a mouse model of leukodystrophy, a disorder characterized by degeneration of the white matter in the brain, GCN2 is essential for protecting glial cells during amino acid deficiency [55]. Together, these results demonstrate several GCN2 protective responses that could be activated during methionine deficiency, thus mediating, in part, the beneficial effect of MR.

MR has been shown to induce drastic genetic changes, mediated by the reduction of histone methylation [56]. It is also possible to consider long-term changes induced by GCN2 activation, driven by epigenetic changes as has been proposed for ATF4 in the context of nutrient deprivation in the fetus with consequences in adulthood [28]. Regarding aging, MR has been shown to extend healthspan and lifespan in progeroid mice [57]. Even though some of the consequences of MR in mammals are quite described [58], even more, its effects on accelerated aging models is also known [57]; still, *in vivo* genetic evidence of GCN2 function in the aging process remains unclear, revealing an information gap in the nutrition sensing scenery.

4. Nutrient Sensing Imbalance and Its Impact in the Redox Status along Aging

Aerobic cells and organisms have developed mechanisms for dealing with the oxidative stress implicated in the cell respiration, because of the reactive oxygen species (ROS) generated as byproducts in the oxidative phosphorylation process. In homeostatic conditions, antioxidants counteract the ROS oxidative damage, which is fundamental for proper mitochondrial, thus cellular function. Throughout aging, the respiratory chain becomes ineffective, leading to electron leakage accompanied by a decrease in ATP production [59]. Thereby, the aging process results in an oxidative imbalance yielded by the increased generation of ROS and/or lessen antioxidant defenses while concomitantly cells tend to accumulate aggregated proteins and dysfunctional mitochondria [2].

Even though the relationship between mitochondrial dysfunction, oxidative stress, and aging, at first sight, might seem intuitive, the experimental evidence has not been as clarifying as expected. Remarkably, the reduction of the antioxidant defenses can accelerate the aging process, speeding up the onset of neuropathological phenotypes related to aging, such as motor dysfunction, neuronal DNA damage, and neurodegeneration in flies and mice [60, 61]. On the other hand, there exist contradictory data, particularly the findings that the increase of ROS can extend the lifespan in invertebrate models like yeast and *C. elegans* [62–64]. While the evidence in mice is intriguing as well, given that the increase mitochondrial ROS and oxidative damage do not accelerate the aging process [65, 66], the increased antioxidant defenses extend longevity [67–71].

Despite the contradictory evidence, as a consensus in the field, the proper oxidative status of the cell, constituted by the fine balance between ROS and antioxidant defenses, is critical for the healthy cell function which in turn is controlled by different regulatory processes. Interestingly, one essential signaling that lately has been shown to be able to change the cell redox status is the nutrient sensing. How nutritional sensing and oxidative stress are integrated in the cell? *In vitro* and *in vivo* studies have demonstrated that the amino acid availability impacts in the intracellular amounts of antioxidants (for instance amino acids or glutathione), resulting in oxidative stress status changes [25, 72, 73]. This new dimension of nutrition as a modulator of the cellular oxidative status has just started to be explored in mammals. Chaveroux et al. identified GCN2 as new redox regulator that prevents oxidative stress *in vivo* [72], specifically through the transcriptional control of one of the main variants of the glutathione peroxidase 1 (GPX1), thus contributing to regulate the amount of oxidized proteins (carbonyl radicals) in the liver. In the same direction, GCN2 also can impact the redox cellular status through the regulation of autophagy in an inflammatory context. Particularly, GCN2 allows the occurrence of autophagy to increase in response to inflammation, which in turn blocks the augmentation of ROS [73]. This last finding is particularly interesting on the context of the latent proinflammatory phenotype accompanying aging [74]. Thus, nutrient sensing signaling pathways triggered by GCN2 could have protective consequences in maintaining a redox balance, in the described altered immune conditions associated with aging [74].

Interestingly, GCN2 also modulates cell cycle progression through the regulation of p53 function in the nucleolus. In conditions of GCN2 deletion, p53 is activated, arresting the cell cycle and inducing canonical transcriptional targets such as p21 [75]. This recently obtained piece of evidence opens a new perspective of the role that nutritional stress controls different cell processes, particularly the integration of amino acid requirements for the cell cycle progression. Considering the above evidence, the modulation of the nutrient sensing along the aging may not only be beneficial for allowing adaptation to metabolic stress *per se* but may also contribute to balance a wide spectrum of cell processes (Figure 2). In this regard, even though the classically described function of GCN2 is to be a kinase that is activated by amino acid deprivation inducing a nutrient stress response transcriptional program through ATF4 action, GCN2 also is able to regulate other cell processes such as autophagy, cell cycle, and redox status, in which GCN2 is being a central coordinator of metabolic homeostasis that integrates the nutritional requirements in a healthy cell balance. In the aging scenery, how GCN2 integrates these cellular processes in a fine-tuned

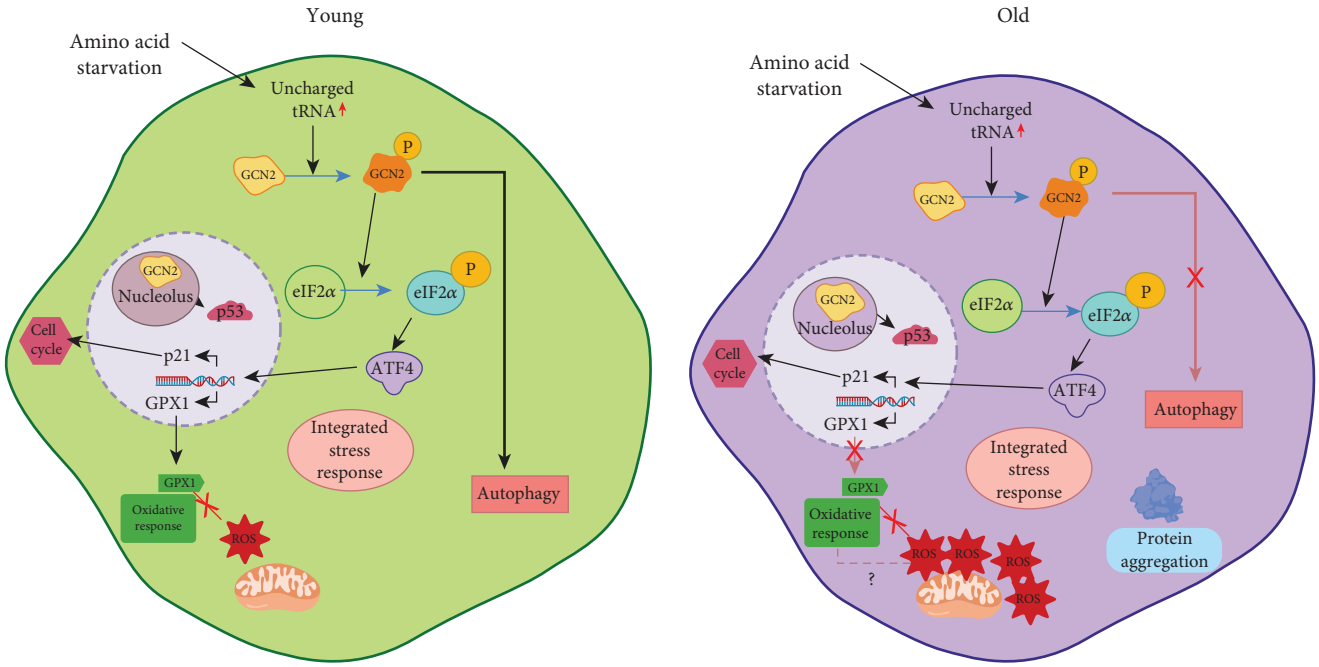

Figure 2: GCN2-dependent nutrient sensing along aging. Amino acid deprivation activates GCN2, a kinase that induces a nutrient stress response transcriptional program through ATF4 action. GCN2 also regulates other cell processes such as autophagy, cell cycle through p53 function, and redox status by transcriptionally modulating GPX1; this homeostatic balance can be altered throughout aging. ROS: reactive oxygen species; GPX1: glutathione peroxidase 1; GCN2: general control nonderepressive 2; eIF2α: eukaryotic initiation factor 2 alpha; ATF4: activating transcription factor 4.

balance and the involvement of its control in the healthy aging or its contribution to the molecular mechanism of age-related disorders remains unclear (Figure 2). Along with this line, given the experimental evidence available, one could speculate regarding the benefits that could implicate the GCN2 function and manipulation throughout the aging. Considering the classic dogma that during aging the accumulation of reactive oxygen species causes cumulative cell damage and senescence, GCN2 manipulation might ameliorate the oxidation observed during aging through the transcriptional control of key enzymes involved in the oxidative counterbalance such as GXP1, diminishing the mitochondrial functional decline and bioenergetics dysregulation commonly exhibited in aging [3]. Autophagy is another process that is regulated by starvation and also by oxidative stress; albeit during aging the autophagy markers has been shown to be both up- and downregulated, autophagy has been related to both normal and pathological conditions of inflammation [76] and the defective autophagy response is one of the causes that may contribute to the accumulation of proinflammatory damage that aged tissue exhibits [74]. In this regard, considering that GCN2 has been shown to be capable of modulating inflammation through autophagy [73, 77], the GCN2 function and intervention might represent an interesting tool for attenuating the autophagy-driven proinflammatory damage observed during normal and pathological aging.

It is important to mention that angiogenesis, namely, the formation of new blood vessels by endothelial cells, even though it is considered to be an adaptive response to oxygen and/or nutrient deprivation upon ischemia or exercise, is not considered a classical aging hallmark. In this regard, different angiogenic alterations observed through the aging process have been broadly reported. Particularly, a decline in microcirculation has been described, given by a reduction in capillary density throughout aging [78]. These functional changes can be explained mostly by decreased levels of angiogenic factors in aged individuals. The abovementioned gave rise to the "angiogenesis hypothesis of aging," in which proangiogenic therapies are proposed for ameliorating age-related symptoms (reviewed in [79]).

The canonical angiogenesis process is orchestrated by the vascular endothelial growth factor (VEGF), triggered by the hypoxia/nutrient deprivation during ischemia. Although both phenomena are difficult to dissect, hypoxia *per se* is a well-described VEGF inductor (through HIF1α transcriptional factor activation). Interestingly, recent studies have shown that nutrient deprivation, specifically amino acid starvation, is able to regulate angiogenesis by GCN2/ATF4 activation, in a hypoxia-independent manner, both *in vitro* and *in vivo* [80]. Particularly, GCN2/ATF4 activation shows to modulate both VEGF and hydrogen sulfide, a proangiogenic effector capable to induce the glucose intake and ATP production during endothelial cell migration [80]. Considering this piece of evidence, GCN2 stands out as a metabolic integrator able to modulate the response to stress in endothelial cells, also highlighting its therapeutic potential in ameliorating aging-related angiogenesis deficiencies, mainly improving the microcirculation condition in aged people.

5. Translational Potential and Therapies, Looking towards the Future

In the aging research field, the efforts now are directed to delay the aging process to diminish the vulnerability to the occurrence of age-related disorders. In terms of interventions, the dietary restriction stands out as an interesting tool. Dietary restriction, namely, the reduction of food intake without malnutrition, has the main advantage that is the less-invasive approach to be used in aged people. On the other hand, the main disadvantage of the dietary restriction is the low adherence to the treatment that might impact its therapeutic efficacy. Among these types of intervention, the most documented is the CR, which has bridged the gap between preclinical studies and human aging studies, demonstrating robustly that is able to increase lifespan in different mammalian models, including nonhuman primates (reviewed in [81]).

Particularly, amino acid deprivation has not been proved, so far, as a therapeutic strategy to modulate aging in humans. However, there exist preclinical and clinical data that show that dietary protein restriction is able to reduce the triglyceride levels in humans [82]. Considering that GCN2 has been shown to be involved in a wide variety of cellular phenomena, its manipulation is a promising tool that could implicate pleiotropic benefits. To date, the only type of intervention that has been done in order to regulate GCN2 function has been its pharmacological modulation through halofuginone (Hfg), an alkaloid originally isolated from the plant *Dichroa febrifuga* [83]. Hfg is an agonist able to activate GCN2; nevertheless, the preclinical studies using this drug have been directed mostly to the cancer research field (reviewed in [83]), evaluating how nutritional sensing modification can impact in cancer cell biology, and finally its therapeutic potential as an anticancer treatment.

However, so far, no treatment has been proven to modulate GCN2-mediated nutritional stress in aging, neither pharmacologically nor genetically.

6. Conclusion

The world is rapidly getting old [84], and as a consequence, aging has become an intense field of study. There is an enormous amount of studies aimed at understanding the molecular and cellular bases of aging seeking to preserve health in old stages of life. Nutritional strategies, including CR or MR, that extend life have given many clues about the signaling pathways involved in the aging process, in particular, the ones associated with nutrient sensing. Several pieces of evidence have shown that the AA deficiency sensor GCN2 and the signaling pathway triggered by its activation could be involved in the beneficial effects of restricted diets.

The GCN2 kinase has been studied in a variety of contexts including liver metabolism, innate immunity, cancer, and memory formation, among others [85]. Studies in nematode *C. elegans* have shown direct involvement of CGN2 in extending life span. However, its contribution to aging process in mammals remains unclear.

More than an AA-deficit sensing kinase, GCN2 appears to be a metabolic reprogramming controller that integrates and regulates key processes including autophagy, inflammation, and redox balance. Those are precisely contributors to aging process, and moreover, the consequences of GCN2 activation could be impacting cognitive function through neuronal and nonneuronal cells.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

All authors equally contributed to this manuscript.

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References

[1] B. J. Allison, J. J. Kaandorp, A. D. Kane et al., "Divergence of mechanistic pathways mediating cardiovascular aging and developmental programming of cardiovascular disease," *The FASEB Journal*, vol. 30, no. 5, pp. 1968–1975, 2016.

[2] M. P. Mattson and T. V. Arumugam, "Hallmarks of brain aging: adaptive and pathological modification by metabolic states," *Cell Metabolism*, vol. 27, no. 6, pp. 1176–1199, 2018.

[3] C. López-Otín, L. Galluzzi, J. M. P. Freije, F. Madeo, and G. Kroemer, "Metabolic control of longevity," *Cell*, vol. 166, no. 4, pp. 802–821, 2016.

[4] M. Kaeberlein, P. S. Rabinovitch, and G. M. Martin, "Healthy aging: the ultimate preventative medicine," *Science*, vol. 350, no. 6265, pp. 1191–1193, 2015.

[5] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, pp. 1194–1217, 2013.

[6] Y. W. Fridell and F. Sierra, "Metabolic aspects of aging," *Progress in Molecular Biology and Translational Science*, vol. 155, pp. 11–23, 2018.

[7] J. L. Kirkland, M. B. Stout, and F. Sierra, "Resilience in aging mice," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 71, no. 11, pp. 1407–1414, 2016.

[8] A. Catic, "Cellular metabolism and aging," *Progress in Molecular Biology and Translational Science*, vol. 155, pp. 85–107, 2018.

[9] M. Tatar, S. Post, and K. Yu, "Nutrient control of *Drosophila* longevity," *Trends in Endocrinology & Metabolism*, vol. 25, no. 10, pp. 509–517, 2014.

[10] N. M. Templeman and C. T. Murphy, "Regulation of reproduction and longevity by nutrient-sensing pathways," *The Journal of Cell Biology*, vol. 217, no. 1, pp. 93–106, 2018.

[11] C. M. McCay, M. F. Crowell, and L. A. Maynard, "The effect of retarded growth upon the length of life span and upon the

ultimate body size," *The Journal of Nutrition*, vol. 10, no. 1, pp. 63–79, 1935.

[12] L. Fontana and L. Partridge, "Promoting health and longevity through diet: from model organisms to humans," *Cell*, vol. 161, no. 1, pp. 106–118, 2015.

[13] N. E. Cummings and D. W. Lamming, "Regulation of metabolic health and aging by nutrient-sensitive signaling pathways," *Molecular and Cellular Endocrinology*, vol. 455, pp. 13–22, 2017.

[14] L. Fontana, L. Partridge, and V. D. Longo, "Extending healthy life span–from yeast to humans," *Science*, vol. 328, no. 5976, pp. 321–326, 2010.

[15] A. Efeyan, W. C. Comb, and D. M. Sabatini, "Nutrient-sensing mechanisms and pathways," *Nature*, vol. 517, no. 7534, pp. 302–310, 2015.

[16] M. Cornu, V. Albert, and M. N. Hall, "mTOR in aging, metabolism, and cancer," *Current Opinion in Genetics & Development*, vol. 23, no. 1, pp. 53–62, 2013.

[17] K. H. Schreiber, M. N. O'Leary, and B. K. Kennedy, "The mTOR Pathway and aging," in *Handbook of the Biology of Aging*, pp. 55–81, 2016.

[18] R. Zoncu, A. Efeyan, and D. M. Sabatini, "mTOR: from growth signal integration to cancer, diabetes and ageing," *Nature Reviews Molecular Cell Biology*, vol. 12, no. 1, pp. 21–35, 2011.

[19] S. C. Johnson, P. S. Rabinovitch, and M. Kaeberlein, "mTOR is a key modulator of ageing and age-related disease," *Nature*, vol. 493, no. 7432, pp. 338–345, 2013.

[20] T. E. Dever, L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch, "Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast," *Cell*, vol. 68, no. 3, pp. 585–596, 1992.

[21] R. C. Wek, H. Y. Jiang, and T. G. Anthony, "Coping with stress: eIF2 kinases and translational control," *Biochemical Society Transactions*, vol. 34, no. 1, Part 1, pp. 7–11, 2006.

[22] A. G. Hinnebusch, "Translational regulation of *GCN4* and the general amino acid control of yeast," *Annual Review of Microbiology*, vol. 59, no. 1, pp. 407–450, 2005.

[23] S. Zhu and R. C. Wek, "Ribosome-binding domain of eukaryotic initiation factor-2 kinase GCN2 facilitates translation control," *The Journal of Biological Chemistry*, vol. 273, no. 3, pp. 1808–1814, 1998.

[24] H. P. Harding, I. Novoa, Y. Zhang et al., "Regulated translation initiation controls stress-induced gene expression in mammalian cells," *Molecular Cell*, vol. 6, no. 5, pp. 1099–1108, 2000.

[25] H. P. Harding, Y. Zhang, H. Zeng et al., "An integrated stress response regulates amino acid metabolism and resistance to oxidative stress," *Molecular Cell*, vol. 11, no. 3, pp. 619–633, 2003.

[26] P. D. Lu, H. P. Harding, and D. Ron, "Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response," *The Journal of Cell Biology*, vol. 167, no. 1, pp. 27–33, 2004.

[27] K. M. Vattem and R. C. Wek, "Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 31, pp. 11269–11274, 2004.

[28] M. S. Kilberg, J. Shan, and N. Su, "ATF4-dependent transcription mediates signaling of amino acid limitation," *Trends in Endocrinology & Metabolism*, vol. 20, no. 9, pp. 436–443, 2009.

[29] K. Pakos-Zebrucka, I. Koryga, K. Mnich, M. Ljujic, A. Samali, and A. M. Gorman, "The integrated stress response," *EMBO Reports*, vol. 17, no. 10, pp. 1374–1395, 2016.

[30] A. G. Hinnebusch, "Translational regulation of yeast *GCN4* A window on factors that control initiator-trna binding to the ribosome," *The Journal of Biological Chemistry*, vol. 272, no. 35, pp. 21661–21664, 1997.

[31] P. P. Mueller and A. G. Hinnebusch, "Multiple upstream AUG codons mediate translational control of GCN4," *Cell*, vol. 45, no. 2, pp. 201–207, 1986.

[32] V. D. Longo, G. S. Shadel, M. Kaeberlein, and B. Kennedy, "Replicative and chronological aging in *Saccharomyces cerevisiae*," *Cell Metabolism*, vol. 16, no. 1, pp. 18–31, 2012.

[33] Z. Wu, L. Song, S. Q. Liu, and D. Huang, "Independent and additive effects of glutamic acid and methionine on yeast longevity," *PLoS One*, vol. 8, no. 11, article e79319, 2013.

[34] Z. Hu, B. Xia, S. D. Postnikoff et al., "Ssd1 and Gcn2 suppress global translation efficiency in replicatively aged yeast while their activation extends lifespan," *eLife*, vol. 7, 2018.

[35] A.-C. Maurin, C. Chaveroux, S. Lambert-Langlais et al., "The amino acid sensor GCN2 biases macronutrient selection during aging," *European Journal of Nutrition*, vol. 51, no. 1, pp. 119–126, 2012.

[36] M. J. Kang, D. Vasudevan, K. Kang et al., "4E-BP is a target of the GCN2–ATF4 pathway during *Drosophila* development and aging," *Journal of Cell Biology*, vol. 216, no. 1, pp. 115–129, 2017.

[37] A. Rousakis, A. Vlassis, A. Vlanti, S. Patera, G. Thireos, and P. Syntichaki, "The general control nonderepressible-2 kinase mediates stress response and longevity induced by target of rapamycin inactivation in *Caenorhabditis elegans*," *Aging Cell*, vol. 12, no. 5, pp. 742–751, 2013.

[38] S. H. Panowski, S. Wolff, H. Aguilaniu, J. Durieux, and A. Dillin, "PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*," *Nature*, vol. 447, no. 7144, pp. 550–555, 2007.

[39] M. Zhong, W. Niu, Z. J. Lu et al., "Genome-wide identification of binding sites defines distinct functions for *Caenorhabditis elegans* PHA-4/FOXA in development and environmental response," *PLoS Genetics*, vol. 6, no. 2, article e1000848, 2010.

[40] C. M. Pereira, E. Sattlegger, H. Y. Jiang et al., "IMPACT, a protein preferentially expressed in the mouse brain, binds GCN1 and inhibits GCN2 activation," *The Journal of Biological Chemistry*, vol. 280, no. 31, pp. 28316–28323, 2005.

[41] R. C. Ferraz, H. Camara, E. A. de-Souza et al., "IMPACT is a GCN2 inhibitor that limits lifespan in *Caenorhabditis elegans*," *BMC Biology*, vol. 14, no. 1, p. 87, 2016.

[42] B. C. Lee, A. Kaya, and V. N. Gladyshev, "Methionine restriction and life-span control," *Annals of the New York Academy of Sciences*, vol. 1363, no. 1, pp. 116–124, 2016.

[43] B. E. Hasek, A. Boudreau, J. Shin et al., "Remodeling the integration of lipid metabolism between liver and adipose tissue by dietary methionine restriction in rats," *Diabetes*, vol. 62, no. 10, pp. 3362–3372, 2013.

[44] B. E. Hasek, L. K. Stewart, T. M. Henagan et al., "Dietary methionine restriction enhances metabolic flexibility and increases uncoupled respiration in both fed and fasted states," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 299, no. 3, pp. R728–R739, 2010.

[45] V. L. Malloy, R. A. Krajcik, S. J. Bailey, G. Hristopoulos, J. D. Plummer, and N. Orentreich, "Methionine restriction

decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction," *Aging Cell*, vol. 5, no. 4, pp. 305–314, 2006.

[46] C. E. Perrone, D. A. L. Mattocks, M. Jarvis-Morar, J. D. Plummer, and N. Orentreich, "Methionine restriction effects on mitochondrial biogenesis and aerobic capacity in white adipose tissue, liver, and skeletal muscle of F344 rats," *Metabolism*, vol. 59, no. 7, pp. 1000–1011, 2010.

[47] E. P. Plaisance, T. M. Henagan, H. Echlin et al., "Role of β-adrenergic receptors in the hyperphagic and hypermetabolic responses to dietary methionine restriction," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 299, no. 3, pp. R740–R750, 2010.

[48] T. Laeger, D. C. Albarado, S. J. Burke et al., "Metabolic responses to dietary protein restriction require an increase in FGF21 that is delayed by the absence of GCN2," *Cell Reports*, vol. 16, no. 3, pp. 707–716, 2016.

[49] T. Laeger, T. M. Henagan, D. C. Albarado et al., "FGF21 is an endocrine signal of protein restriction," *Journal of Clinical Investigation*, vol. 124, no. 9, pp. 3913–3922, 2014.

[50] T. G. Anthony, B. J. McDaniel, R. L. Byerley et al., "Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2," *The Journal of Biological Chemistry*, vol. 279, no. 35, pp. 36553–36561, 2004.

[51] F. Guo and D. R. Cavener, "The GCN2 eIF2α kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid," *Cell Metabolism*, vol. 5, no. 2, pp. 103–114, 2007.

[52] D. Wanders, K. P. Stone, L. A. Forney et al., "Role of GCN2-independent signaling through a noncanonical PERK/NRF2 pathway in the physiological responses to dietary methionine restriction," *Diabetes*, vol. 65, no. 6, pp. 1499–1510, 2016.

[53] G. J. Wilson, P. Bunpo, J. K. Cundiff, R. C. Wek, and T. G. Anthony, "The eukaryotic initiation factor 2 kinase GCN2 protects against hepatotoxicity during asparaginase treatment," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 305, no. 9, pp. E1124–E1133, 2013.

[54] W. Peng, L. Robertson, J. Gallinetti et al., "Surgical stress resistance induced by single amino acid deprivation requires *Gcn2* in mice," *Science Translational Medicine*, vol. 4, no. 118, article 118ra111, 2012.

[55] P. She, P. Bunpo, J. K. Cundiff, R. C. Wek, R. A. Harris, and T. G. Anthony, "General control nonderepressible 2 (GCN2) kinase protects oligodendrocytes and white matter during branched-chain amino acid deficiency in mice," *Journal of Biological Chemistry*, vol. 288, no. 43, pp. 31250–31260, 2013.

[56] X. Tang, M. M. Keenan, J. Wu et al., "Comprehensive profiling of amino acid response uncovers unique methionine-deprived response dependent on intact creatine biosynthesis," *PLoS Genetics*, vol. 11, no. 4, article e1005158, 2015.

[57] C. Bárcena, P. M. Quirós, S. Durand et al., "Methionine restriction extends lifespan in progeroid mice and alters lipid and bile acid metabolism," *Cell Reports*, vol. 24, no. 9, pp. 2392–2403, 2018.

[58] R. S. McIsaac, K. N. Lewis, P. A. Gibney, and R. Buffenstein, "From yeast to human: exploring the comparative biology of methionine restriction in extending eukaryotic life span," *Annals of the New York Academy of Sciences*, vol. 1363, no. 1, pp. 155–170, 2016.

[59] D. R. Green, L. Galluzzi, and G. Kroemer, "Mitochondria and the autophagy–inflammation–cell death axis in organismal aging," *Science*, vol. 333, no. 6046, pp. 1109–1112, 2011.

[60] S. Melov, J. A. Schneider, B. J. Day et al., "A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase," *Nature Genetics*, vol. 18, no. 2, pp. 159–163, 1998.

[61] A. Paul, A. Belton, S. Nag, I. Martin, M. S. Grotewiel, and A. Duttaroy, "Reduced mitochondrial SOD displays mortality characteristics reminiscent of natural aging," *Mechanisms of Ageing and Development*, vol. 128, no. 11-12, pp. 706–716, 2007.

[62] R. Doonan, J. J. McElwee, F. Matthijssens et al., "Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans," *Genes & Development*, vol. 22, no. 23, pp. 3236–3241, 2008.

[63] A. Mesquita, M. Weinberger, A. Silva et al., "Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H_2O_2 and superoxide dismutase activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 34, pp. 15123–15128, 2010.

[64] J. M. Van Raamsdonk and S. Hekimi, "Deletion of the mitochondrial superoxide dismutase *sod-2* extends lifespan in *Caenorhabditis elegans*," *PLoS Genetics*, vol. 5, no. 2, article e1000361, 2009.

[65] H. Van Remmen, Y. Ikeno, M. Hamilton et al., "Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging," *Physiological Genomics*, vol. 16, no. 1, pp. 29–37, 2003.

[66] Y. Zhang, Y. Ikeno, W. Qi et al., "Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 64A, no. 12, pp. 1212–1220, 2009.

[67] D. Edgar, I. Shabalina, Y. Camara et al., "Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice," *Cell Metabolism*, vol. 10, no. 2, pp. 131–138, 2009.

[68] A. Hiona, A. Sanz, G. C. Kujoth et al., "Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice," *PLoS One*, vol. 5, no. 7, article e11468, 2010.

[69] G. C. Kujoth, A. Hiona, T. D. Pugh et al., "Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging," *Science*, vol. 309, no. 5733, pp. 481–484, 2005.

[70] V. I. Pérez, H. Van Remmen, A. Bokov, C. J. Epstein, J. Vijg, and A. Richardson, "The overexpression of major antioxidant enzymes does not extend the lifespan of mice," *Aging Cell*, vol. 8, no. 1, pp. 73–75, 2009.

[71] A. Trifunovic, A. Wredenberg, M. Falkenberg et al., "Premature ageing in mice expressing defective mitochondrial DNA polymerase," *Nature*, vol. 429, no. 6990, pp. 417–423, 2004.

[72] C. Chaveroux, S. Lambert-Langlais, L. Parry et al., "Identification of GCN2 as new redox regulator for oxidative stress prevention *in vivo*," *Biochemical and Biophysical Research Communications*, vol. 415, no. 1, pp. 120–124, 2011.

[73] R. Ravindran, J. Loebbermann, H. I. Nakaya et al., "The amino acid sensor GCN2 controls gut inflammation by inhibiting

inflammasome activation," *Nature*, vol. 531, no. 7595, pp. 523–527, 2016.

[74] A. Salminen, K. Kaarniranta, and A. Kauppinen, "Inflammaging: disturbed interplay between autophagy and inflammasomes," *Aging*, vol. 4, no. 3, pp. 166–175, 2012.

[75] A. Nakamura and H. Kimura, "A new role of GCN2 in the nucleolus," *Biochemical and Biophysical Research Communications*, vol. 485, no. 2, pp. 484–491, 2017.

[76] M. Martinez-Vicente and A. M. Cuervo, "Autophagy and neurodegeneration: when the cleaning crew goes on strike," *The Lancet Neurology*, vol. 6, no. 4, pp. 352–361, 2007.

[77] S. Battu, S. Afroz, J. Giddaluru et al., "Amino acid starvation sensing dampens IL-1β production by activating ribiclustering and autophagy," *PLoS Biology*, vol. 16, no. 4, article e2005317, 2018.

[78] R. S. Sweat, D. C. Sloas, S. A. Stewart et al., "Aging is associated with impaired angiogenesis, but normal microvascular network structure, in the rat mesentery," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 312, no. 2, pp. H275–H284, 2017.

[79] C. T. Ambrose, "Pro-angiogenesis therapy and aging: a mini-review," *Gerontology*, vol. 63, no. 5, pp. 393–400, 2017.

[80] A. Longchamp, T. Mirabella, A. Arduini et al., "Amino acid restriction triggers angiogenesis via GCN2/ATF4 regulation of VEGF and H_2S production," *Cell*, vol. 173, no. 1, pp. 117–129.e14, 2018.

[81] P. Balasubramanian, P. R. Howell, and R. M. Anderson, "Aging and caloric restriction research: a biological perspective with translational potential," *eBioMedicine*, vol. 21, pp. 37–44, 2017.

[82] J. H. Treviño-Villarreal, J. S. Reynolds, A. Bartelt et al., "Dietary protein restriction reduces circulating VLDL triglyceride levels via CREBH-APOA5–dependent and –independent mechanisms," *JCI Insight*, vol. 3, no. 21, 2018.

[83] M. Pines and I. Spector, "Halofuginone — the multifaceted molecule," *Molecules*, vol. 20, no. 1, pp. 573–594, 2015.

[84] B. K. Kennedy and J. K. Pennypacker, "Drugs that modulate aging: the promising yet difficult path ahead," *Translational Research*, vol. 163, no. 5, pp. 456–465, 2014.

[85] B. A. Castilho, R. Shanmugam, R. C. Silva, R. Ramesh, B. M. Himme, and E. Sattlegger, "Keeping the eIF2 alpha kinase Gcn2 in check," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1843, no. 9, pp. 1948–1968, 2014.

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Review Article

Mitochondria- and Oxidative Stress-Targeting Substances in Cognitive Decline-Related Disorders: From Molecular Mechanisms to Clinical Evidence

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Alzheimer's disease (AD) is the most common form of dementia affecting people mainly in their sixth decade of life and at a higher age. It is an extensively studied neurodegenerative disorder yet incurable to date. While its main postmortem brain hallmarks are the presence of amyloid-β plaques and hyperphosphorylated tau tangles, the onset of the disease seems to be largely correlated to mitochondrial dysfunction, an early event in the disease pathogenesis. AD is characterized by flawed energy metabolism in the brain and excessive oxidative stress, processes that involve less adenosine triphosphate (ATP) and more reactive oxygen species (ROS) production respectively. Mitochondria are at the center of both these processes as they are responsible for energy and ROS generation through mainly oxidative phosphorylation. Standardized *Ginkgo biloba* extract (GBE), resveratrol, and phytoestrogens as well as the neurosteroid allopregnanolone have shown not only some mitochondria-modulating properties but also significant antioxidant potential in *in vitro* and *in vivo* studies. According to our review of the literature, GBE, resveratrol, allopregnanolone, and phytoestrogens showed promising effects on mitochondria in a descending evidence order and, notably, this order pattern is in line with the existing clinical evidence level for each entity. In this review, the effects of these four entities are discussed with special focus on their mitochondria-modulating effects and their mitochondria-improving and antioxidant properties across the spectrum of cognitive decline-related disorders. Evidence from preclinical and clinical studies on their mechanisms of action are summarized and highlighted.

1. Introduction

1.1. Alzheimer's Disease: A Well-Known yet Untreatable Age-Related Neurodegenerative Disorder. Alzheimer's disease (AD), the most common neurodegenerative disorder, as well as dementia type, is characterized by extracellular senile beta-amyloid protein (Aβ) plaques and intracellular neurofibrillary tau tangles [1]. There are two types of AD: (i) the sporadic form of AD (SAD) whose onset occurs above the age of 65 and (ii) the familial AD forms (FAD) that are more rare with less than 1% occurrence among the AD cases and whose onset starts at a younger age (<65 years). The biological system of aging is the major risk factor of SAD [2]. The familial forms (FAD) bear inheritable mutations in the amyloid precursor protein (APP) and presenilin 1 and presenilin 2 genes [3, 4]. The symptoms of AD are the same in SAD and FAD [5]. There are different types of age-related cognitive diseases which differ in severity. SMI (subjective memory impairment) is the condition when nondemented aged people express subjective complaints related to their memory but have no organic or identifiable condition [6]. SMI is discussed as an early predictor of dementia [7–10]. The concept of mild cognitive impairment (MCI) defines an intermediate stage between normal aging and dementia. MCI patients show mild but measurable changes in cognitive tests and thinking abilities that are noticeable to the patients and to family members, but they are able to carry out everyday activities. Approximately 15-20% of people aged 65 or older have MCI. This group of people represents a population at increased risk for developing dementia, especially MCI

involving memory problems [11]. The occurrence of MCI in the population is 3.2%, of which 11.1% of the cases convert to dementia within 3 years [12]. It has been indicated, yet not conclusively, that SMI is a precursor of MCI which can then lead to dementia or AD [4, 13]. Dementia is a more severe condition compared to SMI and MCI which affects aged people and interferes negatively in the performance of everyday activities. It is described as a cluster of symptoms related to mental, cognitive, and memory decline [12, 14]. There are different forms of dementia, such as AD, the most common type, and vascular dementia. Vascular dementia (VaD) is the second most common form of dementia and occurs as a cognitive decline due to a reduced blood flow in the brain (e.g., due to brain injury or stroke). However, sometimes different kinds of dementias coexist and their discrimination is difficult due to overlapping clinical symptoms. Moreover, many of these patients also suffer from psychiatric or behavioural problems that are sometimes referred to as BPSD (behavioural and psychological symptoms of dementia) or NPS (neuropsychiatric symptoms), including irritability, anxiety, psychosis, and aggression [15].

1.2. Mitochondria and Neuroplasticity. Mitochondria are essential yet independent organelles contained in eukaryotic cells, and they are responsible for numerous functional activities within the cells. However, they are not always an intrinsic structure of eukaryotic cells. They occur through the endosymbiosis of an alpha-proteobacterium into a prokaryotic progenitor, and this is why they contain their own DNA, namely, the mitochondrial DNA (mtDNA) [16]. Regarding the structural characteristics of these organelles, they contain two structurally and functionally distinct membranes, the outer and the inner membranes. The inner membrane encapsulates the matrix and also carries the electron transport chain (ETC) where oxidative phosphorylation (OXPHOS) is taking place. mtDNA is located in the matrix encoding 13 proteins which are used as structural components of the ETC complexes [17].

Mitochondria have obtained the title of "powerhouse of the cell" due to their ability of producing the energy, mainly through OXPHOS, required for the survival and functioning of the cell. Actually, they are more than just a "powerhouse" as they are the ultimate multitaskers which define the cell fate. Apart from the production of energy in the form of ATP, mitochondria are the key modulators of brain cell survival and death by controlling calcium (Ca^{2+}) and redox equilibrium (which in turn affects neurotransmitter release and neuronal plasticity), by producing reactive oxygen species (ROS), and by controlling cell apoptosis [17–19]. The brain is an organ which requires a considerable amount of energy in order to operate, maintain, and enhance neuronal functions and plasticity. Neurons are postmitotic polarized cells with significant energy demands. OXPHOS, taking place in mitochondria, is the main energy provider in the form of ATP, and neurons depend almost solely on this procedure in order to satisfy their energy needs [20]. In particular, neurons direct this energy into the formation of interconnections, the synapses. The number and strength of these neuron interconnections define synaptic plasticity, which is responsible for cognitive function [21]. Synaptic plasticity is a crucial mechanism by which the neural activity generated by an experience alters synaptic transmission and therefore modifies brain function [22]. Neurite outgrowth is a process wherein developing neurons generate new projections as they grow in response to guidance cues. Nerve growth factors (NGF), or neurotrophins, are one family of such stimuli that regulate neurite growth [23]. Brain-derived neurotrophic factor (BDNF) exerts several actions on neurons ranging from the acute enhancement of transmission to long-term promotion of neurite outgrowth and synaptogenesis [24, 25]. Synaptic plasticity includes the dynamic regulation of long-term potentiation (LTP), spine density, and the number and length of dendrites and axons (neuritogenesis), as well as neurogenesis. Adult neurogenesis generates functional neurons from adult neural precursors in restricted brain regions throughout life [26]. These plasticity processes need a high energy requirement, and this is why mitochondria play such a pivotal role in the well-being of neurons especially when neurons need to adapt to periods of pathologically reduced functions.

1.3. Mitochondria, Oxidative Stress, Aging, and AD. However, while mitochondria regulate the functions of healthy neurons, they are also largely affected during aging and pathological states such as age-related neurodegenerative diseases. Mitochondria are not only the regulators of energy metabolism but are also the main ROS generators. ROS are immensely reactive species which are produced in mitochondria mainly by complexes I and III of the ETC when there is a leak of electrons. They are chemical species including hydroxyl radical ($^{\bullet}OH$), superoxide anion (O_2-), and hydrogen peroxide (H_2O_2) which can interact with and damage DNA and proteins and lipids which can compromise cell survival leading to aging and to vulnerability to several diseases [27, 28]. When they exist at normal levels, they constitute signalling agents in many physiological processes, such as redox homeostasis, cellular death, cellular senescence, and cell proliferation, and they can also trigger immune responses, synaptic plasticity, and cognitive enhancement [20, 27]. ROS are neutralized by antioxidant enzymes such as superoxide dismutase, which transforms the radicals (O_2-) into H_2O_2, and by catalase, glutathione peroxidase, and thioredoxin peroxidase, which diffuse H_2O_2 [27]. In a healthy state, there is a balance between ROS production and neutralization. Nevertheless, when ROS are produced in excess, e.g., during aging, they directly affect mitochondria since mitochondrial membranes consist of long polyunsaturated fatty acids which are easily oxidized. Also, mtDNA is found in close proximity to the ROS source and is susceptible to mutations resulting in the production of faulty ETC proteins, leading back to the production of more ROS [18, 28]. It could be said that mitochondria are the main organelles in aging and neurodegeneration by being both generators and targets of ROS. It has been shown that aging is characterized by a rise in oxidative stress, a decline in antioxidant defense systems, and an impairment of the OXPHOS. So, aging is characterized by energy deprivation and a shift of the redox state towards oxidation. Mitochondria are at the

center of these hallmarks [20]. Neurons, which highly depend on OXPHOS to satisfy their energy demands, are particularly susceptible to energy hypometabolism [20]. In addition, taking into account that they are nondividing cells, neurons are almost as old as the entire organism and are not replaced during life with the exception of the hippocampus that continuously generates new neurons during adulthood [20, 29]. This means that neurons accumulate oxidative stress and therefore defective mitochondria during aging [20, 30]. This is particularly important since mitochondrial dysfunction represents an early event in AD pathogenesis [20, 28, 31].

Intense oxidative stress and decreased brain energy metabolism are common characteristics of both normal aging and AD, although to different extents [20]. Of note, mitochondrial abnormalities are observed in FAD and SAD brains [32, 33]. On one hand, recent data obtained from AD models, in which mitochondrial failure is a prominent feature, implicate tau hyperphosphorylation as well as Aβ overproduction and deposition. On the other hand, Aβ and tau target mitochondria synergistically, thereby possibly amplifying each other's toxic effects. This interrelationship of Aβ, tau, and mitochondrial function constitutes a vicious cycle [34]. The mitochondrial cascade hypothesis postulates that mitochondrial dysfunction represents the most upstream pathology in AD [28]. According to this hypothesis, arresting brain aging will prevent the development of AD [32].

1.4. Mitochondria-Directed Natural Compounds. The current mitochondrial cascade hypothesis postulates mitochondrial dysfunction as a central pathomechanism in age-related degenerative disorders [28, 35, 36]. Taking into account their primary role in aging and in the early stages of AD, mitochondria constitute promising targets for therapeutic strategies. For this reason, pharmacological studies are directed in enhancing mitochondrial functions such as ATP production and respiration or in reducing mitochondrial harmful by-products such as ROS [36]. To date no drugs are able to cure or stop the progression of age-related neurodegenerative disorders. Most of them may be beneficial in delaying the progression of AD and only partially treat some of its symptoms (e.g., confusion and memory loss).

Many drugs including whole plant extracts and single compounds originate from natural and botanical sources. Two single compound AD drugs are derived from plants: (i) the acetylcholine-esterase inhibitor, galanthamine, derived from the *Galanthus* species (*Galanthus caucasicus* and *Galanthus woronowii*) and (ii) rivastigmine, a physostigmine analogue (physistigmine was isolated from the Calabar bean, *Physostigma venenosum*) [37, 38]. In addition, the phytopharmacon GBE that is used as antidementia medicine was shown to improve mitochondrial function emphasizing the concept of targeting mitochondria as an emerging and promising therapeutic approach [35, 39]. Therefore, we focused our search on natural compounds that possess mitochondria-enhancing properties based on our own past and ongoing research as well as on research of other groups. Standardized *Gingko biloba* extract (GBE), resveratrol, phytoestrogens, and the natural neurosteroid allopregnanolone fulfilled our criteria. Common targets of these agents (Figure 1) have been reported, such as ROS, mitochondrial membrane potential (MMP), Aβ, tau protein, antiapoptotic protein (Bcl-2), and OXPHOS (Figures 2 and 3). Accordingly, in this review we aimed to summarize the molecular modes of action of these natural agents with special focus on mitochondria, their mitochondrial function-enhancing properties, and their antioxidant properties. We discuss evidence on their mechanism of action from preclinical as well as clinical studies. Especially regarding clinical trials, there is a different level of existing evidence for each phytochemical. GBE, resveratrol, phytoestrogens, and allopregnanolone appear in a descending order according to their clinical evidence level. The databases PubMed and Google Scholar, as well as the database ClinicalTrials.gov were used for our search with a focus on the years 2000–2018. For the clinical evidence, we considered randomized, double-blind, placebo-controlled trials as well as ongoing trials, systematic reviews, meta-analyses, and Cochrane analyses.

2. Pharmacologic Features of Natural Substances in Alzheimer's Disease

2.1. Gingko biloba. Gingko biloba has existed for over 250 million years and is a native from Japan, Korea, and China; however, it can be found worldwide. Traditional Chinese clinicians originally utilized GBE for a variety of applications [40]. There are several *Ginkgo biloba* extracts sold on the market, including standardized and nonstandardized extracts, which are also used in studies. The standardized extracts have to meet specific criteria regarding their manufacturing process, the quality of the plant material, and their composition, which is not the case with the nonstandardized extracts. Many products have been reported on the market which are not standardized and are even adulterated. These products not only reduce the efficacy of GBE, but they can be potentially harmful [41]. GBE contains two main groups of active constituents ensuring its medicinal effects: terpenes (including bilobalide and ginkgolides A, B, and C) and flavonoids (including meletin, isorhamnetin, and kaempferol). Both the United States Pharmacopoeia and the European Pharmacopoeia define as standardized only extracts that contain the active components of *Ginkgo* in a certain and defined content. In particular, the standardized extracts should contain 5-7% triterpene lactones, 22-27% flavonoids, and less than 5 ppm of ginkgolic acids, which are toxic ingredients of *Ginkgo*. [42]. Most toxicological, pharmacological, and clinical investigations have focused on the neuroprotective value of two main standardized extracts labeled EGb761 and LI 1370 [43–45]. The EGb761 extract consists of 24% flavone glycosides (mainly quercetin, kaempferol, and isorhamnetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B, and C and 2.6-3.2% bilobalide), while the extract LI 1370 is composed of 25% ginkgo flavone glycosides as well as 6% terpenoids. Several terpene lactones (ginkgolides and bilobalide) show substantial mitochondria-protecting properties, while the flavonoid fraction seems to play an important role in the free radical scavenging properties [46]. In

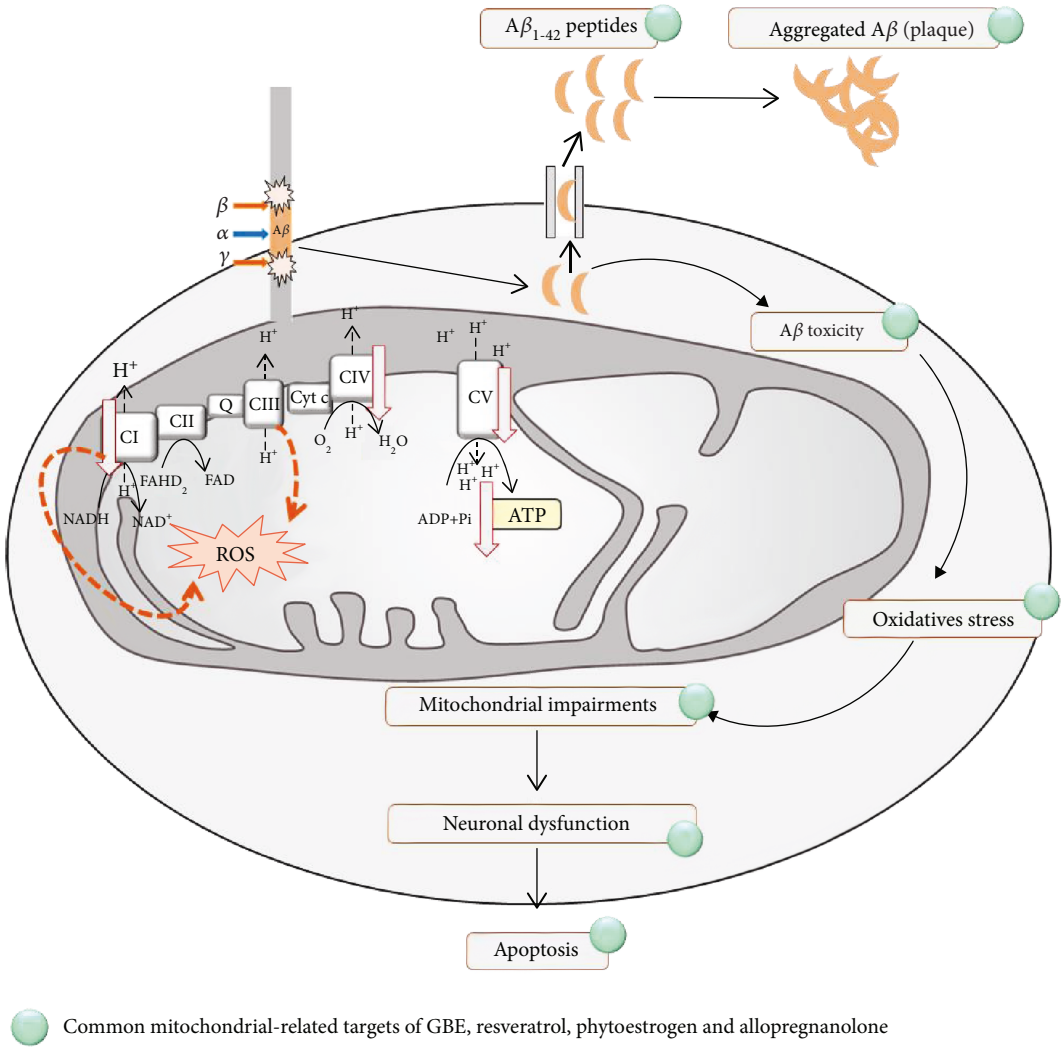


Figure 1: Common mitochondria-related targets of natural substances in neuroprotection. In AD, the precursor of amyloid protein APP is cleaved sequentially by β- and γ-secretases leading to the production of Aβ peptides, their aggregation, and the formation of extracellular plaques. Different Aβ species exist, but A$\beta_{1\text{-}42}$ is one of the most abundant and is the one that is mainly deposited in the brain due to its hydrophobic and fibrillogenic nature. AD is associated with electron transport chain (ETC) impairments leading to decreased ATP levels and basal respiration, with a decrease of antioxidant defenses and an increase of ROS production by complex I and complex III (orange dashed arrows). Globally, *Gingko biloba*, resveratrol, and phytoestrogens have been shown to protect against cell death in AD through a common mechanism of action by reducing abnormal aggregation of Aβ, amyloid beta (Aβ) toxicity, oxidative stress, mitochondrial impairments leading to neuronal dysfunction, and apoptosis. *Gingko biloba*, resveratrol, and phytoestrogens are suggested to exert a beneficial effect in AD affected neurons, but their specific mechanisms of mitochondrial interaction are not fully described yet. ↓: AD-related decrease. The green circle indicates the common mitochondria-related targets of GBE, resveratrol, phytoestrogen, and allopregnanolone.

the following parts, only the effects of standardized GBE will be discussed.

2.1.1. Mechanisms of Action Based on Preclinical Evidence

2.1.1.1. Direct Effects of GBE on Mitochondria. Several findings demonstrate the mitochondria-modulating effect of GBE, mainly in cellular and animal models of AD. In particular, GBE has been shown to attenuate effectively mitochondrial dysfunction through several mechanisms of action, such as antioxidant effect and free radical scavenging properties, with all the evidence leading to this conclusion having been reviewed extensively [35, 47–49]. *In vitro*, GBE was shown to ameliorate mitochondrial function by improving MMP and ATP levels at a low concentration of 0.01 mg/ml in pheochromocytoma cells (PC12) cells [46]. In amyloid precursor protein- (APP-) transfected human neuroblastoma cells, an AD cellular model with increased Aβ generation, GBE improved respiration of mitochondria, stimulated mitochondrial biogenesis, and increased ATP production [50]. Mitochondria-related modes of action of GBE are summarized in Figure 2.

2.1.1.2. Effects of GBE on Oxidative Stress, Aβ, and Tau Toxicity Related to Damage of Mitochondria. Aβ plaque deposition is one of the main hallmarks of AD. The

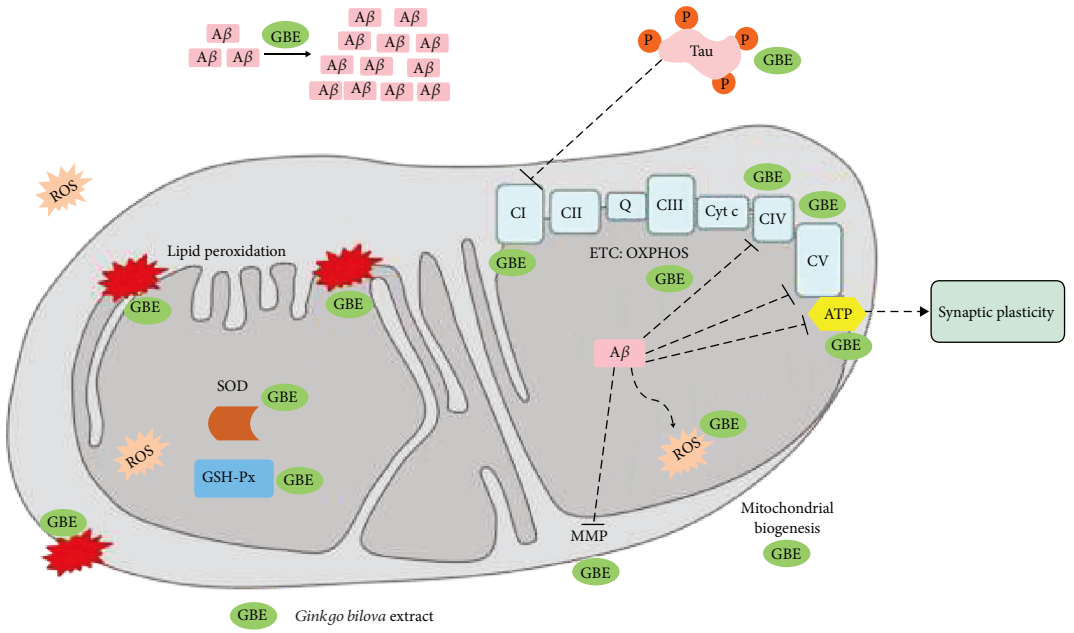


FIGURE 2: The effects of Aβ, hyperphosphorylated tau, and standardized *Ginkgo biloba* extract (GBE) on mitochondrial function in AD. It has been shown that mitochondrial dysfunction is a key feature in AD and plays a pivotal role on the onset of the disease. While defining the chronologically first hallmark of the disease can be puzzling, there is evidence about mitochondrial dysfunction being the first hallmark at the early stages of AD with Aβ occurring as a result. Aβ has been shown to cause a decline in OXPHOS, taking place at the ETC, which leads to defective complexes IV and V and decreased ATP production. Faulty OXPHOS function results in the production of ROS which, when in excess, cannot be counterbalanced by the antioxidant enzymes like GSH-Px and SOD. ROS can cause membrane lipid peroxidation and instable MMP. Hyperphosphorylated tau inhibits complex I activity. However, GBE has been proven to reduce Aβ aggregation and tau hyperphosphorylation and to enhance OXPHOS, activities of complexes, and ATP levels, as well as to restore MMP. ROS and consequently lipid peroxidation are reduced due to GBE, while the extract has the ability to enhance SOD and GSH-Px activity and also induce mitochondrial biogenesis. $\downarrow$: represents increase; $\perp$: represents inhibition.

overexpression of both Aβ itself and its precursor protein, the amyloid precursor protein (APP), has been used to create cellular and animal models of AD. GBE has been shown to be effective in reducing both the deposition of Aβ and its toxicity. In detail, the prooxidant $A\beta_{25\text{-}35}$ peptide treatment was shown to decrease complex I and IV activities and to increase the level of reactive oxygen/reactive nitrogen species (ROS/RNS) in SH-SY5SY cells [51]. Thus, pretreatment with GBE was able to reduce the Aβ-related increase in ROS/RNS levels as well as to ameliorate the complex I and IV activities [51]. GBE protected against $A\beta_{1\text{-}42}$ oligomer-induced neurotoxicity and cell damage with an indirect effect on SH-SY5Y neuroblastoma cells by improving Hsp70 protein expression and subsequently by activating the Akt (protein kinase B) pathways as well as ER stress [52]. GBE also attenuated $A\beta_{1\text{-}42}$ oligomer-induced cell damage and protected against Aβ toxicity and oxidative stress [53, 54], as well as apoptosis [52]. GBE was also able to reduce Aβ production [55]. In terms of animal models, a chronic treatment with GBE improved cognitive defects in a transgenic mouse model of AD (Tg2576), a model that overexpresses a mutant form of APP [53]. GBE was also shown to decrease Aβ oligomers and to significantly increase neuronal proliferation in the hippocampus of young (6 months) and old (22 months) mice in a double transgenic mouse model (TgAPP/PS1) [54]. A chronic daily treatment with GBE for 6 months improved the cognitive function and alleviated amyloid plaque deposition in two-month-old APP/PS1 mice. Of note, GBE treatment seems to decrease the level of insoluble Aβ, while the soluble content of Aβ was unchanged [56]. GBE reduced the hyperphosphorylation of tau at AD-specific Ser262, Ser404, Ser396, and Thr231 sites, rescued the activity of tau phosphatase PP2Ac and kinase GSK3β, and reduced the oxidative stress in the hippocampus and prefrontal cortex on a hyperhomocysteinemia-treated rat model of AD. Memory lesions were also restored, and the expression of synapse-associated protein PSD95 and synapsin-1 protein was upregulated [57].

2.1.1.3. Effects of GBE on Neuroplasticity Pathways. GBE exerts its beneficial effects not only by acting on the Akt pathway, as aforementioned, but also by acting on the cyclic AMP response element-binding protein (CREB) [54, 58, 59]. CREB phosphorylation induces transcriptional activation which results in the expression of BDNF, and therefore, in synaptic plasticity and cognitive enhancement. Conversely, lack of CREB phosphorylation is a pathological ailment of neurodegenerative diseases such as AD [60].

In detail, the administration of GBE restored CREB phosphorylation in the hippocampus of TgAPP/PS1 mice [54]. Quercetin and bilobalide are the major constituents that have contributed to GBE-induced neurogenesis [58]. Both constituents promoted dendritic processes in hippocampal neurons

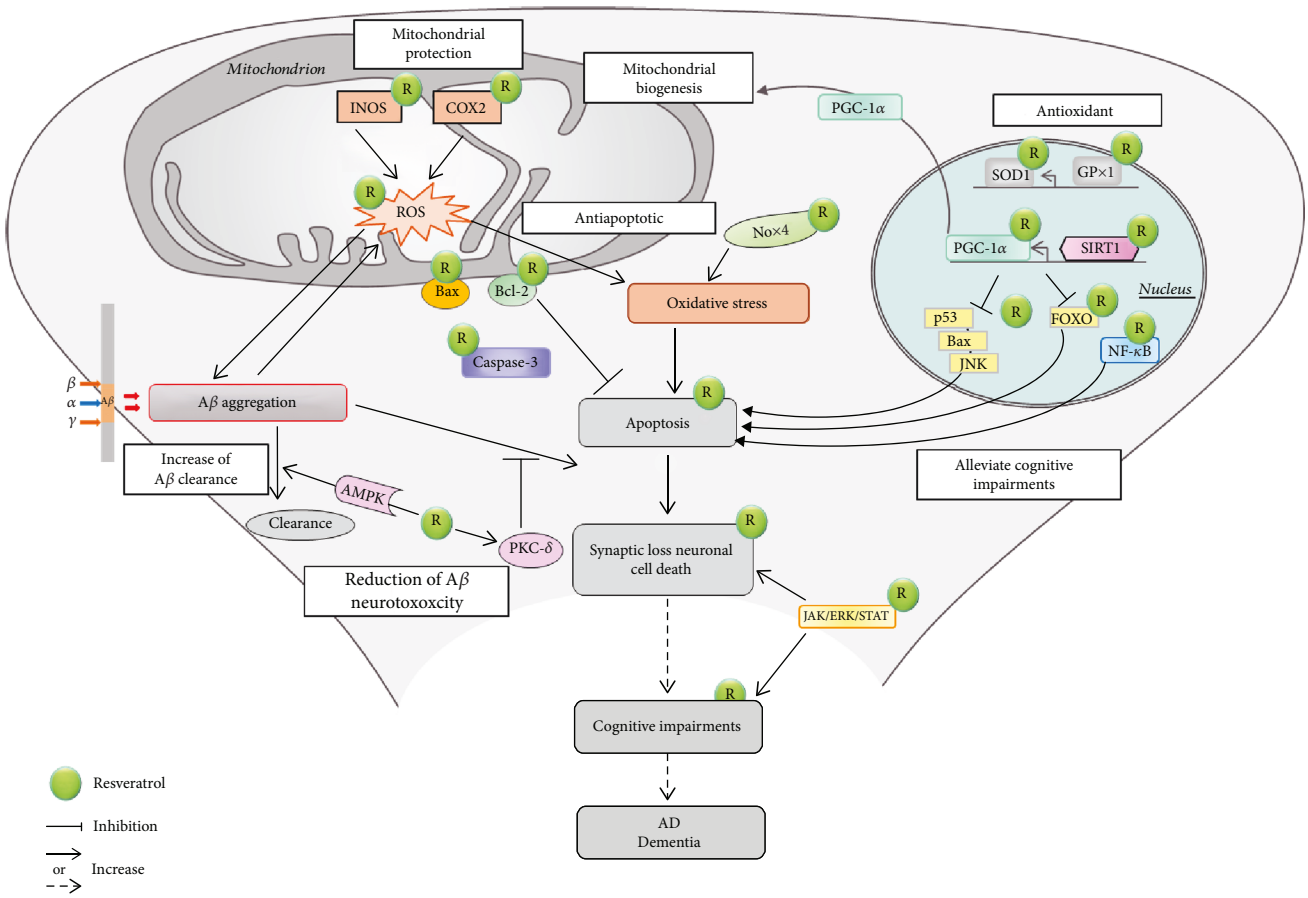
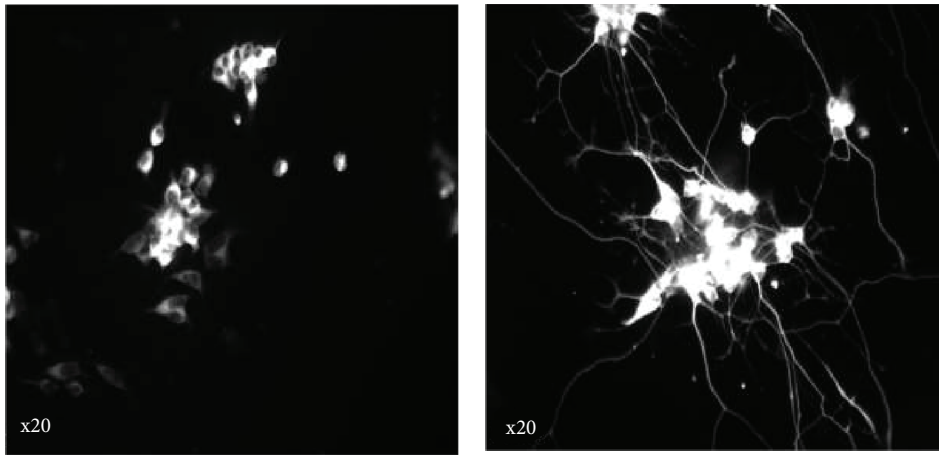


Figure 3: Neuroprotective effects of resveratrol in AD. The precursor of amyloid protein APP is cleaved sequentially by β- and γ-secretases leading to the production of Aβ and their aggregation. Resveratrol increases the clearance of Aβ peptides through the activation of AMPK. Resveratrol plays an important role in the neuroprotective properties as it reduces Aβ neurotoxicity by phosphorylating PKC-δ. Damaged mitochondria generate ROS which are implicated in apoptosis. iNOS and COX-2 also enhance the production of ROS. Resveratrol exerts antioxidant properties and attenuates oxidative damage by decreasing iNOS and COX-2 levels. Resveratrol also protects mitochondria by increasing the expression of ROS-inactivating enzymes GPx1 as well as SOD1 and by reducing the expression of the ROS-producing enzyme Nox4. Resveratrol also influences the Aβ-induced apoptotic signalling pathway by inhibiting the expression of caspace-3, Bax, FOXO, and p53 by blocking the activation of JNK and by restoring the decrease of Bcl-2 expression, as well as by inhibiting the increase of NF-κB DNA binding. Mitochondrial biogenesis is induced by resveratrol through SIRT1 activation and deacetylation of PGC-1α. Resveratrol was also able to protect hippocampal neurons by alleviating cognitive impairment and reducing neuronal loss via modulating the janus kinases, extracellular signal-regulated kinases, and signal transducers, as well as the signalling pathway of the activators of transcription (JAK/ERK/STAT).

and restored Aβ oligomer-induced synaptic loss, as well as restored CREB phosphorylation [58]. Ginkgo flavonols quercetin and kaempferol have been shown to stimulate BDNF and phosphorylation of CREB in neurons isolated from double transgenic AD mouse (TgAPPswe/PS1e9) [59]. Recently, our team could confirm the neurite outgrowth stimulating effects of GBE in a 3D cell culture model (Figure 4).

2.1.2. Clinical Evidence. Apart from the preclinical studies, the extract has been largely investigated in clinical trials in a range of age-associated cognitive conditions from SMI and MCI to dementia and AD. GBE has been suggested for both the symptomatic treatment and prevention of those cognitive decline-related diseases. The standardized GBE is considered a phytopharmacon, and the dose of 240 mg/day is recommended as the most effective by the guidelines for biological treatment of dementias [12]. There are 9 categories (A, B, C, C1, C2, C3, D, E, and F) and 5 grades (1-5) of pharmaceuticals used for AD and other dementias according to the level of existing clinical evidence and the occurrence of side effects, respectively. GBE belongs to category B of the level of evidence (limited positive evidence from controlled studies) and to grade 3 [12]. Here, we are going to highlight evidence on the extract's efficacy on subgroups of age-associated cognitive conditions in an ascending severity order (Table 1).

2.1.2.1. Patients with SMI and MCI. Three randomized, double-blind, placebo-controlled, parallel-group trials were conducted for patients with memory complaints, one in SMI and two in MCI patients. In total, data from 61 SMI and 460 MCI patients were evaluated. One trial conducted in healthy aged patients with SMI showed that GBE enhanced cognitive flexibility without changes in brain



(a) (b)

Figure 4: Standardized *Ginkgo biloba* extract (GBE) LI 1370 (Vifor SA, Switzerland) (100 μg/ml) increased neurite outgrowth of SH-SY5Y neuroblastoma cells after 3 days of treatment in 3D cell culture. Pictures were taken using a cell imaging multimode reader Cytation3 (Biotek Instruments Inc., X20 in black and white) after immunostaining (IMS, βIII-tubuline/Alexa488). Compared to the untreated SH-SY5Y cells (CTRL, (a)), 100 μg/ml of GBE (b) was efficient in increasing the formation of neurites.

activation and that it mildly increased prefrontal dopamine [61]. Two trials showed that GBE ameliorated neuropsychiatric symptoms (NPS) and cognitive ability in patients with MCI [62] as well as improved cognitive functioning and aspects of quality of life in subjects with very mild cognitive impairment [63].

2.1.2.2. Patients with Dementia. GBE has been found particularly efficacious in demented people with neuropsychiatric symptoms (NPS) [64, 65]. In total, 3 original papers, 1 systemic review, 6 meta-analyses, and 1 Cochrane analysis involving 14974 demented patients were evaluated. In detail, the pooled analyses of 4 published trials in a systemic review, involving outpatients with mild to moderate dementia and BPSD, demonstrated the efficacy of GBE at a daily dose of 240 mg [66]. Six meta-analyses (3 trials included in these meta-analyses were conducted in 1997 [67–69]) of 32 controlled, randomized, double-bind clinical trials and one bivariate meta-analysis of 6 trials come to the conclusion that GBE is efficacious and well tolerated in patients with a diagnosis of AD, VaD, or mixed dementia in three typical domains of assessment in dementia, i.e., cognition, activities of daily living (ADL), and clinical global judgment [65, 70–74]. However, there are also the studies with inconclusive or contrasting results to the efficacious effect of GBE in demented subjects [75–77].

2.1.2.3. Patients with Specific Dementia Type: AD and Vascular Dementia. In total, data from 1 original paper, 1 review, and 3 systematic reviews and meta-analyses involving 6880 patients with AD and VaD were evaluated. In detail, in an original paper, low doses of GBE administered to patients with vascular cognitive impairment in a randomized, double-blind, placebo-controlled trial showed significant deceleration of cognitive decline versus placebo only in one (Clinical Global Impression) of the four tests conducted in the trial [78]. The systematic reviews and meta-analyses (3 trials included in these meta-analyses were conducted in 1997 [67–69]) concluded that GBE exerts potentially beneficial effects on the improvement of activities of daily living, cognitive function, and on global clinical assessment in patients with MCI or AD, in mainly the AD type of dementia and in aged people with VaD having NPS [79–82].

2.1.2.4. Prevention. The preventive effect of GBE was reported in 14812 patients in three original papers and one systematic review and meta-analysis. In contrast, there are 4 studies that do not support the efficacious effect of GBE in preventing the onset of AD in either healthy aged or aged with MCI people [83–86]. The outcome for the efficacy of GBE in preventing the onset of AD in healthy individuals varies among different studies. However, there is also high variability in the design of the studies in terms of GBE doses, duration of the treatment, sample size, statistical tools, and compliance with the medication. Therefore, there is space for criticism regarding the methodological design of studies and the interpretation of the outcome. There are two large studies which form good examples of scepticism towards their negative outcome: the GEM study and the GuidAge study [83, 84, 87]. The GEM study was conducted in healthy old people (80 years old or more) and found no efficacy of GBE in preventing the onset of AD. In this study, the compliance of subjects with the treatment was nonadequate, yet this parameter was not taken into account in the interpretation of the results. In the GuidAge study, the conversion rate from memory complaints to dementia was examined in aged people with memory complaints and no difference was found between GBE and placebo. However, the statistical power for the analysis of hazards was found low. The selection of suitable statistical methods to take into account increasing hazards overtime is crucial for meaningful results and increased significance [35].

Table 1: Clinical trials on the effects of GBE.

Study design	GBE dose/preparation	Duration	Subjects	Purpose	Main results	References
Patients with memory complaints, SMI, and MCI						
R, DB, PC	240 mg of GBE once daily or placebo	56 ± 4 days	(61) Healthy aged patients with subjective memory decline (SMI)	Test the effect of GBE on cognitive functions associated with prefrontal dopamine	GBE caused a mild increase in prefrontal dopamine; there were indications for enhanced cognitive flexibility and for ameliorated response inhibition results	Beck et al., 2016 [61]
R, DB, PC	240 mg of GBE once daily or placebo	12 weeks	(300) Patients 45-65 years old with very mild cognitive impairment (MCI)	Evaluate the effects of GBE on cognition and quality of life in patients with very mild cognitive impairment	GBE improved the cognitive ability and quality of life of patients	Grass-Kapanke, 2011 [63]
R, PC, DB, MC	240 mg of GBE once daily or placebo	24 weeks	(160) Patients with MCI	Test the effect of GBE on NPS and cognition in patients with MCI	GBE improved NPS and cognition; the extract was safe and well tolerated	Gavrilova et al., 2014 [62]
Patients with dementia						
R, DB, PC	240 mg of GBE once daily	22 weeks	(400) Demented patients with NPS	Test the efficacy of GBE on NPS of dementia	GBE statistically superior to placebo in ameliorating NPS (e.g., irritability, apathy, and anxiety)	Scripnikov et al., 2007 [64]
Systematic review	240 mg of GBE once daily	22 weeks	(1628) Demented patients with behavioural and psychological symptoms (BPSD)	Demonstrate efficacy of GBE in dementia with BPSD	Improvements of quality of life, cognition, and BPSD activities of daily living clinical global impression	Von Gunten et al., 2016 [66] ([12, 166–168])
Meta-analysis and systematic review	Different dosages of GBE	Not available	Demented patients	Test the efficacy of GBE in ameliorating symptoms of demented patients	GBE improved cognitive function and activities of everyday life in patients with dementia	Brondino et al., 2013 [72] ([12, 67–69, 79, 166, 169, 170])
Meta-analysis of randomized placebo controlled trials	120 mg or 240 mg of GBE per day or placebo	22-26 weeks	(2684) Demented patients	Evaluate evidence for efficacy of GBE in dementia	Confirmation of efficacy of GBE and good tolerability	Gauthier and Schlaefke, 2014 [70] ([12, 69, 166, 167, 169, 171])
Systematic review and meta-analysis of randomized controlled trials	240 mg of GBE once daily	22-26 weeks	(2561) Demented patients	Evaluate the clinical efficacy and adverse effects of GBE in dementia and cognitive decline	GBE was found more effective than placebo in decelerating cognition deficits and in improving daily life activities and NPS in dementia	Tan et al., 2015 [65] ([12, 62, 69, 167, 169–174])
Meta-analysis of randomized controlled clinical trials	240 mg/day	22 or 24 weeks	Old patients aged over 60 years	Effects of GBE on anxiety, dementia, and depression in aging patients	Improvements in dementia, anxiety, and depression	Kasper, 2015 [73] ([12, 166–168])

Table 1: Continued.

Study design	GBE dose/preparation	Duration	Subjects	Purpose	Main results	References
Meta-analysis of randomized controlled trials	240 mg of GBE once daily	22 or 24 weeks	(1628) Demented patients with behavioural and psychological symptoms (BPSD)	Test the effects of GBE on BPSD of demented patients	Significant superiority of GBE to placebo in improving BPSD and therefore caregiver experience	Savaskan et al., 2017 [74] ([12, 166–168])
Bivariate meta-analysis	Different dosages of GBE	Approximately 6 months	Demented patients	Evaluate baseline risk on the treatment effect and assess the efficacy of GBE on cognitive symptoms of dementia	GBE was effective at improving cognitive functions in dementia after 6 months of treatment	Wang et al., 2010 [71] ([12, 67, 69, 166, 169, 170])
R, DB, PC, PG, MC	160 mg or 240 mg of GBE daily	24 weeks	(214) Patients with dementia or age-related memory loss	To assess the efficacy of GBE in aged demented patients or patients with age-related memory loss	No beneficial effect of GBE for demented or age-related memory-impaired patients	Van Dongen, 2000 [75]
R, DB, PC, PG	120 mg of GBE daily	6 months	176 mildly to moderately demented patients	Assess the efficacy and safety of GBE for treating dementia in early stages	GBE not beneficial in mild to moderate dementia after a 6-month treatment	McCarney et al., 2008 [76]
Cochrane analysis of R, DB, PC trials	Different GBE doses ranging from low to high	Different treatment periods	Aging with dementia or cognitive impairment	Assess the efficacy and safety of GBE in dementia and cognitive impairment	GBE displays unreliable and inconsistent evidence in being beneficial for demented people	Birks and Evans, 2009 [77]
			Patients with AD and vascular dementia			
R, DB, PC	120 mg of GBE, 60 mg of GBE, or placebo	6 months	(90) Patients with vascular dementia (VaD)	Evaluate the efficacy and safety of GBE in vascular demented patients	GBE slowed down the cognitive deterioration in vascular demented patients, effect shown in only one of the four neuropsychological tests	Demarin et al., 2017 [78]
Review of R, PC	120 mg of GBE twice daily or 240 mg of GBE once daily	22 or 24 weeks	(1294) Demented patients (AD or VaD) with NPS	Test the efficacy of GBE in older patients with AD/vascular dementia with NPS	Confirmation of efficacy of GBE and good tolerability	Ihl, 2013 [79] ([12, 166, 167, 175])
Systematic review and meta-analysis	GBE extract	12-52 weeks	(2372) Patients with AD or vascular or mixed dementia	Evaluate the effects of GBE in AD and vascular and mixed dementias	Superiority of GBE to placebo in improving everyday life activities in mainly the AD type of dementia	Weinmann et al., 2010 [80] ([67–69, 166, 169, 173, 175])
Systematic review and meta-analysis	240 mg and 120 mg of GBE daily	24 weeks	Patients with MCI or AD	Assess the effectiveness and safety of GBE in treating MCI and AD	There is an indication for the beneficial effect of GBE in MCI and AD but the results were inconsistent	Yang et al., 2016 [81] (AD: [67, 68, 169, 170, 174–176]; MCI: [62])

Table 1: Continued.

Study design	GBE dose/preparation	Duration	Subjects	Purpose	Main results	References
Systematic review of randomized controlled trials	240 mg of GBE daily	Period ≥ 16 weeks	Patients with mildly to moderately severe and severe AD	Assess the beneficial effect of GBE in AD	Evidence of beneficial effects of GBE in amelioration cognition, every day activities, and psychopathological symptoms but great heterogeneity among the results	Janssen et al., 2010 [82] ([67, 69, 166, 169])
			Prevention			
R, DB, PC, PG	120 mg of GBE twice daily	5 years	Adults 70 years or older with occasional memory problems	Efficacy of long-term use of GBE for the prevention of AD in aging with memory complaints	GBE did not reduce the incidence of AD compared to placebo	GuidAge study, Vellas et al., 2012 [83]
R, DB, PC	120 mg of GBE twice daily	Every 6 months from 2000 to 2008	(3069) Healthy old people or people with MCI aged 72 to 96 years	Test whether GBE delays or prevents global or domain-specific cognitive impairment in aging	GBE did not prevent cognitive decline in aging	Snitz et al., 2009 [84]
R, DB, PC	120 mg of GBE twice daily	5 years	(3000) Healthy subjects aging over 80 years old	Assess the ability of GBE in the prevention of dementia in normal aging or those with MCI	GBE does not prevent dementia	GEM study, DeKosky et al., 2006 [87]
Systematic review and meta-analysis	240 mg of GBE daily	Not available	Nondemented patients aged 70 years or older	Evaluate the efficacy of GBE for the prevention of dementia in nondemented adults	GBE is not able to prevent the development of dementia	Charemboon and Jaisin, 2015 [86] ([83, 85])

SMI, subjective memory impairment; MCI, mild cognitive impairments; AD, Alzheimer's disease; VaD, vascular dementia; R, randomized; DB, double blind; PC, placebo controlled; MC, multicenter; PG, parallel group; BPSD, behavioural psychological symptoms; VCI: vascular cognitive impairment. The number of patients involved in the trials is indicated in parentheses.

Based on the included studies, GBE has been reported in only a few studies that show no effect. The majority of the recent studies demonstrated that the treatment with doses up to 240 mg/day was safe, well-tolerated, and efficacious against age-related disorders.

In summary, GBE has been proven more effective in patients with cognitive impairment at baseline than preventing the onset of cognitive impairment in healthy aged subjects. As mentioned before (see Introduction), mitochondrial dysfunction is more profound in cognitive disorders than in normal aging. Similarly, GBE shows increasing promising effects with increasing cognitive impairment. This, again, represents an indicator that GBE exerts its effects clinically by acting on mitochondria [35]. Thus, we can conclude that GBE can potentially improve mitochondrial dysfunction across the aging spectrum.

2.2. Resveratrol. Resveratrol, known as a polyphenol from white hellebore (*Veratrum grandiflorum*), was discovered by Takaoka (1939) as a component of several dietary sources such as berries, peanuts, and red grape skin or wine. Siemann and Creasy discovered that resveratrol is present at high concentration in red wine [88]. Resveratrol has been reported to possess several benefits, including antitumor, antioxidant, antiaging, anti-inflammatory, cardioprotective, and neuroprotective properties. This polyphenol has emerged as a novel natural agent in the prevention and possible therapy of AD [89].

2.2.1. Mechanisms of Action Based on Preclinical Evidence

In vitro and *in vivo*, the direct molecular targets of resveratrol are not known in detail. However, there is evidence that resveratrol exerts a complex mode of actions through the protection of mitochondrial function and the activation of biogenesis, through its effect on certain signalling pathways, through its antioxidant effects, through the increase of Aβ clearance, and through the reduction of Aβ neurotoxicity [90] (Figure 3).

2.2.1.1. Direct Effects of Resveratrol on Mitochondria. Dietary supplementation with 0.2% (w/w) resveratrol suppressed the aging-associated decline in physical performance in senescence-accelerated mice (SAMP1) at 18 weeks of age by improving several mitochondrial functions such as the activity of respiratory enzymes, oxygen consumption, and mitochondrial biogenesis, as well as the activity of lipid-oxidizing enzymes [91]. In 18-month-old aged mice, resveratrol (15 mg/kg/day) and/or exercise for 4 weeks were able to counteract aging-associated oxidative damage targeting mitochondrial biogenesis and function by causing overexpression of peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) mRNA and by increasing citrate synthase enzyme activity [92]. Mitochondrial biogenesis is induced by resveratrol through SIRT1 activation and deacetylation of PCG-1α [90] (Figure 3).

2.2.1.2. Effects of Resveratrol on Oxidative Stress. Damaged mitochondria activate ROS production during oxidative stress which is involved in apoptosis [93]. ROS may damage the mitochondrial and cellular proteins and nucleic acids, causing lipid peroxidation and resulting in the loss of membrane integrity [94] (Figure 3). Resveratrol also protects mitochondria by increasing the expression of the ROS-inactivating enzymes glutathione peroxidase 1 (GPx1) and superoxide dismutase 1 (SOD1) and by reducing the expression of the ROS-producing enzyme NADPH oxidase 4 (Nox4) [93, 95] (Figure 3). In line with this, resveratrol rescued Aβ-treated human neural stem cells (hNSCs) from oxidative stress by increasing the mRNA of antioxidant enzyme genes such as SOD-1, nuclear factor erythroid 2-related factor 2 (NRF-2), Gpx1, catalase, and heme oxigenase 1 (HO-1) [96]. In addition, resveratrol exerted antioxidant properties and attenuated oxidative damage by decreasing iNOS and COX-2 levels [93].

2.2.1.3. Effects of Resveratrol on Aβ Toxicity Related to Damage of Mitochondria. Thanks to its natural antioxidant properties and/or by sirtuin1 (SIRT1) activation, resveratrol shows a neuroprotective effect because it counteracts Aβ toxicity. In more details, resveratrol increases the clearance of Aβ through the activation of AMPK [90] (Figure 3). This natural molecule plays an important role in reducing Aβ neurotoxicity by phosphorylating protein kinase C delta (PKC-δ) [90] (Figure 3). Resveratrol also influences the Aβ-induced apoptotic signalling pathway through SIRT1 activation, including inhibiting the expression of caspase protein 3 (caspase-3), apoptotic regulator Bax, Forkhead box O (FOXO), and tumor protein p53, through blocking the activation of c-Jun N-terminal kinase (JNK) and restoring the decrease of B-cell lymphoma 2 (Bcl-2) expression, as well as through inhibiting the increase of the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) DNA binding [90] (Figure 3). Resveratrol (20 μM) protected PC12 cells against neurotoxicity caused by $A\beta_{25\text{-}35}$ by provoking autophagy which was proven dependent on the tyrosyl tRNA synthetase-poly(-ADP-ribose) polymerase 1 (TyrRS-PARP1) and SIRT1 pathway (TyrRS-PARP1-SIRT1 pathway) [97]. A very low concentration of resveratrol (0.2 mg/l) significantly attenuated Aβ neuropathology and AD-type deterioration of spatial memory function in Tg2576 mice compared to control [98]. In a transgenic mouse model of AD (Tg19959), dietary supplementation with resveratrol (300 mg/kg) decreased amyloid plaque formation [93]. In order to translate the animal doses into ones that are relevant in humans, a scaling factor of 0.08 is used to calculate the human equivalent dose (http://www.fda.gov/cber/gdlns/dose.htm). For resveratrol, this is about 24 mg/kg or 1.68 g per day for a 70 kg individual [93]. Resveratrol is also known to act as a phytoestrogen (this mode of action of resveratrol is discussed in more detail in Phytoestrogens).

2.2.1.4. Effects of Resveratrol on Metabolic and Signalling Pathways. Resveratrol has been suggested to regulate cellular processes by activating key metabolic proteins such

as SIRT1, 5′ adenosine monophosphate-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG-1α) [99–101]. Sirtuins and nicotinamide adenine dinucleotide- (NAD^+-) dependent protein deacetylases are described as novel therapeutic agents against neurodegenerative disease pathogenesis [102]. In fact, the essential neuroprotective effect of resveratrol is based on the action of SIRT1 and AMPK and on the phosphorylation/acetylation status of PGC-1α that consequently activates the mitochondrial biogenesis leading to the improvement of the mitochondrial activity [103] (Figure 3).

In a study using Aβ-treated hNSCs, the neuroprotective effect of (10 μM) resveratrol was demonstrated by the activation of the AMPK-dependent pathway by rescuing the expression levels of inhibitory kappa B kinase (IKK) and by restoring iNOS and COX-2 levels [104]. In the inducible p25 transgenic mouse model of tauopathy and AD, resveratrol-mediated (5 μg/μl) SIRT1 activation reduced learning impairment and hippocampal neurodegeneration [105]. The JAK/ERK/STAT signalling pathway (janus kinases, extracellular signal-regulated kinases, and signal transducers and activators of transcription) is implicated in cell survival, proliferation, and differentiation, while the dysregulation of the JAK/STAT pathway in neurodegenerative disorders contributes to neuronal loss, cognitive impairment, and brain damage [96]. Treatment with 20 mg/kg resveratrol exerted a neuroprotective effect via the JAK/ERK/STAT signalling pathway in a rat model of ischemia-reperfusion injury. In detail, resveratrol attenuated the increase in phosphorylation of JAK, ERK, STAT, and JNK caused by ischemia-reperfusion [96] (Figure 3).

2.2.2. Clinical Evidence. Only eight clinical trials and four ongoing trials on resveratrol aim at evaluating the effects of this compound on cognitive function in humans [106] (Table 2). Efficacy results of resveratrol are based only on one clinical trial in MCI and one in AD patients.

2.2.2.1. Young and Old Healthy Subjects. Witte et al. conducted a study to evaluate the effect of resveratrol (200 mg/day) supplementation in a formulation with quercetin 320 mg in 23 healthy overweight older individuals versus placebo during 26 weeks. They showed that resveratrol supplementation is able to improve memory performances and glucose metabolism and is able to increase hippocampal functional connectivity in older adults for the maintenance of brain health during aging [107]. No effect on cognitive function was detected in young healthy people [94, 95].

2.2.2.2. Patients with Cognitive Decline and MCI. Lee et al. examined the effects of grape consumption (which contains resveratrol) on cognitive function and metabolism in the brain of patients with mild cognitive decline and demonstrated a protective effect of the grape extract against pathologic metabolic decline [108]. In a more recent 14-week study carried out on 80 postmenopausal women aged 45-85 years, it was proven that a regular consumption of a modest dose of resveratrol (75 mg twice daily) is able to enhance cerebrovascular function and cognition and to reduce their heightened risk of accelerated cognitive decline [109].

Clinical studies are underway to explore the beneficial effect of resveratrol on MCI. In the ongoing trials, one four-month resveratrol supplementation study in phase 1 aims at evaluating the efficacy and safety of bioactive dietary preparation (BDPP) at low, moderate, and high doses in treating mild cognitive impairment on 48 MCI subjects (55-85 years) [110]. The purpose of another study in phase 4 is to test the effect of a six-month administration of resveratrol on brain functions in MCI subjects (50-80 years) (National Institutes of Health, ClinicalTrials.gov) [111]. In a randomized, double-blind interventional study, resveratrol intake (200 mg/day, 26 weeks) reduced glycated hemoglobin A1c, preserved hippocampus volume, and improved hippocampus resting-state functional connectivity (RSFC) in 40 well-characterized patients with MCI (21 females, 50-80 years) [112].

2.2.2.3. Patients with Moderate AD and Dementia. Class II evidence provided by the study of Turner et al. on patients with AD showed that resveratrol (500 mg/day to 2 g/day, 52 weeks) is well-tolerated, safe, and able to decrease $A\beta_{40}$ levels in cerebrospinal fluid (CSF) and plasma but had no significant effects on cognitive score [113]. Recently, a phase 2 study was conducted investigating the effect of resveratrol (500 mg) in individuals with mild to moderate AD confirming its tolerability and safety as well as its modulation of AD biomarker pathways [114]. Currently, an ongoing study in phase 3 tests the effect of resveratrol supplementation (215 mg/day for 52 weeks) on cognitive and global functioning in mild-to-moderate AD patients (50-90 years) [115]. A second ongoing study in phase 3 aims at evaluating the effect of resveratrol combined with glucose and malate in slowing down the progression of AD after 12 months in mild-to-moderate AD (50-90 year old patients) [116].

On the basis of the results from the very few clinical trials in MCI and AD, no conclusion about the efficacy of resveratrol on cognition can be drawn at the current time, but promising trials are underway.

2.3. Neurosteroids. Neurosteroids offer therapeutic opportunities through their pleiotropic effects on the nervous system. They are a subcategory of steroids synthetized de novo from cholesterol in the central nervous system independently of supply by peripheral steroidogenic glands [117, 118] and accumulate within the brain in neurons or glial cells [119, 120]. Neurosteroids are derived from cholesterol which is translocated from the outside to the inside of mitochondria via the translocator protein (TSPO). In the inner mitochondrial membrane, cholesterol is then converted by the cytochrome cholesterol side-chain cleavage enzyme (P450scc) to pregnenolone, the precursor of all the neurosteroids [121]. In particular, pregnenolone and allopregnanolone play an essential role in aging, in the performance of memory, and in physiopathology. Indeed, the age-related drop of neurosteroids gives rise to neuronal degeneration and dysfunction

Table 2: Clinical trials on the effects of resveratrol. Ongoing trials are italicized.

Study design	Resveratrol dose/preparation	Duration	Subjects	Purpose	Main results	References
Young and aged healthy individuals						
R, DB, PC, CO	*Trans*-resveratrol from Biotivia Bioceuticals 250 mg or 500 mg	21 days	(24) 18-25 years healthy	Ability to increase cerebral blood flow and modulate mental function	Increase in cerebral flow, no effect in cognitive function	Kennedy et al., 2010 [94]
R, DB, PC, CO	*Trans*-resveratrol 250 mg/day or *trans*-resveratrol 250 mg/day with 20 mg piperine	21 days	(23) Healthy subjects aged 19-34 years	Effect of piperine on the efficacy and bioavailability of resveratrol	Piperine enhances the effect of resveratrol on cerebral blood flow but no effect on bioavailability and cognition	Wightman et al., 2014 [95]
Study in older adults	200 mg of resveratrol per day	26 weeks	(46) Healthy overweight subjects aged 50-75 years	Test whether resveratrol would improve memory performance in older adults	Resveratrol ameliorates memory performance in combination with improved glucose metabolism and increased hippocampal functional connectivity in healthy overweight old people	Witte et al., 2014 [107]
Patients with cognitive decline and postmenopausal women						
R, DB, PC	72 g of active grape formulation	6 months	(10) Adults with mild cognitive decline with mean age of 72.2 years	Evaluate the effects of grapes on regional cerebral metabolism	Grapes could possess a protective effect against early pathologic metabolic decline	Lee et al., 2017 [108]
R, PC, intervention trial	75 mg twice daily of *trans*-resveratrol	14 weeks	(80) Postmenopausal women between 45 and 85 years old	Test the effects of resveratrol on cognition, mood, and cerebrovascular function in postmenopausal women	Resveratrol was well tolerated and able to improve cognition which was related to the improvement of cerebrovascular function. Mood was improved but not significantly.	Evans et al., 2017 [109]
Patients with MCI						
R, DB, interventional study	200 mg of resveratrol per day	26 weeks	(40) Old patients with MCI	Assess if resveratrol improves long-term glucose control, resting-state functional connectivity of the hippocampus, and memory function in patients with MCI	Resveratrol supplementation decreased glycated hemoglobin A1c, preserved hippocampus volume, and improved hippocampus RSFC in patients with MCI	Koebe et al., 2017 [112]
R, DB Phase 1	Bioactive dietary polyphenol preparation (BDPP) at low, moderate, and high doses	4 months	(48) 55-85 years MCI	Safety and efficacy in treating mild cognitive impairment	—	*NCT02502253* [110]
R, DB, PC Phase 4	Resveratrol or omega-3 supplementation or caloric restriction	6 months	(330) 50-80 years MCI	Effects on brain function	—	*NCT01219244* [111]

Table 2: Continued.

Study design	Resveratrol dose/preparation	Duration	Subjects	Purpose	Main results	References
			Patients with mild to moderate AD			
R, DB, PC, MC Phase 2	Resveratrol 500 mg/day with escalation by 500 mg increments ending with 2 g/day	52 weeks	(119) Over 49 years mild to moderate AD	Assess efficacy and safety	No effect on cognitive score, decrease of CSF and plasma Aβ40 levels	Turner et al., 2015 [113]
R, DB, PC Phase 2	Resveratrol 500 mg daily (orally) with a dose elevation by 500 mg every 13 weeks until a final dose of 1000 mg twice daily was reached for the final 13 weeks.	52 weeks	(119) Adults older than 49 years old with a diagnosis of mild to moderate dementia due to AD	Evaluation of safety and tolerability of resveratrol and its effects on AD biomarkers and also on clinical outcomes	Resveratrol was well tolerated and safe, it was detected in the cerebrospinal fluid (nM), it changed the AD biomarker paths, it modified the CNS immune response, and it maintained the BBB integrity; however, more research is needed	*Sawda et al., 2017* [114]
R, DB, PC Phase 3	Longevinex brand resveratrol supplement (resveratrol 250 mg/day)	52 weeks	(50) 50-90 years mild to moderate AD on standard therapy	Effects on cognitive and global functioning	—	NCT00743743 [115]
R, DB, PC Phase 3	Resveratrol with malate and glucose	12 months	(27) 50-90 years mild to moderate AD	Ability to slow the progression of AD	—	NCT00678431 [116]

MCI, mild cognitive impairment; AD, Alzheimer's disease; R, randomized; DB, double blind; PC, placebo controlled; CO, cross over; MC, multicenter; CSF, cerebrospinal fluid. The number of patients involved in the trials is indicated in parentheses.

in human and animal models owing to the loss of neurosteroid neuroregenerative and protective effects [122, 123]. Allopregnanolone is used in several studies as a plasmatic biomarker for AD because of its reduced level in the plasma of demented patients [122]. It is known to be a regenerative agent in the brain [124]. Several neurosteroids were quantified and were found decreased in postmortem brains of aged nondemented controls and aged AD patients. The transgenic mice model of AD (APPswe+PSEN1Δ9 mice) presents a decreased ability to form allopregnanolone in the hippocampus [125].

2.3.1. Allopregnanolone

2.3.1.1. Mechanisms of Action Based on Preclinical Evidence.
2.3.1.1.1. Direct Effects of Allopregnanolone on Mitochondria. In control and APP/Aβ SH-SY5Y cells, allopregnanolone improved basal respiration and glycolysis as well as increased the bioenergetic activity and ATP production [126]. In APP-transfected cells, a pretreatment with allopregnanolone exerted a neuroprotective effect against oxidative stress-induced cell death via the amelioration of the cellular and mitochondrial energy, the reduction of ROS, and the improvement of mitochondrial respiration [126]. Thereby, it exerted its beneficial effect by improving the mitochondrial redox environment, such as MnSOD activity and mitochondrial ROS levels [127]. Moreover, allopregnanolone increased ATP levels and respiration in mouse primary cortical neurons [127]. In addition, *in vitro*, allopregnanolone potentiated mitochondrial respiration in both adult neural stem cells (NSCs), neurons, and mixed glia [128]. *In vivo*, allopregnanolone was able to restore the ovarectomized-(OVX-) induced decrease in mitochondrial respiration in both non-Tg and 3xTgAD mice [128]. Moreover, allopregnanolone also improved the activity of bioenergetic enzymes such as pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGDH) [128].

2.3.1.1.2. Effects of Allopregnanolone on Aβ Toxicity Related to Damage of Mitochondria. In a recent study, allopregnanolone was shown to exert an increased neuroprotective activity against Aβ_{42}-induced cell death in neural stem cells [129] (Figure 5). *In vivo*, the natural neurosteroid allopregnanolone appears to be a promising therapeutic tool for the development of neurogenic and/or neuroprotective strategies, but diverse points have to be taken into account, including the dosing regimen, the treatment regimen, bioavailability, solubility, route of administration, and sex differences. Acute single administration of allopregnanolone promoted neurogenesis in the subgranular zone (SGZ) in the triple transgenic mouse model of AD (3xTgAD) at 3 months of age prior to the appearance of AD [71]. Allopregnanolone reversed memory and learning deficits in these mice. Chen et al. showed that allopregnanolone administration (once/week for 6 months) decreased Aβ generation and promoted survival of newly generated neurons in the brain of 3xTgAD [130]. They also demonstrated that allopregnanolone increased oligodendrocyte myelin markers and ameliorated cholesterol homeostasis and clearance from the brain by increasing the expression of PXR and Liver-X-receptor (LXR). Singh et al. reported that allopregnanolone is able to restore cognitive performance in the preplaque phase of AD as well as memory and learning in aging 3xTgAD mice [131]. All these studies demonstrated the neuroprotective effects of allopregnanolone against the Aβ toxicity in 3xTgAD mice and also its capacity to stimulate rodent and human neural progenitor cell proliferation and to compensate the cell loss [130, 132]. Continuous infusions of allopregnenanolone were antiregenerative, while intermittent administration promoted repair and renewal in a mouse model of AD [124]. The mode of action of allopregnanolone is summarized in Figure 5.

2.3.1.2. Clinical Evidence. Currently, there is only one phase I ongoing clinical trial testing the safety and the tolerability of allopregnanolone in patients with mild cognitive impairment and early AD [133] (Table 3). The primary aim of this phase 1 study is to evaluate the maximally tolerated dose after intravenous injection of allopregnanolone (2, 4, or 6 mg, once per week for 12 weeks). Thus, no clinical evidence is currently available.

The natural neurosteroid allopregnanolone appears to be a promising therapeutic tool with specific regard to its neurogenic properties besides its mitochondria-directed effects. However, more trials are urgently needed to prove that.

2.4. Phytoestrogens. Phytoestrogens are the most bioactive molecules of soy and present structural similarity to the 17β-estradiol, which is the major circulating estrogen. Specific estrogen receptors have been shown to localize in mitochondria in the frontal lobe and the hippocampus of men and women suggesting a role of estrogen in controlling cognitive functions and memory processes via energy supply [134]. Estrogen plays a neuroprotective role during the aging process, especially through its beneficial impact upon mitochondrial metabolism by increasing glucose utilization by cells as well as by enhancing ETC activity, by stabilizing the MMP, and by preventing ROS production and calcium-induced excitotoxicity [135]. Moreover, females live longer than males and this can be attributed in part to the antioxidant effect of estrogen and the upregulation of life longevity-related genes [19, 136]. The phytoestrogens are characterized by their ability to bind to estrogen receptor α and estrogen receptor β and to exert similar responses to endogenous estrogens [137]. Isoflavones are a subclass of phytoestrogens and are contained abundantly in soy and soybeans. Soy presents estrogenic effects attributed to genistein, daidzein, and glycitein. The most potent isoflavone is genistein, while daidzein and glycitein present an affinity to the estrogen receptor, 100-500 times lower than genistein [138]. Estrogen receptors are localized in the important brain areas, including the prefrontal cortex and the hippocampus that are also known to be vulnerable to age-related decline [139–142].

2.4.1. Mechanisms of Action Based on Preclinical Evidence

2.4.1.1. Effects of Phytoestrogens on Aβ and Tau Toxicity and Cognitive Performance Related to Damage of Mitochondria. One of the most important phytoestrogens is resveratrol, an estrogen receptor agonist/antagonist. In

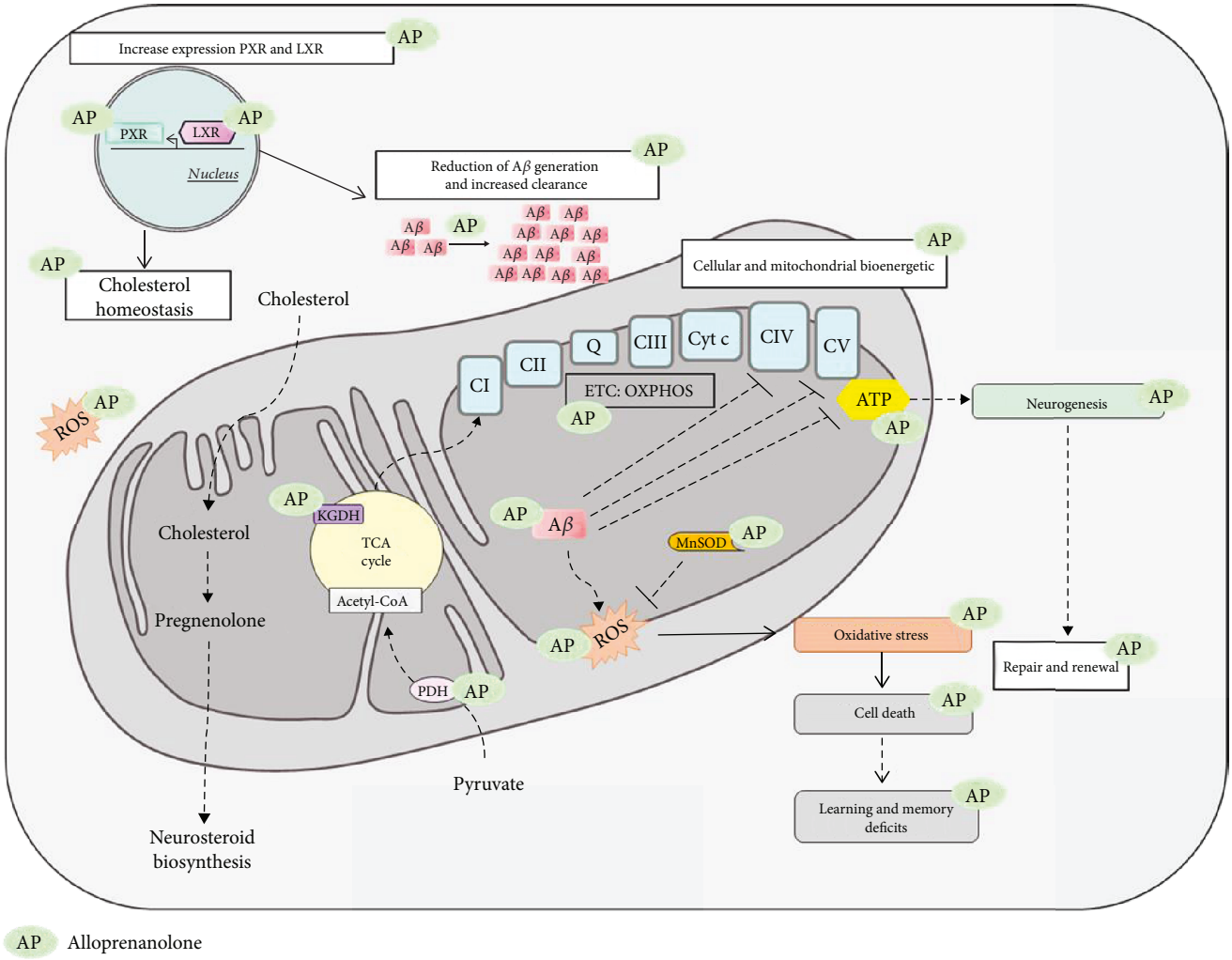


Figure 5: Neuroprotective effects of allopregnanolone (AP) in AD. AP has been proven to reduce Aβ aggregation-induced cell death. It exerts a neuroprotective effect against oxidative stress-induced cell death via the improvement of the cellular and mitochondrial energy by enhancing the OXPHOS and ATP levels. AP ameliorates the mitochondrial redox environment by decreasing ROS and by improving the activity of the enzyme MnSOD. AP also has beneficial effects on bioenergetic enzymes such as PDH and αKGDH implicated in the TCA cycle. AP ameliorates cholesterol homeostasis and clearance for the biosynthesis of neurosteroids by raising the expression of PXR and LXR. AP promotes repair and renewal of neurons leading to restored cognitive performances in AD.

Table 3: Ongoing clinical trial on the effects of allopregnanolone in MCI and mild AD.

Study design	Allopregnanolone dose/preparation	Duration	Subjects	Purpose	Main results	References
R, DB, parallel assignment Phase 1	Allopregnanolone 2, 4, or 6 mg intravenous injection once per week or placebo intravenous injection once per week	12 weeks	(8) For each dose group, 55 years and older, both genders MCI or mild AD (6) Randomized to AP (2) Randomized to placebo	Determine the maximally tolerated dose, safety and tolerability, pharmacokinetic profile, and effects on cognitive function	Not available	NCT02221622 [133]

The number of patients involved in the trials is indicated in parentheses.

particular, resveratrol acts on estrogen receptor β, whose activation is known to play a major role in cognitive processes, leading to the improvement of cognitive impairment in AD [143]. The soybean is a source of vegetable proteins and contains also other functional ingredients including phytoestrogens. The isoflavones genistein and daidzein have been shown to present protective effects against tau protein phosphorylation [144]. Animal models confirmed

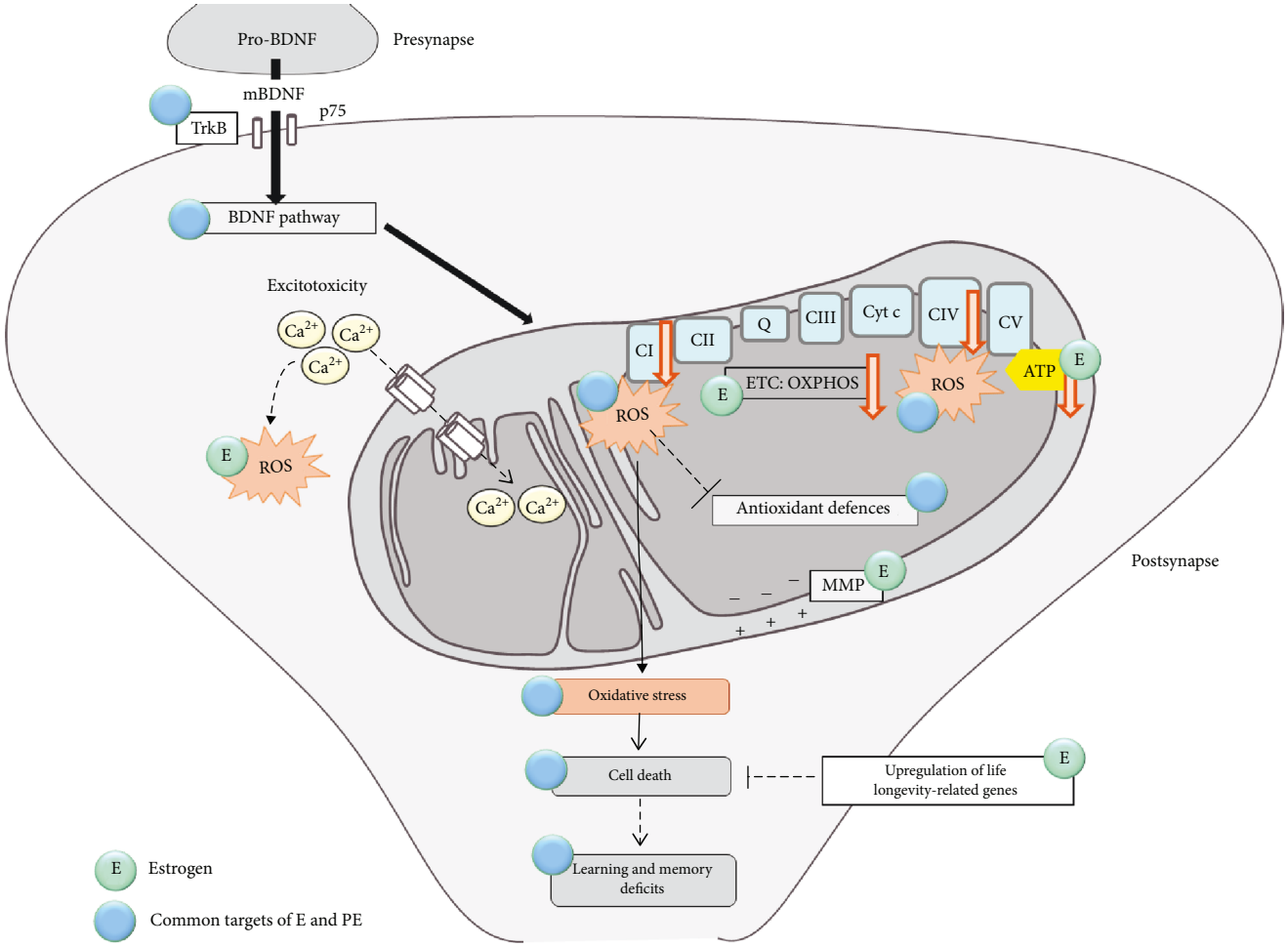


Figure 6: Modulation of mitochondrial function by estrogen and phytoestrogen. Less evidence is provided for the direct effects of phytoestrogen on mitochondria compared to estrogen, but antioxidant properties were demonstrated.

the neuroprotective effects of phytoestrogens. Genistein, the most active molecule of soy isoflavones, improved Aβ-induced cell death and reduced neuronal loss in rats [145–147]. In OVX female rats, dietary supplementation of soy phytoestrogens (0.4 g/kg or 1.6 g/kg) or 17β-estradiol (0.15 g/kg) for 12 weeks has been shown to increase the expression of brain neurotrophic factors such as BDNF and tropomyosin receptor kinase B (TrkB) and, as a result, to ameliorate hippocampal learning [148]. In normal and OVX transgenic AD mice, a selection of phytoestrogens in combination, composed of genistein, daidzein, and equol, has been shown to improve spatial working memory performance and to reduce mortality, as well as to delay neuropathological changes associated with AD [149].

2.4.1.2. Effects of Phytoestrogens on Oxidative Stress. The phytoestrogens are also known for their neuroprotective antioxidant effects in neuronal cell models after exposure to neurotoxic substances [150–152]. Phytoestrogens are able to reduce ROS within a cell and to protect from cellular damage [153]. In aged mice, soybean supplementation has been shown to prevent cognitive deficits by decreasing free radical generation, by enhancing scavenging of free radicals, and by increasing GSH levels [154]. Compared to estrogen itself, less evidence is provided for the direct effects of phytoestrogens on mitochondria, but antioxidant properties were demonstrated [155–158]. The molecular effects of phytoestrogens are summarized in Figure 6.

2.4.2. Clinical Evidence. Until today, no clinical trials in MCI and AD were performed. Thus, currently there is no clinical evidence.

2.4.2.1. Healthy and Postmenopausal Women. Among five randomized controlled trials, four recent studies reported the beneficial effect of phytoestrogens on cognitive function in healthy individuals (Table 4). In a study with young healthy adults of both sexes, a high soya or a low soya diet for 10 weeks had a beneficial effect and showed significant improvements in short-term and long-term memory as well as in mental flexibility [159]. In another cross-over design study, the administration of 4 capsules/day containing soya isoflavones during 6 weeks improved the spatial working memory of men aged 30-80 years [160]. In postmenopausal women, 6 months of treatment duration with isoflavone supplementation provoked better learning, mental flexibility, and increased attention, as well as caused improvement in mood and lower depressive symptoms [161]. In a small

Table 4: Clinical trials on the effects of phytoestrogens.

Study design	Phytoestrogens dose/preparation	Duration	Subjects	Purpose	Main results	References
			Healthy individuals and postmenopausal women			
Randomized control trial	High soya (100 mg total isoflavones/day) or a low soya (0.5 mg total isoflavones/day) diet	10 weeks	(27) Young healthy adults (15 men, 12 women)	Effects on memory, attention, and frontal lobe function	Improvements in short-term memory, long-term memory and mental flexibility	File et al., 2015 [159]
DB, CO, PC	4 capsules/day containing soya isoflavones (116 mg isoflavone equivalents/day: 68 mg daidzein, 12 mg genistein, and 36 mg glycitin) or placebo	6 weeks	(34) Men aged 30-80 years	Effects on cognitive function	Improvements of spatial working memory but no effect on auditory and episodic memory and executive function and visual-spatial processing	Thorp et al., 2009 [160]
18 R, DB, CO, PC	Isoflavone supplementation 60 mg/day or placebo	6 months	(78) Postmenopausal women (mean age 49.5 years)	Effects of soy isoflavones on mood and cognitive function in postmenopausal women	Improvements in mental flexibility, attention, mood, and lower depressive symptoms	Casini et al., 2006 [161]
R, DB, PC	100 mg/day soy isoflavones (glycoside weight) or matching placebo tablets	6 months	Older nondemented men and women (age 62-89 years)	Examination of safety, feasibility, and cognitive efficacy of soy isoflavone administration	Improvements of visual-spatial memory and construction of verbal fluency and speeded dexterity	Gleason et al., 2009 [162]
R, DB, PC	20 g of soy protein containing 160 mg of total isoflavones	12 weeks	(93) Healthy postmenopausal women (mean age 56 years)	Effect of a high-dose isoflavones on cognition, quality of life, lipoproteins, and androgen status in postmenopausal women	Significant improvement in the quality of life versus placebo. No significant effects in cognition. The testosterone and HDL levels were significantly lower at the end of the study.	Basaria et al., 2009 [163]

The number of patients involved in the trials is indicated in parentheses.

mixed gender sample of older adults, soy supplementation ameliorated the visuospatial memory and the construction of verbal fluency and speeded dexterity [162]. All these studies demonstrated that phytoestrogens may affect human cognition. However, no clinical trials of phytoestrogens are known for the prevention or the treatment of AD.

Inconclusive findings have also been reported from randomized controlled trials and observational studies in humans. In fact, these discrepant data could have several possible reasons. Investigation in European cohorts showed that a low dietary consumption of phytoestrogens had a significant effect on the improvement of the quality of life but no effect on cognition [163].

Mediating variables in the characteristics of the study population such as gender, age, ethnicity, and menopausal status appears to play an important role [164]. Phytoestrogens have been shown to have time-limited positive effects on cognition. These findings are in line with estrogen treatment which also exerts an initially positive short-term effect on cognition and a reversion after a long-term continuous use in aged women [164].

Globally, the effects of phytoestrogens can be dependent upon a window of opportunity for treatment and can affect males differentially than females due to the diminished presence of ER-mediated protective mechanisms and the tyrosine kinase activity with a potentially deleterious outcome of the supplements [165]. An age-dependent effect of phytoestrogen supplements is suggested in postmenopausal women [165]. In males, the findings are equivocal and sparse, and more investigations are needed to determine whether the effects will be deleterious or beneficial [165].

3. Conclusion

In this article, the efficacy of standardized *Ginkgo biloba* extract, resveratrol, allopregnanolone, and phytoestrogens in combatting age-related cognitive decline has been reviewed. The mechanisms of action as well as preclinical and clinical evidence for each of those entities have been discussed. The four entities share common mechanisms of action but also diverse ones. In terms of the main AD features, Aβ and tau, all four categories were able to reduce the Aβ accumulation but only GBE and phytoestrogens seem to reduce tau hyperphosphorylation. Similarly (and quite predictably due to their phenolic character), all four act as antioxidants either by reducing ROS and oxidative stress (GBE, phytoestrogens, and allopregnanolone) or by enhancing the activity of antioxidant enzymes such as SOD and GPx1 (GBE, resveratrol, and phytoestrogens) and by reducing lipid peroxidation (GBE) and prooxidant enzymes such as Nox4 (resveratrol). GBE, resveratrol, and allopregnanolone target mitochondria by enhancing their functions (activities of complexes, oxidative phosphorylation, oxygen consumption, respiration, mitochondrial membrane potential, and ATP production), while in addition to this, GBE and resveratrol promote mitochondrial biogenesis. This is particularly important since mitochondria play a pivotal role in synaptic plasticity that is reduced in pathological states in the brain. However, there are also some differences in the mechanisms of action of the four discussed substances and mainly in the pathways through which they exert their beneficial effects. Based on our review of the literature, GBE rescues the Aβ neurotoxicity through the activation of the Akt pathway and through phosphorylation of CREB. Neurotrophic factors such as BDNF are stimulated both by GBE and by phytoestrogens. Resveratrol leads to Aβ clearance, enhancement of mitochondrial biogenesis and metabolism, and reduction of inflammation and ROS mainly through the activation of SIRT 1 and AMPK pathways as well as through the deacetylation of PGC-1α and the modulation of the JAK/ERK/STAT pathway. Phytoestrogens act as ER receptor modulators. Resveratrol can additionally act as a phytoestrogen and bind to the ERβ receptor. In terms of *in vitro* assays, it should be taken into account that the extract and the substances should be tested in meaningful, physiologically relevant concentrations and not in irrationally high ones.

Regarding clinical trials, there is a different level of evidence for the four phytochemicals. Standardized GBE, resveratrol, allopregnanolone, and phytoestrogens appear in a descending order according to the level of existing clinical evidence. According to the World Federation of Societies of Biological Psychiatry (WFSBP) Guidelines, GBE has been classified in category B and grade 3 in terms of the outcome of existing studies. Therefore, there is sufficient and good clinical evidence for the efficacy of GBE. There is increasing and promising clinical evidence for resveratrol, but more studies of larger sample size are definitively needed. Lastly, there are no clinical trials indicating the beneficial effect of allopregnanolone and phytoestrogen in age-related cognitive decline disorders. There is only promising evidence from preclinical data regarding allopregnanolone and phytoestrogen. Notably, the four entities follow the same descending order regarding the existing level of clinical evidence and their mitochondria-improving properties. All in all, the effect on mitochondria goes hand in hand with the clinical effect and this highlights one more time the importance of these organelles not only in the pathogenesis of AD but also in aging in general.

Abbreviations

17β-estradiol:	Estradiol
2A PP2Ac:	Catalytic subunit of protein phosphatase
3xTgAD:	Triple transgenic mouse model of AD
AD:	Alzheimer's disease
Akt:	Protein kinase B
AMPK:	5′ adenosine monophosphate-activated protein kinase
APP:	Amyloid precursor protein
ATP:	Adenosine triphosphate
Aβ:	beta-Amyloid protein
αKGDH:	α-Ketoglutarate dehydrogenase
Bax:	Apoptotic regulator
BBB:	Blood-brain barrier
Bcl-2:	Anti-B-cell lymphoma 2
BDNF:	Brain-derived neurotrophic factor

BDPP: Bioactive dietary preparation
Ca^{2+}: Calcium
CMRglc: Cerebral metabolic rates of glucose
COX-2: Cyclooxygenase-2
CREB: Cyclic AMP response element-binding protein
CSF: Cerebrospinal fluid
CTRL: Untreated SH-SY5Y cells
DAT: Dopamine transporters
E2: Estrogen
ER: Endoplasmic reticulum
ERT: Estrogen replacement therapies
ETC: Electron transport chain
FAD: Familial Alzheimer's disease
FOXO: Forkhead box O
GBE: *Gingko biloba* extract
GPx1: Glutathione peroxidase 1
GSK3β: Glycogen synthase kinase 3 beta
hNSCs: Human neural stem cells
HO-1: Heme oxigenase 1
I_A: Transient potassium channel
IBO: Ibotenic acid
IKK: Inhibitory kappa B kinase
IMM: Inner mitochondrial membrane
IMR-32: Human neuroblastoma cells
iNOS: Nitric oxide synthase
JAK/ERK/STAT: Janus kinases/extracellular signal-regulated kinases/signal transducers and activators of transcription
JNK: c-Jun N-terminal kinase
LXR: Liver-X-receptor
MCI: Mild cognitive impairment
MDA: Malondialdehyde
MMP: Mitochondrial membrane potential
MnSOD: Manganese superoxide dismutase
mPTP: Mitochondrial permeability transition pore
MRI: Magnetic resonance imaging
MTDLs: Multitarget-directed ligands
mtDNA: Mitochondrial DNA
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
Nox4: Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4
NPS: Neuropsychiatric symptoms
NRF-2: Nuclear factor erythroid 2-related factor 2
NRs: Nuclear receptors
OVX: Ovarectomized
OXPHOS: Oxidative phosphorylation
p53: Tumor protein
P450scc: Cytochrome cholesterol side-chain cleavage enzyme
PAF-AH-1: Platelet-activating factor-acetylhydrolase-1
PCG-1α: Peroxisome proliferator-activated receptor γ coactivator-1α
PC12: Pheochromocytoma cells
PDH: Pyruvate dehydrogenase
PKC-δ: Protein kinase C delta
PPARγ: Peroxisome proliferator-activated receptor gamma
PSD95: Synapse-associated protein
PXR: Pregnane xenobiotic receptor
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
RSFC: Resting-state functional connectivity
SAD: Sporadic Alzheimer's disease
SAMP1: Senescence-accelerated mice
SGZ: Subgranular zone
SH-SY5Y: Human neuroblastoma cells
SIRT1: Sirtuin1
SMI: Subjective memory impairment
SOD: Superoxide dismutase
SOD1: Superoxide dismutase 1
TH: Tyrosine hydroxylase
TNF-α: Tumor necrosis factor alpha
TrkA: Tropomyosin receptor kinase A
TrkB: Tropomyosin receptor kinase B
TSPO: The translocator protein
TyrRS-PARP1: Tyrosyl tRNA synthetase-poly(ADP-ribose) polymerase 1
VaD: Vascular dementia
YY-1224: A terpene trilactone-enhanced GBE.

Disclosure

AE has served as a consultant or on advisory boards for Vifor Pharma and Schwabe.

Conflicts of Interest

With the relevance to this review, there is no direct conflict of interest to declare.

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References

[1] A. Kumar and A. Singh, "A review on mitochondrial restorative mechanism of antioxidants in Alzheimer's disease and other neurological conditions," *Frontiers in Pharmacology*, vol. 6, p. 206, 2015.

[2] A. Kumar and J. W. Tsao, "Alzheimer Disease," StatPearls, Treasure Island (FL), 2018.

[3] N. S. Ryan and M. N. Rossor, "Correlating familial Alzheimer's disease gene mutations with clinical phenotype," *Biomarkers in Medicine*, vol. 4, no. 1, pp. 99–112, 2010.

[4] F. Jessen, B. Wiese, C. Bachmann et al., "Prediction of dementia by subjective memory impairment: effects of severity and temporal association with cognitive impairment," *Archives of General Psychiatry*, vol. 67, no. 4, pp. 414–422, 2010.

[5] R. A. Sperling, P. S. Aisen, L. A. Beckett et al., "Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for

Alzheimer's disease," *Alzheimer's & Dementia*, vol. 7, no. 3, pp. 280–292, 2011.

[6] K. Abdulrab and R. Heun, "Subjective memory impairment. A review of its definitions indicates the need for a comprehensive set of standardised and validated criteria," *European Psychiatry*, vol. 23, no. 5, pp. 321–330, 2008.

[7] M. I. Geerlings, C. Jonker, L. M. Bouter, H. J. Adèr, and B. Schmand, "Association between memory complaints and incident Alzheimer's disease in elderly people with normal baseline cognition," *American Journal of Psychiatry*, vol. 156, no. 4, pp. 531–537, 1999.

[8] A. F. Jorm, H. Christensen, A. E. Korten, P. A. Jacomb, and A. S. Henderson, "Memory complaints as a precursor of memory impairment in older people: a longitudinal analysis over 7-8 years," *Psychological medicine*, vol. 31, no. 3, pp. 441–449, 2001.

[9] P. St John and P. Montgomery, "Are cognitively intact seniors with subjective memory loss more likely to develop dementia?," *International Journal of Geriatric Psychiatry*, vol. 17, no. 9, pp. 814–820, 2002.

[10] L. Wang, G. van Belle, P. K. Crane et al., "Subjective memory deterioration and future dementia in people aged 65 and older," *Journal of the American Geriatrics Society*, vol. 52, no. 12, pp. 2045–2051, 2004.

[11] J. Xue, J. Li, J. Liang, and S. Chen, "The prevalence of mild cognitive impairment in China: a systematic review," *Aging and Disease*, vol. 9, no. 4, pp. 706–715, 2018.

[12] R. Ihl, L. Frölich, B. Winblad et al., "World Federation of Societies of Biological Psychiatry (WFSBP) guidelines for the biological treatment of Alzheimer's disease and other dementias," *The World Journal of Biological Psychiatry*, vol. 12, no. 1, pp. 2–32, 2011.

[13] A. C. van Harten, M. M. Mielke, D. M. Swenson-Dravis et al., "Subjective cognitive decline and risk of MCI: the Mayo Clinic Study of Aging," *Neurology*, vol. 91, no. 4, pp. e300–e312, 2018.

[14] M. Ganguli, "Depression, cognitive impairment and dementia: why should clinicians care about the web of causation?," *Indian journal of psychiatry*, vol. 51, Supplement 1, pp. S29–S34, 2009.

[15] J. Cerejeira, L. Lagarto, and E. B. Mukaetova-Ladinska, "Behavioral and psychological symptoms of dementia," *Frontiers in neurology*, vol. 3, p. 73, 2012.

[16] L. Sagan, "On the origin of mitosing cells," *Journal of Theoretical Biology*, vol. 14, no. 3, pp. 225–IN6, 1967.

[17] J. Nunnari and A. Suomalainen, "Mitochondria: in sickness and in health," *Cell*, vol. 148, no. 6, pp. 1145–1159, 2012.

[18] A. Grimm, K. Friedland, and A. Eckert, "Mitochondrial dysfunction: the missing link between aging and sporadic Alzheimer's disease," *Biogerontology*, vol. 17, no. 2, pp. 281–296, 2016.

[19] I. Lejri, A. Grimm, and A. Eckert, "Mitochondria, estrogen and female brain aging," *Frontiers in Aging Neuroscience*, vol. 10, p. 124, 2018.

[20] A. Grimm and A. Eckert, "Brain aging and neurodegeneration: from a mitochondrial point of view," *Journal of Neurochemistry*, vol. 143, no. 4, pp. 418–431, 2017.

[21] G. Turrigiano, "Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 1, p. a005736, 2012.

[22] A. Citri and R. C. Malenka, "Synaptic plasticity: multiple forms, functions, and mechanisms," *Neuropsychopharmacology*, vol. 33, no. 1, pp. 18–41, 2008.

[23] D. G. Drubin, "Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors," *The Journal of Cell Biology*, vol. 101, no. 5, pp. 1799–1807, 1985.

[24] R. H. Ring, J. Alder, M. Fennell, E. Kouranova, I. B. Black, and S. Thakker-Varia, "Transcriptional profiling of brain-derived-neurotrophic factor-induced neuronal plasticity: a novel role for nociceptin in hippocampal neurite outgrowth," *Journal of Neurobiology*, vol. 66, no. 4, pp. 361–377, 2006.

[25] P. S. Murray and P. V. Holmes, "An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus," *International Journal of Peptides*, vol. 2011, Article ID 654085, 12 pages, 2011.

[26] G. L. Ming and H. Song, "Adult neurogenesis in the mammalian brain: significant answers and significant questions," *Neuron*, vol. 70, no. 4, pp. 687–702, 2011.

[27] H. Cui, Y. Kong, and H. Zhang, "Oxidative stress, mitochondrial dysfunction, and aging," *Journal of Signal Transduction*, vol. 2012, Article ID 646354, 13 pages, 2012.

[28] P. Mecocci, V. Boccardi, R. Cecchetti et al., "A long journey into aging, brain aging, and Alzheimer's disease following the oxidative stress tracks," *Journal of Alzheimer's Disease*, vol. 62, no. 3, pp. 1319–1335, 2018.

[29] A. Terman, T. Kurz, M. Navratil, E. A. Arriaga, and U. T. Brunk, "Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging," *Antioxidants & Redox Signaling*, vol. 12, no. 4, pp. 503–535, 2010.

[30] A. Kowald and T. B. L. Kirkwood, "Accumulation of defective mitochondria through delayed degradation of damaged organelles and its possible role in the ageing of post-mitotic and dividing cells," *Journal of Theoretical Biology*, vol. 202, no. 2, pp. 145–160, 2000.

[31] Q. Yu, F. du, J. T. Douglas, H. Yu, S. S. D. Yan, and S. F. Yan, "Mitochondrial dysfunction triggers synaptic deficits via activation of p38 MAP kinase signaling in differentiated Alzheimer's disease *trans*-mitochondrial cybrid cells," *Journal of Alzheimer's Disease*, vol. 59, no. 1, pp. 223–239, 2017.

[32] R. H. Swerdlow, J. M. Burns, and S. M. Khan, "The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives," *Biochimica et Biophysica Acta*, vol. 1842, no. 8, pp. 1219–1231, 2014.

[33] R. H. Swerdlow and S. M. Khan, "A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease," *Medical Hypotheses*, vol. 63, no. 1, pp. 8–20, 2004.

[34] L. M. Ittner and J. Götz, "Amyloid-β and tau — a toxic pas de deux in Alzheimer's disease," *Nature Reviews Neuroscience*, vol. 12, no. 2, pp. 65–72, 2011.

[35] W. E. Muller et al., "Therapeutic efficacy of the *Ginkgo* special extract EGb761((R)) within the framework of the mitochondrial cascade hypothesis of Alzheimer's disease," *The World Journal of Biological Psychiatry*, vol. 20, no. 3, pp. 1–17, 2019.

[36] V. Boccardi et al., "Of energy and entropy: the ineluctable impact of aging in old age dementia," *International Journal of Molecular Sciences*, vol. 18, no. 12, article 2672, 2017.

[37] P. Russo, A. Frustaci, A. del Bufalo, M. Fini, and A. Cesario, "Multitarget drugs of plants origin acting on Alzheimer's disease," *Current Medicinal Chemistry*, vol. 20, no. 13, pp. 1686–1693, 2013.

[38] E. E. Elgorashi, G. I. Stafford, and J. Van Staden, "Acetylcholinesterase enzyme inhibitory effects of amaryllidaceae alkaloids," *Planta Medica*, vol. 70, no. 3, pp. 260–262, 2004.

[39] A. Eckert, "Mitochondrial effects ofGinkgo biloba extract," *International Psychogeriatrics*, vol. 24, Supplement 1, pp. S18–S20, 2012.

[40] T. Isah, "Rethinking *Ginkgo biloba* L.: medicinal uses and conservation," *Pharmacognosy Reviews*, vol. 9, no. 18, pp. 140–148, 2015.

[41] A. Booker, D. Frommenwiler, E. Reich, S. Horsfield, and M. Heinrich, "Adulteration and poor quality of *Ginkgo biloba* supplements," *Journal of Herbal Medicine*, vol. 6, no. 2, pp. 79–87, 2016.

[42] C. Ude, M. Schubert-Zsilavecz, and M. Wurglics, "*Ginkgo biloba* extracts: a review of the pharmacokinetics of the active ingredients," *Clinical Pharmacokinetics*, vol. 52, no. 9, pp. 727–749, 2013.

[43] K. C. M. de Lima, C. L. R. Schilichting, L. A. C. Junior, F. M. da Silva, A. Benetoli, and H. Milani, "The*Ginkgo biloba* extract, EGb 761, fails to reduce brain infarct size in rats after transient, middle cerebral artery occlusion in conditions of unprevented, ischemia-induced fever," *Phytotherapy Research*, vol. 20, no. 6, pp. 438–443, 2006.

[44] Z. A. Shah, S. E. Nada, and S. Dore, "Heme oxygenase 1, beneficial role in permanent ischemic stroke and in *Gingko biloba* (EGb 761) neuroprotection," *Neuroscience*, vol. 180, pp. 248–255, 2011.

[45] S. Y. Chung, F. C. Cheng, M. S. Lee, J. Y. Lin, M. C. Lin, and M. F. Wang, "*Ginkgo biloba*leaf extract (EGb761) combined with neuroprotective agents reduces the infarct volumes of gerbil ischemic brain," *The American Journal of Chinese Medicine*, vol. 34, no. 05, pp. 803–817, 2006.

[46] R. Abdel-Kader, S. Hauptmann, U. Keil et al., "Stabilization of mitochondrial function by *Ginkgo biloba* extract (EGb 761)," *Pharmacological Research*, vol. 56, no. 6, pp. 493–502, 2007.

[47] W. Zuo, F. Yan, B. Zhang, J. Li, and D. Mei, "Advances in the studies of *Ginkgo biloba* leaves extract on aging-related diseases," *Aging and Disease*, vol. 8, no. 6, pp. 812–826, 2017.

[48] C. Shi, J. Liu, F. Wu, and D. Yew, "*Ginkgo biloba* extract in Alzheimer's disease: from action mechanisms to medical practice," *International Journal of Molecular Sciences*, vol. 11, no. 1, pp. 107–123, 2010.

[49] A. Eckert, "Stabilization of mitochondrial membrane potential and improvement of neuronal energy metabolism by *Ginkgo biloba* extract EGb 761," *Annals of the New York Academy of Sciences*, vol. 1056, no. 1, pp. 474–485, 2005.

[50] V. Rhein, M. Giese, G. Baysang et al., "Ginkgo Biloba Extract Ameliorates Oxidative Phosphorylation Performance and Rescues Aβ-Induced Failure," *PLoS One*, vol. 5, no. 8, article e12359, 2010.

[51] N. Kaur, M. Dhiman, J. R. Perez-Polo, and A. K. Mantha, "Ginkgolide B revamps neuroprotective role of apurinic/apyrimidinic endonuclease 1 and mitochondrial oxidative phosphorylation against Aβ25-35-induced neurotoxicity in human neuroblastoma cells," *Journal of Neuroscience Research*, vol. 93, no. 6, pp. 938–947, 2015.

[52] L. Liu, C. Zhang, B. Kalionis et al., "EGb761 protects against Aβ1-42 oligomer-induced cell damage via endoplasmic reticulum stress activation andHsp70 protein expression increase in SH-SY5Y cells," *Experimental Gerontology*, vol. 75, pp. 56–63, 2016.

[53] R. W. Stackman, F. Eckenstein, B. Frei, D. Kulhanek, J. Nowlin, and J. F. Quinn, "Prevention of age-related spatial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment," *Experimental Neurology*, vol. 184, no. 1, pp. 510–520, 2003.

[54] F. Tchantchou, Y. Xu, Y. Wu, Y. Christen, and Y. Luo, "EGb 761 enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer's disease," *The FASEB Journal*, vol. 21, no. 10, pp. 2400–2408, 2007.

[55] C. Rendeiro, J. S. Rhodes, and J. P. E. Spencer, "The mechanisms of action of flavonoids in the brain: direct versus indirect effects," *Neurochemistry International*, vol. 89, pp. 126–139, 2015.

[56] W. Wan, C. Zhang, M. Danielsen et al., "EGb761 improves cognitive function and regulates inflammatory responses in the APP/PS1 mouse," *Experimental Gerontology*, vol. 81, pp. 92–100, 2016.

[57] K. Zeng, M. Li, J. Hu et al., "*Ginkgo biloba* extract EGb761 attenuates hyperhomocysteinemia-induced AD like tau hyperphosphorylation and cognitive impairment in rats," *Current Alzheimer Research*, vol. 15, no. 1, pp. 89–99, 2017.

[58] F. Tchantchou, P. N. Lacor, Z. Cao et al., "Stimulation of neurogenesis and synaptogenesis by bilobalide and quercetin via common final pathway in hippocampal neurons," *Journal of Alzheimer's Disease*, vol. 18, no. 4, pp. 787–798, 2009.

[59] Y. Hou, M. A. Aboukhatwa, D. L. Lei, K. Manaye, I. Khan, and Y. Luo, "Anti-depressant natural flavonols modulate BDNF and beta amyloid in neurons and hippocampus of double TgAD mice," *Neuropharmacology*, vol. 58, no. 6, pp. 911–920, 2010.

[60] R. Scott Bitner, "Cyclic AMP response element-binding protein (CREB) phosphorylation: a mechanistic marker in the development of memory enhancing Alzheimer's disease therapeutics," *Biochemical Pharmacology*, vol. 83, no. 6, pp. 705–714, 2012.

[61] S. M. Beck, H. Ruge, C. Schindler et al., "Effects of Ginkgo biloba extract EGb 761® on cognitive control functions, mental activity of the prefrontal cortex and stress reactivity in elderly adults with subjective memory impairment - a randomized double-blind placebo-controlled trial," *Human Psychopharmacology: Clinical and Experimental*, vol. 31, no. 3, pp. 227–242, 2016.

[62] S. I. Gavrilova, U. W. Preuss, J. W. M. Wong et al., "Efficacy and safety of*Ginkgo biloba*extract EGb 761 in mild cognitive impairment with neuropsychiatric symptoms: a randomized, placebo-controlled, double-blind, multi-center trial," *International Journal of Geriatric Psychiatry*, vol. 29, no. 10, pp. 1087–1095, 2014.

[63] B. Grass-Kapanke, A. Busmane, A. Lasmanis, R. Hoerr, and R. Kaschel, "Effects of Ginkgo Biloba Special Extract EGb 761& 174; in Very Mild Cognitive Impairment (vMCI)," *Neuroscience and Medicine*, vol. 02, no. 01, pp. 48–56, 2011.

[64] A. Scripnikov, A. Khomenko, O. Napryeyenko, and for the GINDEM-NP Study Group, "Effects of *Ginkgo biloba* extract EGb 761 on neuropsychiatric symptoms of dementia:

findings from a randomised controlled trial," *Wiener Medizinische Wochenschrift*, vol. 157, no. 13-14, pp. 295–300, 2007.

[65] M. S. Tan, J. T. Yu, C. C. Tan et al., "Efficacy and adverse effects of *Ginkgo biloba* for cognitive impairment and dementia: a systematic review and meta-analysis," *Journal of Alzheimer's Disease*, vol. 43, no. 2, pp. 589–603, 2014.

[66] A. von Gunten, S. Schlaefke, and K. Uberla, "Efficacy ofGinkgo bilobaextract EGb 761®in dementia with behavioural and psychological symptoms: A systematic review," *The World Journal of Biological Psychiatry*, vol. 17, no. 8, pp. 622–633, 2016.

[67] P. L. le Bars, M. M. Katz, N. Berman, T. M. Itil, A. M. Freedman, and A. F. Schatzberg, "A placebo-controlled, double-blind, randomized trial of an extract of *Ginkgo biloba* for dementia. North American EGb Study Group," *JAMA: The Journal of the American Medical Association*, vol. 278, no. 16, pp. 1327–1332, 1997.

[68] K. Maurer, R. Ihl, T. Dierks, and L. Frölich, "Clinical efficacy of *Ginkgo biloba* special extract EGb 761 in dementia of the Alzheimer type," *Journal of Psychiatric Research*, vol. 31, no. 6, pp. 645–655, 1997.

[69] S. Kanowski, W. M. Herrmann, K. Stephan, W. Wierich, and R. Hörr, "Proof of efficacy of the *Ginkgo biloba* special extract EGb 761 in outpatients suffering from mild to moderate primary degenerative dementia of the Alzheimer type or multi-infarct dementia," *Phytomedicine*, vol. 4, no. 1, pp. 3–13, 1997.

[70] S. Gauthier and S. Schlaefke, "Efficacy and tolerability of Ginkgo biloba extract EGb 761® in dementia: a systematic review and meta-analysis of randomized placebo-controlled trials," *Clinical Interventions in Aging*, vol. 9, pp. 2065–2077, 2014.

[71] B. S. Wang, H. Wang, Y. Y. Song et al., "Effectiveness of StandardizedGinkgo bilobaExtract on Cognitive Symptoms of Dementia with a Six-Month Treatment: A Bivariate Random Effect Meta-Analysis," *Pharmacopsychiatry*, vol. 43, no. 03, pp. 86–91, 2010.

[72] N. Brondino, A. de Silvestri, S. Re et al., "A systematic review and meta-analysis of *Ginkgo biloba* in neuropsychiatric disorders: from ancient tradition to modern-day medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, 11 pages, 2013.

[73] S. Kasper, "Phytopharmaceutical treatment of anxiety, depression, and dementia in the elderly: evidence from randomized, controlled clinical trials," *Wiener Medizinische Wochenschrift*, vol. 165, no. 11-12, Article ID 915691, pp. 217–228, 2015.

[74] E. Savaskan, H. Mueller, R. Hoerr, A. von Gunten, and S. Gauthier, "Treatment effects ofGinkgo bilobaextract EGb 761® on the spectrum of behavioral and psychological symptoms of dementia: meta-analysis of randomized controlled trials," *International Psychogeriatrics*, vol. 30, no. 3, pp. 285–293, 2018.

[75] M. C. J. M. van Dongen, E. van Rossum, A. G. H. Kessels, H. J. G. Sielhorst, and P. G. Knipschild, "The efficacy of ginkgo for elderly people with dementia and age-associated memory impairment: new results of a randomized clinical trial," *vJournal of the American Geriatrics Society*, vol. 48, no. 10, pp. 1183–1194, 2000.

[76] R. McCarney, P. Fisher, S. Iliffe et al., "*Ginkgo biloba* for mild to moderate dementia in a community setting: a pragmatic, randomised, parallel-group, double-blind, placebo-controlled trial," *International Journal of Geriatric Psychiatry*, vol. 23, no. 12, pp. 1222–1230, 2008.

[77] J. Birks and J. Grimley Evans, "*Ginkgo biloba* for cognitive impairment and dementia," *Cochrane Database of systematic reviews*, vol. 1, article CD003120, 2009.

[78] V. Demarin, V. Bašić Kes, Z. Trkanjec et al., "Efficacy and safety of *Ginkgo biloba* standardized extract in the treatment of vascular cognitive impairment: a randomized, double-blind, placebo-controlled clinical trial," *Neuropsychiatric Disease and Treatment*, vol. Volume 13, pp. 483–490, 2017.

[79] R. Ihl, "Effects of Ginkgo biloba extract EGb 761®in dementia with neuropsychiatric features: review of recently completed randomised, controlled trials," *International Journal of Psychiatry in Clinical Practice*, vol. 17, Supplement 1, pp. 8–14, 2013.

[80] S. Weinmann, S. Roll, C. Schwarzbach, C. Vauth, and S. N. Willich, "Effects of Ginkgo biloba in dementia: systematic review and meta-analysis," *BMC Geriatrics*, vol. 10, no. 1, p. 14, 2010.

[81] G. Yang, Y. Wang, J. Sun, K. Zhang, and J. Liu, "*Ginkgo biloba* for mild cognitive impairment and Alzheimer's disease: a vsystematic review and meta-analysis of randomized controlled trials," *Current Topics in Medicinal Chemistry*, vol. 16, no. 5, pp. 520–528, 2015.

[82] I. M. Janßen, S. Sturtz, G. Skipka, A. Zentner, M. V. Garrido, and R. Busse, "Ginkgo biloba in Alzheimer's disease: a systematic review," *Wiener Medizinische Wochenschrift*, vol. 160, no. 21-22, pp. 539–546, 2010.

[83] B. Vellas, N. Coley, P. J. Ousset et al., "Long-term use of standardised *Ginkgo biloba* extract for the prevention of Alzheimer's disease (GuidAge): a randomised placebo-controlled trial," *The Lancet Neurology*, vol. 11, no. 10, pp. 851–859, 2012.

[84] B. E. Snitz, E. S. O'Meara, M. C. Carlson et al., "*Ginkgo biloba* for preventing cognitive decline in older adults: a randomized trial," *JAMA*, vol. 302, no. 24, pp. 2663–2670, 2009.

[85] S. T. DeKosky, J. D. Williamson, A. L. Fitzpatrick et al., "*Ginkgo biloba* for prevention of dementia: a randomized controlled trial," *JAMA*, vol. 300, no. 19, pp. 2253–2262, 2008.

[86] T. Charemboon and K. Jaisin, "*Ginkgo biloba* for prevention of dementia: a systematic review and meta-analysis," *Journal of the Medical Association of Thailand*, vol. 98, no. 5, pp. 508–513, 2015.

[87] S. T. DeKosky, A. Fitzpatrick, D. G. Ives et al., "The Ginkgo Evaluation of Memory (GEM) study: design and baseline data of a randomized trial of *Ginkgo biloba* extract in prevention of dementia," *Contemporary Clinical Trials*, vol. 27, no. 3, pp. 238–253, 2006.

[88] E. H. Siemann and L. L. Creasy, "Concentration of the phytoalexin resveratrol in wine," *American Journal of Enology and Viticulture*, vol. 43, no. 1, pp. 49–52, 1992.

[89] T. Richard, A. D. Pawlus, M. L. Iglésias et al., "Neuroprotective properties of resveratrol and derivatives," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 103–108, 2011.

[90] T. Ma, M. S. Tan, J. T. Yu, and L. Tan, "Resveratrol as a therapeutic agent for Alzheimer's disease," *BioMed Research International*, vol. 2014, 13 pages, 2014.

[91] T. Murase, S. Haramizu, N. Ota, and T. Hase, "Suppression of the aging-associated decline in physical performance by a

combination of resveratrol intake and habitual exercise in senescence-accelerated mice," *Biogerontology*, vol. 10, no. 4, pp. 423–434, 2009.

[92] M. H. Muhammad and M. M. Allam, "Resveratrol and/or exercise training counteract aging-associated decline of physical endurance in aged mice; targeting mitochondrial biogenesis and function," *The Journal of Physiological Sciences*, vol. 68, no. 5, pp. 681–688, 2018.

[93] S. S. Karuppagounder, J. T. Pinto, H. Xu, H. L. Chen, M. F. Beal, and G. E. Gibson, "Dietary supplementation with resveratrol reduces plaque pathology in a transgenic model of Alzheimer's disease," *Neurochemistry International*, vol. 54, no. 2, pp. 111–118, 2009.

[94] D. O. Kennedy, E. L. Wightman, J. L. Reay et al., "Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation," *The American Journal of Clinical Nutrition*, vol. 91, no. 6, pp. 1590–1597, 2010.

[95] E. L. Wightman, J. L. Reay, C. F. Haskell, G. Williamson, T. P. Dew, and D. O. Kennedy, "Effects of resveratrol alone or in combination with piperine on cerebral blood flow parameters and cognitive performance in human subjects: a randomised, double-blind, placebo-controlled, cross-over investigation," *British Journal of Nutrition*, vol. 112, no. 2, pp. 203–213, 2014.

[96] C. Chang, Y. Zhao, G. Song, and K. She, "Resveratrol protects hippocampal neurons against cerebral ischemia-reperfusion injury via modulating JAK/ERK/STAT signaling pathway in rats," *Journal of Neuroimmunology*, vol. 315, pp. 9–14, 2018.

[97] H. Deng and M. T. Mi, "Resveratrol Attenuates Aβ25–35 Caused Neurotoxicity by Inducing Autophagy Through the TyrRS-PARP1-SIRT1 Signaling Pathway," *Neurochemical Research*, vol. 41, no. 9, pp. 2367–2379, 2016.

[98] J. Wang, L. Ho, Z. Zhao et al., "Moderate consumption of Cabernet Sauvignon attenuates Aβ neuropathology in a mouse model of Alzheimer's disease," *The FASEB Journal*, vol. 20, no. 13, pp. 2313–2320, 2006.

[99] C. Canto and J. Auwerx, "Caloric restriction, SIRT1 and longevity," *Trends in Endocrinology & Metabolism*, vol. 20, no. 7, pp. 325–331, 2009.

[100] C. Canto and J. Auwerx, "PGC-1α, SIRT1 and AMPK, an energy sensing network that controls energy expenditure," *Current Opinion in Lipidology*, vol. 20, no. 2, pp. 98–105, 2009.

[101] P. Zou, X. Liu, G. Li, and Y. Wang, "Resveratrol pretreatment attenuates traumatic brain injury in rats by suppressing NLRP3 inflammasome activation via SIRT1," *Molecular Medicine Reports*, vol. 17, no. 2, pp. 3212–3217, 2018.

[102] M. Schiedel, D. Robaa, T. Rumpf, W. Sippl, and M. Jung, "The current state of NAD(+)-dependent histone deacetylases (sirtuins) as novel therapeutic targets," *Medicinal Research Reviews*, vol. 38, no. 1, pp. 147–200, 2018.

[103] S. S. Kulkarni and C. Canto, "The molecular targets of resveratrol," *Biochimica et Biophysica Acta*, vol. 1852, no. 6, pp. 1114–1123, 2015.

[104] M. C. Chiang, C. J. Nicol, and Y. C. Cheng, "Resveratrol activation of AMPK-dependent pathways is neuroprotective in human neural stem cells against amyloid-beta-induced inflammation and oxidative stress," *Neurochemistry International*, vol. 115, pp. 1–10, 2018.

[105] D. Kim, M. D. Nguyen, M. M. Dobbin et al., "SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis," *The EMBO Journal*, vol. 26, no. 13, pp. 3169–3179, 2007.

[106] G. Mazzanti and S. Di Giacomo, "Curcumin and resveratrol in the management of cognitive disorders: what is the clinical evidence?," *Molecules*, vol. 21, no. 9, p. 1243, 2016.

[107] A. V. Witte, L. Kerti, D. S. Margulies, and A. Floel, "Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults," *Journal of Neuroscience*, vol. 34, no. 23, pp. 7862–7870, 2014.

[108] J. Lee, N. Torosyan, and D. H. Silverman, "Examining the impact of grape consumption on brain metabolism and cognitive function in patients with mild decline in cognition: a double-blinded placebo controlled pilot study," *Experimental Gerontology*, vol. 87, Part A, pp. 121–128, 2017.

[109] H. Evans, P. Howe, and R. Wong, "Effects of resveratrol on cognitive performance, mood and cerebrovascular function in post-menopausal women; a 14-week randomised placebo-controlled intervention trial," *Nutrients*, vol. 9, no. 1, p. 27, 2017.

[110] National Institutes of Health, CTg, "BDPP treatment for mild cognitive impairment (MCI) and prediabetes (BDPP)," June 2016, https://clinicaltrials.gov/ct2/show/NCT02502253.

[111] National Institutes of Health, CTg, "Effects of dietary interventions on the brain in mild cognitive impairment (MCI)," June 2016, https://clinicaltrials.gov/ct2/show/NCT01219244.

[112] T. Köbe, A. V. Witte, A. Schnelle et al., "Impact of resveratrol on glucose control, hippocampal structure and connectivity, and memory performance in patients with mild cognitive impairment," *Frontiers in Neuroscience*, vol. 11, p. 105, 2017.

[113] R. S. Turner, R. G. Thomas, S. Craft et al., "A randomized, double-blind, placebo-controlled trial of resveratrol for Alzheimer disease," *Neurology*, vol. 85, no. 16, pp. 1383–1391, 2015.

[114] C. Sawda, C. Moussa, and R. S. Turner, "Resveratrol for Alzheimer's disease," *Annals of the New York Academy of Sciences*, vol. 1403, no. 1, pp. 142–149, 2017.

[115] National Institutes of Health, CTg, "Pilot study of the effects of resveratrol supplement in mild-to-moderate Alzheimer's disease," June 2016, https://clinicaltrials.gov/ct2/show/NCT00743743.

[116] National Institutes of Health, CTg, "Randomized trial of a nutritional supplement in Alzheimer's disease," June 2016, https://clinicaltrials.gov/ct2/show/NCT00678431.

[117] E. E. Baulieu, "Neurosteroids: a new function in the brain," *Biology of the Cell*, vol. 71, no. 1-2, pp. 3–10, 1991.

[118] A. G. Mensah-Nyagan, J. L. Do-Rego, D. Beaujean, V. Luu-The, G. Pelletier, and H. Vaudry, "Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system," *Pharmacological reviews*, vol. 51, no. 1, pp. 63–81, 1999.

[119] K. Tsutsui, "Neurosteroids in the Purkinje cell: biosynthesis, mode of action and functional significance," *Molecular Neurobiology*, vol. 37, no. 2-3, pp. 116–125, 2008.

[120] J. L. Do Rego, J. Y. Seong, D. Burel et al., "Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides," *Frontiers in Neuroendocrinology*, vol. 30, no. 3, pp. 259–301, 2009.

[121] W. L. Miller and R. J. Auchus, "The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders," *Endocrine Reviews*, vol. 32, no. 1, pp. 81–151, 2011.

[122] C. D. Smith, D. R. Wekstein, W. R. Markesbery, and C. A. Frye, "3α,5α-THP: a potential plasma neurosteroid biomarker in Alzheimer's disease and perhaps non-Alzheimer's dementia," *Psychopharmacology*, vol. 186, no. 3, pp. 481–485, 2006.

[123] D. Caruso, A. M. Barron, M. A. Brown et al., "Age-related changes in neuroactive steroid levels in 3xTg-AD mice," *Neurobiology of Aging*, vol. 34, no. 4, pp. 1080–1089, 2013.

[124] R. D. Brinton, "Neurosteroids as regenerative agents in the brain: therapeutic implications," *Nature Reviews Endocrinology*, vol. 9, no. 4, pp. 241–250, 2013.

[125] C. A. Frye and A. A. Walf, "Effects of progesterone administration and APPswe+PSEN1Δe9 mutation for cognitive performance of mid-aged mice," *Neurobiology of Learning and Memory*, vol. 89, no. 1, pp. 17–26, 2008.

[126] I. Lejri, A. Grimm, M. Miesch, P. Geoffroy, A. Eckert, and A. G. Mensah-Nyagan, "Allopregnanolone and its analog BR 297 rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement," *Biochimica et Biophysica Acta*, vol. 1863, no. 3, pp. 631–642, 2017.

[127] A. Grimm, K. Schmitt, U. E. Lang, A. G. Mensah-Nyagan, and A. Eckert, "Improvement of neuronal bioenergetics by neurosteroids: implications for age-related neurodegenerative disorders," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1842, no. 12, Part A, pp. 2427–2438, 2014.

[128] J. Yao, S. Chen, K. Wong, R. Irwin, and R. Brinton, "Allopregnanolone as a regenerative therapeutic for Alzheimer's disease, 3: evidence for potentiation of brain mitochondrial function," *Alzheimer's & Dementia*, vol. 9, no. 4, pp. P709–P710, 2013.

[129] M. Karout, M. Miesch, P. Geoffroy et al., "Novel analogs of allopregnanolone show improved efficiency and specificity in neuroprotection and stimulation of proliferation," *Journal of Neurochemistry*, vol. 139, no. 5, pp. 782–794, 2016.

[130] S. Chen, J. M. Wang, R. W. Irwin, J. Yao, L. Liu, and R. D. Brinton, "Allopregnanolone Promotes Regeneration and Reduces β-Amyloid Burden in a Preclinical Model of Alzheimer's Disease," *PLoS One*, vol. 6, no. 8, article e24293, 2011.

[131] C. Singh, L. Liu, J. M. Wang et al., "Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xTgAD and nonTg mice," *Neurobiology of Aging*, vol. 33, no. 8, pp. 1493–1506, 2012.

[132] J. M. Wang, P. B. Johnston, B. G. Ball, and R. D. Brinton, "The neurosteroid allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell-cycle gene and protein expression," *Journal of Neuroscience*, vol. 25, no. 19, pp. 4706–4718, 2005.

[133] National Institutes of Health, Cg, "Allopregnanolone for mild cognitive impairment due to Alzheimer's disease or mild AD (Allo)," June 2018, https://clinicaltrials.gov/ct2/show/NCT02221622.

[134] A. R. Genazzani, N. Pluchino, S. Luisi, and M. Luisi, "Estrogen, cognition and female ageing," *Human Reproduction Update*, vol. 13, no. 2, pp. 175–187, 2007.

[135] A. Grimm, Y. A. Lim, A. G. Mensah-Nyagan, J. Götz, and A. Eckert, "Alzheimer's disease, oestrogen and mitochondria: an ambiguous relationship," *Molecular Neurobiology*, vol. 46, no. 1, pp. 151–160, 2012.

[136] J. Viña, J. Gambini, F. J. García-García, L. Rodriguez-Mañas, and C. Borrás, "Role of oestrogens on oxidative stress and inflammation in ageing," *Hormone Molecular Biology and Clinical Investigation*, vol. 16, no. 2, pp. 65–72, 2013.

[137] J. L. Bowers, V. V. Tyulmenkov, S. C. Jernigan, and C. M. Klinge, "Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta," *Endocrinology*, vol. 141, no. 10, pp. 3657–3667, 2000.

[138] L. Pilsakova, I. Riecansky, and F. Jagla, "The physiological actions of isoflavone phytoestrogens," *Physiological Research*, vol. 59, no. 5, pp. 651–664, 2010.

[139] S. N. Burke and C. A. Barnes, "Neural plasticity in the ageing brain," *Nature Reviews Neuroscience*, vol. 7, no. 1, pp. 30–40, 2006.

[140] S. M. Resnick, M. A. Espeland, S. A. Jaramillo et al., "Postmenopausal hormone therapy and regional brain volumes: the WHIMS-MRI Study," *Neurology*, vol. 72, no. 2, pp. 135–142, 2009.

[141] P. M. Maki, L. Dennerstein, M. Clark et al., "Perimenopausal use of hormone therapy is associated with enhanced memory and hippocampal function later in life," *Brain Research*, vol. 1379, pp. 232–243, 2011.

[142] S. Shanmugan and C. N. Epperson, "Estrogen and the prefrontal cortex: towards a new understanding of estrogen's effects on executive functions in the menopause transition," *Human Brain Mapping*, vol. 35, no. 3, pp. 847–865, 2014.

[143] M. A. Collins, E. J. Neafsey, K. J. Mukamal et al., "Alcohol in moderation, cardioprotection, and neuroprotection: epidemiological considerations and mechanistic studies," *Alcoholism: Clinical and Experimental Research*, vol. 33, no. 2, pp. 206–219, 2009.

[144] Y. J. Park, Y. Jang, and Y. H. Kwon, "Protective effect of isoflavones against homocysteine-mediated neuronal degeneration in SH-SY5Y cells," *Amino Acids*, vol. 39, no. 3, pp. 785–794, 2010.

[145] H. Zeng, Q. Chen, and B. Zhao, "Genistein ameliorates β-amyloid peptide (25–35)-induced hippocampal neuronal apoptosis," *Free Radical Biology and Medicine*, vol. 36, no. 2, pp. 180–188, 2004.

[146] Y. H. Huang and Q. H. Zhang, "Genistein reduced the neural apoptosis in the brain of ovariectomised rats by modulating mitochondrial oxidative stress," *British Journal of Nutrition*, vol. 104, no. 09, pp. 1297–1303, 2010.

[147] M. Bagheri, M. Roghani, M. T. Joghataei, and S. Mohseni, "Genistein inhibits aggregation of exogenous amyloid-beta1–40 and alleviates astrogliosis in the hippocampus of rats," *Brain Research*, vol. 1429, pp. 145–154, 2012.

[148] M. Pan, Z. Li, V. Yeung, and R. J. Xu, "Dietary supplementation of soy germ phytoestrogens or estradiol improves spatial memory performance and increases gene expression of BDNF, TrkB receptor and synaptic factors in ovariectomized rats," *Nutrition & Metabolism*, vol. 7, no. 1, p. 75, 2010.

[149] L. Zhao, Z. Mao, and R. D. Brinton, "A Select Combination of Clinically Relevant Phytoestrogens Enhances Estrogen Receptor β-Binding Selectivity and Neuroprotective Activitiesin Vitroandin Vivo," *Endocrinology*, vol. 150, no. 2, pp. 770–783, 2009.

[150] M. Sonee, T. Sum, C. Wang, and S. K. Mukherjee, "The soy isoflavone, genistein, protects human cortical neuronal cells

from oxidative stress," *Neurotoxicology*, vol. 25, no. 5, pp. 885–891, 2004.

[151] I. Azcoitia, A. Moreno, P. Carrero, S. Palacios, and L. M. Garcia-Segura, "Neuroprotective effects of soy phytoestrogens in the rat brain," *Gynecological Endocrinology*, vol. 22, no. 2, pp. 63–69, 2006.

[152] L. Zhao, Q. Chen, and R. D. Brinton, "Neuroprotective and neurotrophic efficacy of phytoestrogens in cultured hippocampal neurons," *Experimental Biology and Medicine*, vol. 227, no. 7, pp. 509–519, 2002.

[153] J. H. Mitchell, P. T. Gardner, D. B. McPhail, P. C. Morrice, A. R. Collins, and G. G. Duthie, "Antioxidant efficacy of phytoestrogens in chemical and biological model systems," *Archives of Biochemistry and Biophysics*, vol. 360, no. 1, pp. 142–148, 1998.

[154] N. Bansal and M. Parle, "Soybean supplementation helps reverse age- and scopolamine-induced memory deficits in mice," *Journal of Medicinal Food*, vol. 13, no. 6, pp. 1293–1300, 2010.

[155] J. Hwang, J. Wang, P. Morazzoni, H. N. Hodis, and A. Sevanian, "The phytoestrogen equol increases nitric oxide availability by inhibiting superoxide production: an antioxidant mechanism for cell-mediated LDL modification," *Free Radical Biology and Medicine*, vol. 34, no. 10, pp. 1271–1282, 2003.

[156] R. C. M. Siow, F. Y. L. Li, D. J. Rowlands, P. de Winter, and G. E. Mann, "Cardiovascular targets for estrogens and phytoestrogens: transcriptional regulation of nitric oxide synthase and antioxidant defense genes," *Free Radical Biology and Medicine*, vol. 42, no. 7, pp. 909–925, 2007.

[157] K. Mizutani, K. Ikeda, T. Nishikata, and Y. Yamori, "Phytoestrogens attenuate oxidative DNA damage in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats," *Journal of Hypertension*, vol. 18, no. 12, pp. 1833–1840, 2000.

[158] A. E. Valsecchi, S. Franchi, A. E. Panerai, P. Sacerdote, A. E. Trovato, and M. Colleoni, "Genistein, a natural phytoestrogen from soy, relieves neuropathic pain following chronic constriction sciatic nerve injury in mice: anti-inflammatory and antioxidant activity," *Journal of Neurochemistry*, vol. 107, no. 1, pp. 230–240, 2008.

[159] S. File, N. Jarrett, E. Fluck, R. Duffy, K. Casey, and H. Wiseman, "Eating soya improves human memory," *Psychopharmacology*, vol. 157, no. 4, pp. 430–436, 2001.

[160] A. A. Thorp, N. Sinn, J. D. Buckley, A. M. Coates, and P. R. C. Howe, "Soya isoflavone supplementation enhances spatial working memory in men," *British Journal of Nutrition*, vol. 102, no. 09, pp. 1348–1354, 2009.

[161] M. L. Casini, G. Marelli, E. Papaleo, A. Ferrari, F. D'Ambrosio, and V. Unfer, "Psychological assessment of the effects of treatment with phytoestrogens on postmenopausal women: a randomized, double-blind, crossover, placebo-controlled study," *Fertility and Sterility*, vol. 85, no. 4, pp. 972–978, 2006.

[162] C. E. Gleason, C. M. Carlsson, J. H. Barnet et al., "A preliminary study of the safety, feasibility and cognitive efficacy of soy isoflavone supplements in older men and women," *Age and Ageing*, vol. 38, no. 1, pp. 86–93, 2008.

[163] S. Basaria, A. Wisniewski, K. Dupree et al., "Effect of high-dose isoflavones on cognition, quality of life, androgens, and lipoprotein in post-menopausal women," *Journal of Endocrinological Investigation*, vol. 32, no. 2, pp. 150–155, 2009.

[164] M. Soni, T. B. W. Rahardjo, R. Soekardi et al., "Phytoestrogens and cognitive function: a review," *Maturitas*, vol. 77, no. 3, pp. 209–220, 2014.

[165] N. Sumien, K. Chaudhari, A. Sidhu, and M. J. Forster, "Does phytoestrogen supplementation affect cognition differentially in males and females?," *Brain Research*, vol. 1514, pp. 123–127, 2013.

[166] O. Napryeyenko, I. Borzenko, and G-NS Group, "*Ginkgo biloba* special extract in dementia with neuropsychiatric features. A randomised, placebo-controlled, double-blind clinical trial," *Arzneimittelforschung*, vol. 57, no. 1, pp. 4–11, 2007.

[167] H. Herrschaft, A. Nacu, S. Likhachev, I. Sholomov, R. Hoerr, and S. Schlaefke, "Ginkgo biloba extract EGb 761® in dementia with neuropsychiatric features: A randomised, placebo-controlled trial to confirm the efficacy and safety of a daily dose of 240 mg," *Journal of Psychiatric Research*, vol. 46, no. 6, pp. 716–723, 2012.

[168] N. G. Yancheva et al., "*Ginkgo biloba* extract in dementia: a 22-week randomised, placebo-controlled, double-blind trial," *Bulgarian Neurology*, vol. 14, no. 3, pp. 139–143, 2013.

[169] L. Schneider, S. DeKosky, M. Farlow, P. Tariot, R. Hoerr, and M. Kieser, "A Randomized, Double-Blind, Placebo-Controlled Trial of Two Doses of Ginkgo Biloba Extract in Dementia of the Alzheimers Type," *Current Alzheimer Research*, vol. 2, no. 5, pp. 541–551, 2005.

[170] M. Mazza, A. Capuano, P. Bria, and S. Mazza, "*Ginkgo biloba* and donepezil: a comparison in the treatment of Alzheimer's dementia in a randomized placebo-controlled double-blind study," *European Journal of Neurology*, vol. 13, no. 9, pp. 981–985, 2006.

[171] P. L. Le Bars, M. Kieser, and K. Z. Itil, "A 26-Week Analysis of a Double-Blind, Placebo-Controlled Trial of the Ginkgo biloba Extract EGb 761® in Dementia," *Dementia and Geriatric Cognitive Disorders*, vol. 11, no. 4, pp. 230–237, 2000.

[172] S. Kanowski and R. Hoerr, "*Ginkgo biloba* extract EGb 761 in dementia: intent-to-treat analyses of a 24-week, multi-center, double-blind, placebo-controlled, randomized trial," *Pharmacopsychiatry*, vol. 36, no. 6, pp. 297–303, 2003.

[173] M. van Dongen, E. van Rossum, A. Kessels, H. Sielhorst, and P. Knipschild, "Ginkgo for elderly people with dementia and age-associated memory impairment: a randomized clinical trial," *Journal of Clinical Epidemiology*, vol. 56, no. 4, pp. 367–376, 2003.

[174] R. Ihl, M. Tribanek, N. Bachinskaya, and for the GOTADAY Study Group, "Efficacy and Tolerability of a Once Daily Formulation of Ginkgo biloba Extract EGb 761® in Alzheimer's Disease and Vascular Dementia: Results from a Randomised Controlled Trial," *Pharmacopsychiatry*, vol. 45, no. 02, pp. 41–46, 2012.

[175] S. Yancheva, R. Ihl, G. Nikolova et al., "Ginkgo bilobaextract EGb 761®, donepezil or both combined in the treatment of Alzheimer's disease with neuropsychiatric features: A randomised, double-blind, exploratory trial," *Aging & Mental Health*, vol. 13, no. 2, pp. 183–190, 2009.

[176] N. M. Nasab et al., "Efficacy of rivastigmine in comparison to ginkgo for treating Alzheimer's dementia," *Journal of Pakistan Medical Association*, vol. 62, no. 7, pp. 677–680, 2012.

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Research Article

Indoxyl Sulfate Induces Renal Fibroblast Activation through a Targetable Heat Shock Protein 90-Dependent Pathway

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Indoxyl sulfate (IS) accumulation occurs early during chronic kidney disease (CKD) progression and contributes to renal dysfunction by inducing fibrosis, inflammation, oxidative stress, and tissue remodeling. Renal toxicity of high IS concentrations (250 μM) has been widely explored, particularly in resident tubular and glomerular cells, while the effect of a moderate IS increase on kidneys is still mostly unknown. To define the effects of IS accumulation on renal fibroblasts, we first analyzed kidneys of C57BL/6 mice receiving IS (0.1%) in drinking water for 12 weeks. As a next step, we treated renal fibroblasts (NRK-49F) with IS (20 μM) with or without the HSP90 inhibitor 17-AAG (1 μM). In mouse kidneys, IS increased the collagen deposition and HSP90 and α-SMA expression (immunohistochemistry) in interstitial fibroblasts and caused tubular necrosis (histological H&E and picrosirius red staining). In NRK-49F cells, IS induced MCP1, TGF-β, collagen I, α-SMA, and HSP90 gene/protein expression and Smad2/3 pathway activation. IS had no effects on fibroblast proliferation and ROS production. 17-AAG counteracted IS-induced MCP1, TGF-β, collagen I, and α-SMA expression and Smad2/3 phosphorylation. Our study demonstrates that the IS increase promotes renal fibroblast activation by a HSP90-dependent pathway and indicates HSP90 inhibition as a potential strategy to restrain IS-induced kidney inflammation and fibrosis in CKD.

1. Introduction

In patients with chronic kidney disease (CKD), the progressive decline of the glomerular filtration rate (GFR) and kidney metabolic function hinders the removal of several endogenous toxins which are normally cleared by the kidney. A current hypothesis is that these toxic compounds, accumulating in blood and tissues, become triggers for CKD progression and contribute to CKD-related complications.

Indoxyl sulfate (IS) has been extensively studied as a putative uremic toxin [1, 2]. Circulating IS increases rather precociously in CKD patients [3] and reaches very high plasma levels in patients with stage 5-5D CKD, exceeding 500 μM/l as compared to 0.1–2.39 μM/L in the healthy population [4]. Remarkably, previous in vitro observations reveal that even a moderate increase in IS affects cell homeostasis and induces tissue remodeling [5], and several clinical studies point out that IS levels predict the progression of CKD [6].

Renal toxicity of high IS concentrations (exceeding 250 μM) has been widely explored, in particular in resident tubular and glomerular cells. Both in proximal tubule cells and in podocytes [7], IS has profibrotic [8], prooxidant [9], and proinflammatory [10] action, while the effect of a moderate increase in circulating IS levels in kidneys is far to be

defined. Renal fibrosis is a common adaptive response to a variety of pathological triggers, and fibroblast activation in the kidney contributes to tissue remodeling by collagen production and release of profibrotic factors [11], being involved in the activation of multiple pathways which include the TGF-β and the Smad downward signaling [12].

Heat shock proteins (HSPs) are a family of molecular chaperone proteins; among them, HSP90 is one of the most abundant and is involved in protein folding and stabilization [13]. Various stressful conditions induce the activation of HSP90, which has been found to be upregulated during the ischemia-reperfusion injury in the kidney [14] and in models of dermal [15] and pulmonary fibrosis [16]. IS is a ligand of the aryl hydrocarbon receptor (AhR); upon binding, IS and AhR form a complex with HSP90, which translocates to the nucleus and promotes proinflammatory and fibrotic target gene transcription.

In this paper, we initially observed the effect of IS supplementation on kidney histology, HSP90 expression, and fibroblast phenotype in mice. Next, we characterized *in vitro* the effects of 20 μM IS, a concentration found in early stages of CKD, on the renal fibroblast phenotype and inflammatory profile through activation of Smad 2/3 and HSP90. Finally, we demonstrated that pretreatment with 17-N-allylamino-17demethoxygeldanamycin (17-AAG), a selective HSP90 inhibitor, is able to reverse the IS-induced fibroblast activation, suggesting HSP90 inhibition as an option to restrain fibrosis in kidneys during CKD progression.

2. Materials and Methods

2.1. Animal Model. Male C57BL/6 mice (bred in-house from stock originally obtained from Harlan Laboratories, S. Pietro al Natisone, UD, Italy), 8 to 12 weeks old, were housed in a pathogen-free environment. Water and regular mouse diet were available ad libitum. Two separate groups were used to acquire data: the IS group (IS, $N = 6$), receiving 0.1% IS in drinking water, and the control group (CTR, $N = 6$). Water consumption was recorded every second day, when replacing the IS solution, and the body weight weekly. The study lasted for 12 weeks (Supplementary 1, Figure 1A). At the termination of the in vivo study, mice were killed in a CO_2 chamber. Immediately after explantation, kidneys were dissected and kept in cold PBS for 40 minutes, changing the buffer solution 3 times, and then fixed in cold 2% paraformaldehyde.

2.2. Histopathological Examination and Fibrosis Quantification. Standard histopathological techniques were followed for processing the fixed kidney tissue and the preparation of paraffin blocks. Hematoxylin and eosin (H&E) staining was performed to detect tissue damage and tubular necrosis. Specimens were examined in a blinded manner by two pathologists independently under light microscopy. Briefly, six high-power fields (40x magnification) were checked for confluent cell necrosis or sloughing of the tubular epithelium and loss of nuclei and of cytoplasm (evidenced as light areas), as described by Speir and colleagues [17].

Picrosirius red staining was performed to quantify collagen deposition. The paraffin sections were dewaxed, hydrated, and stained with a picrosirius red solution (0.1% sirius red F3B in saturated picric acid) for 1 h, washed twice in acidified water, and counterstained with Carazzi's hematoxylin.

2.3. Immunohistochemistry. Paraffin sections (5 μm) of 2% paraformaldehyde-fixed tissue were analyzed for an HSP90 and nitrotyrosine mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) and α-SMA mouse monoclonal antibody (Dako Agilent Pathology Solution, Santa Clara, USA). Immunostaining was performed as described previously [18].

2.4. Cell Cultures and Treatments. Rat kidney fibroblast cells (NRK-49F) were obtained from the American Type Culture Collection (ATCC, Carlsbad, California, USA). NRK-49F cells were maintained in DMEM (EuroClone, Milan, Italy) containing 2 mmol L-glutamine and 100 U/mL penicillin-streptomycin (EuroClone), with 5% FBS, and incubated at 37°C with 5% of CO_2.

NRK-49F cells were incubated with 20 μM IS, diluted in ultrapure H_2O, for different time lags, depending on the experimental requirements: the cells were incubated for 15, 30, and 120 minutes to assess the activation of HSP90 and p- (phospho-) Smad 2/3, for 5 hours to evaluate the mRNA levels of MCP1, TGF-β, and collagen I, for 1, 3, and 16 hours to evaluate the mRNA levels of NOX4, and for 24 hours to evaluate α-SMA and collagen I protein expression. Finally, fibroblasts were treated with 20 μM IS for 60 minutes to assess the ROS production and for 24 and 48 hours to analyze the cell proliferation. In selected experiments, 1 μM HSP90 inhibitor (tanespimycin (17-AAG); Selleckchem, Munich, Germany) was added to the culture medium 1 hour before stimulation with IS.

2.5. mRNA Analysis. The total RNA was extracted using the QIAzol Lysis Reagent (Qiagen Sciences, Maryland, USA), and the concentration and integrity of each sample were evaluated on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). 1 μg RNA was used for cDNA synthesis.

2.6. cDNA Reverse Transcription and Quantitative Real-Time PCR. cDNA synthesis was performed using the iScript™ cDNA synthesis kit RT (Bio-Rad Laboratories Inc., Hercules, California, USA). MCP1, TGF-β, collagen I, NOX4, and GAPDH primers were obtained from Tib Molbiol Srl (Genoa, Italy), and sequences are reported in Table 1. PCR amplification was carried out in a total volume of 10 μl, containing 1 μl of cDNA solution, 5 μl of SYBR Master Mix solution (Eppendorf, Hamburg, Germany), 0.03 μl of each primer, and 3.94 μl of nuclease-free water. GAPDH was quantified and used for the normalization of expression values of the other genes. Fluorescence signals measured during the amplification were considered positive if the fluorescence intensity was more than 20-fold greater than the standard deviation of the baseline fluorescence. The $\Delta\Delta$CT method of relative quantification was used to determine the fold change in expression. Assays were run in

Table 1: Primer sequences.

Name	Species	Accession number	Forward	Reverse
GAPDH	Rat	NM_017008.4	ctctctgctcctccctgttct	atacggccaaatccgttcaca
Collagen I	Rat	NM_053304.1	tcacctacagcacgcttg	ggtctgtttccagggttg
TGF-β	Rat	NM_021578.2	tggaagtggatccacgcgcccaagg	gcaggagcgcacgatcatgttggac
MCP1	Rat	NM_031530.1	cagttaatgccccactcacct	tgacaaatactacagcttctttggg
NOX4	Rat	NM_053524.1	gatgactggaaaccatacaagctaag	catagagcaagtctgcaaaccaactg

triplicate using Universal PCR Master Mix on a MasterCycler RealPlex (Eppendorf) PCR system.

2.7. Proliferation and ROS Production. Proliferation was evaluated by cell labeling with carboxyfluorescein succinimidyl ester (CFDA-SE; Invitrogen, Carlsbad, California, USA). Data were analyzed with the Proliferation Wizard module of the ModFit LT 4.0 software (Verity Software House, Topsham, ME, USA), and the results were expressed as the proliferation index.

Intracellular ROS production was evaluated using the CellROX Deep Red kit from Life Technologies (Carlsbad, California, USA). Following treatments, a CellROX reagent was added for 30 minutes. Cells were directly analyzed on FACSCanto II.

2.8. Western Blot Analysis. Cells were lysed in cold buffer (20 mM HEPES, 150 mM NaCl, 10% (*v*/*v*) glycerol, 0.5% (*v*/*v*) NP-40, 1 mM EDTA, 2.5 mM DTT, 10 μg/l aprotinin, leupeptin, pepstatin A, 1 mM PMSF, and Na3VO4). Protein concentration was determined by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; Rockford, IL, USA), and 10–20 μg was resolved on SDS-polyacrylamide gels and electrotransferred to a PVDF membrane (Merck Group). Blots were probed using an anti-HSP90 (AC88) monoclonal antibody (Santa Cruz Biotechnology), anti-p-SMAD 2 (Ser 465/467) polyclonal antibody (Cell Signaling Technology; Danvers, USA), anti-p-SMAD 3 (Ser 423/425) monoclonal antibody (Cell Signaling Technology), anti-α-SMA monoclonal antibody (Dako Agilent Pathology Solution; Santa Clara, CA, USA), anti-TGF-β monoclonal antibody (Santa Cruz Biotechnology), and anti-CCL2/MCP1 polyclonal antibody (Novus Biologicals). The reference proteins were detected with an anti-β-actin mouse monoclonal antibody (Santa Cruz Biotechnology), anti-Smad rabbit polyclonal antibody (Cell Signaling Technology), or anti-histone 3 rabbit polyclonal antibody (Cell Signaling Technology) and incubated with horseradish peroxidase secondary antibodies (Cell Signaling Technology). Immunoblots were developed with the Immobilon Western Chemiluminescent HRP Substrate (Merck Group, Darmstadt, Germany). Band intensities were determined using the Alliance system (Uvitec; Cambridge, UK).

2.9. Immunocytochemistry and Immunofluorescence. NRK-49F cells were grown on chamber slides to subconfluence, treated with IS with or without 17-AAG and fixed in cold methanol. For immunocytochemistry, after a 24-hour treatment, fixed cells had undergone quenching of the endogenous peroxidase with H_2O_2 in PBS and incubation with an anti-collagen I polyclonal antibody (Proteintech; Manchester, UK) and with an anti-α-SMA monoclonal antibody (Dako Agilent Pathology Solution); immunostaining was then performed as previously described [19]. For immunofluorescence, treatments lasted for 15, 45, and 120 min. Cells were incubated with an anti-HSP90 monoclonal antibody (AC88, Santa Cruz Biotechnology) and then with the secondary anti-mouse antibody goat-anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA).

2.10. Image Analysis. In immunohistochemical/immunocytochemical staining, the positivity was evaluated by image analysis performed using the Leica Q500 MC Image Analysis System (Leica, Cambridge, UK) as previously described [18]. For picrosirius red staining, a total of 10 fields were randomly chosen per mouse and images were viewed with brightfield illumination as well as with polarization contrast illumination at 40x. Picrosirius red is a birefringent molecule that binds to collagens. The complex fibrillar collagen/sirius red can be detected under polarized light, and the collagen bundles are red, yellow, and green. Collagen expression was quantified under brightfield illumination.

2.11. Statistics. In vitro experiments were performed at least 3 times. Summary data are expressed as mean ± SEM and compared by Student's *t*-test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. IS Induces Fibrosis and HSP90 Activation in Mice. Kidneys of C57BL/6 mice receiving continuous supplementation of 0.1% IS in drinking water displayed signs of tubular necrosis (Figure 1(a)) and interstitial fibrosis whose severity was evaluated by picrosirius red staining (Figure 1(b)). As shown in Figure 1(b), a significant increase in staining intensity was noticeable in the interstitial zone of kidneys obtained from IS-treated mice (3 fold in respect top untreated control mice, $p < 0.01$). α-SMA expression in the interstitium is associated with the progression of kidney disease, and the accumulation of α-SMA-positive fibroblasts represents the earliest histological marker of fibrosis progression [20]. We found a significant increase in α-SMA protein in interstitial fibroblasts (2-fold vs. control mice, $p < 0.05$) (Figure 1(c)) and upregulation of HSP90 in the tubular-interstitial compartment (1.4-fold increase vs. control mice, $p < 0.05$) (Figure 1(d)).

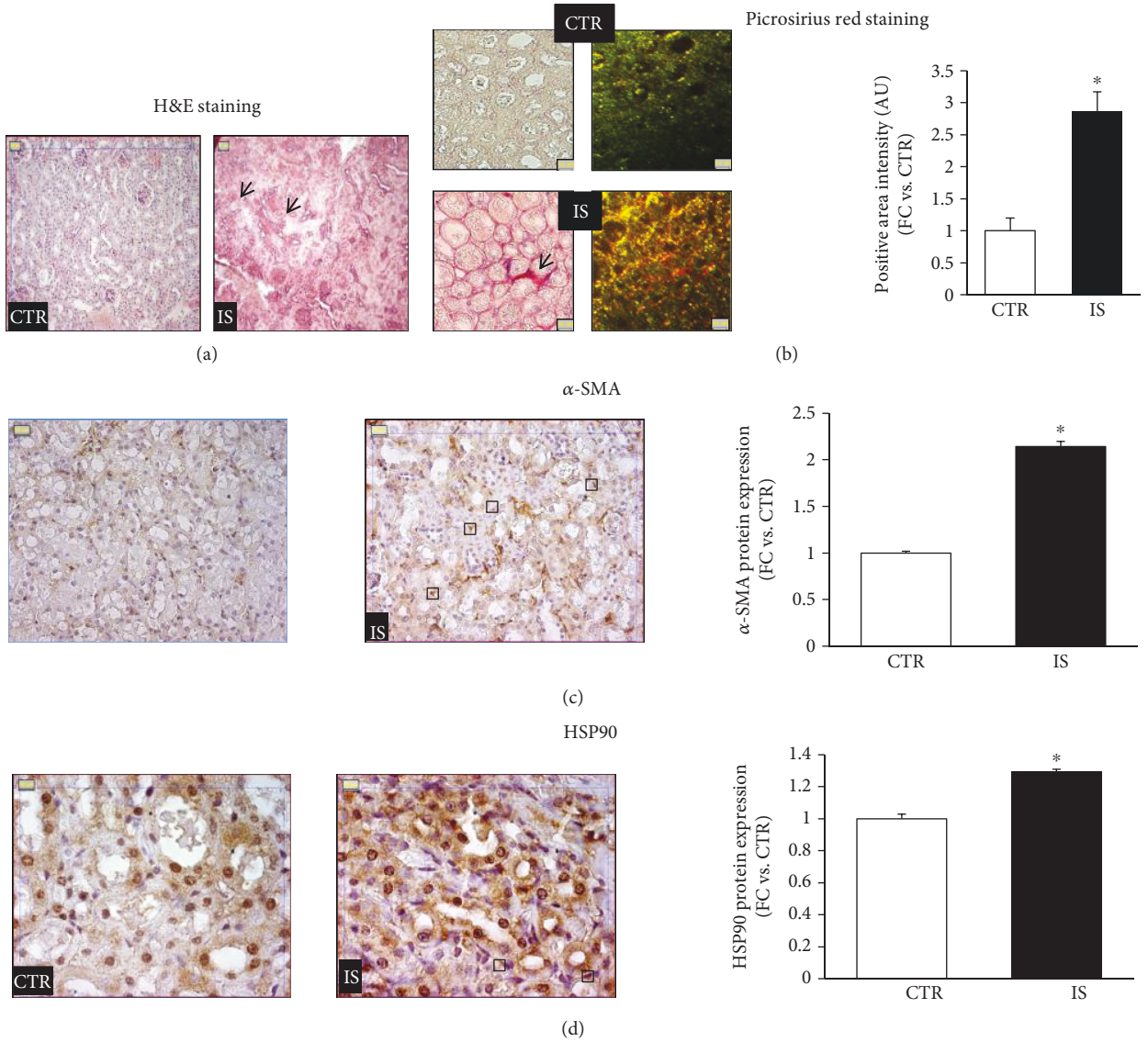

Figure 1: Mouse kidney analysis after a 12-week exposure to 0.1% IS. (a) H&E staining; arrows indicate areas of tubular necrosis. (b) Collagen deposition as revealed by picrosirius red staining, brightfield illumination (left side) and polarization contrast illumination (right side); the graph represents the quantification of red staining intensity under brightfield illumination. (c, d) Images of immunochemistry of α-SMA and HSP90; boxes specify the positive nuclei of interstitial fibroblasts found in the kidney from IS-treated mice. The histograms indicate the immunopositivity quantification (fold change: FC). (magnification: (a)–(c), ×400; (d), ×1000); $^*p < 0.05$.

These data revealed that even moderate IS increases may induce HSP90 overexpression and prime interstitial fibroblasts to an activated phenotype.

3.2. IS Induces a Profibrotic and Proinflammatory Phenotype in NRK49F Cells. Next, we examined the effects of 20 μM IS on the profibrotic and proinflammatory phenotype in the NRK-49F cell line.

Both collagen I mRNA and protein were increased 3-fold ($p < 0.05$ and 0.001, respectively) (Figure 2(a)), and α-SMA protein level arose significantly, as shown by western blot analysis and immunocytochemistry (Figure 2(b): 9.5-fold, $p < 0.01$), in respect to untreated control cells.

IS also induced expression of TGF-β (Figure 2(c): 2.8-fold for mRNA and 2-fold for protein expression vs. untreated cells; $p < 0.05$ and 0.01, respectively), a potent mediator in renal fibrosis, and MCP1, one of the inflammatory cytokines involved in tubular-interstitial injury (3-fold for mRNA and 1.5-fold for protein expression vs. untreated cells, $p < 0.05$) (Figure 2(d)).

3.3. IS Does Not Stimulate Proliferation and Oxidative Stress in NRK49 Cells. Proliferation of fibroblasts and oxidative stress are critical pathological processes during initiation and maintenance of fibrotic lesions; however, in our experimental setting, 20 μM IS did not significantly affect the

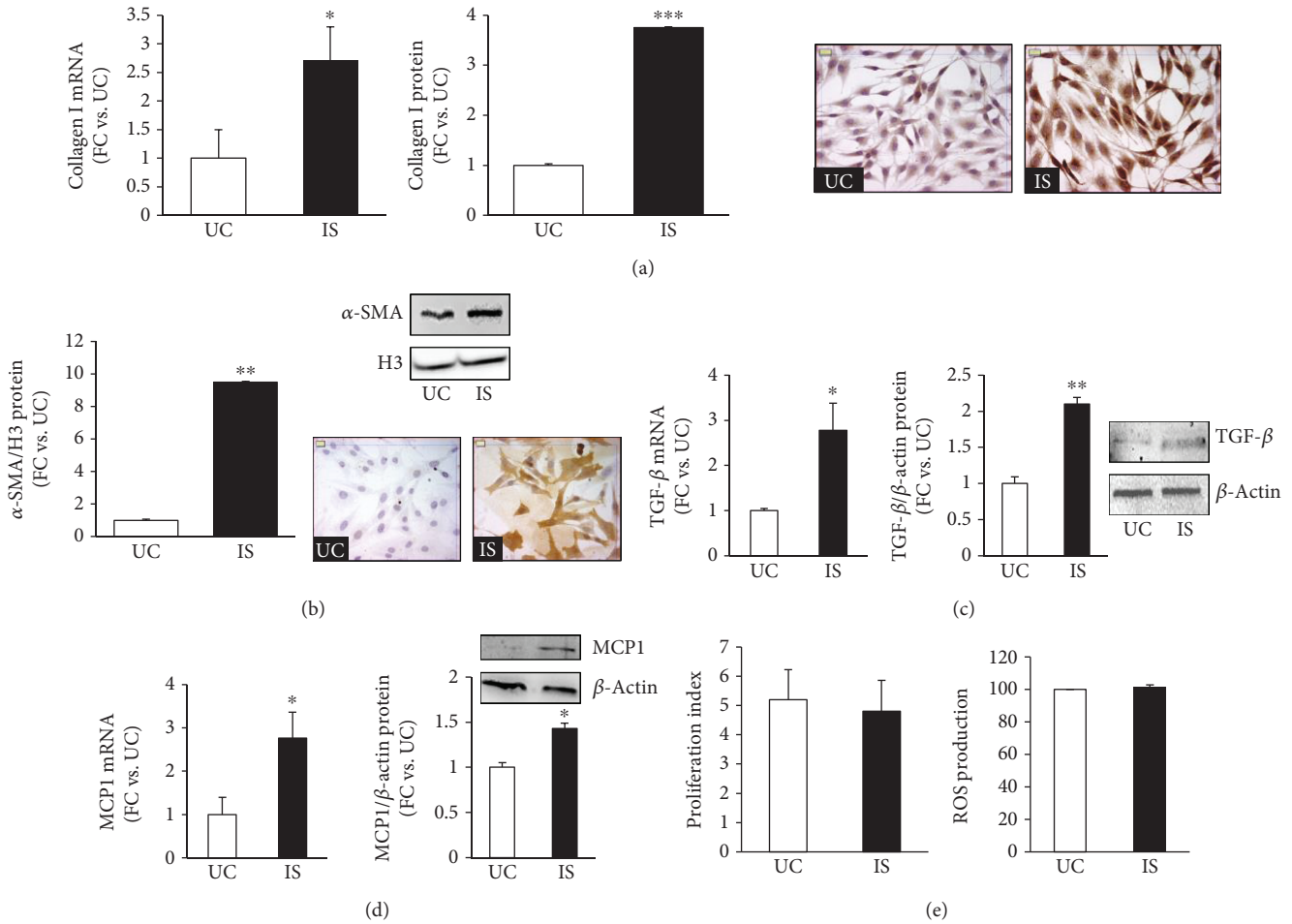

Figure 2: Effects of IS on the NRK-49F rat kidney fibroblast cell line. (a) Collagen I mRNA and protein in IS-treated and untreated NRK-49F cells. Data are expressed as fold change (FC) ± SEM versus untreated cells ($^{*}p < 0.05$ and $^{***}p < 0.001$, respectively). Photos are representative of collagen I expression and pattern evaluated by immunocytochemistry (magnification ×400). (b) α-SMA protein expression in IS-treated and untreated NRK-49F cells. Data, obtained by western blot analysis, are expressed as fold change (FC) ± SEM versus untreated cells ($^{**}p < 0.01$). Pictures are representative of α-SMA expression evaluated by western blot and immunocytochemistry. (c) TGF-β and (d) MCP1 mRNA and protein levels in IS-treated or untreated NRK-49F cells. mRNA expression is evaluated by real-time PCR, normalized for GAPDH mRNA. Western blot analysis is normalized for β-actin; values are expressed as fold increase ± SEM versus untreated cells ($^{*}p < 0.05$). (e) Flow cytometry analysis of the proliferation index, after 48 hours of exposure, and ROS production, after 60 minutes of exposure, as detected by the probes CFSE and CellRox, respectively, in NRK-49F cells treated or not with 20 μM IS. UC: untreated cells; IS: indoxyl sulfate-treated cells.

proliferation rate neither the redox status as revealed by ROS production (Figure 2(e)) and NOX4 mRNA (Supplementary 2, Figure 2).

3.4. Effects of IS on Cell Signaling. To confirm our observation on mouse kidneys, we verified in NRK-49F cells the effects of IS on HSP90 activation. IS induced a 70% increase in HSP90 protein expression after a 15-minute treatment; this increase in respect to the control level lasted 2 hours ($p < 0.01$-0.05 vs. baseline referred to as T0) (Figure 3(a)). By looking at the intracellular distribution, a marked perinuclear concentration of HSP90 was noticeable already after a 15-minute IS treatment (Figure 3(b)); looking at the downstream signaling, we examined the effects of IS on the Smad pathway (Figure 4). Both Smad 2 and Smad 3 are activated in respect to T0 after 120 minutes ($p < 0.05$ and $p < 0.001$, respectively) with Smad 3 phosphorylation occurring early within 15 minutes ($p < 0.01$).

3.5. Effects of HSP90 Inhibition on the Profibrotic and Proinflammatory Phenotype. We next examined the effects of IS treatment in the presence of 1 μM 17-AAG, a selective inhibitor of HSP90. 17-AAG counteracted the increase in mRNA and protein collagen I synthesis (-70 and -80%, respectively, $p < 0.05$-0.001) (Figure 5(a)) and in α-SMA protein expression ($p < 0.05$) as depicted in Figure 5(b). The inhibition of HSP90 also blunted TGF-β and MCP1 mRNA (-90%, $p < 0.001$) and protein expression (-60%

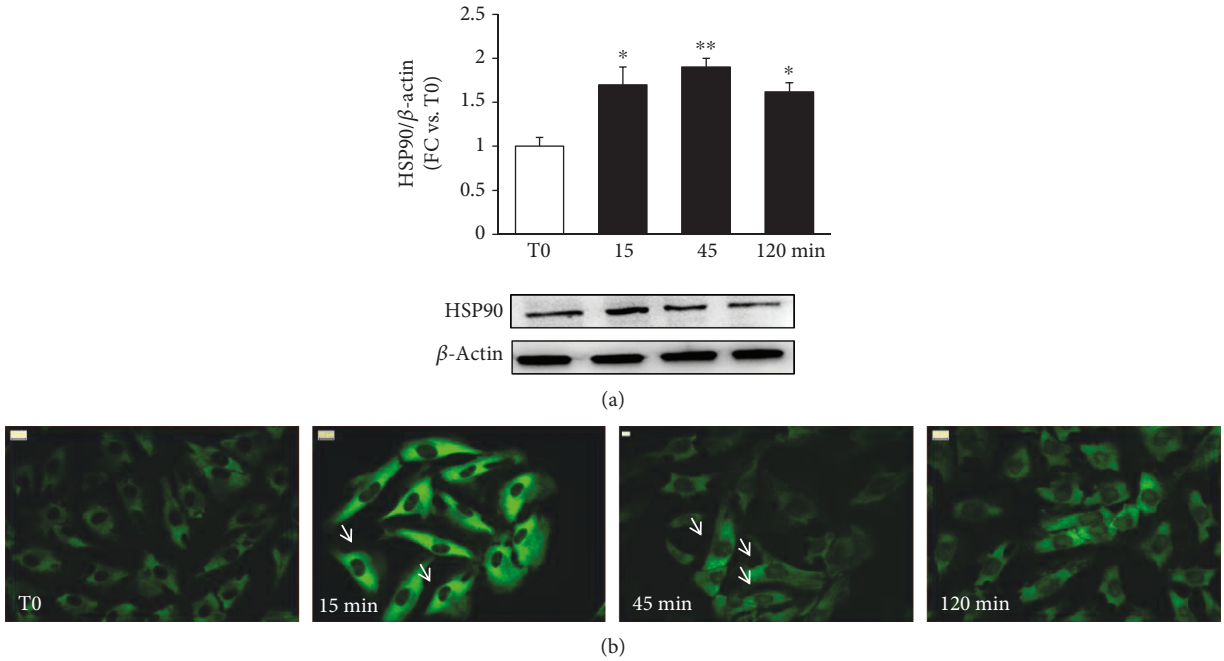

Figure 3: Effects of IS on HSP90 after different times of IS exposure (0, 15, 45, and 120 min). (a) Total protein expression, evaluated by western blot, and (b) intracellular localization, detected by immunofluorescence. Arrows indicate perinuclear localization ($^{*}p < 0.05$, $^{**}p < 0.01$).

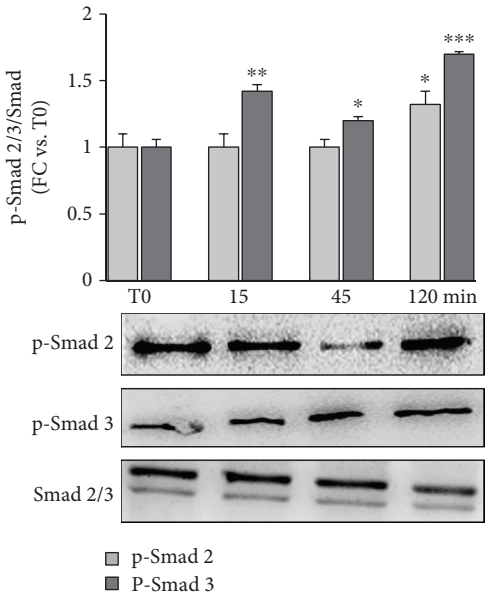

Figure 4: Smad 2 and 3 phosphorylation after different times of IS exposure (15, 45, and 120 min) in respect to untreated cells (T0), normalized for total Smad 2/3. Measures are represented as fold changes ± SEM versus untreated cells ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

and -40%, respectively, $p < 0.01$) (Figures 5(c) and 5(d)). Lastly, 17-AAG lowered NRK-49F cells proliferation ($p < 0.05$ vs. IS-treated cells) (Figure 5(e)).

3.6. 17-AAG Blocks IS-Induced Activation of the Smad Pathway. We then evaluated the effect of 17-AAG on HSP90 and Smad 2/3 activation. As shown in Figure 6, the pretreatment with 17-AAG blocked, stably over time, the perinuclear and nuclear HSP90 translocation (Figure 6(a)) and the IS-induced phosphorylation of Smad 2/3 (-80% and -60%, $p < 0.05$-0.01) (Figure 6(b)).

4. Discussion

The purpose of the present study was essentially to better understand whether moderate IS levels contribute to renal fibroblast activation. First, we set up an in vivo experimental design consisting in a chronic supplementation of moderate amount of IS to mice with a preserved renal function (Supplementary 1); then, to buttress our ex vivo observation, we evaluated in vitro the potential for moderate IS concentration, such as 20 μM, to prime the renal fibroblast phenotype. Finally, since the cytoplasmic receptor of IS, AhR, is tightly regulated by the molecular complex with the chaperone HSP90 [21], we tested whether the profibrotic effects of IS occur through the HSP90/Smad 2/3 pathway.

Our results highlight that even the modest increase in IS, administered continuously for 12 weeks, promotes HSP90 upregulation and α-SMA expression, a hallmark of fibroblast transition from a resting phenotype to an activated phenotype, as well as interstitial collagen deposition. When tested on renal fibroblast cell culture, 20 μM IS was able to induce a profibrotic and proinflammatory phenotype, by upregulating the TGF-β signal and collagen I synthesis. We also demonstrated that IS-induced fibroblast activation occurs through the HSP90/Smad 2/3-dependent pathway, as proven by the efficacy of the HSP90 inhibitor (17-AAG) in inhibiting IS-induced TGF-β signaling and collagen I synthesis.

Among a variety of uremic toxins, IS is of particular interest because its levels are markedly elevated in CKD [6]

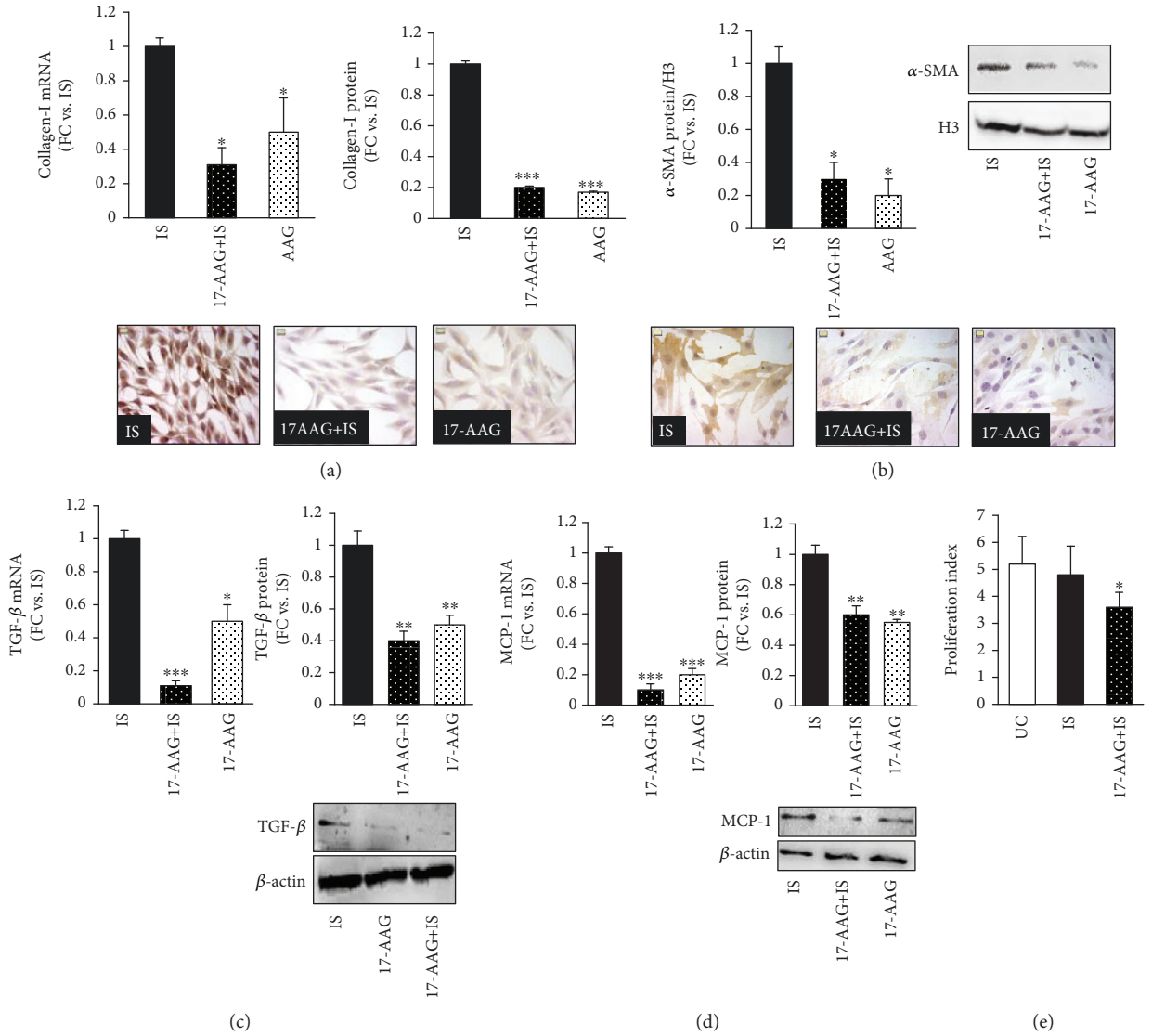

Figure 5: Effects of HSP90 inhibition on the profibrotic and proinflammatory phenotype of NRK-49F cells. (a) Collagen I mRNA and protein levels and representative images (magnification 400x). The protein expression is evaluated by immunocytochemistry, as shown in the pictures. Values are expressed as fold changes ± SEM versus cells treated with IS. (b) Western blot and immunocytochemistry for α-SMA protein expression. The graph reports the measure obtained from western blot analysis. (c) TGF-β and (d) MCP1 mRNA and protein expression. (e) Proliferation index of IS and 17-AAG+IS cells vs. untreated cells ($^{*}p < 0.05$, $^{***}p < 0.001$). mRNA expression is tested by real-time PCR and normalized to GAPDH mRNA. UC: untreated cells; IS: indoxyl sulfate-treated cells; 17-AAG+IS: cells pretreated with the HSP90 inhibitor 17-AAG and then treated with IS.

and it is hardly removed by conventional dialysis due to its protein-binding capacity [22]. Several studies have demonstrated that IS, at uremic concentration (100–500 μM), exerts profibrotic and proinflammatory effects on mesangial [23] and tubular cells [24] and induces epithelial-to-mesenchymal transition in NRK-52E renal proximal tubular cells [25].

Previous animal models on mice and rats were aimed at obtaining the features of a severe renal failure, which include the accumulation of not only IS but also other endogenous compounds [26, 27]; although these models are useful experimental tools to understand the mechanism operating in the uremic condition, they cannot be suggestive when considering a progressive accumulation of IS for small, chronic intake, as occurs in the first stages of the renal disease.

So far, IS has been scarcely investigated in relation to renal fibroblasts and, mostly, when considering moderate IS increases, as those utilized in our experimental setting and found during transition from early to moderate kidney damage [28]. These few studies demonstrate that the exposure to 1-5 mg/lt IS can induce inflammation in renal tubular cells [29] and kidney tissue remodeling through binding and activation of the renal EGF receptor [30]. On this regard, we believe that our animal and in vitro experimental design might offer a good platform to investigate its contribution to renal fibrosis onset in all those conditions that, beside renal

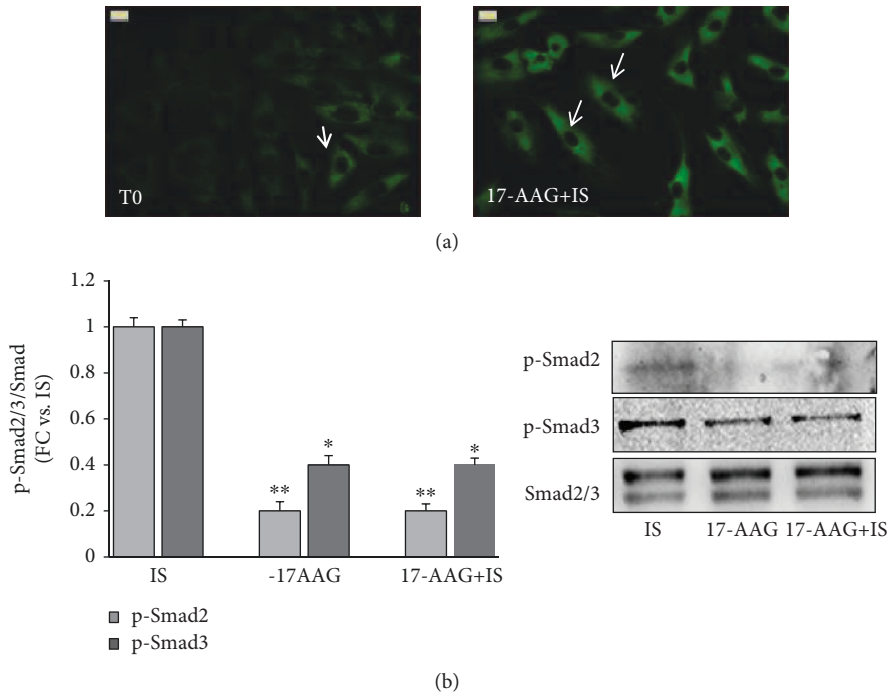

Figure 6: 17-AAG blocks HSP90-dependent activation of the Smad pathway induced by IS in NRK-49F cells. (a) HSP90 intracellular localization after a 120-minute IS treatment with or without 17-AAG. Arrows indicate nuclear sites, where no immunopositivity occurred in the presence of 17-AAG. (b) Western blot of Smad 2/3 phosphorylation in cells treated for 120 minutes with IS, 17-AAG, and 17-AAG +IS, respectively. Results are normalized for total Smad 2/3. Values are expressed as fold change ± SEM versus cells treated with IS ($^{*}p < 0.05$ and $^{**}p < 0.01$). IS: indoxyl sulfate-treated cells; 17-AAG+IS: cells pretreated with the HSP90 inhibitor 17-AAG and then treated with IS.

failure, may lead to increased level of circulating IS, such as gut microbiota disequilibrium, reduced plasma albumin, and unbalanced IS/albumin ratio for binding competition with other uremic compounds [31].

In the pathogenesis of CKD, resident fibroblasts are key players and renal interstitial fibrosis is considered the hallmark of progressive renal disease. Several studies demonstrate that renal impairment correlates better with interstitial changes than with glomerular changes in most forms of CKD, indicating that renal function is also influenced by the interstitial cell behavior. In 5/6-nephrectomized uremic rats, administration of IS upregulated TGF-β, tissue inhibitor of metalloproteinase-1 (TIMP-1), and proalpha 1(I) collagen in the renal cortex and provoked a significant decline in renal function and worsening of renal sclerosis [32].

High IS levels induce ROS production in different cell types, such as vascular endothelial cells, vascular smooth muscle cells, renal tubular cells, monocytes, and macrophages [33–37]. In our setting, the NOX4 mRNA level and ROS production are unchanged by IS treatment, indicating that the IS detrimental effects on our cell line are not induced by oxidative stress damage. This result is reinforced also by ex vivo immunostaining of kidneys for nitrotyrosine, a marker for inflammation and ROS production suitable to detect oxidative damage: increased positivity is detectable in kidneys from IS-treated mice and is mainly localized in tubular cells, while interstitial fibroblasts are negative (Supplementary 3).

A similar observation was obtained from monocytes exposed to different IS concentrations: moderate levels of IS (1-20 μM) evoked only a transient rise in ROS production, but sufficient to promote monocyte differentiation toward a profibrotic and proinflammatory phenotype [5].

The TGF-β/Smad 2/3-mediated damage has been found to be operative in several renal fibrotic models [38, 39] and human nephropathies [40]; in the present study, we identify the HSP90/Smad 2/3 activation as the pathway of IS profibrotic induction.

Fibrosis and inflammation constitute a deleterious loop, and the development of fibrosis with loss of renal function often follows renal inflammation [41].

The IS proinflammatory effect was shown in several different cell types: endothelial cells [42], adipocytes [43], and glomerular [23] and renal tubular cells [29]; these studies recognized IS as an important mediator of cell dysfunction in promoting a persistent and systemic inflammatory state in CKD patients. In our experimental setting, we demonstrated that IS can stimulate MCP1 expression in renal fibroblasts and identified HSP90 as a possible shared pawn between IS-induced fibrosis and inflammation in renal fibroblasts.

HSP90 is one of several stress proteins, and as such, its modulation is a potential therapeutic target under stressful conditions. Previous studies demonstrated that modulation of HSP90 affects TGF-β-induced collagen synthesis in dermal fibroblasts, [15], attenuates renal fibrosis through degradation of the TGF-β type II receptor in TGF-β1-treated renal

tubular cells and in a murine CKD model [44], regulates the fibroblast activation in pulmonary and hepatic fibrosis [16, 45], and hampers the inflammatory response in atherosclerosis [46] and in ischemia-reperfusion injury in the kidney [47].

To date, the link between HSP90 activation/inhibition and IS effects was scarcely studied. First of all, we have observed that IS induces HSP90 expression; accordingly, pretreatment with 17-AAG blunted Smad 2/3 signaling, inflammatory (MCP1) and fibrotic molecule expression (collagen I, α-SMA, and TGF-β), and proliferation of renal fibroblasts, suggesting HSP90 activity as a crossroad for IS-induced inflammation and fibrosis.

In conclusion, our report demonstrates that moderate levels of IS cause fibrosis and inflammation by upregulating HSP90 in renal fibroblasts and suggests HSP90 inhibition as an effective tool for reducing IS-induced damage and slowing the progression of renal disease.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Part of the in vitro data has been reported as a poster presentation at the ERA-EDTA Congress, Copenhagen, Denmark, May 24-27, 2018 (https://academic.oup.com/ndt/article/33/suppl_1/i58/4997136), and an oral presentation at the ICRNM, Genoa, Italy, June 26-30, 2018.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplementary 1. Figure 1: (A) in vivo experimental design, (B) body weight, and (C) weekly liquid consumption records.

Supplementary 2. Figure 2: the NOX4 mRNA levels are not modified by 1-hour treatment with IS.

Supplementary 3. Figure 3: immunostaining for nitrotyrosine to detect oxidative damage in vivo. Images A-B: magnification 20x; in image B, white boxes indicate the necrotic area. Images C-D: magnification 40x; in image D, black arrows indicate interstitial fibroblasts (negative to the immunostaining).

References

[1] R. Vanholder, E. Schepers, A. Pletinck, E. V. Nagler, and G. Glorieux, "The uremic toxicity of indoxyl sulfate and p-cresyl sulfate: a systematic review," *American Society of Nephrology*, vol. 25, no. 9, pp. 1897–1907, 2014.

[2] C. Barisione, G. Ghigliotti, M. Canepa, M. Balbi, C. Brunelli, and P. Ameri, "Indoxyl sulfate: a candidate target for the prevention and treatment of cardiovascular disease in chronic kidney disease," *Current Drug Targets*, vol. 16, no. 4, pp. 366–372, 2015.

[3] K. Atoh, H. Itoh, and M. Haneda, "Serum indoxyl sulfate levels in patients with diabetic nephropathy: relation to renal function," *Diabetes Research and Clinical Practice*, vol. 83, no. 2, pp. 220–226, 2009.

[4] S. Lekawanvijit, A. R. Kompa, B. H. Wang, D. J. Kelly, and H. Krum, "Cardiorenal syndrome: the emerging role of protein-bound uremic toxins," *Circulation Research*, vol. 111, no. 11, pp. 1470–1483, 2012.

[5] C. Barisione, S. Garibaldi, A. L. Furfaro et al., "Moderate increase of indoxyl sulfate promotes monocyte transition into profibrotic macrophages," *PLoS One*, vol. 11, no. 2, article e0149276, 2016.

[6] I. W. Wu, K. H. Hsu, C. C. Lee et al., "p-Cresyl sulphate and indoxyl sulphate predict progression of chronic kidney disease," *Nephrology, Dialysis, Transplantation*, vol. 26, no. 3, pp. 938–947, 2011.

[7] O. Ichii, S. Otsuka-Kanazawa, T. Nakamura et al., "Podocyte injury caused by indoxyl sulfate, a uremic toxin and aryl-hydrocarbon receptor ligand," *PLoS One*, vol. 9, no. 9, article e108448, 2014.

[8] H. Shimizu, D. Bolati, A. Adijiang et al., "NF-κB plays an important role in indoxyl sulfate-induced cellular senescence, fibrotic gene expression, and inhibition of proliferation in proximal tubular cells," *American Journal of Physiology. Cell Physiology*, vol. 301, no. 5, pp. C1201–C1212, 2011.

[9] H. Shimizu, S. Saito, Y. Higashiyama, F. Nishijima, and T. Niwa, "CREB, NF-κB, and NADPH oxidase coordinately upregulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells," *American Journal of Physiology. Cell Physiology*, vol. 304, no. 7, pp. C685–C692, 2013.

[10] H. Shimizu, D. Bolati, Y. Higashiyama, F. Nishijima, K. Shimizu, and T. Niwa, "Indoxyl sulfate upregulates renal expression of MCP-1 via production of ROS and activation of NF-κB, p 53, ERK, and JNK in proximal tubular cells," *Life Sciences*, vol. 90, no. 13-14, pp. 525–530, 2012.

[11] J. Varga and D. Abraham, "Systemic sclerosis: a prototypic multisystem fibrotic disorder," *The Journal of Clinical Investigation*, vol. 117, no. 3, pp. 557–567, 2007.

[12] M. Sato, Y. Muragaki, S. Saika, A. B. Roberts, and A. Ooshima, "Targeted disruption of TGF-β1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction," *The Journal of Clinical Investigation*, vol. 112, no. 10, pp. 1486–1494, 2003.

[13] W. B. Pratt and D. O. Toft, "Regulation of signaling protein function and trafficking by the HSP90/hsp70-based chaperone machinery," *Experimental Biology and Medicine*, vol. 228, no. 2, pp. 111–133, 2016.

[14] S. O'Neill, J. A. Ross, S. J. Wigmore, and E. M. Harrison, "The role of heat shock protein 90 in modulating ischemia-reperfusion injury in the kidney," *Expert Opinion on Investigational Drugs*, vol. 21, no. 10, pp. 1535–1548, 2012.

[15] S. B. Lee, A. Lim, D. K. Rah, K. S. Kim, and H. J. Min, "Modulation of heat shock protein 90 affects TGF-β-induced collagen synthesis in human dermal fibroblast cells," *Tissue and Cell*, vol. 48, no. 6, pp. 616–623, 2016.

[16] V. Sontake, Y. Wang, R. K. Kasam et al., "Hsp90 regulation of fibroblast activation in pulmonary fibrosis," *JCI Insight*, vol. 2, no. 4, article e91454, 2017.

[17] R. W. Speir, J. D. Stallings, J. M. Andrews, M. S. Gelnett, T. C. Brand, and S. K. Salgar, "Effects of valproic acid and dexamethasone administration on early bio-markers and gene expression profile in acute kidney ischemia-reperfusion injury in the rat," *PLoS One*, vol. 10, no. 5, article e0126622, 2015.

[18] D. Verzola, S. Milanesi, M. Bertolotto et al., "Myostatin mediates abdominal aortic atherosclerosis progression by inducing vascular smooth muscle cell dysfunction and monocyte recruitment," *Scientific Reports*, vol. 7, no. 1, article 46362, 2017.

[19] D. Verzola, M. T. Gandolfo, F. Salvatore et al., "Testosterone promotes apoptotic damage in human renal tubular cells," *Kidney International*, vol. 65, no. 4, pp. 1252–1261, 2004.

[20] S. Meran and R. Steadman, "Fibroblasts and myofibroblasts in renal fibrosis," *International Journal of Experimental Pathology*, vol. 92, no. 3, pp. 158–167, 2011.

[21] I. Kudo, M. Hosaka, A. Haga et al., "The regulation mechanisms of AhR by molecular chaperone complex," *Journal of Biochemistry*, vol. 163, no. 3, pp. 223–232, 2018.

[22] T. L. Sirich, K. Fong, B. Larive et al., "Limited reduction in uremic solute concentrations with increased dialysis frequency and time in the Frequent Hemodialysis Network Daily Trial," *Kidney International*, vol. 91, no. 5, pp. 1186–1192, 2017.

[23] S. Li, S. Cheng, Z. Sun et al., "Indoxyl sulfate induces mesangial cell proliferation via the induction of COX-2," *Mediators of Inflammation*, vol. 2016, Article ID 5802973, 10 pages, 2016.

[24] W. J. Wang, C. H. Chang, M. F. Sun, S. F. Hsu, and C. S. Weng, "DPP-4 inhibitor attenuates toxic effects of indoxyl sulfate on kidney tubular cells," *PLoS One*, vol. 9, no. 4, article e93447, 2014.

[25] S. H. Kim, M. A. Yu, E. S. Ryu, Y. H. Jang, and D. H. Kang, "Indoxyl sulfate-induced epithelial-to-mesenchymal transition and apoptosis of renal tubular cells as novel mechanisms of progression of renal disease," *Laboratory Investigation*, vol. 92, no. 4, pp. 488–498, 2012.

[26] N. Neirynck, R. Vanholder, E. Schepers, S. Eloot, A. Pletinck, and G. Glorieux, "An update on uremic toxins," *International Urology and Nephrology*, vol. 45, no. 1, pp. 139–150, 2013.

[27] Y. Enoki, H. Watanabe, R. Arake et al., "Indoxyl sulfate potentiates skeletal muscle atrophy by inducing the oxidative stress-mediated expression of myostatin and atrogin-1," *Scientific Reports*, vol. 6, no. 1, article 32084, 2016.

[28] C. J. Lin, H. H. Chen, C. F. Pan et al., "p-Cresylsulfate and indoxyl sulfate level at different stages of chronic kidney disease," *Journal of Clinical Laboratory Analysis*, vol. 25, no. 3, pp. 191–197, 2011.

[29] C. Y. Sun, H. H. Hsu, and M. S. Wu, "p-Cresol sulfate and indoxyl sulfate induce similar cellular inflammatory gene expressions in cultured proximal renal tubular cells," *Nephrology, Dialysis, Transplantation*, vol. 28, no. 1, pp. 70–78, 2013.

[30] C. Y. Sun, G. H. Young, Y. T. Hsieh et al., "Protein-bound uremic toxins induce tissue remodeling by targeting the EGF receptor," *Journal of the American Society of Nephrology*, vol. 26, no. 2, pp. 281–290, 2015.

[31] H. Watanabe, Y. Miyamoto, M. Otagiri, and T. Maruyama, "Update on the pharmacokinetics and redox properties of protein-bound uremic toxins," *Journal of Pharmaceutical Sciences*, vol. 100, no. 9, pp. 3682–3695, 2011.

[32] T. Miyazaki, M. Ise, H. Seo, and T. Niwa, "Indoxyl sulfate increases the gene expressions of TGF-β1, TIMP-1 and pro-alpha 1(I) collagen in uremic rat kidneys," *Kidney International. Supplement*, vol. 62, pp. S15–S22, 1997.

[33] M. Rossi, K. L. Campbell, D. W. Johnson et al., "Protein-bound uremic toxins, inflammation and oxidative stress: a cross-sectional study in stage 3-4 chronic kidney disease," *Archives of Medical Research*, vol. 45, no. 4, pp. 309–317, 2014.

[34] S. Ito, M. Osaka, Y. Higuchi, F. Nishijima, H. Ishii, and M. Yoshida, "Indoxyl sulfate induces leukocyte-endothelial interactions through up-regulation of E-selectin," *The Journal of Biological Chemistry*, vol. 285, no. 50, pp. 38869–38875, 2010.

[35] Y. Adelibieke, H. Shimizu, G. Muteliefu, D. Bolati, and T. Niwa, "Indoxyl sulfate induces endothelial cell senescence by increasing reactive oxygen species production and p53 activity," *Journal of Renal Nutrition*, vol. 22, no. 1, pp. 86–89, 2012.

[36] S. Adesso, A. Popolo, G. Bianco et al., "The uremic toxin indoxyl sulphate enhances macrophage response to LPS," *PLoS One*, vol. 8, no. 9, article e76778, p. 30, 2013.

[37] H. Shimizu, M. Yisireyili, Y. Higashiyama, F. Nishijima, and T. Niwa, "Indoxyl sulfate upregulates renal expression of ICAM-1 via production of ROS and activation of NF-κb and p53 in proximal tubular cells," *Life Sciences*, vol. 92, no. 2, pp. 143–1148, 2013.

[38] M. Fujimoto, Y. Maezawa, K. Yokote et al., "Mice lacking Smad3 are protected against streptozotocin-induced diabetic glomerulopathy," *Biochemical and Biophysical Research Communications*, vol. 305, no. 4, pp. 1002–1007, 2003.

[39] Z. Liu, X. R. Huang, and H. Y. Lan, "Smad3 mediates ANG II-induced hypertensive kidney disease in mice," *American Journal of Physiology. Renal Physiology*, vol. 302, no. 8, pp. F986–F997, 2012.

[40] L. H. Thomsen, M. Fog-Tonnesen, L. Nielsen Fink et al., "Disparate phospho-Smad2 levels in advanced type 2 diabetes patients with diabetic nephropathy and early experimental db/db mouse model," *Renal Failure*, vol. 39, no. 1, pp. 629–642, 2017.

[41] S. B. Lee and R. Kalluri, "Mechanistic connection between inflammation and fibrosis," *Kidney International. Supplement*, vol. 78, pp. S22–S26, 2010.

[42] W. C. Shen, C. J. Liang, T. M. Huang et al., "Indoxyl sulfate enhances IL-1β-induced E-selectin expression in endothelial cells in acute kidney injury by the ROS/MAPKs/NFκB/AP-1 pathway," *Archives of Toxicology*, vol. 90, no. 11, pp. 2779–2792, 2016.

[43] M. B. Stockler-Pinto, J. F. Saldanha, D. Yi, D. Mafra, D. Fouque, and C. O. Soulage, "The uremic toxin indoxyl sulfate exacerbates reactive oxygen species production and inflammation in 3T3-L1 adipose cells," *Free Radical Research*, vol. 50, no. 3, pp. 337–344, 2016.

[44] H. Noh, H. J Kim, M. R Yu et al., "Heat shock protein 90 inhibitor attenuates renal fibrosis through degradation of transforming growth factor-β type II receptor," *Laboratory Investigation*, vol. 92, no. 11, pp. 1583–1596, 2012.

[45] S. J. Myung, J. H. Yoon, B. H. Kim, J. H. Lee, E. U. Jung, and H. S. Lee, "Heat shock protein 90 inhibitor induces apoptosis and attenuates activation of hepatic stellate cells," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 330, no. 1, pp. 276–282, 2009.

[46] J. Madrigal-Matute, O. López-Franco, L. M. Blanco-Colio et al., "Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis," *Cardiovascular Research*, vol. 86, no. 2, pp. 330–337, 2010.

[47] S. O'Neill, D. Humphries, G. Tse et al., "Heat shock protein 90 inhibition abrogates TLR4-mediated NF-κB activity and reduces renal ischemia-reperfusion injury," *Scientific Reports*, vol. 5, no. 1, article 12958, 2015.

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Review Article

Exercise for Prevention and Relief of Cardiovascular Disease: Prognoses, Mechanisms, and Approaches

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This review is aimed at summarizing the new findings about the multiple benefits of exercise on cardiovascular disease (CVD). We pay attention to the prevalence and risk factors of CVD and mechanisms and recommendations of physical activity. Physical activity can improve insulin sensitivity, alleviate plasma dyslipidemia, normalize elevated blood pressure, decrease blood viscosity, promote endothelial nitric oxide production, and improve leptin sensitivity to protect the heart and vessels. Besides, the protective role of exercise on the body involves not only animal models in the laboratory but also clinical studies which is demonstrated by WHO recommendations. The general exercise intensity for humans recommended by the American Heart Association to prevent CVD is moderate exercise of 30 minutes, 5 times a week. However, even the easiest activity is better than nothing. What is more, owing to the different physical fitness of individuals, a standard exercise training cannot provide the exact treatment for everyone. So personalization of exercise will be an irresistible trend and bring more beneficial effects with less inefficient physical activities. This paper reviews the benefits of exercise contributing to the body especially in CVD through the recent mechanism studies.

1. Cardiovascular Diseases: Prevalence and Risk Factors

CVD is a class of diseases which are related to the heart or blood vessels including stroke, heart failure, hypertension, coronary artery diseases, heart arrhythmia, peripheral artery disease, and atherosclerosis [1]. Individuals with CVD are found to have the accompanying raised blood pressure, elevated glucose, smoking, obesity, lack of exercise, excessive alcohol consumption, and dyslipidemia. Fortunately, CVD can be properly managed and prevented by controlling blood pressure, glucose, lipid, smoking, and alcohol drinking and through lifestyle modifications for sleep, emotion, exercise, and diet, which are called SEED intervention [2]. With the aging of population in the world, CVD has become the leading cause of death globally. Approximately 17.9 million deaths in 2015 were caused by CVD in the world [3]. The percentage of Chinese older than 60 has increased to 16% in 2015 which directly leads to a consequence that cardiovascular disease is becoming the leading cause of death in China [4]. Multiple risk factors contributing to CVD include obesity, high blood pressure, diabetes, aging, male sex, metabolic syndrome, and physical inactivity.

Determined by body mass index (BMI), obesity refers to the condition of those who have over 30 BMI and overweight includes persons with BMI more than 25. The prevalence among people of obesity has exceeded 50% in most countries and is increasing in both adults and children over the past few decades worldwide [5]. Obesity is found to increase blood volume, CRP, and TNFα which cause cardiac remodeling and inflammation. Obesity also aggravates the risks of high blood pressure, stroke, myocardial infarction (MI), and insulin resistance, which are all risk factors of CVD [6]. Furthermore, mortality and morbidity of CVD have been shown increasing in overweight populations, especially in those with abdominal obesity [7].

As the most common form of diabetes, type 2 diabetes is a chronic metabolic disease which is characterized by high

blood glucose, low insulin sensitivity. With the incidence of diabetes continuing to rise, the number of patients diagnosed with diabetes has swelled from 30 million in 1985 to 382 million in 2014, and scientists predict 592 million people will have diabetes by 2035, almost 1 in 10 persons suffering from this disease [8]. Besides affecting aging people, this disease also influences an increasing number of young populations and even children [9]. Lots of evidences and studies demonstrate that type 2 diabetes acts as an independent risk factor for CVD. The patients who are diagnosed with type 2 diabetes have a worse prognosis and therapeutic effects of CVD compared with those without diabetes. CVD death rates with diabetes in the United States adults are 1.7 times higher than those without diabetes [10]. The huge medical care burden of type 2 diabetes generally attributes to vascular complications like MI, hypertension, peripheral vascular disease, and coronary artery disease [11].

Aging is a gradual, systematic, irreversible, degenerative process in the body, which results in weakness, disease, and even death. As there is an average increasing lifespan for humans, there will be approximately 20% of the population over 65 by 2030. Aging is an inevitable and important determinant for CVD which leads to the decline of mitochondrial functions, excess reactive oxygen species (ROS) production, and disorder of Ca^{2+} levels [12]. Aging is relevant to the progressive damage in various physiological processes and increases incidence of atherosclerosis, hypertension, and stroke [13], thus inducing an elevating risk of cardiac and arterial systematic disorders. Studies show parts of the key genes regulating lifespan including AMPK, m-TOR, and IGF-1; sirtuins are closely related to CVD progress [14].

Epidemiologists demonstrate the different CVD occurrence rates between males and females. Clinical studies show that the onset of heart attack is delayed 9 years in women compared with men [15]. In a cross-sectional survey on hospitalized patients with coronary artery disease, women were found 3.1 years older than men [16]. It is widely believed that estrogen is the prominent protective element for females [17]. Besides, women tend to have a better awareness of weight measurement and their waist circumference if they have central obesity, which would lower the risk of adiposity and dyslipidemia. Smoking and alcohol drinking are severe risk factors for CVD, which are more common lifestyle behaviors in men compared to women [18].

Metabolic syndrome is a condition characterized by low high-density lipoprotein (HDL), high triglycerides (TG), high blood pressure, high blood glucose, and central obesity, associating with the risks of developing to CVD and type 2 diabetes. Studies show that metabolic syndrome affects 30-40% of people older than 65 by doubling the risk of getting CVD [19]. Low HDL and high TG are associated with elevated levels of low-density lipoprotein (LDL), which increase the risk of having atherosclerosis [20]. High glucose links to dysfunctions of glucose uptake and catabolism and induces high oxidative stress, dysfunction of endothelial cells. High blood pressure leads to cardiac and vascular injury. Central obesity is related to maldistribution of free fatty acid, overproduction of inflammatory molecules, and leptin resistance [21].

Physical inactivity has been identified as the fourth risk factor of death worldwide, leading to approximately 3.2 million deaths annually. Various studies show an obvious dose-response relationship between increased physical activity and decreased occurrence rate of CVD including reduced blood pressure, body weight, ox-LDL, and elevated glucose tolerance. A systematic review estimates that the lack of exercise leads to 6% of coronary heart disease occurrence worldwide. Deficiency in physical activity leads to obesity, increasing endogenous inflammatory molecules and coagulation factors. In addition, there is coordinated protective effects to decrease the overall risk of incident CVD by exercise [22, 23]. So having a healthy diet, avoiding smoking, and keeping regular physical activity are the three pieces of advice the WHO recommend to avoid CVD.

2. Mechanisms of Action for Physical Exercise

Many considerable evidences support the therapeutic and protective effects of exercise on the body, including improvement of insulin sensitivity of diabetic mice, attenuating sympathetic activity, arterial pressure, and heart rate in the spontaneously hypertensive rats [24]. Mitochondrial biogenic response, components of the electron transport chain, mtDNA, and related lipid metabolic pathways are all increased after exercise training [25]. Here, we talk about the benefits of exercise on cardiovascular disease from the following aspects.

2.1. Insulin Sensitivity and Blood Glucose Control. Type 2 diabetes mellitus is a kind of chronic disease characterized by obesity, hyperglycemia, impaired insulin secretion, and insulin resistance [26]. Studies show that diabetic rats with exercise training present reduced body weight, decreased TG levels, and diminished blood glucose levels compared with those sedentary rats [27, 28]. PPARγ known as "energy-balanced receptor," is well studied in metabolic disorders. Carnitine palmitoyl transferase-1 (CPT-1) mainly transports fatty acids into mitochondria for medium-chain acyl-CoA dehydrogenase (MCAD) catalyzing β-oxidation. Through upregulating PPARγ and its target genes, CPT-1 and MCAD, exercise alleviates hepatic steatosis, promotes glucose uptake, and improves insulin sensitivity in nonalcoholic fatty liver disease mice [29]. Exercise stimulates the translocation of glucose transporter type 4 (GLUT4) from the cytoplasm to the cell membrane, thus promoting glucose uptake and improving insulin resistance [30]. Besides, improved insulin sensitivity is independent with exercise modality. High or low intensity of exercise and aerobic or anaerobic training lead to improvement in glucose clearance curve and insulin sensitivity [31].

Besides improving insulin sensitivity, exercise facilitates glucose uptake and usage via insulin-independent mechanisms. Once glucose enters muscular and adipose cells, it will be phosphorylated by hexokinase which is an irreversible catalytic reaction to form glucose 6-phosphate (G-6-P) that cannot diffuse back out of cells. Glycolysis and glycogenesis started from G-6-P and promote glucose uptake by cells and usage in cells to affect blood glucose. Exercise increases

G-6-P level in the skeletal muscles accompanying increased GLUT4, hexokinase level, and glycogen synthase activity, which finally improve glucose tolerance and decrease blood glucose level [32].

2.2. Lipid Profile. Cholesterol is a soft waxy fat that our body needs to function well. But too much cholesterol will become risk factors for human diseases like heart disease, stroke, and atherosclerosis [33]. For those who have been diagnosed with diabetes, heart disease, and stroke or people who are taking medicine to control cholesterol level, taking cholesterol test every year is necessary [34]. Generally, a cholesterol test includes total cholesterol, LDL, HDL, and TG [35]. Standard management strategies like drug therapy and diet control are generally used to lower serum cholesterol to prevent heart disease. However, some people are insensitive to statins or cannot tolerate statins. Hence, other ways need to replace or be used together with statins. More and more evidences support aerobic exercise as a positive method for alleviating plasma dyslipidemia and improving the prognosis of cardiovascular diseases [36]. Through using meta-analyses to investigate exercise and lipid profiles, Pedersen et al. concluded that exercise led to benefits of physical health [37]. A prospective cohort study lasting for 10 years about exercise and lipid metabolism shows that the risk of mortality is significantly reduced by combining statins with exercise, especially compared to other therapy alone [38]. Comparing with LDL and TG, HDL is more sensitive to exercise. Studies indicate that HDL is increasing more or less both in humans and rats after exercise [39, 40]. For the "bad" cholesterol LDL, the effects of exercise reduce the serum levels significantly in rats [40]. However, the effects are not consistent in humans, which may be due to the different dietary habits and living conditions [41, 42]. It is strongly accepted and reported that exercise leads a high requirement of energy which induces decreasing of plasma TG concentrations [43].

2.3. Blood Pressure. Blood pressure is elicited by the force exerted by the blood against the blood vessels, which depends on the ejection of the heart and resistance of the blood vessels. Hypertension is another name of high blood pressure, a disease related to heart attack, stroke, heart failure, and other problems [44]. Exercise always leads to a postexercise hypotension, and both normotensive and hypertensive persons experience a transient reduction in blood pressure. The reduced magnitude may achieve the point wherein patients with hypertension recover to the normal blood pressure levels. In a meta-analysis, they investigated the effects of acute exercise on blood pressure response. There were significant changes, reduction of 4.8 mmHg for systolic blood pressure (SBP) and 3.2 mmHg for diastolic blood pressure (DBP). The epidemiological study demonstrates that 2 mmHg decline in SBP leads to 6% reduction of stroke mortality and 4% reduction of coronary heart disease mortality, and a decrease of 5 mmHg causes the reduction of mortality of these diseases by 14% and 9%, respectively [45]. So the meta-analysis results confirm the undoubted place of noninvasive therapy method, acute exercise.

The transient reduction only lasts for a few hours and would recover after rest. However, the benefits of physical activity cannot be ignored because of chronic treatment of exercise showing significant changes among subjects. The vasodilator activity leads to the decrease of blood pressure. On the contrary, increased blood pressure is caused by vasoconstriction. Moderate-intensity exercise causes a vasodilatory response and decreases the vasoconstricting response and lipid in rat aortas, which exhibits a decrease in diastolic blood pressure [46, 47]. In addition to the vascular tone, exercise decreases blood pressure through lowering oxidative stress and inflammation levels. In the spontaneous hypertension rats (SHR), exercise normalizes the increased collagen deposition and diminished fenestra size in the internal elastic lamina, meaning that exercise shows the benefit roles in normalizing the increased vascular stiffness and decreased vascular distensibility in both small mesenteric arteries and coronary arteries [48]. However, high-intensity exercise leads to the opposite effects that increased oxidative stress, elevated blood pressure, and high vasoconstrictor activity are found [49]. Some studies report that males tend to achieve greater reductions than females from the exercise training [50]. However, the authors ignore the factors of menstrual cycle in the female subjects which affect the regulation of the autonomic nervous system [51]. Studying the effects of exercise on circadian rhythms using ambulatory blood pressure monitoring, there are significant reductions of daytime BP, but no obvious changes are observed at nighttime BP [44].

2.4. Blood Viscosity, Platelet Aggregation, and Thrombosis Profile. Blood viscosity in normal conditions is like a Newtonian fluid which is influenced by hematocrit, shear rate of blood flow, vascular caliber, and temperature. Elevated blood viscosity which is associated with blood resistance increases risks of cardiovascular complications. Blood viscosity is decreasing during exercise accompanied by decreased systemic vascular resistance [52]. More nitric oxide (NO) is produced attributed to greater shear stress in exercise and promote vasodilation [53, 54]. During exercise, erythrocyte volume is slightly increased; however, a much higher increase of plasma volume is generated which finally results in lower blood viscosity. Decline in plasma fibrinogen level is observed under the effect of exercise which plays important roles in declining erythrocyte aggregation and decreasing blood viscosity [55].

Platelet is a small volume component in blood which has no cell nucleus generated from megakaryocytes. Through forming thrombus, the platelets exert primary function of maintaining hemostasis of blood flow. Once an injury occurs, the platelets in the circulation will be activated and aggregated to the interrupted endothelial site to plug the hole [56]. So abnormality in platelet activation leads to a variety of atherosclerotic diseases mainly through excess thrombosis in small arteries like coronary arteries and blood vessels of the brain [57]. Exercise training presents an antithrombotic manner through platelet functional regulation. Through enhancing blood flow, exercise enhances endothelial NO production which counteracts platelet activation [58]. The moderate physical activity decreases both platelet adhesion

and aggregation through downregulating intracellular calcium levels and increasing cGMP levels [59]. It is recommended by government guidelines that physical activity is an effective way to prevent thrombosis [60]. Exercise is also used to improve chronic complications of deep venous thrombosis, postthrombotic syndrome. A six-month exercise training markedly increases leg strength, hamstring and gastrocnemius flexibility, and overall fitness [61]. However, studies showed that the strenuous short-term exercise activated platelets and promoted aggregation of platelets, thus increasing the risk of MI or cardiac arrest. This suggests that acute exhausting exercise may trigger clot formation, but the mechanisms remain to be clarified [62].

2.5. Endothelial NO Production. NO is a gaseous signaling molecule playing an irreplaceable role in a variety of biological processes. It is catalyzed by various nitric oxide synthase (NOS) enzymes by using substrate L-arginine. Known as an endothelial-derived relaxing factor, NO contributes to not only endothelial-dependent relaxation (EDR) but also to the maintenance of endothelial function [63]. The endothelium is a single layer of cells in the intima of vessels separating blood from the tissue. The functions of the endothelium involve regulating angiogenesis, balancing vasoconstriction and vasodilation, adjusting smooth muscle cell proliferation, and excreting endocrine. The intact endothelium acts an indispensable role in vessel homeostasis [64]. In a cross-sectional study with 184 healthy individuals, L-arginine was reduced and production of NO was increased after exercise training [46]. The mechanisms may be partial that exercise training leads to increased blood flow and shear stress, contributing to endothelial NOS (eNOS) expression, NO release, and artery relaxation. NO activates soluble guanylate cyclase which increases cGMP and therefore activates protein kinase G (PKG). In blood vessels, PKG activation always induces relaxation and regulates blood pressure. In the heart, PKG works as a brake on stress response signaling [65]. Through increasing vascular AMPK/PPARδ, exercise suppresses endoplasmic reticulum stress, thus increasing endothelial NO bioavailability. Exercise shows preserved EDR in the aorta and mesenteric artery in high-fat diet rats and db/db mice [66]. In the aging-induced downregulation of VEGF signaling cascade in the heart, exercise upregulates VEGF, its receptors Flt-1 and Flk-1, and the downstream signaling pathway Akt/eNOS [67]. Increased NO production usually facilitates angiogenesis and vascular permeability.

Dysfunction of EDR and decrease of cGMP in both plasma and vascular tissue are found in diabetic rats. However, chronic exercise improves endothelial function through releasing NO in the aorta. Interestingly, exercise does not affect the vascular function in normal rats administrated with L-arginine but improves vascular function in the aortas from diabetic rats [68]. In the high fat-induced obese mice, impaired EDR, reduced NO bioavailability, and decreased phosphorylation of eNOS are found in the coronary arteries which can be normalized by exercise. Through injecting β-adrenoceptor agonists, isoproterenol for 8 days, Yang recapitulated cardiac hypertrophy which was regarded as the primary risk factor for heart failure. After 4 weeks of exercise training, the eNOS expression did not change, but the phosphorylation of serine residue 1177 with an activating impact on eNOS was increased and the phosphorylation at threonine residue 495 with an inhibitory impact on eNOS was decreased. As a result, exercise promoted NO production and attenuated cardiac remodeling, echocardiographic and hemodynamic changes after β-adrenergic overload [69].

2.6. Leptin Sensitivity. Leptin is a 16 kDa circulating hormone which is predominantly released by adipocytes to exert regulation of food intake and energy metabolism. Through binding to leptin receptors on hypothalamic cells, leptin inhibits hunger, prevents weight gain, and promotes positive energy balance [70]. So leptin deficiency or leptin resistance promote diabetes, obesity, and other metabolic disorders. Exercise improves leptin resistance and sensitivity, attenuates body weight, and promotes homeostatic control of energy balance through influencing the leptin receptor in the ventromedial hypothalamic nucleus of obese mice [71, 72]. It also directly works on leptin receptors to induce NO-dependent vasodilation expressed in endothelial cells [73]. A decreased leptin sensitivity and hyperleptinaemia are found in obese mice coronary arteries, demonstrating leptin resistance and low leptin sensitivity. Exercise maintains leptin sensitivity of obese mice and preserves leptin receptor, thus exerting endothelial protection [74]. Exercise facilitates SOCS3 expression and improves leptin resistance in the liver and muscle of high-fat diet-induced rats [75]. Leptin sensitivity is restored by exercise manifested as facilitating fatty acid toward oxidation and away from triacylglycerol stores [76]. Through activating leptin, exercise also initiates the downstream JAK/STAT signal transduction pathway in obese mice to protect against myocardial ischemia-reperfusion injury [77]. Shapiro et al. found that leptin overexpression failed to reduce body weight in obese rats, and chronic leptin treatment aggravated the diet-induced obesity. Besides, wheel running for obese rats was insufficient to lower body weight. However, combinational administration of exercise and exogenous leptin dramatically induced weight loss and improved leptin sensitivity [78]. That means exercise may directly activate leptin signaling pathway or improve leptin sensitivity via coordinating with other therapeutic methods.

2.7. Modulation of Autonomic Function. The autonomic nervous system, regulated by the hypothalamus, consists of sympathetic nervous system, parasympathetic nervous system, and enteric nervous system. The primary autonomic functions include cardiac regulation, control of respiration, and vasomotor activity, which act largely unconsciously [79]. Exercise training has beneficial roles in autonomic function, as indicated by improved heart rate recovery (HRR) and heart rate variability (HRV) in various populations, such as in sedentary individuals and chronic heart failure patients [80]. HRR refers to the declining rate of heart rate and is recognized as an indicator of cardiac prognosis [81]. HRV is defined as consecutive heart beat variations in heart rate of sinus rhythm. Reduced HRV represents attenuation of autonomic regulation of sinoatrial node [82]. Exercise works as an intervention for autonomic dysfunction in type 2 diabetes

by preserving HRV, HRR, and baroreflex sensitivity (BRS). BRS is regulated by sympathetic and parasympathetic autonomic nerves and is downregulated when there is cardiac autonomic neuropathy [83].

Electrocardiogram is a noninvasive way to determine cardiac conditions. Elevated R wave amplitudes and widened QT intervals are reliable predictors of autonomic neuropathy. Exercise training lowers heart rate and reduces QT interval and R wave amplitudes on electrocardiogram in the diabetic fatty rat model [84]. In myocardial-infarcted rats, structural remodeling leads to heterogeneity which causes slow conduction and creates the generation of arrhythmias. However, exercise training increases ratio of parasympathetic over sympathetic tones and decreases probability of ventricular arrhythmias of the MI rats [85]. The mechanism of exercise-induced improvement of arrhythmia might be related to intrinsic electrophysiological cardioadaptive mechanisms because of decreased action potential duration gradient between epicardial and endocardial cells in the exercise-trained rats [86]. The mechanisms by which exercise improves autonomic function and preserves neurovascular perfusion might be related to increasing NO bioavailability, lowering angiotensin II (AngII) levels, and suppressing chronic inflammation [87–90]. It is controversial that some studies show that MI, stroke, ventricular tachycardia, or fibrillation can be elevated during the progress of physical activity, so further studies about the duration, frequency, and intensity need to be specifically investigated.

2.8. Antioxidant Defense. ROS of physiological levels are responsible for signaling molecules to regulate normal physical activities. On the contrary, excessively generated ROS plays a crucial role in the initiation and progression of CVD [91]. ROS overproduction, decreased antioxidant enzymes, and the downstream targets damage the subcellular organelles, thus impairing the cardiovascular system. Excessive ROS may decrease NO bioavailability and NO/cGMP-dependent pathway which result in pathological vasoconstriction and hypertension [92]. ROS also initiates inflammation with the activation of redox-sensitive transcription factors and promotes expression of inflammatory molecules [93]. DNA is oxidized by ROS to form 8-oxo-dG which can be detected in plasma and urine [94]. AngII-induced insulin resistance is relevant to ROS, which can impair insulin signaling by decreasing phosphorylation of IRS1 and Akt and translocation of GLUT4 to the plasma membrane [24].

Exercise attenuates oxidative damage in the blood vessel, heart, brain, liver, skeletal muscle, and other organs. Exercise reduces age-related mitochondrial oxidative damage and increases mitochondrial NADH-cytochrome C reductase activity in the heart of aged rats [95]. By improving the antioxidant enzymes and redox status in many organs and pathologies, the chronic treadmill exercise has an anti-inflammatory effect. Exercise alleviates oxidative stress through inactivating ERK/AMPK signaling pathway and decreases expression of inflammatory factors like IL-6, high-sensitivity CRP, TNFα, and leucocyte differentiation antigens in diabetes [96]. In renovascular hypertensive rats, ROS released by vascular endothelial cells impairs EDR by decreasing NO production and facilitating vasoconstriction. Evidences indicate that exercise reduces ROS, improves endothelial function, and increases levels of elastin, fibrillin, and NO. The elevated cardiac MDA levels and MPO activity and depleted GSH and catalase expression in hypertensive rats are totally reversed by exercise training [97]. BRS is improved by physical activity through reducing oxidative stress in the rostral ventrolateral medulla to inhibit the sympathetic nerve [98, 99]. In diabetic rats, the overproduction of ROS leads to the abnormal aortic function and metabolic disorder. Through exercise training, enzymes producing ROS such as p67phox protein decrease and the enzymes taking part in scavenging oxygen-free radicals, like superoxide dismutase (SOD) 1, SOD2, catalase, and glutathione peroxidase (GPX), are normalized, even increased [48, 100]. That means, all the proteins taking part in oxidative stress tend to recover to normal levels after exercise treatment [101].

Exercise upregulates the antioxidant defense mechanisms through redox-sensitive transcription factors, NF-κB and activator protein-1, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Exercise normalizes the aging-induced decline in mitochondrial oxidative capacity through upregulating PGC-1α, redox-sensitive transcription factors PPARγ, and the target antioxidant genes like GPX and SOD2 [46, 102]. Through elevating laminar shear stress, exercise upregulates SOD and downregulates NADPH oxidase and VCAM-1. Through activating AMPK, exercise training increases sirt3 and PPARγ and the targets SOD1, SOD2, GPX1, and catalase, thus resulting in less ROS production and more ROS clearance in skeletal muscles [103]. Exercise increases thioredoxin reductase 1 and decreases thioredoxin-interacting protein (TXNIP) in blood mononuclear cells and skeletal muscles, which promote antioxidant ability and eliminate free radical [104, 105]. TXNIP is a regulator of cellular redox signaling by binding thioredoxin to inhibit the neutralization of ROS, indirectly enhancing the oxidative stress [106]. Nuclear factor erythroid 2-like factor 2 (Nrf2) is reported to be an important transcription factor that performs antioxidant defense mechanisms through Nrf2-ARE signaling to activate antioxidant gene expression [107]. Mounting evidences demonstrate that the Nrf2-ARE signaling is downregulated in the aging heart, ischemia/reperfusion injury, and MI. Researchers find that through chronic exercise treatment, Nrf2 is upregulated and consequently activates the key antioxidant enzyme expression such as hemoxygenase-1, NADPH quinone oxidase-1, GPX1, GPX2, glutathione reductase, γ-glutamyl cysteine ligase-catalytic, γ-glutamyl cysteine ligase-modulatory, glucose-6-phosphate dehydrogenase (G6PD), and catalase in the heart [108, 109]. Based on these risk factors and studies, all major cardiovascular societies recommend that a minimum of 5 days a week of exercise, with at least 30 minutes of moderate-intensity aerobic activity, is needed to prevent CVD [110].

3. The Recommendations for Physical Activity

Because of low-cost, low-risk, and nondrug intervention, the European Society of Cardiology recommended that exercise

training should be provided by cardiac rehabilitation programs in patients with non-ST elevation acute coronary syndrome in 2015 [111]. Physical activity is a part of everybody's life. However, the exercise intensity differs between people depending on the physical condition of individuals [112]. Although lots of studies show a positive correlation between exercise and good health, a thorough physical evaluation is necessary before an intensive exercise training program. The intensity, mode, duration, and frequency of exercise can strongly affect outcome.

Aerobic exercise is defined as using aerobic metabolism to extract energy in muscles, mainly referring to low- to moderate-intensity physical activities. As discussed above, aerobic exercise has favorable effects on lipid metabolism, cardiac remodeling, post-MI heart failure, insulin resistance, and endothelial function. Anaerobic exercise is an activity that synthesize energy sources without using oxygen as energy sources but glycolysis and fermentation. Anaerobic exercise usually refers to high-intensity training, including sprinting and power lifting. In several studies, high-intensity exercise is recommended to lower TG and LDL. Similar to aerobic exercise, anaerobic exercise also shows positive influences on body mass index and blood pressure [113]. In some cases, high-intensity training shows more beneficial effects on the cardiovascular system and EDR compared to low-intensity training [114]. The advantages of high-intensity intermittent exercise refer to the fact that the shorter time as 3-4 sessions/week will produce significant changes [115]. However, there is a paradox of disadvantages about anaerobic exercise training that the elevated mortality and sudden death are brought by high-intensity activity. A well-recognized viewpoint is that acute strenuous exercise increases the risks for cardiovascular diseases, like MI, by upregulating blood pressure [57]. In conclusion, intensive exercise should be intermittent especially for a long-term program. Professional supervision and guidance are indispensable when you conduct high-intensity exercise.

The general recommended exercise intensity for humans from the American Heart Association to prevent CVD is 30 minutes, 5 times a week to reach at least 150 minutes per week of moderate exercise, or 25 minutes, 3 times a week to reach at least 75 minutes per week of vigorous activity. Individuals can choose one way of physical activity or combine moderate and vigorous activities. They will also be benefited even if they divide the entire time into several parts of 10 to 15 minutes per day. For those who want to lower the risk for heart attack and stroke, 40 minutes of moderate to vigorous intensity aerobic activity, 3 or 4 times a week, is recommended [116]. Moderate-intensity exercise is more widely performed among people who are interested in exercise and enjoy rest time. Various studies show that the duration of physical activity but not intensity is the primary factor leading to benefits for humans [117].

Physical activity is regarded as an efficient way to prevent and counteract age-related changes in muscle and organic function [118]. As we know, any activity is better than none and it is never too late to start for the elders [119]. The American College of Sports Medicine and the American Heart Association recommend detailed description for elder people that 30 minutes, 5 times a week, of moderate intensity, or 20 minutes, 3 times a week, of vigorous intensity activity is good for aging-related diseases. Strength training should be included to enhance muscle groups and prevent falls as 8-10 exercises with 10-15 repetitions, twice a week [120]. For the elders, joining exercise classes to improve balance and prevent falls are strongly suggested [121]. Exercise is generally safe for older people unless for those elders who already suffered from health problems doing resistance training [122].

Examples of aerobic exercise include cycling, dancing, hiking, treadmill, climbing stairs, swimming, walking, and any activities (the criterion is that you can talk without breathing too hard). Anaerobic exercise refers to sprinting and power lifting accompanied by accumulation of lactic acid causing muscular fatigue [123]. However, the definition of moderate and vigorous exercise varies between individuals because the indispensability to measure the level of intensity bases on your fitness and overall health.

4. Future Perspectives

Many people are robbed by the thinking all-or-nothing when doing exercise. However, the low-level intensity training program could benefit you as long as you begin to change. Even the easiest activity is better than nothing and whenever you start is not too late [124].

There are also some limits for the exercise training when applied to real patients' treatment. The existing studies do not provide an exact guidance on the diverse intensity, duration, and frequency of exercise that may be suitable for the different kinds of diseases. In the future, personalization of exercise will be an irresistible trend. For those young and in healthy condition, it may be optional to consult a doctor before they start an exercise program. However, personalized exercise enables you to perform more specifically based on your present fitness level, interest, age, and gender [125]. For those old who have been inactive for a long period, it is necessary to consult physicians who will measure some indexes, like cardiorespiratory endurance, muscular strength, muscular endurance, and flexibility, and make graded exercise tests for individuals [126]. Also, for someone who has a baseline disease, the extreme exercise training seems to trigger the progression of disease. So tailored exercise would bring benefits not only for healthy humans but also for the patients with chronic disease [127]. For slow disease-related declines of muscle strength, tailored exercise may focus on the training to improve muscle strength and reduce the risk of falls. For those who has type 2 diabetes, body weight control and enhancement of peripheral circulation are the first two goals to realize. For people with arthritis, tailored exercise helps reduce joint stiffness and enhances muscle strength [125, 128]. The systematical and overall exercise guidance from a professional instructor is totally necessary. Personalization of exercise training will be a huge demand and replace the random mode of exercise today.

Although we get to know that exercise protects against CVD through attenuating sympathetic activity, arterial pressure, and heart rate, increasing blood flow and endothelial

NO production, causing vessel dilation, decreasing inflammatory cytokine and reactive oxygen species formation, the exact mechanisms leading to transcriptional factor changes or transcriptional modifications are not studied. So future studies may be applied to the mechanisms of protective effects of exercise on the heart and vessels.

Conflicts of Interest

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

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References

[1] J. Yong, D. Lin, and X. R. Tan, "Primary prevention of cardiovascular disease in older adults in China," *World Journal of Clinical Cases*, vol. 5, no. 9, pp. 349–359, 2017.

[2] C. S. Hu, Q. H. Wu, D. Y. Hu, and T. Tkebuchava, "Novel strategies halt cardiovascular, diabetes, and cancer strips," *Chronic Diseases and Translational Medicine*, vol. 3, no. 3, pp. 159–164, 2017.

[3] GBD 2015 Mortality and Causes of Death Collaborators, "Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the global burden of disease study 2015," *The Lancet*, vol. 388, no. 10053, pp. 1459–1544, 2016.

[4] Y. Jiang, F. Mao, Y. Li et al., "Construction of China cardiovascular health index," *BMC Public Health*, vol. 18, no. 1, p. 937, 2018.

[5] F. B. Ortega, C. J. Lavie, and S. N. Blair, "Obesity and cardiovascular disease," *Circulation Research*, vol. 118, no. 11, pp. 1752–1770, 2016.

[6] L. Akil and H. A. Ahmad, "Relationships between obesity and cardiovascular diseases in four southern states and Colorado," *Journal of Health Care for the Poor and Underserved*, vol. 22, no. 4A, pp. 61–72, 2011.

[7] M. Abdelaal, C. W. le Roux, and N. G. Docherty, "Morbidity and mortality associated with obesity," *Annals of Translational Medicine*, vol. 5, no. 7, p. 161, 2017.

[8] B. M. Leon and T. M. Maddox, "Diabetes and cardiovascular disease: epidemiology, biological mechanisms, treatment recommendations and future research," *World Journal of Diabetes*, vol. 6, no. 13, pp. 1246–1258, 2015.

[9] "Diabetes in China: mapping the road ahead," *The Lancet Diabetes & Endocrinology*, vol. 2, no. 12, p. 923, 2014.

[10] S. M. Grundy, I. J. Benjamin, G. L. Burke et al., "Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association," *Circulation*, vol. 100, no. 10, pp. 1134–1146, 1999.

[11] M. Kosiborod, M. B. Gomes, A. Nicolucci et al., "Vascular complications in patients with type 2 diabetes: prevalence and associated factors in 38 countries (the DISCOVER study program)," *Cardiovascular Diabetology*, vol. 17, no. 1, p. 150, 2018.

[12] M. Steenman and G. Lande, "Cardiac aging and heart disease in humans," *Biophysical Reviews*, vol. 9, no. 2, pp. 131–137, 2017.

[13] B. J. North and D. A. Sinclair, "The intersection between aging and cardiovascular disease," *Circulation Research*, vol. 110, no. 8, pp. 1097–1108, 2012.

[14] A. J. P. O. de Almeida, T. P. Ribeiro, and I. A. de Medeiros, "Aging: molecular pathways and implications on the cardiovascular system," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 7941563, 19 pages, 2017.

[15] A. O'Neil, A. J. Scovelle, A. J. Milner, and A. Kavanagh, "Gender/sex as a social determinant of cardiovascular risk," *Circulation*, vol. 137, no. 8, pp. 854–864, 2018.

[16] D. De Smedt, D. De Bacquer, J. De Sutter et al., "The gender gap in risk factor control: effects of age and education on the control of cardiovascular risk factors in male and female coronary patients. *The EUROASPIRE IV study by the European Society of Cardiology*," *International Journal of Cardiology*, vol. 209, pp. 284–290, 2016.

[17] C. Vitale, M. Fini, G. Speziale, and S. Chierchia, "Gender differences in the cardiovascular effects of sex hormones," *Fundamental & Clinical Pharmacology*, vol. 24, no. 6, pp. 675–685, 2010.

[18] I. Wakabayashi, "Gender differences in cardiovascular risk factors in patients with coronary artery disease and those with type 2 diabetes," *Journal of Thoracic Disease*, vol. 9, no. 5, pp. E503–E506, 2017.

[19] T. S. Han and M. E. Lean, "A clinical perspective of obesity, metabolic syndrome and cardiovascular disease," *JRSM Cardiovascular Disease*, vol. 5, 2016.

[20] G. A. Bray, "Medical consequences of obesity," *The Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 6, pp. 2583–2589, 2004.

[21] S. A. Ritchie and J. M. C. Connell, "The link between abdominal obesity, metabolic syndrome and cardiovascular disease," *Nutrition, Metabolism, and Cardiovascular Diseases*, vol. 17, no. 4, pp. 319–326, 2007.

[22] J. Li and J. Siegrist, "Physical activity and risk of cardiovascular disease–a meta-analysis of prospective cohort studies," *International Journal of Environmental Research and Public Health*, vol. 9, no. 2, pp. 391–407, 2012.

[23] P. D. Thompson, D. Buchner, I. L. Pina et al., "Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease: a statement from the Council on Clinical Cardiology (Subcommittee on Exercise, Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity)," *Circulation*, vol. 107, no. 24, pp. 3109–3116, 2003.

[24] M. P. Lemos, G. R. D. Mota, M. Marocolo, C. C. Sordi, R. S. Chriguer, and O. Barbosa Neto, "Exercise training attenuates sympathetic activity and improves morphometry of splenic arterioles in spontaneously hipertensive rats," *Arquivos Brasileiros de Cardiologia*, vol. 110, no. 3, pp. 263–269, 2018.

[25] R. B. Vega, J. L. Horton, and D. P. Kelly, "Maintaining ancient organelles: mitochondrial biogenesis and maturation," *Circulation Research*, vol. 116, no. 11, pp. 1820–1834, 2015.

[26] R. A. DeFronzo, E. Ferrannini, L. Groop et al., "Type 2 diabetes mellitus," *Nature Reviews Disease Primers*, vol. 1, article 15019, 2015.

[27] E. Teixeira de Lemos, F. Reis, S. Baptista et al., "Exercise training decreases proinflammatory profile in Zucker diabetic (type 2) fatty rats," *Nutrition*, vol. 25, no. 3, pp. 330–339, 2009.

[28] E. Teixeira-Lemos, S. Nunes, F. Teixeira, and F. Reis, "Regular physical exercise training assists in preventing type 2 diabetes development: focus on its antioxidant and anti-inflammatory properties," *Cardiovascular Diabetology*, vol. 10, no. 1, p. 12, 2011.

[29] F. Zheng and Y. Cai, "Concurrent exercise improves insulin resistance and nonalcoholic fatty liver disease by upregulating PPAR-γ and genes involved in the beta-oxidation of fatty acids in ApoE-KO mice fed a high-fat diet," *Lipids in Health and Disease*, vol. 18, no. 1, p. 6, 2019.

[30] S. Lund, G. D. Holman, O. Schmitz, and O. Pedersen, "Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 13, pp. 5817–5821, 1995.

[31] K. E. Hall, M. W. McDonald, K. N. Grisé, O. A. Campos, E. G. Noble, and C. W. J. Melling, "The role of resistance and aerobic exercise training on insulin sensitivity measures in STZ-induced type 1 diabetic rodents," *Metabolism*, vol. 62, no. 10, pp. 1485–1494, 2013.

[32] C. H. Kim, J. H. Youn, J. Y. Park et al., "Effects of high-fat diet and exercise training on intracellular glucose metabolism in rats," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 278, no. 6, pp. E977–E984, 2000.

[33] H. Pereira, "The importance of cholesterol in psychopathology: a review of recent contributions," *Indian Journal of Psychological Medicine*, vol. 39, no. 2, pp. 109–113, 2017.

[34] American Diabetes Association, "Standards of medical care in diabetes—2008," *Diabetes Care*, vol. 31, Supplement 1, pp. S12–S54, 2008.

[35] Y. Wang and D. Xu, "Effects of aerobic exercise on lipids and lipoproteins," *Lipids in Health and Disease*, vol. 16, no. 1, p. 132, 2017.

[36] J. F. Trejo-Gutierrez and G. Fletcher, "Impact of exercise on blood lipids and lipoproteins," *Journal of Clinical Lipidology*, vol. 1, no. 3, pp. 175–181, 2007.

[37] B. K. Pedersen and B. Saltin, "Evidence for prescribing exercise as therapy in chronic disease," *Scandinavian Journal of Medicine & Science in Sports*, vol. 16, Supplement 1, pp. 3–63, 2006.

[38] P. F. Kokkinos, C. Faselis, J. Myers, D. Panagiotakos, and M. Doumas, "Interactive effects of fitness and statin treatment on mortality risk in veterans with dyslipidaemia: a cohort study," *The Lancet*, vol. 381, no. 9864, pp. 394–399, 2013.

[39] L. Nybo, E. Sundstrup, M. D. Jakobsen et al., "High-intensity training versus traditional exercise interventions for promoting health," *Medicine and Science in Sports and Exercise*, vol. 42, no. 10, pp. 1951–1958, 2010.

[40] F. Kazeminasab, M. Marandi, K. Ghaedi, F. Esfarjani, and J. Moshtaghian, "Endurance training enhances LXRα gene expression in Wistar male rats," *European Journal of Applied Physiology*, vol. 113, no. 9, pp. 2285–2290, 2013.

[41] W. E. Kraus, J. A. Houmard, B. D. Duscha et al., "Effects of the amount and intensity of exercise on plasma lipoproteins," *The New England Journal of Medicine*, vol. 347, no. 19, pp. 1483–1492, 2002.

[42] G. O'Donovan, A. Owen, S. R. Bird et al., "Changes in cardiorespiratory fitness and coronary heart disease risk factors following 24 wk of moderate- or high-intensity exercise of equal energy cost," *Journal of Applied Physiology*, vol. 98, no. 5, pp. 1619–1625, 2005.

[43] L. M. LeMura, S. P. von Duvillard, J. Andreacci, J. M. Klebez, S. A. Chelland, and J. Russo, "Lipid and lipoprotein profiles, cardiovascular fitness, body composition, and diet during and after resistance, aerobic and combination training in young women," *European Journal of Applied Physiology*, vol. 82, no. 5-6, pp. 451–458, 2000.

[44] K. M. Diaz and D. Shimbo, "Physical activity and the prevention of hypertension," *Current Hypertension Reports*, vol. 15, no. 6, pp. 659–668, 2013.

[45] E. Carpio-Rivera, J. Moncada-Jiménez, W. Salazar-Rojas, and A. Solera-Herrera, "Acute effects of exercise on blood pressure: a meta-analytic investigation," *Arquivos Brasileiros de Cardiologia*, vol. 106, no. 5, pp. 422–433, 2016.

[46] Y. Tsukiyama, T. Ito, K. Nagaoka, E. Eguchi, and K. Ogino, "Effects of exercise training on nitric oxide, blood pressure and antioxidant enzymes," *Journal of Clinical Biochemistry and Nutrition*, vol. 60, no. 3, pp. 180–186, 2017.

[47] T. V. Lewis, A. M. Dart, J. P. F. Chin-Dusting, and B. A. Kingwell, "Exercise training increases basal nitric oxide production from the forearm in hypercholesterolemic patients," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 11, pp. 2782–2787, 1999.

[48] F. R. Roque, A. M. Briones, A. B. García-Redondo et al., "Aerobic exercise reduces oxidative stress and improves vascular changes of small mesenteric and coronary arteries in hypertension," *British Journal of Pharmacology*, vol. 168, no. 3, pp. 686–703, 2013.

[49] A. F. Brito, A. S. Silva, I. L. L. Souza, J. C. Pereira, and B. A. Silva, "Intensity of swimming exercise influences aortic reactivity in rats," *Brazilian Journal of Medical and Biological Research*, vol. 48, no. 11, pp. 996–1003, 2015.

[50] D. D. Christou, P. P. Jones, J. Jordan, A. Diedrich, D. Robertson, and D. R. Seals, "Women have lower tonic autonomic support of arterial blood pressure and less effective baroreflex buffering than men," *Circulation*, vol. 111, no. 4, pp. 494–498, 2005.

[51] V. L. Brooks, P. A. Cassaglia, D. Zhao, and R. K. Goldman, "Baroreflex function in females: changes with the reproductive cycle and pregnancy," *Gender Medicine*, vol. 9, no. 2, pp. 61–67, 2012.

[52] B. Y. S. Vázquez, J. Martini, A. G. Tsai, P. C. Johnson, P. Cabrales, and M. Intaglietta, "The variability of blood pressure due to small changes of hematocrit," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 299, no. 3, pp. H863–H867, 2010.

[53] J. Martini, B. Carpentier, A. C. Negrete, J. A. Frangos, and M. Intaglietta, "Paradoxical hypotension following increased hematocrit and blood viscosity," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 289, no. 5, pp. H2136–H2143, 2005.

[54] P. Connes, A. Pichon, M. D. Hardy-Dessources et al., "Blood viscosity and hemodynamics during exercise," *Clinical Hemorheology and Microcirculation*, vol. 51, no. 2, pp. 101–109, 2012.

[55] S. Immanuel, S. R. Bororing, and R. S. Dharma, "The effect of aerobic exercise on blood and plasma viscosity on cardiac health club participants," *Acta Medica Indonesiana*, vol. 38, no. 4, pp. 185–188, 2006.

[56] M. Holinstat, "Normal platelet function," *Cancer Metastasis Reviews*, vol. 36, no. 2, pp. 195–198, 2017.

[57] S. Heber and I. Volf, "Effects of physical (in)activity on platelet function," *BioMed Research International*, vol. 2015, Article ID 165078, 11 pages, 2015.

[58] C. Di Massimo, P. Scarpelli, M. Penco, and M. G. Tozzi-Ciancarelli, "Possible involvement of plasma antioxidant defences in training-associated decrease of platelet responsiveness in humans," *European Journal of Applied Physiology*, vol. 91, no. 4, pp. 406–412, 2004.

[59] J. S. Wang, Y. S. Li, J. C. Chen, and Y. W. Chen, "Effects of exercise training and deconditioning on platelet aggregation induced by alternating shear stress in men," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 2, pp. 454–460, 2005.

[60] J. Yamamoto, Y. Ijiri, H. Ikarugi, K. Otsui, N. Inoue, and K. S. Sakariassen, "Prevention of thrombotic disorders by antithrombotic diet and exercise: evidence by using global thrombosis tests," *Future Science*, vol. 4, no. 4, article FSO285, 2018.

[61] S. R. Kahn, I. Shrier, S. Shapiro et al., "Six-month exercise training program to treat post-thrombotic syndrome: a randomized controlled two-centre trial," *CMAJ*, vol. 183, no. 1, pp. 37–44, 2011.

[62] A. M. Hvas and S. Neergaard-Petersen, "Influence of exercise on platelet function in patients with cardiovascular disease," *Seminars in Thrombosis and Hemostasis*, vol. 44, no. 8, pp. 802–812, 2018.

[63] Y. Zhao, P. M. Vanhoutte, and S. W. S. Leung, "Vascular nitric oxide: beyond eNOS," *Journal of Pharmacological Sciences*, vol. 129, no. 2, pp. 83–94, 2015.

[64] R. J. Widmer and A. Lerman, "Endothelial dysfunction and cardiovascular disease," *Global Cardiology Science and Practice*, vol. 2014, no. 3, pp. 291–308, 2014.

[65] P. P. Rainer and D. A. Kass, "Old dog, new tricks: novel cardiac targets and stress regulation by protein kinase G," *Cardiovascular Research*, vol. 111, no. 2, pp. 154–162, 2016.

[66] W. S. Cheang, W. T. Wong, L. Zhao et al., "PPARδ is required for exercise to attenuate endoplasmic reticulum stress and endothelial dysfunction in diabetic mice," *Diabetes*, vol. 66, no. 2, pp. 519–528, 2017.

[67] M. Iemitsu, S. Maeda, S. Jesmin, T. Otsuki, and T. Miyauchi, "Exercise training improves aging-induced downregulation of VEGF angiogenic signaling cascade in hearts," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 291, no. 3, pp. H1290–H1298, 2006.

[68] A. Heidarianpour, "Does detraining restore influence of exercise training on microvascular responses in streptozotocin-induced diabetic rats?," *Microvascular Research*, vol. 80, no. 3, pp. 422–426, 2010.

[69] L. Yang, Z. Jia, L. Yang et al., "Exercise protects against chronic β-adrenergic remodeling of the heart by activation of endothelial nitric oxide synthase," *PLoS One*, vol. 9, no. 5, article e96892, 2014.

[70] H. Pan, J. Guo, and Z. Su, "Advances in understanding the interrelations between leptin resistance and obesity," *Physiology & Behavior*, vol. 130, pp. 157–169, 2014.

[71] Y. Zhou and L. Rui, "Leptin signaling and leptin resistance," *Frontiers in Medicine*, vol. 7, no. 2, pp. 207–222, 2013.

[72] K. A. Krawczewski Carhuatanta, G. Demuro, M. H. Tschöp, P. T. Pfluger, S. C. Benoit, and S. Obici, "Voluntary exercise improves high-fat diet-induced leptin resistance independent of adiposity," *Endocrinology*, vol. 152, no. 7, pp. 2655–2664, 2011.

[73] J. D. Knudson, Ü. D. Dincer, C. Zhang et al., "Leptin receptors are expressed in coronary arteries, and hyperleptinemia causes significant coronary endothelial dysfunction," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 289, no. 1, pp. H48–H56, 2005.

[74] Y. Park, F. W. Booth, S. Lee, M. J. Laye, and C. Zhang, "Physical activity opposes coronary vascular dysfunction induced during high fat feeding in mice," *The Journal of Physiology*, vol. 590, no. 17, pp. 4255–4268, 2012.

[75] S. Kang, K. B. Kim, and K. O. Shin, "Exercise training improves leptin sensitivity in peripheral tissue of obese rats," *Biochemical and Biophysical Research Communications*, vol. 435, no. 3, pp. 454–459, 2013.

[76] D. J. Dyck, "Leptin sensitivity in skeletal muscle is modulated by diet and exercise," *Exercise and Sport Sciences Reviews*, vol. 33, no. 4, pp. 189–194, 2005.

[77] C. C. T. Smith, R. A. Dixon, A. M. Wynne et al., "Leptin-induced cardioprotection involves JAK/STAT signaling that may be linked to the mitochondrial permeability transition pore," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 299, no. 4, pp. H1265–H1270, 2010.

[78] A. Shapiro, M. Matheny, Y. Zhang et al., "Synergy between leptin therapy and a seemingly negligible amount of voluntary wheel running prevents progression of dietary obesity in leptin-resistant rats," *Diabetes*, vol. 57, no. 3, pp. 614–622, 2008.

[79] L. K. McCorry, "Physiology of the autonomic nervous system," *American Journal of Pharmaceutical Education*, vol. 71, no. 4, p. 78, 2007.

[80] G. R. H. Sandercock, R. Grocott-Mason, and D. A. Brodie, "Changes in short-term measures of heart rate variability after eight weeks of cardiac rehabilitation," *Clinical Autonomic Research*, vol. 17, no. 1, pp. 39–45, 2007.

[81] K. Imai, H. Sato, M. Hori et al., "Vagally mediated heart rate recovery after exercise is accelerated in athletes but blunted in patients with chronic heart failure," *Journal of the American College of Cardiology*, vol. 24, no. 6, pp. 1529–1535, 1994.

[82] L. C. Vanderlei, C. M. Pastre, R. A. Hoshi, T. D. Carvalho, and M. F. Godoy, "Basic notions of heart rate variability and its clinical applicability," *Revista Brasileira de Cirurgia Cardiovascular*, vol. 24, no. 2, pp. 205–217, 2009.

[83] G. Dimitropoulos, A. A. Tahrani, and M. J. Stevens, "Cardiac autonomic neuropathy in patients with diabetes mellitus," *World Journal of Diabetes*, vol. 5, no. 1, pp. 17–39, 2014.

[84] L. VanHoose, Y. Sawers, R. Loganathan et al., "Electrocardiographic changes with the onset of diabetes and the impact of aerobic exercise training in the Zucker diabetic fatty (ZDF) rat," *Cardiovascular Diabetology*, vol. 9, no. 1, p. 56, 2010.

[85] W. Zareba, A. J. Moss, and S. le Cessie, "Dispersion of ventricular repolarization and arrhythmic cardiac death in coronary artery disease," *The American Journal of Cardiology*, vol. 74, no. 6, pp. 550–553, 1994.

[86] H. Dor-Haim, C. Lotan, M. Horowitz, and M. Swissa, "Intensive exercise training improves cardiac electrical stability in

myocardial-infarcted rats," *Journal of the American Heart Association*, vol. 6, no. 7, 2017.

[87] B. A. Kingwell, "Nitric oxide as a metabolic regulator during exercise: effects of training in health and disease," *Clinical and Experimental Pharmacology & Physiology*, vol. 27, no. 4, pp. 239–250, 2000.

[88] E. Juttler, V. Tarabin, and M. Schwaninger, "Interleukin-6 (IL-6): a possible neuromodulator induced by neuronal activity," *The Neuroscientist*, vol. 8, no. 3, pp. 268–275, 2002.

[89] J. N. Townend, M. al-Ani, J. N. West, W. A. Littler, and J. H. Coote, "Modulation of cardiac autonomic control in humans by angiotensin II," *Hypertension*, vol. 25, no. 6, pp. 1270–1275, 1995.

[90] R. Wada and S. Yagihashi, "Role of advanced glycation end products and their receptors in development of diabetic neuropathy," *Annals of the New York Academy of Sciences*, vol. 1043, no. 1, pp. 598–604, 2005.

[91] T. P. Devasagayam, J. C. Tilak, K. K. Boloor, K. S. Sane, S. S. Ghaskadbi, and R. D. Lele, "Free radicals and antioxidants in human health: current status and future prospects," *The Journal of the Association of Physicians of India*, vol. 52, pp. 794–804, 2004.

[92] K. M. Naseem, "The role of nitric oxide in cardiovascular diseases," *Molecular Aspects of Medicine*, vol. 26, no. 1-2, pp. 33–65, 2005.

[93] R. Zhou, A. Tardivel, B. Thorens, I. Choi, and J. Tschopp, "Thioredoxin-interacting protein links oxidative stress to inflammasome activation," *Nature Immunology*, vol. 11, no. 2, pp. 136–140, 2010.

[94] H. Nojima, H. Watanabe, K. Yamane et al., "Effect of aerobic exercise training on oxidative stress in patients with type 2 diabetes mellitus," *Metabolism*, vol. 57, no. 2, pp. 170–176, 2008.

[95] A. Navarro, C. Gomez, J. M. López-Cepero, and A. Boveris, "Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress, and mitochondrial electron transfer," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 286, no. 3, pp. R505–R511, 2004.

[96] M. Wang, S. Li, F. Wang, J. Zou, and Y. Zhang, "Aerobic exercise regulates blood lipid and insulin resistance via the toll-like receptor 4-mediated extracellular signal-regulated kinases/AMP-activated protein kinases signaling pathway," *Molecular Medicine Reports*, vol. 17, no. 6, pp. 8339–8348, 2018.

[97] Z. N. Kumral, G. Sener, S. Ozgur et al., "Regular exercise alleviates renovascular hypertension-induced cardiac/endothelial dysfunction and oxidative injury in rats," *Journal of Physiology and Pharmacology*, vol. 67, no. 1, pp. 45–55, 2016.

[98] T. Kishi, Y. Hirooka, M. Katsuki et al., "Exercise training causes sympathoinhibition through antioxidant effect in the rostral ventrolateral medulla of hypertensive rats," *Clinical and Experimental Hypertension*, vol. 34, no. 4, pp. 278–283, 2012.

[99] N. F. B. Alves, S. K. P. Porpino, M. M. O. Monteiro, E. R. M. Gomes, and V. A. Braga, "Coconut oil supplementation and physical exercise improves baroreflex sensitivity and oxidative stress in hypertensive rats," *Applied Physiology, Nutrition, and Metabolism*, vol. 40, no. 4, pp. 393–400, 2015.

[100] L. K. Oharomari, N. F. Garcia, E. C. . Freitas et al., "Exercise training and taurine supplementation reduce oxidative stress and prevent endothelium dysfunction in rats fed a highly palatable diet," *Life Sciences*, vol. 139, pp. 91–96, 2015.

[101] T. I. Lima, I. C. Monteiro, S. Valença et al., "Effect of exercise training on liver antioxidant enzymes in STZ-diabetic rats," *Life Sciences*, vol. 128, pp. 64–71, 2015.

[102] J. St-Pierre, S. Drori, M. Uldry et al., "Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators," *Cell*, vol. 127, no. 2, pp. 397–408, 2006.

[103] M. Ristow, K. Zarse, A. Oberbach et al., "Antioxidants prevent health-promoting effects of physical exercise in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 21, pp. 8665–8670, 2009.

[104] I. Belaya, M. Suwa, T. Chen et al., "Long-term exercise protects against cellular stresses in aged mice," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 2894247, 10 pages, 2018.

[105] M. R. Beltran Valls, I. Dimauro, A. Brunelli et al., "Explosive type of moderate-resistance training induces functional, cardiovascular, and molecular adaptations in the elderly," *Age*, vol. 36, no. 2, pp. 759–772, 2014.

[106] D. Tian, J. Dong, S. Jin, X. Teng, and Y. Wu, "Endogenous hydrogen sulfide-mediated MAPK inhibition preserves endothelial function through TXNIP signaling," *Free Radical Biology & Medicine*, vol. 110, pp. 291–299, 2017.

[107] S. S. Gounder, S. Kannan, D. Devadoss et al., "Impaired transcriptional activity of Nrf 2 in age-related myocardial oxidative stress is reversible by moderate exercise training," *PLoS One*, vol. 7, no. 9, article e45697, 2012.

[108] W. O. Osburn and T. W. Kensler, "Nrf 2 signaling: an adaptive response pathway for protection against environmental toxic insults," *Mutation Research*, vol. 659, no. 1-2, pp. 31–39, 2008.

[109] V. R. Muthusamy, S. Kannan, K. Sadhaasivam et al., "Acute exercise stress activates Nrf 2/ARE signaling and promotes antioxidant mechanisms in the myocardium," *Free Radical Biology & Medicine*, vol. 52, no. 2, pp. 366–376, 2012.

[110] S. C. Smith Jr., E. J. Benjamin, R. O. Bonow et al., "AHA/ACCF secondary prevention and risk reduction therapy for patients with coronary and other atherosclerotic vascular disease: 2011 update: a guideline from the American Heart Association and American College of Cardiology Foundation endorsed by the World Heart Federation and the Preventive Cardiovascular Nurses Association," *Journal of the American College of Cardiology*, vol. 58, no. 23, pp. 2432–2446, 2011.

[111] M. Roffi, C. Patrono, J. P. Collet et al., "2015 ESC guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: task force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC)," *European Heart Journal*, vol. 37, no. 3, pp. 267–315, 2016.

[112] H. S. Buttar, T. Li, and N. Ravi, "Prevention of cardiovascular diseases: role of exercise, dietary interventions, obesity and smoking cessation," *Experimental and Clinical Cardiology*, vol. 10, no. 4, pp. 229–249, 2005.

[113] H. Patel, H. Alkhawam, R. Madanieh, N. Shah, C. E. Kosmas, and T. J. Vittorio, "Aerobic *vs* anaerobic exercise training effects on the cardiovascular system," *World Journal of Cardiology*, vol. 9, no. 2, pp. 134–138, 2017.

[114] R. B. Batacan Jr., M. J. Duncan, V. J. Dalbo, G. L. Buitrago, and A. S. Fenning, "Effect of different intensities of physical activity on cardiometabolic markers and vascular and cardiac function in adult rats fed with a high-fat high-carbohydrate diet," *Journal of Sport and Health Science*, vol. 7, no. 1, pp. 109–119, 2018.

[115] U. Kannan, K. Vasudevan, K. Balasubramaniam, D. Yerrabelli, K. Shanmugavel, and N. A. John, "Effect of exercise intensity on lipid profile in sedentary obese adults," *Journal of Clinical and Diagnostic Research*, vol. 8, no. 7, pp. BC08–BC10, 2014.

[116] F. Lobelo, D. Rohm Young, R. Sallis et al., "Routine assessment and promotion of physical activity in healthcare settings: a scientific statement from the American Heart Association," *Circulation*, vol. 137, no. 18, pp. e495–e522, 2018.

[117] M. Y. Cortez, C. E. Torgan, Brozinick JT Jr, and J. L. Ivy, "Insulin resistance of obese Zucker rats exercise trained at two different intensities," *The American Journal of Physiology*, vol. 261, 5, Part 1, pp. E613–E619, 1991.

[118] J. S. McPhee, D. P. French, D. Jackson, J. Nazroo, N. Pendleton, and H. Degens, "Physical activity in older age: perspectives for healthy ageing and frailty," *Biogerontology*, vol. 17, no. 3, pp. 567–580, 2016.

[119] J. Cvecka, V. Tirpakova, M. Sedliak, H. Kern, W. Mayr, and D. Hamar, "Physical activity in elderly," *European Journal of Translational Myology*, vol. 25, no. 4, pp. 249–252, 2015.

[120] M. E. Nelson, W. J. Rejeski, S. N. Blair et al., "Physical activity and public health in older adults: recommendation from the American College of Sports Medicine and the American Heart Association," *Medicine and Science in Sports and Exercise*, vol. 39, no. 8, pp. 1435–1445, 2007.

[121] D. Forbes, E. J. Thiessen, C. M. Blake, S. C. Forbes, and S. Forbes, "Exercise programs for people with dementia," *Cochrane Database of Systematic Reviews*, vol. 4, no. 12, article CD006489, 2013.

[122] C. J. Liu and N. Latham, "Adverse events reported in progressive resistance strength training trials in older adults: 2 sides of a coin," *Archives of Physical Medicine and Rehabilitation*, vol. 91, no. 9, pp. 1471–1473, 2010.

[123] P. F. Saint-Maurice, R. P. Troiano, D. Berrigan, W. E. Kraus, and C. E. Matthews, "Volume of light versus moderate-to-vigorous physical activity: similar benefits for all-cause mortality?," *Journal of the American Heart Association*, vol. 7, no. 23, article e03714, 2018.

[124] L. J. Geneen, R. A. Moore, C. Clarke et al., "Physical activity and exercise for chronic pain in adults: an overview of Cochrane reviews," *Cochrane Database of Systematic Reviews*, vol. 4, article CD011279, 2017.

[125] T. W. Buford, M. D. Roberts, and T. S. Church, "Toward exercise as personalized medicine," *Sports Medicine*, vol. 43, no. 3, pp. 157–165, 2013.

[126] A. N. Sullivan and M. E. Lachman, "Behavior change with fitness technology in sedentary adults: a review of the evidence for increasing physical activity," *Frontiers in Public Health*, vol. 4, p. 289, 2017.

[127] L. W. Jones, N. D. Eves, and J. M. Scott, "Bench-to-bedside approaches for personalized exercise therapy in cancer," *American Society of Clinical Oncology Educational Book*, vol. 37, pp. 684–694, 2017.

[128] T. W. Buford, S. D. Anton, D. J. Clark, T. J. Higgins, and M. B. Cooke, "Optimizing the benefits of exercise on physical function in older adults," *PM & R : The Journal of Injury, Function, and Rehabilitation*, vol. 6, no. 6, pp. 528–543, 2014.