UNIVERSITÀ DEGLI STUDI DELL'AQUILA

DIPARTIMENTO DI MEDICINA CLINICA, SANITÀ PUBBLICA, SCIENZE DELLA VITA E DELL'AMBIENTE

Dottorato di Ricerca in Medicina Clinica e Sanità Pubblica

Curriculum Epidemiologia, medicina preventiva e scienze infermieristiche

XXXVIII ciclo

Titolo della tesi

Effects of hydroalcoholic Saffron extract in an *in vitro* model of neural senescence

SSD: BIOS-11/A

Dottorando

Erjola Bej

Coordinatore del corso

Prof. Stefano Necozione

Tutor

Prof. ssa Vanessa Castelli

a.a. 2024/2025

Index

Abstract

Brain aging is presented by progressive loss and cognitive decline. Age is the most crucial factor that contributes to brain senescence, which is mainly defined as oxidative stress, neuroinflammation, and neurodegeneration. Age itself contributes to changes in the mitochondria, mostly causing an overproduction of reactive oxygen species (ROS). The free radical aging theory, which is largely studied and researched, reveals how cells go through damage oxidative stress related. Cells themselves are provided with antioxidants such as superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2), glutathione peroxidase and catalase (CAT) capable of removing ROS. When a disbalance between the inner anti-oxidative defense system and ROS happens, active molecules that might counteract the oxidative stress can be qualified as helpful.

Our study was aimed in the evaluation of anti-aging effects presented by the Saffron extract (SE) in human SH-SY5Y neuroblastoma cells after D-GAL induced aging model using assessment of cell proliferation, live-cell cytotoxicity, Beta-Galactosidase (β-GAL), lipid peroxidation (MDA), intracellular reactive oxygen species, advanced glycation end products (AGEs) and malondialdehyde (MDA) levels. After D-GAL induction, what was observed were high levels of oxidative stress associated with endoplasmic reticulum (ER) stress and elevated activity of lysosomal enzyme SA-β-gal that can be considered as a biomarker of senescence. Pretreatment with SE succeeded in reducing oxidative stress conditions and ER stress.

Introduction

Chapter I

Overview of the thesis

Neurological disorders are a presented by diseases/conditions that target part of the nervous system, like brain, spine or nerves and are classified as one of the main causes leading to long-term disabilities or in the worst case to death [1]. Neurological diseases are an increasing global issue recently and from the neurodegenerative disorders Parkinson's disease (PD) and Alzheimer's disease (AD) have shown a noticeable increase in frequency. The main risk factors influencing the neurological disorders include high blood pressure, body-mass index and low birth weight plus short gestational period and environmental pollution. Smoking is a major cause and also contributes significantly in triggering the disorders [2]. Neurological disorders can weaken cognitive motor function, increase the amyloid deposition, damage the Blood Brain Barrier (BBB) and disrupt nerve conduction. Since nowadays the population is reaching a certain age, more and more people presenting neurological disorders are being observed. Therefore, PD and dementia have almost multiplied by two in the recent years [3,4]. Patients that suffer from neurological disorders often require special care, social and also economic assistance because of the cognitive and physical disabilities leading to social distancing [5]. From the main neurological disorders we should mention stroke, migraine, meningitis, AD, PD, idiopathic epilepsy, neural tube defects, spinal injuries, encephalitis, multiple sclerosis [2]. Since for some of the neurological disorders there is no cure or treatment, it is very important to take precautions after the absolute increase of cases each year.

Chapter II: The hallmarks of aging

2.1 Brain aging

Brain aging is presented by loss of neurophysiologic functions as a result of neurodegeneration [6]. One of its main characteristics is progressive loss and decline [7]. Another important feature of aging is the disturbance of BBB losing this way the homeostasis [8]. In physiological conditions, aging includes biological changes of the brain related to alterations of cognitive ability [9]. For many years it was thought that cognitive changes happening during aging could not affect functional abilities [10]. These beliefs were based on hypothesis according to which brain aging might affect only some of the cognitive skills [11,12]. Brain aging does not involve only functional alterations but also structural ones that occur over time. One of the most studied theories about aging is the one concerning the free radicals [13]. The free radical aging theory implies a cellular damage due to oxidative stress [14]. This theory is also reveals how mitochondrial DNA (mtDNA) mutations are related to mitochondrial dysfunction causing therefore higher free radical production [15]. According to Payene at al., mtDNA mutations over time can not only accumulate but also lead to proliferation of the already existing mtDNA age-related mutations [16]. One of the *Drosophila* studies indicated how the quantity of ROS produced during aging increases leading to a collapse of mitochondria on one hand but on the other hand, the ROS production on a specific site on the electron transport chain (ETC) can contribute significantly in signaling in order to protect mitochondria and increase the lifespan [17]. Other studies of *Caenorhabditis elegans* showed how low levels of mitochondrial stress can increment the longevity [18].

Studies are now trying to confirm if the presence of Aβ plaques is related to brain aging independent of AD or it might be a marker of a juvenile form of AD being developed [19]. Increased life longevity has given risen to difficulties in distinguishing the presence of Aβ plaques AD related with those corresponding to brain aging [10,20]. The most observed brain aging changes comprehend functional decline, DNA alteration and toxic aggregations [21].

Senescent cells, which main characteristic is preventing cells from dividing and replicating, produce a recognizably different senescence-associated secretory phenotype (SASP) concerning proteases, cytokines and growth factors and they tend to accumulate with time in order to degenerate the tissue [22,23]. The upregulation of the proinflammatory molecules correlates aging as a result of a chronic inflammation [24]. These types of cells, in the content of neurodegenerative diseases, promote dysfunction [25]. DNA damage and ROS are capable of inducing senescence and according to researchers, DNA damage is mostly observed in the mitochondria of neurons of seniors [26–28]. In the meantime, chronic and acute injuries and stress might be one of the causes [29,30]. One fundamental feature of senescent cells, is the accumulation of dysfunctional mitochondria [31]. Senescent cells have the tendency to display β-gal activity which can be an indicator and be measured with a colorimetric assay in order to identify the senescent cells. According to researchers, neurons of aged mice present DNA damage and high expression of pro-inflammatory cytokines plus a higher activity of β-gal [26].The inflammatory activity happening in the brain of seniors takes place in the absence of a pathogen [32]. Microglia of the older mice displays a boost to overproduce cytokines like IL-6, IL-1β and TNF-α [33,34]. In the *in vitro* studies, the activated microglia present characteristics of senescence like growth arrest and β-gal activity [35]. Astrocytes interactions with the endothelial cells are very crucial in

maintaining the unification of the BBB [36]. Astrocytes also present signs of senescence *in vitro* after ROS exposure and ionizing radiation exposure [37,38]. Targeting senescent cells might be a promising strategy to delay dysfunction and to increase longevity [39]. DNA damage, dysfunctional mitochondria and the accumulation of the lipofuscin aggregates are mainly attributed to cellular senescence [40]. One of the characteristic phenotypic changes during normal aging is excess of neuromelanin that is responsible for activation of microglial, damage to dopaminergic neurons, mitochondrial dysfunction and induction of oxidative stress [41,42]. One of the consequences of senescence might be the demyelination which causes damage to axons [43]. Neurodegeneration itself can be classified as a boost of neuronal senescence phenotypes like misfolded proteins or altered synaptic transmission [44]. Tau pathology might also be related to cellular senescence [45].

2.2 Age related changes in the brain

The aging process is a continuous deterioration process that is related to irrevocable changes to all the molecules, cells, tissues and organs [46]. Brain senescence as a biological phenomenon leading to gradual deterioration of functional characteristics in living organisms is indeed a contributing factor for developing different degenerative neurological diseases such as AD and PD and for that reason, developing successful approaches to prevent or slow it down is of vital importance especially in ameliorating the quality of living of older generations [47]. Neurons, if compared to other cells have a higher probability to modify their function and metabolism if subjected to insults or diseases like the neurodegenerative ones [48]. If high levels of oxidative stress are

presented, neurons have the tendency to morphologically change the structure, especially of their dendrites with the passing of time. But even so, dendritic growth is still possible even in case of aging neurons [48]. While dendritic shortening is observed, axons tend to increase with the passing of time. Mitochondria have a major impact in aging and axon growth [49,50]. During aging and neurodegeneration as pathological condition, axons tend to deteriorate due to a selective destruction process [51]. The self-destructive process begins when mitochondrial permeability transition pore (mPTP) found between both membranes of the mitochondria, the inner and the outer one, is activated. The indicated process leading to axonal degeneration can be a possible target with the main aim to contrast neurodegeneration [52].

Dendritic shrinkage is not the only process observed during aging, but also dendritic degradation, demyelination and microglial activation during all stages of the aging natural process [53]. Microglial activation, in particular, can cause neuroinflammation [54]. All these physiological age-related variations result in irregularities in synaptic transmission [55,56]. Neuronal survival is mostly regulated by neurotransmitters including dopamine, serotonin (5-HT) as well as by brain-derived neurotrophic factor (BDNF), all of which tend to decline with age. In parallel, with the reduction in neurotransmitter levels, aging is also linked to a marked lowering of glucose and oxygen rates in cerebral blood flow [57].

Overall, aging is mainly related to neuronal loss, cognitive deficits and impairment of the central nervous system (CNS) functions [58]. Cellular senescence is distinguished by alterations in morphology, metabolic activity and transcriptional and epigenetic alterations in gene expression [47,59,60]. Senescent cells are identified by inflammation and intensified oxidative stress resulting in DNA damage and cell cycle arrest developing and SASP [60]. Brain

senescence is mainly characterized by oxidative stress, neuroinflammation, neurodegeneration and cognitive impairment. Based on the fact that senescence is characterized by neuroinflammation, using anti-inflammatory drugs, for example ibuprofen, markers of aging according to studies conducted on genetic mouse models, are decreased [61]. The first steps of aging include only cells, but, with the passing of time, morphological alterations in tissue and organs start to appear [62]. Studies confirm the existence of a correlation between cognitive performance and degeneration of age-related white matter sections [9]. The neurofibrillary tangles (NFTs) and the senile plaques are established in physiological and pathological states of the organism while accumulation of Aβ protein and α-synuclein is observed mostly in aged brain or during pathological conditions [63]. Accumulation of Aβ protein is a primary determinant of mitochondrial dysfunction and oxidative stress [55].

The heat shock proteins (HSPs) normally protect the neurons and glia. Acting as molecular chaperones, these proteins regulate protein folding and prevent the excessive aggregation of hyperphosphorylated tau and amyloid peptide in case of neurodegenerative disorders. HSPs are also responsible for controlling the apoptotic and inflammatory pathway. In case of nonfunctioning HSPs, the production of hyperphosphorylated inclusions is elevated [64]. The proteostasis is an important process that maintains the protein homeostasis between the synthesis, folding and in the end the degradation. Another target for fighting neurodegenerative diseases or improve the symptoms can also be modifying the conformational structure of the proteins [65]. These biological and pharmacological small molecules called chaperones can activate the refolding of damaged proteins [66]. Not only refolding of proteins can be target by the proteolytic enzymes like peptidases or proteases but also the aggregation or degradation

processes can be modified. Chaperones can prevent impaired aggregation [67]. The synthesis of chaperones is reduced or damaged with aging and therefore with the senescence, what comes along is also a variation of proteostasis [68,69]. We find the proteases in both intracellular and extracellular compartments and as a result they may play the role of signaling molecules [67].

2.3 Mitochondrial dysfunction in senescent brain

Mitochondria contributes to energy production and cell cycle regulation starting from growth to apoptosis [70]. Changes that affect the mitochondria during aging are mostly characterized by functional decline and overproduction of ROS [71,72]. As mitochondria age, their energy output decreases, while ROS generation increases, damaging cellular components, especially mtDNA. Some studies suggest that mtDNA mutations can be a start of aging or speed up the process and appearance of the first symptoms [73]. The content of mtDNA is increased during aging while seniors present elevated neuron mtDNA levels [74]. The oxidation of mtDNA makes it more open to further mutations. The mitochondrial changes in the brain that are related to aging involve a reduction in both complex I (NADH dehydrogenase/ubiquinone oxidoreductase) and complex IV (cytochrome oxidase; COX) while the activity of complex II (succinate dehydrogenase) remains stable [72]. Increased ROS levels induces autophagy that is a catalytic process able to maintain cellular homeostatic stability [75]. In the meantime, the activated autophagy presents a pivotal role in reducing the quantity of oxidized molecules contributing therefore in minimizing the damage of oxidative stress [76]. Studies suggest that superoxide is the main ROS that guides the ROS-regulating autophagy [77]. Autophagy is a very special process because it can

remove not only oxidized and damaged products but also the organelles that produce ROS like mitochondria and peroxisome to reduce the production of more ROS [78,79]. In case of nutrient starvation or high quantity of ROS produced, autophagy permits a cell to eat a part of itself especially protein aggregates or damaged organelles [80]. However, the mechanism through which ROS activate autophagy and its protective role remain unclear [81].

Proteins are among the primary targets of ROS or other radicals *in vivo* [82]. Disulfide bonds, covalent links between sulfur atoms of cysteine residues, are critical for stabilizing protein structure. Irreversible protein oxidation disrupt these structure, but molecules like glutathione or other antioxidants can reduce the disulfide bridges [83]. Moreover, cells are equipped with antioxidant compounds such as SOD1, SOD2, CAT and glutathione peroxidase able to remove ROS. Results of different studies show how the antioxidant chaperones proteins can be selective and bind with the crucial proteins of autophagy to activate the process [81]. Autophagy systems are helpful when the first line of antioxidant defenses like protein and non-protein thiols cannot control the irreversible oxidation of macromolecules and damaged organelles [38].

Mitochondria and ROS are essential in apoptosis induction activating the caspase 3-like protease, a cysteine protease that splits specific proteins, causing cells shrinking and DNA fragmentation [84,85]. While ROS activate several pathways that induce apoptosis [86]. On the other hand, damaged DNA leads to overproduction of ROS [87]. Both damaged DNA and ROS production are associated with caspase-9- dependent apoptosis, a mechanism playing a role in the intrinsic apoptotic pathway that results in programmed cell death [88]. Caspase 8 and caspase 9 are initiator caspases; caspase 3 on the other hand is part of the terminal apoptotic process [89].

During aging, the ATP production is reduced [90]. The cells are presented with the option to work using little energy or find a secondary strategy to gain energy from non-oxidative metabolisms. This back up plan, is going to have an impact on other signaling pathways [91].More mutations of mtDNA in aging will lead to a more dysfunctional mitochondria creating a vicious cycle further damaging mtDNA [92]. During phosphorylation of macromolecules like proteins, lipids and particularly mtDNA, ROS are produced. The produced ROS are the cause of mtDNA mutations in aging [93,94]. mtDNA itself is mainly the motive of encoding some of the most important components in the cascade of oxidative phosphorylation and so if it is damaged, more ROS will be produced, altering the activity of the respiratory mitochondrial chain effecting cell death [95]. In pathological conditions or during aging, mitochondria is not capable of maintaining the same levels of synthesis of mitochondrial proteins like mitochondrial ATP synthase or the same oxidative enzymatic activity and therefore the ATP produced is indirectly reduced [96].

During senescence, mitochondrial dysfunction is presented by reduced respiratory function and also a decline in mitochondrial membrane potential (MMP). To maintain constant ATP production and to preserve the MPP, mitochondria must maintain the best equilibrium between NAD+ and NADH. The production and consumption determine the levels of NAD+ [97]. During aging or in cases of excessive stress, NAD+ consumption is higher implying an altered NAD+/NADH balance and this entails a malfunctional mitochondrial membrane depolarization and also a modified mitochondrial permeability [98].

2.4 Mitochondrial electron transport chain during aging

4 protein complexes are constituent part of the mitochondrial ETC. The mentioned complexes are effective in facilitating transfers of electrons while inducing the process of oxidative phosphorylation using ATP synthase to get ATP produced [99]. The first complex (NADH dehydrogenase) is presented as an electron acceptor transferring them from NADH to ubiquinone while the second complex (succinate dehydrogenase) accepts electrons from succinate with the main aim of also transferring them to ubiquinone. The third complex (Q-cytochrome c oxidoreductase) receives electrons from the reduced form of ubiquinone called ubiquinol so that to transfer them to cytochrome c. The fourth complex (cytochrome c oxidase) instead shifts the received electrons to oxygen which is later reduced to water. According to studies, if we inhibit complex I, complex III or complex IV hydrogen peroxide formation will be intensified. With the intention of enhancing ROS generation, it is necessary to inhibit the complex I with 25-30% while in order to power up the ROS generation, the complex III and the complex IV must be inhibited by more than 70% [100].

During aging, dysfunctional mitochondria tend to create an inflammation chronic process that persists even when the insult to cells or tissues is over [101]. The mitochondrial release in the cytoplasm and/or extracellular space can cause chronic inflammation, therefore, to avoid it, it is very crucial to assemble the damaged mitochondria or the aged one, in a double membrane autophagosome via mitophagy [102]. $Ca^{2+}$is a major key in synaptic activity and the transmission of signals. Its homeostasis can be maintained by protein channels that are found in the mitochondrial membranes, both inner and outer and also by the endoplasmic reticulum [103]. Defected age-related mitochondria can unbalance the homeostasis of Ca^{2+} influencing the levels of

cytoplasmatic Ca^{2+} and interfering with neurotransmitter release, synaptic plasticity and neuron excitability [104].

The proteases ATP-dependent present some main functions like ribosome assembly, adaptation to hypoxia, transcription and translation and also functions that include macromolecules like protein import or lipid transferring. All these function seem to be reduced with the passing of time and therefore, during aging we observe an unhealthy and non-completely functioning mitochondria [105]. During aging, an assemblement of senescent cells is verified and this process causes morbidity and mortality [106].

Protein turnover in the brain is a process like all the others that suffer from age-related changes. In case of oxidation of proteins and aggregation, different forms may result like Lewy bodies [107]. We observe the gathering of lipofuscin granules not only during senescence but also in neurodegenerative diseases like AD, where their presence disrupts cellular functioning due to toxic compounds [108–110].

2.5 Morphological rearrangement and oxidative stress in aging brain

Over the years, myelin undergoes some structural irregularities and alterations while axons tend to lose nerve fibers. This can result in weak conduction speed or faulty signals conduction through the axons. The study of Sandell on monkeys revealed that old monkeys present less myelinated fibers compared to the young monkeys [111,112].

The brain is one of the organs that uses the most quantity of oxygen and therefore is at more risk of undergoing cellular damage. Inflammaging is this process of inflammation happening in the brain

not because of a pathogen but because of ROS generation during aging [113,114]. Aging is characterized by a high number of senescent cells that release pro-inflammatory molecules and mediators, like cytokines [115,116].

2.6 Free radical aging theory

The most studied theory related to aging is the free radical hypothesis according to which senior people have more oxidized products if compared to the rising generations [117]. As described in the free radical theory, the accumulation of mtDNA mutation during aging will cause loss of function leading to a speed up of cell death. Also, based on this theory, it is very crucial for the physiological functions to maintain a balance between antioxidants products and oxidants [118]. According to some studies, an overexpression of antioxidant enzymes can increase longevity. ROS contribute as signaling molecules and modulators [119].

The lifespan of an individual is mainly inversely proportional to the quantity of ROS; more ROS we find in the mitochondria, reduced the lifespan of the person will theoretically will be [120]. The redox imbalance is mainly caused by a debilitated anti-oxidative defense system that is not able to counteract the higher levels of the ROS produced like hydrogen peroxide, superoxide and reactive nitric oxide [47]. The reduction of ROS may take place in different cellular compartment other than the mitochondria [121]. In case of damage to the mitochondrial membranes, the content can be released in the cytoplasm and/or extracellular environment triggering an inflammatory responses [122]. Rotenone is a mitochondrial complex I inhibitor that, if used in treating human THP1 macrophages, can cause a dose-related increment of the secretion of IL-1β,loss of MMP and ROS overproduction [123]. MPTP (1-

methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is also an inhibitor of complex I. It causes increased levels of inflammatory cytokines and in conclusion we observe microglial activation in both monkeys and mice according to recent studies [124]. Angiotensin is a peptide that is considered to be related to inflammatory response in the MPTP model [125]. Angiotensin is also responsible for maintaining the iron homeostasis which is crucial in case of disbalances because it can boost the microglial inflammatory response [126]. During aging processes, mitochondria are the origin of ROS [127]. Henry J. H. Fenton explained that hydrogen peroxide might power the genesis of hydroxyl radicals [128]. SOD1 and SOD2 enzymes, found in mitochondria and cytosol, can help in transforming the superoxide radical into hydrogen peroxide which is less harmful. Hydrogen peroxide is then transformed into water and oxygen [129] (**Figure 1**).

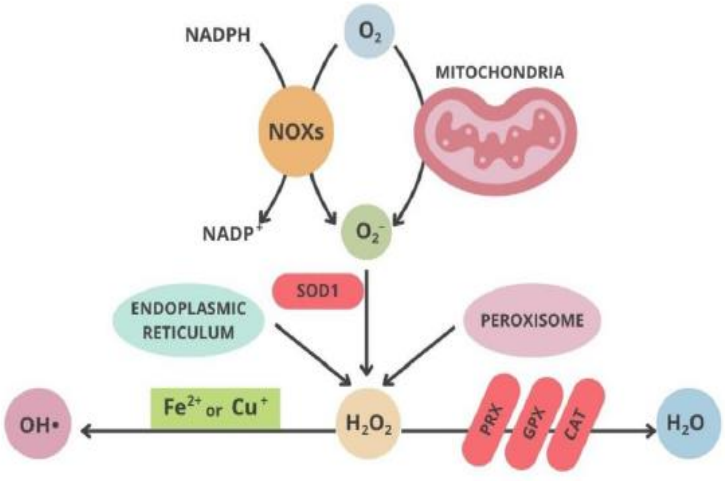


Figure 1: *Illustration of different pathways of oxidative stress*

The fact that ROS can accumulate is a leading sign to probable cell death that might have different mechanisms that might be presented through autophagic and cytoplasmic cell death and apoptosis [130,131]. ROS assemblage can cause conformational changes of pro-apoptotic proteins including the family of Bcl-2 proteins [132]. The release of apoptogenic factors mentioning

cytochrome c is attributable to the shift of these pro-apoptotic proteins into the mitochondrial membranes [133].

2.7 Mitochondrial membrane potential during aging

During aging, cells present a reduced MMP. This decrease is associated with a reduction of response given to agents that induce both the opening or closure of mPTP [134]. The mPTP is a protein complex which we find in the mitochondrial inner membrane [135,136]. In physiological conditions, the activation happens because of the calcium overload in the mitochondrial matrix. One of the mechanisms thought to activate the mPTP is the oxidation of the pyridine by the oxidative stress. The activation of voltage-gate dependent mPTP depolarizes the mitochondria and inhibits the oxidative phosphorylation. Consequently, a great quantity of ROS are released amplifying the risk of neurodegenerative diseases and early aging. Studies report that the inhibition of mPTP can retard aging [137,138]. A partial opening might be helpful for the release of the excessive quantity of ROS produced and calcium. A long-lasting opening on the other hand, might release most of the substrates of the matrix including calcium, mitochondrial ROS and glutathione resulting in membrane rupture, disturbed homeostasis, increased oxidative stress related to nuclear DNA, proteins, and membrane phospholipids enhancing aging [139]. Superabundance of ROS enhances the activity of mPTP leading to an accelerated aging process [140].

Senescent cells in aging might cause inflammation and loss of specific functions therefore clearance is very beneficial [141]. Among the main characteristic of the senescent cells is the ER stress [142]. Senescent cells secrete inflammatory cytokines that have an impact also on adjacent cells causing oxidative stress [143]. The

presence of senescent cells in aging phase is also related to neurodegenerative diseases like AD or PD [144,145].

2.8 Telomere attrition

Telomeres are repeated hexamers presented in the terminal part of chromosomes, composed from repetitive sequences of DNA that have the main aim to guard genetic information during cell division. Since telomere shortening is present in cellular senescence, the length of telomere's can be considered a biomarker related to aging [146]. Telomere attrition is a powerful process leading to block of cellular division and it is believed to be a sign of aging and its related processes predicting also mortality [147]. Telomerase is a specific telomere-synthesizing enzyme that controls telomere's length. Telomerase plays a controlling role in guaranteeing that the replicative process does not generate genomic instability due to uncapped telomeres. This enzyme preserves the telomere length through the insertion of nucleotides [148]. The telomere hypothesis predicts that, when somatic cells extend to a certain length, senescence is activated [149]. Telomere attrition alongside with ROS generation are both related to the development and advancement of geriatric diseases [150,151]. Not only so but shortened telomere are connected to aggravating of cognition in women affected by dementia [152]. The preclinical studies conducted on mice revealed that when mice are susceptible to aging, they present age-related learning and memory deficits. As a result, telomere length can be associated to neurodegenerative diseases and therefore, shortened telomeres can be used as a biomarker although future investigation is indispensable with the main aim to examine the connection between the neurodegenerative processes and telomeres [153–155].

Strategies to restore the length of telomeres include procedures that restore telomerase enzyme activity, reduce oxidative stress and promote the decrease of pro-inflammatory cytokines concentrations [156,157].

Lapham *et al.,* has reported that women present longer telomeres [158]. One of the causes influencing the length of telomeres in women is maternal age and childbirth weight [159]. Lifestyle factors that can have an impact on telomere shortening include smoking, insomnia, physical activity and alcohol consumption [160,161].

2.9 Organelle crosstalk in aging: ER-mitochondria interactions and metabolic control

Some theories present an alternative mitochondrial decline during aging that is related to a partially failure of other organelles such as vacuole/lysosomes [162]. When vacuolar depository declines, what is observed is an increase in cysteine levels in other compartments and that causes interference with redox homeostasis and iron. Cysteine can act as a toxin, disrupting the mitochondrial functioning during aging [163]. Mitochondria are not the only organelles that suffer from defects of vacuolar/lysosomal compartmentalization. Failure of amino acids lysosomal storage has an impact also on the dysfunction of ER [164].

One of the most cited mechanisms of cellular senescence is mitochondrial dysfunction and cellular calcium imbalance and both can be caused by a malfunction of organelle interactions [165]. The intracellular calcium homeostasis is maintained by ER [166]. During senescence, calcium is released by ER increasing therefore the intracellular calcium levels [167]. ER is not the only organelle maintaining calcium homeostasis. Mitochondria and lysosomes also have an instrumental role [168,169]. In order to transfer

calcium from ER to mitochondria an appropriate distance of these two organelles is required [170]. In the meantime, the imbalance of the lysosomal calcium leads to impaired proteolytic activity of lysosomes reflected subsequently in a negative way on lysosome-mediated autophagy proteolytic system causing therefore cellular senescence [171,172].

In senescent cells, the number of contact sites between mitochondria and ER is presented lower if compared to normal cells leading to think that the interruption of contacts between the two organelles influences the cell longevity [173,174]. In the connections between mitochondria and ER, parkin, α-synuclein and other toxin age-related proteins are present [175–177].

2.10 Metabolic syndrome in the elderly: the role of mitochondrial impairment

Metabolic syndrome as a definition is presented by a group of metabolic risk factors such as visceral obesity, high arterial blood pressure, dysglycemia, high triglyceride concentrations, and low HDL cholesterol, which lead to obesity, insulin resistance and cardiovascular pathologies [178]. Metabolic syndrome is also related to early aging. Recent evidence is showing an association of metabolic syndrome with oxidative stress [179]. Oxidative stress triggers the biological reactions that are involved in the metabolic syndrome [180]. In the metabolic syndrome cases of the oxidative stress might result intensified due to alterations of antioxidants defenses [181]. During aging, factors that might influence the metabolic syndrome include inflammation and stress [182].

2.11 Mitochondrial dynamics (Fission and Fusion) and their contribution to cellular senescence

Mitochondria, as highly motile organelles, undergo continuous fusion and fission processes that regulate cellular redox status and cell death [183]. In case of a genetic defect in the proteins associated with fusion and fission in the mitochondria, increased levels of apoptotic cell death can be observed [184]. This shows us how mitochondrial dynamic processes like fusion and fission proteins contribute to cell death [185]. Therefore, manipulating the proteins involved with these two processes might result in improved unwanted cell death and postponed age-related symptoms [186]. While increasing mitochondrial fusion, mitochondrial membrane depolarization is prevented and resistance to apoptosis is manifested [187,188]. Fission is a process that contributes to isolating damaged fragments of mitochondria and activating their autophagy. If these defense mechanisms go wrong, mitochondrial fission can promote apoptosis [189,190]. Alterations of fusion and fission processes might decrease mitochondrial movement and cause defects in neurons [184].

2.12 The interplay between nuclear and mitochondrial genomes in age-associated decline

The normal functioning of mitochondria along with the aging process might be regulated by a signaling process transmitted by nucleus to the mitochondria. The cause of activation of this signal is thought to be nuclear DNA damage during aging [191]. Modified levels of mitochondrial metabolites or stress signals can be the cause of epigenetic changes affecting aging [192]. During aging, mitochondria itself communicate their state of metabolic activation and function to the nucleus as a response to various environmental and intracellular signals [193]. If a failure of MMP takes place, ROS

generation in mitochondria can serve as signals to initiate the mitochondrial nuclear retrograde response [194]. Accumulation of unfolded or misfolded proteins activate this retrograde signaling that turns on a nuclear transcriptional response inducing as a result the mitochondria proteins chaperones [195].

Chapter III: Strategies to prevent or delay brain aging

3.1.1 Caloric restriction

Through the process of caloric restriction, we aim to prolong lifespan and health-span of the nervous system of an individual, improve cognitive performance and delay aging. Not only so, caloric restriction has also been proved to delay the neurodegenerative diseases such as AD and PD and improve long-term health [196–198]. Caloric restriction influences aging and affects age-related changes to the brain improving also cognitive function [199–201]. One of the possible mechanisms of the caloric strategy is influencing energy and oxygen radical metabolism in order to protect neurons during aging. Caloric restriction can boost the formation of protein chaperones and antioxidant enzymes able to protect cells against stress in order to resist longer to neurological disorders [197]. It is possible that through the reduction of calories, an anti-inflammatory mechanism and a process of synaptic plasticity protect neurons from oxidative damage. Caloric restriction might also be helpful in blocking and arresting β-amyloid neuropathology in Alzheimer's transgenic models [202]. Lowering the oxidative stress generated by mitochondria while reducing the calories can increase maximum lifespan in many species [203]. One of the main effects of caloric restriction is promoting the survival of individual cells downregulating in the meantime, cell proliferation [204]. According to what studies reveal, neurons of the brains of

mice and rats maintained on caloric restriction, performed a better resistance to oxidative stress [197]. During low states of energy in case of caloric restriction, neurotrophic factors such as BDNF show increased concentrations probably due to a compensatory mechanism opposed to the physiological stress [205]. Caloric restriction results useful in decreasing brain concentrations of pro-inflammatory cytokines protecting the brain from acute damage and aging [206]. Since caloric restriction counteracts aging and has a neuroprotective effect, it can be a potential target for future research [207].

3.1.2 Intermittent fasting

During fasting periods, we notice a switch from liver-derived glucose to adipose cell-derived ketone. Effects depend on diet, sex and genetic factors although, studies on mice and non-human primates promise a concordant effect of health span [208,209]. Studies in animals and humans have proved that intermittent fasting not only reduces the production of ROS but also improves stress resistance and represses inflammation. Not only so but during fasting periods, cells trigger mechanisms that boost intrinsic defenses against oxidative and metabolic syndrome [210]. According to studies in animals, fasting helps in improving cognition [200]. Through intermittent fasting neuronal stress resistance is observed. Fasting stimulates autophagy, antioxidants defenses, DNA repair, the production of neurotrophic factors, synaptic plasticity and neurogenesis [208,211]. One of the studies conducted on individuals with mild cognitive impairment revealed how the patients that practiced intermittent fasting, presented a higher score and an improved cognitive function after a follow up of 36 months [212]. Another study based on intermittent fasting

regimen was performed on C. elegans and Drosophila melanogaster. The result demonstrated a proven extension on lifespan [213,214].

3.1.3 Ketogenic diet

Ketogenic diet that presents low carbohydrate intake, shifts the metabolism from carbohydrates to proteins and fatty acids restriction, causing an increase in longevity and health span of mice [215,216]. This dietary approach manifests neuroprotective effects while repressing the glycolysis and nurturing the production of ketone bodies [217,218]. The ketone bodies present neuroprotective activity, activate autophagy, improve mitochondrial functioning, increase the amount of endogenous antioxidants and suppress the inflammation [219]. When there is no glucose available, the brain operates with ketones as a substitute form of energy since they can be transformed in acetyl Coenzyme A (CoA), which after oxidative phosphorylation can generate ATP [220]. Ketogenic diet dietary approach also results in reduced cognition decline [221]. In elderly mice, ketogenic diet resulted in a reduced mitochondrial ROS production, amplified mitochondrial production of ATP and enhanced MMP [222]. Ketogenic diet weakens the decline age-related in physiological functions present in old mice [216,218].

3.2 Supplementation

Dietary interventions and nutritional supplements can play an important role, alongside with pharmacological therapies, in cognitive impairments during aging [223]. Based on the free radical aging theory, the administration of exogenous antioxidants might

be helpful in order to reduce the reactions of free radicals [224,225]. The antioxidant supplementation is thought to be a future intervention in age-related disease [226]. The modern technologies use nanoparticles as antioxidants delivery systems in order to attenuate oxidative stress while penetrating to specific target organs or tissues. The concentration of the nanoparticles during administration does not influence in any way the final result which is increase of the lifespan of model organisms [227].

From among the main antioxidant compounds, we mention vitamin C, curcumin, resveratrol, coenzyme Q10, vitamin E and melatonin. Vitamin C is a potent antioxidant able to inhibit the peroxidation of lipids [224]. Tetrahydrocurcumin which is the active metabolite of curcumin turns out to be a potential antioxidant possessing anti-inflammatory effects [228]. Resveratrol presents a crucial protective effect against oxidative damage [224]. Coenzyme Q10 is a vital antioxidant found in cell membranes and lipoproteins [229]. The production of pro-inflammatory cytokines is decreased due to the use of Coenzyme Q10 [230]. Vitamin E also presents powerful antioxidant properties required for counteracting the damaging activity of variety of oxygen radicals [231]. Vitamin E also presents anti-inflammatory properties inhibiting the expression and/or function of the cyclooxygenase 2 (Cox-2) known for its crucial role in the inflammatory process [232]. Melatonin energizes the activity of glutathione peroxidase and SOD [233].

Chapter IV: Aim of the study

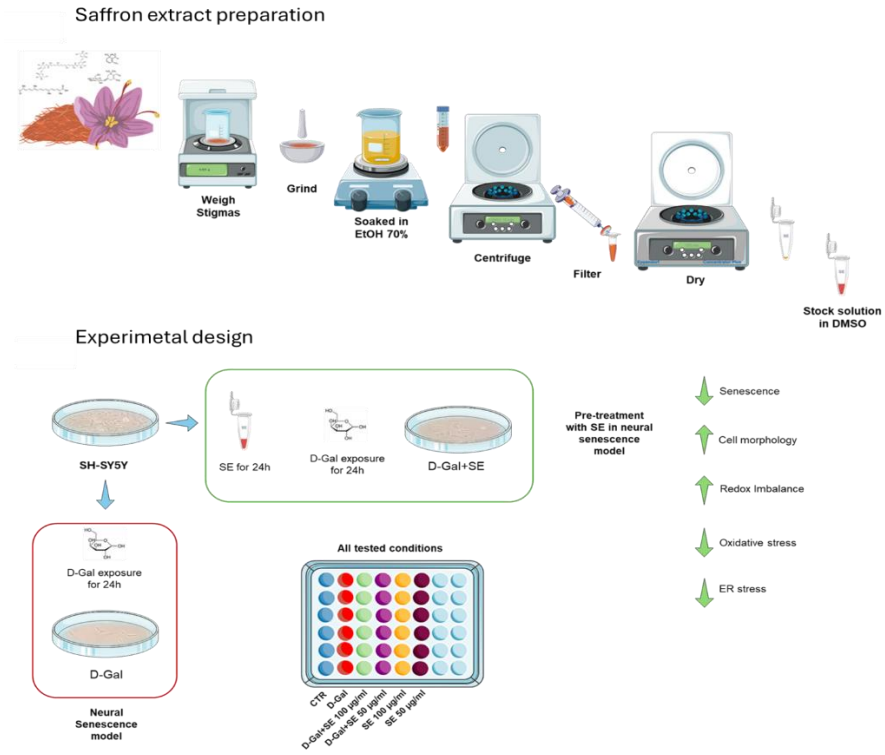
4.1 Effects of hydroalcoholic saffron extract in an *in vitro* model of neural senescence

Aging is presented as a chronic physiological process that causes irreversible alterations and modifications in most of the cases to all

molecules, tissues and organs [47]. Brain senescence is mostly characterized by chronic inflammation, high levels of oxidative stress and gradual decline of its functions. Not only so but brain senescence is related to a higher vulnerability of developing neurodegenerative diseases such as AD and PD [59,60]. For that reason, detecting active molecules or finding strategies to oppose or at least postpone the brain senescence is of high significance for the lifespan of individuals. This study was based on the evaluation of anti-aging effects of SE in human SH-SY5Y neuroblastoma cells after D-Gal induced aging model using analysis that evaluate of cell proliferation, live-cell cytotoxicity, Beta-Galactosidase (β-GAL), lipid peroxidation (MDA), intracellular ROS, advanced glycation end products (AGEs) and malondialdehyde (MDA) levels. The last step was using holotomography to observe changes in morphology.

Human SH-SY5Y cells are a well employed cellular model for observing the mechanistic features of neurodevelopment and neurodegeneration. D-galactose (D-GAL), a sugar that can be used in experimental studies to induce aging, can cause oxidative stress, DNA damage and inflammation and as a result it can stimulate the senescence in many brain cell types [234]. For this reason, using exogenous antioxidants compounds such as saffron might be helpful.

Figure 2: *Graphical abstract of saffron extract preparation and experimental design*



Saffron (*Crocus Sativus L.)* is part of the Iridaceae family [235]. Saffron is represented by its bioactive constituents like crocin, crocetin, safranal, picrocrocin and kaempferol that are distinguished for their beneficial effects in particular in cognitive functions [236]. Crocin (**Figure 3A**) and crocetin (**Figure 3B**) are the carotenoids that are responsible for the color while safranal (**Figure 3C)** the aldehyde responsible for the characteristic smell. Picrocrocin (**Figure 3D**) is a monoterpene glycoside giving the saffron the bitter taste [237].

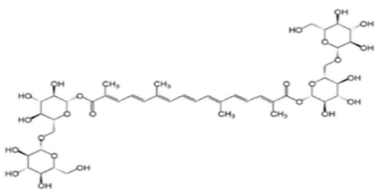


Figure 3A: *Crocin*

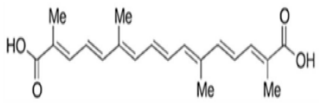
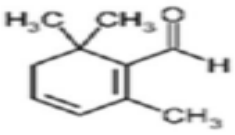


Figure 3B: *Crocetin*



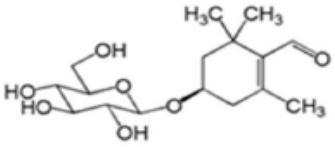


Figure 3C: *Safranal* **Figure 3D**: *Picrocrocin*

Saffron's constituents have been studied considering their potential neuroprotective effects against brain senescence and other neurological diseases [238]. Not only so, saffron and its constituents exert their neuroprotective effects through many mechanisms like modulating neurotransmitters, supplement the neurogenesis, control oxidative stress and manage inflammation [239]. Saffron's chemical composition is mainly sugar 63% and proteins 12%. Fat, minerals and fibers are the remaining part [238]. Since crocin finds it difficult to pass the BBB, it is converted into crocetin by hydrolysis in the intestine. Crocetin can reach the brain more easily and due to this peculiar behavior it can be used in case of neurodegenerative diseases [240]. Two of the major components of saffron, which are crocin and crocetin can inhibit the generation of the α-synuclein aggregates which we find accumulated in PD and also, segregate the already formed ones improving therefore the symptoms and the quality of life and supporting people with PD [241]. AD, being part of the neurodegenerative diseases, is also strongly impacted by oxidative stress which is one of the main factors contributing to neuronal death and loss of synaptic transmission [242]. The study of Khalili et al, demonstrated that crocin is capable of improving the cognitive impairment in three weeks after the streptozocin injection inducing a sporadic AD in male rats [243]. One *in vitro* study suggested crocin can be a driving force in inhibiting the tau protein filament formation in AD [244]. Another double blind clinical trial which included patients suffering from AD, after 22 weeks of giving casual administrations to one group saffron and to

the second group donepezil, revealed that if treated with 30mg of saffron per day, saffron is as efficient as donepezil in treating the symptoms of AD without side effects [245]. While in the meantime, another study of a double-blind placebo controlled trial at the same dose of 30mg/day, showed how an improvement of the cognitive enhancement in the group being administered saffron was achieved [246]. Safranal on the other hand is considered to be an antioxidant molecule that is capable of reducing oxidative stress while scavenging free radicals [247]. Safranal can reduce ROS levels and as a result, there is a reduction in the apoptosis through the mitogen-activated protein kinases (MAPKs), extracellular regulated protein kinase ERK 1/2 and PI3K/AKT pathways [248].

The mechanisms through which saffron along with its constituents play important beneficial roles in various neurological disorders are multiple. Saffron is responsible for modulating and increasing the levels of the neurotransmitters like serotonin, glutamate and dopamine involved in memory, learning and movement [249]. Saffron is not only capable of boosting the neurogenesis but also stimulate the maturation and specialization of stem cells and while intensifying the expression of BDNF reinforces the growth of neurons and their survival [250]. Since neuroinflammation can be the cause of brain damage and impaired functioning, reducing it can be beneficial. Saffron can suppress the production of pro-inflammatory cytokines like interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) [249,250]. Saffron can work with different mechanisms to scavenge free radicals such as ROS or reactive nitrogen species (RNS) and in the meantime, to boost the activity of antioxidant enzymes like CAT and SOD [251].

Mitochondrial dysfunction is thought to be the cause of many neurodegenerative diseases and brain senescence [252,253]. The brain is an organ very sensitive to oxidative stress and therefore if the balance between the defense of the exogenous antioxidants

and the production of free radicals changes, it may result in neuronal death [254]. The effect that *Crocus Sativus L*. has in preventing neuronal cell loss counteracting the formation of ROS is demonstrated in different studies and according to them, saffron has a dose-depending effect [255]. The main cause of oxidative stress are unpaired electrons of atoms or different groups that might lead to damage to macromolecules like DNA, RNA and proteins [256]. Cognitive impairment streptozocin-induced in rats can be counteracted by the administration of crocetin. It increases the levels of glutathione peroxidase, which is a powerful antioxidant reducing the damage caused by oxidative stress [257].

Chapter V: Methods

5.1 Saffron extract preparation

We collected the stigmas of Saffron (*Crocus sativus L*.) in November 2022, in L'Aquila, Abruzzo, Italy. The cultivation soil prepared was left resting from November 2022 till August 2023. After the germination, the bulbs were planted and the harvesting process started from mid-October to November in the flowering period of Saffron, being careful to steer clear of the flowers opening to the sun. The saffron stigmas, dried the same day after being harvested, are finally stored in room temperature in sterilized pots, away from sunlight.

The hydroalcoholic extract of saffron is prepared with dried stigmas (250mg of stigmas in 5ml ethanol), weighed, ground and soaked in ethanol (EtOH) 70% in room temperature, away from sunlight for about 2h while stirring and then centrifugation for 10min at 13000 g at +4°C. Supernatants were then filtered with 0.45 μm polyethersulfone filter (PES) and after the division into 4 small tubes, evaporated using Speed Vac system (Concentrator,

Eppendorf) for 2h. The dried extract divided in 4 tubes was kept in the fridge at -20°C. We obtained the stock solution of SE by dissolving the dried extract in dimethyl solfoxide (DMSO). The stock solution of SE was then diluted using cell culture medium obtaining the wanted concentration which was 200 μg/ml- 25 μg/ml.

5.2 Characterization of saffron

We identified the quality of our Saffron with the UV-visible spectrophotometry analysis of SE [258,259].

5.3 *In vitro* model of neural senescence

We used neuroblastoma SH-SY5Ycells, well employed cellular model which were cultured as mentioned in the protocol using Dulbecco's Modified Eagle's Medium (DMEM) completed with 10% fetal bovine serum and also 1% Glutamine (ATCC, USA). The cells were then preserved at 37 °C at 95% humidified air at 5% CO_2 (Eppendor, UK). As mentioned before, D-Gal is a monosaccharide sugar useful in inducing aging in *in vitro* and *in vivo* models. According to some research, cells were seeded in a 96 wells plate of 7000cells/well for the night after being treated with SE 25–200 μg/ml for 24 h and after being subjected to D-Gal induction of senescence (SigmaAldrich, USA; 200 mM, 24 h) [260,261].

5.4 MTS assay

As mentioned before, cells were put on a 96-well plate, and we added Cell Titer based on the manufacturer's instructions (Promega Corporation Madison, USA) in order to determinate the cell viability

of our senescence model using the MTS colorimetric assay. Then, using the Enzyme-linked Immunosorbent Assay (ELISA) reader, Infinite F200 (Tecan, Swiss) we evaluate the index of viability. The tests were executed in quadruplicate at an absorbance of 492nm [262].

5.5 Live cell imaging Cytotox Assay

Cells are always plated in a 96-well plate and in order to calculate the cytotoxic index in live-cell imaging, Cytotox Incucyte assay was used. To count the dead cells, we used 250nM of IncuCyteCytotox Red reagent in each condition (Essen Bioscience, Newark, UK). The plate was then placed in the IncuCyte machine with 20 x objectives for the recording of the cytotoxicity every 3h. The fluorescence scanning and phase contrast were used in measuring the cytotoxicity activation for 72h at 37 °C and 5% CO2. Incucyte Zoom software (Newark, UK) helped in scanning the images while the data were reported as red object count for each image [262].

5.6 β-galactosidase (β-GAL) assay

To measure the β-GAL colorimetric assay Pierce β-GAL reagent was added (ThermoScientific, USA). β-GAL can play the role of a biomarker for induced senescence. The well plate was incubated at 37 °C for half an hour while the β-GAL was quantified by the use of a microplate reader at 405nm [262].

5.7 ROS assay

The main aim was to measure in the *in vitro* model the ROS production therefore DFCDA assay (Abcam, UK), a technique able to quantify ROS within live cells, was used as stated by the manufacturer's instructions. Cells were prepared and treated as mentioned before and were then incubated with 10 µM DFCDA in obscure, at 37 °C for 30minutes. PBS is commonly used for washing because being a water-based salt solution it can act as buffer in a biological surrounding to maintain a stable pH and to have a similar salt concentration as the live cells. After the washing, using a Tecan Spark (Tecan, Switzerland), we can detect the fluorescent DCF and therefore measure the ROS at 485nm excitation and 535nm emission. As a positive control we used hydrogen peroxide [262].

5.8 Malondialdehyde assay

Through this assay we measure the malondialdehyde (MDA) levels in biological samples. In case of lipid peroxidation, lipids are damaged by oxidative stress and MDA can serve as a biomarker. MDA in biological sample interacts with thiobarbituric acid (TBA) resulting in the formation of this MDA-TBA adduct. To quantify the adduct fluorometrically, excitation/emission at 532/553 nm can be used. According to the protocol, TBA solution should be added to the biological sample before incubation for 60minutes at 95 °C. In order to cool the samples, they are put in an ice bath for 10minutes, before being moved to microplate wells were they are examined making use of a microplate reader (Spark, Tecan) [262].

5.9 SOD enzyme activity

The SOD assay kit was obtained from Abcam, UK, and was applied in measuring the SOD activity. This enzymatic colorimetric method

measures the rate of reduction of WST-1 (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-dis-ulfophenyl]-2H-tetrazolium, monosodium salt) producing a dissolved formazan product. The absorbance of WST-1 was calculated at 450nm. To determine the SOD activity we evaluate the reduction of color development at 450nm if compared to control [262].

5.10 CAT enzyme activity

Using the Kit which we purchased from Abcam, we were also able to determine CAT enzyme activity. Basically, the enzyme found in the Kit interacts with hydrogen peroxide forming water and oxygen. The probe reacting with the non-used hydrogen peroxide can generate a product that can be evaluated colorimetrically at outer diameter 570nm.

This procedure includes samples and standards loaded to wells. To the wells we add hydrogen peroxide. Incubation at 25 °C lasts for about 30minutes. The stop solution, used to halt the reaction between CAT and hydrogen peroxide after the incubation, is added, accompanied by developer mixer and incubated again for 10 minutes using the same temperature. The plate was subsequently read in Spark microplate reader at outer diameter 570nm [262].

5.11 Western Blotting

The treated cells along with the control were lysed in ice-cold Radio immunoprecipitation Assay buffer (Sigma) a specialized lysis buffer used to break open cells that contain phosphatase and protease inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA, USA) very crucial in maintaining the protein integrity and preventing its

degradation. The proteins that we extracted are then quantified by the use of Bicinchoninic acid (BCA) assay helpful in determining the total protein concentration in a solution (Thermo Fischer Scientific). The sample buffer and the denaturing agent are used in diluting the protein lysates according to what described by the manufacturer (Thermo Fischer Scientific) and Thermo Block (Eppendorf, Hamburg, Germany) is used for 10min in 70 °C temperature. The next step was to put 30 µg of protein in a gradient precast gel (Invitrogen, USA) in order to be electroblotted into a thin, porous membrane of polyvynil difluoride (PVDF, Millipore, Darmstadt, Germany) using Biorad system for the protein analyses. While using the 5% milk in TBS-T buffer solution for 1h at room temperature, we are able to block the binding sites. Membranes were put in incubation during the night at 4 °C temperature with the primary antibodies, mentioned below, that are diluted in a blocking buffer: anti-P21 1:1000; anti-CyclinD1 1:200; anti-survivin 1:1000; anti-phospo AKT 1:1000; anti-Actin 1:2000. We used peroxidase-conjugated anti-rabbit or anti-mouse IgG as secondary antibody. We detected the immunoreactive bands that were formed through the chemiluminescent substrate (Thermo,USA) emitting light and detected with the Uvitec digital system (Cambridge, UK). We normalized the densities of the bands to actin [262].

5.12 RT-PCR

The cells after being controlled and treated were lysed in Trizol reagent, a monophasic solution containing phenol and guanidine, (Invitrogen) with the main aim to extract RNA which concentrations were estimated and quantified by the use of Qubit Fluorometers (Thermo Fisher Scientific).

We obtained the first strand of the DNA complementary (cDNA) synthesis from 1 µg of total RNA using SuperScript™ IV VILO™ Master Mix (Invitrogen) used for reverse transcription in converting RNA to cDNA while the ezDNase enzyme resulted helpful in detaching any genomic DNA (gDNA) from the sample. qRT-PCR for measuring the abundance of specific gene transcription within a sample was executed in a resultant volume of 20 µL including 25 ng of the cDNA product, specific and reverse primers (500nm) that are short, synthetic DNA sequences used in polymerase chain reaction (PCR) and Power up SYBR Green Master Mix (Applied Biosystems), preformulated and optimized master for real time PCR (qPCR) according to the manufacturer's indications.

The following step was performing the amplification reaction in an Applied Biosystems 7300 system (ThermoFisher Scientific, Rockford, IL, USA), based on the characteristics: 2 min at 50 °C and 2 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Using the 2^DDCt method, we calculated relative gene expression changes in quantitative real time PCR. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is a protein coding gene that was selected to be a reference gene while the Control was used as a calibrator. Two biological replicated samples were conducted. The samples were produced in duplicate.

The genes that were examined were CAT, SOD1, Survivin, CHOP, GRP 78.

The primers were supplied by: a) from Bio-Rad (Italy) i.e CAT (Unique Assay ID qHsaCED0043914) and SOD1 (Unique Assay ID qHsaCID0008628) b) from Eurofins Genomics (Italy) i.e. CHOP (forward AGG CAC TGA GCG TAT CAT GTT reverse CTG TTT CCG TTT CCT GGT TC), GRP 78 (forward GTT CTT GCC GTT CAA GGT GG reverse TGG TAC AGT AAC AAC TGC ATG), survivin (forward AGA ACT GGC CCT TCT TGG AGG reverse CTT TTT ATG TTC CTC TAT GGG

GTC), Gapdh (forward TGC ACC ACC AAC TGC TTA GC reverse GGC ATG GAC TGT GGT CAT GAG) [262].

5.13 Label-free holotomography

Cells were inoculated in a black 96-well plate of glass bottom and treated with lysine which acts as a building block for proteins. The images of live cell time lapses were taken every 10min. Cells were further analyzed using a 3D Cell Explorer-fluo microscope (Nanolive) equipped with a dry 60×/0.8 objective and CMOS camera Sony and able to combine holotomography (3D images) with fluorescence microscopy. The 3D images were then subdivided and examined through built-in Eve and Steve software (Nanolive). The Fiji software was used as a closing process of(i.e., cropping, video, etc) analyzing the images [262].

5.14 Statistical analysis

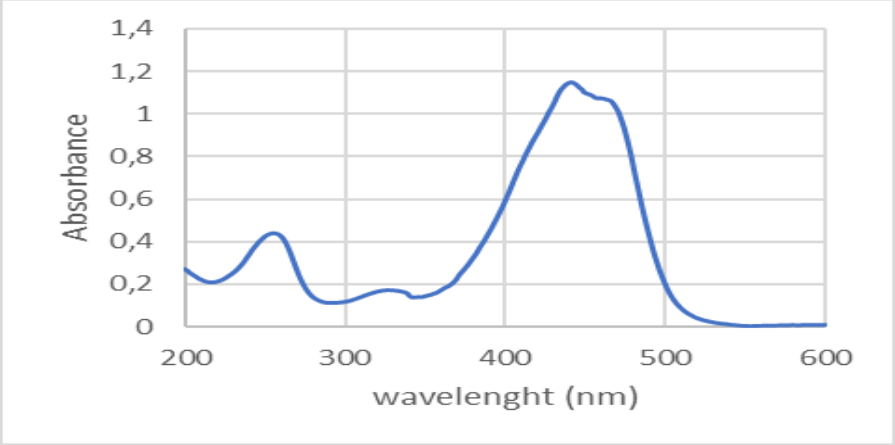
The statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) while one-way ANOVA with Tukey's post hoc multiple comparison test was helpful in determining significant differences between the groups. As a result, *P*values < 0.05 were considered to be statistically significant [262].

Chapter VI: Results

6.1 Saffron extraction and characterization

The UV spectrophotometric analysis of saffron hydroalcoholic solution, used for the ascertainment of the quality of saffron,

revealed an intense band which center is at about 440nm. This band derivates from the absorption of crocin and crocetin. The second bands on the other hand, at around 257nm and 330nm are linked to by ISO-3632 Technical Specifications to the picrocrocin and safranal respectively [263,264]. According to Carmona et al., the absorption of picrocrocin and picrocrocin derivates with a maximum that is around 250nm and the absorption of kaempferol glucosides which maximum is around 265-349nm, also fall in UV spectra [265]. Both the isomers of crocetin, cis and trans, present absorption maximums at 250-260nm while, only the cis-crocin has an absorption peak at about 324-327 nm [258,265]. According to the analysis (**Figure 4**) and based on ISO-3632.1/2 standard, the saffron we used is of grade I, therefore the best quality.



Sample	E_{440}	E_{257}
	237	90
Reference values for saffron quality grade I (dry weight)	≥200	≥70

Figure 4: *Spectrophotometric analysis UV-Vis*

6.2 Effects on cell viability

The following step was to try out the SE on human SH-SY5Y cells exposed previously to 200mM of D-GAL for 24h. The pretreated cells with SE 100 μg/ml and 50 μg/ml, 24h before being exposed to D-Gal, resulted in a weakening of the D-GAL effect (p=0.0078 and p=0.0322, respectively) in comparison to cells treated with D-GAL alone (**Figure 5A**) while the treatment of cells with variable concentrations of SE alone (25–200 μg/ml) for 24h did not effect in any form the cell viability.

We determined the cytotoxicity induced by the use of D-GAL 200mM for 24h on human SH-SY%Y cells using incucyte live-cell Cytotox assay. According to the results (**Figure 5B-C**), the pretreated cells with SE 100 μg/ml and 50 μg/ml, 24h before being subjected to D-Gal, proceed with a weakening of the D-GAL effect when contrasted with cells treated only with D-GAL. The processing of cells with different concentrations of SE alone (25–200 μg/ml) for 24h did not affect in any form the cell viability or did not present any toxic effect. Therefore, the concentration we used during our study was of 50 and 100 μg/ml of SE [262].

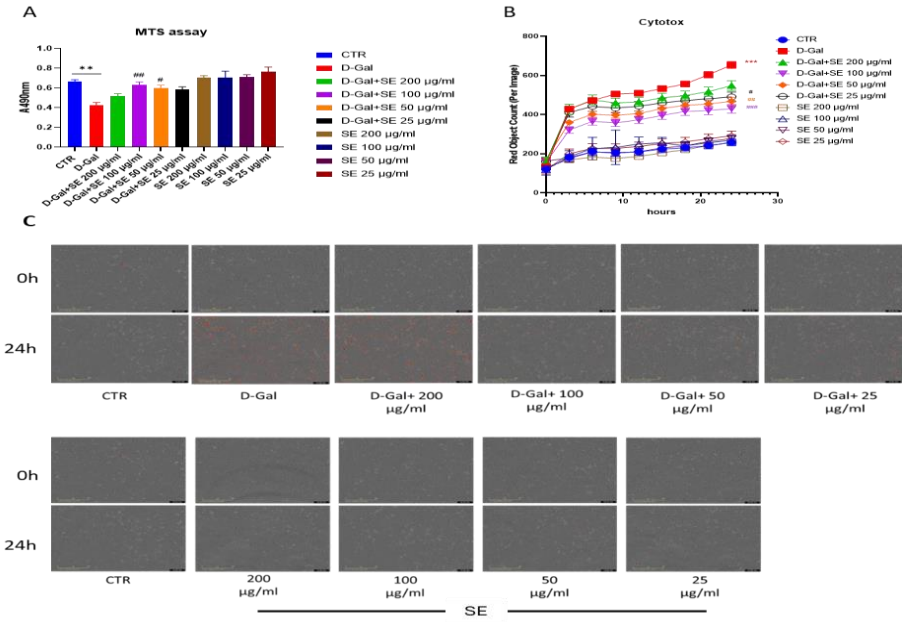


Figure 5: ***A****) MTS assay in human SH-SY5Y upon different treatments at 24h. ANOVA one-way: **, P ≤ 0.01 vs CTR; ##, P ≤ 0.01, #, P ≤ 0.05 vs D-Gal. N=6;* ***B****) Cytotox Incucyte Assay graph 0-24h and* ***C****) representative images at 0h and 24h in human SH-SY5Y upon different treatments. ANOVA two-way: ***, P ≤ 0.001 vs CTR; ##, P ≤ 0.001, ##, P ≤ 0.01, #, P ≤ 0.05 vs D-Gal. N=9.*

6.3 Effects on senescence

β-GAL levels senescence related are an important biomarker in cells senescence-induced. The SH-SY5Y cells, treated previously with D-Gal, revealed a notable increment in β-GAL biomarker in comparison to the control group. The preliminary treatment of cells with SE in the two mentioned concentrations showed a reduction in the senescence biomarker while SE alone had no influence on β-GAL levels (**Figure 6**).

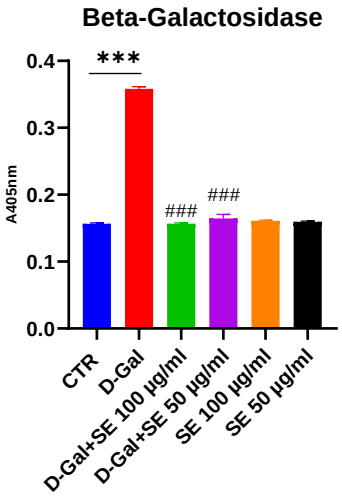


Figure 6: *Senescence-associated β-GAL levels in human SH-SY5Y upon different treatments; ANOVA two-way: ***, P ≤ 0.001 vs CTR; ###, P ≤ 0.001, vs D-Gal. N=4.*

6.4 Oxidative stress

From among the principal focuses of our study was the activity of antioxidants enzymes like SOD, CAT and ROS production (**Figure 7**). These enzymes maintain the cellular redox homeostasis and as a consequence they protect macromolecules like DNA, lipids and proteins from oxidative damage. ROS production was analyzed with DCFDA assay, a widely used method for detecting reactive oxygen species implicated in various cellular processes (**Figure 7A**). ROS levels were higher after D-GAL- induced senescence while the pretreatment at both concentrations 50 µg/ml and 100 µg/ml of SE lowered noticeably ROS concentrations in comparison with D-GAL aging-induced ($p < 0.001$). Malondialdehyde (MDA) levels, another marker of oxidative stress, were also higher when D-GAL is used in inducing senescence while the pretreatment with SE in the two tested concentrations reduced the MDA levels relatively to the D-Gal group (**Figure 7B**). The use of only SE does not have an impact on the parameters [262].

D-GAL was able to induce a limitation in the activity of enzymes SOD and CAT (**Figure 7 C-D**). SE counteracted this reduction; the higher the dose of SE, the higher the neutralization (##). Using the RT-PCR we revealed that both the expression of enzymes SOD and CAT were reduced in the senescence induced model. The pre-incubation with SE restored the control conditions.

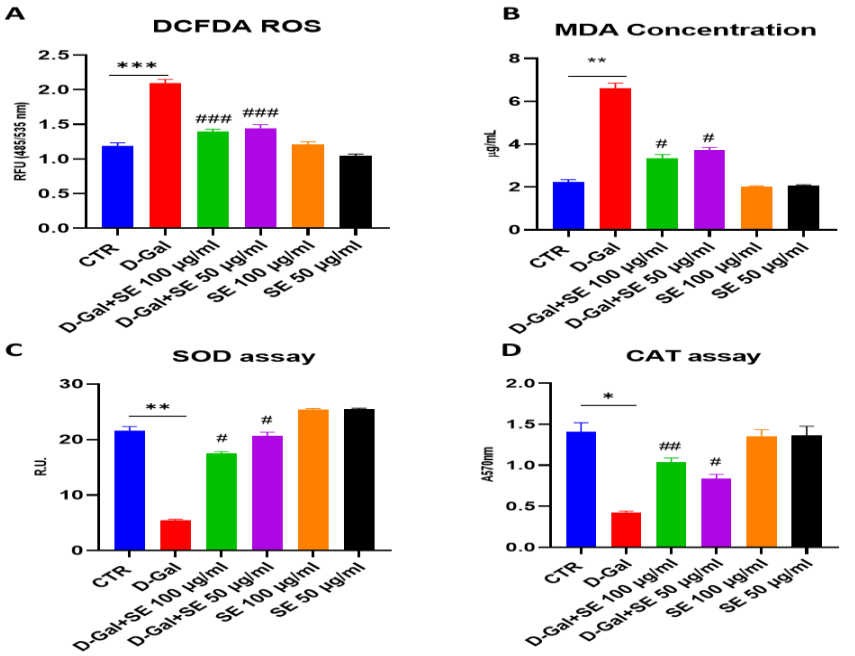


Figure 7: *Oxidative stress analysis on D-Gal-induced senescence upon SE treatment.* ***A****) ROS assay ANOVA one-way: ***, P ≤ 0.001 vs CTR; ###, P ≤ 0.001 vs D-Gal. N=4;* ***B****) MDA levels* ***C****) SOD enzymatic activity assay, ANOVA one-way: **, P ≤ 0.01 vs CTR; ##, P ≤ 0.01, #, P ≤ 0.05 vs D-Gal. N=4.* ***D****) CAT enzymatic activity assay, ANOVA one-way: **, P ≤ 0.01 vs CTR; ##, P ≤ 0.01, #, P ≤ 0.05 vs D-Gal. N=4.*

6.5 Effects on cell morphology

Executing label-free holotomographic microscopy we obtained high resolution 3D imaging over extended time periods in order to observe the changes induced by D-GAL in SH-SY%Y cells and the

impact that SE might have (**Figure 8**). Dry mass is a particular parameter helpful in interpreting the state of the population cells taken into consideration. The dry mass refers to all cellular content in dry state, excluding water and it is very helpful in understanding the state of cells in stress situations. The group of cells treated with D-GAL demonstrated a reduced dry mass if compared to the control group. In the meantime, the pretreated SE cells showed how the dry mass parameter can be preserved while compared to the non-induced senescent cells (**Figure 8**). Induced D-GAL senescent cells indicated less interaction between them and other cells. Moreover, they presented vacuoles accumulation as a morphological change. SE can definitely contrast this situation [262].

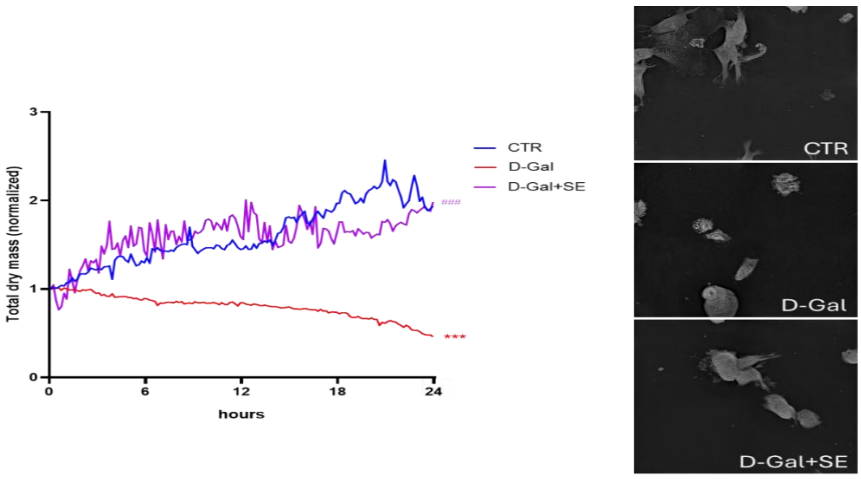


Figure 8*: Live-cell label-free holotomographic microscopy. Graph reports Total dry mass of cells upon different conditions. ANOVA one-way: ***, P ≤ 0.001 vs CTR; ###, P ≤ 0.001 vs D-Gal. N=4.*

6.6 Effects on cell cycle

The induction of P21 protein has an impact on cell cycle and leads to cell arrest. This activation is only temporary because P21 drops after growth cell process is arrested [266,267]. In our experimental study, evaluation with Western Blotting, P21 levels were decreased

while the use of SE counteracted this effect (**Figure 9A**). Cyclin D1 protein is also a protein able to regulate the cell cycle especially helping the passage from the G1 to the S phase. The quantity of this protein in our study, after senescent induction with D-GAL were reduced while SE could restore the initial state (**Figure 9B**). Surviving reduction, a protein having an impact in both cell division and preventing cell apoptosis, is counteracted in both protein levels and expression, by the use of SE (**Figure 9C and 9E**). P-Akt or phosphorylated Akt is a form of Akt protein kinase essential in different processes like cell growth, proliferation, survival and metabolism. In senescent induced D-Gal cells, the levels of active form Akt tend to get reduced suggesting inhibition in PI3K/Akt pathway which is a crucial intracellular signaling pathway. SE can counteract this reduction (**Figure 9D**).

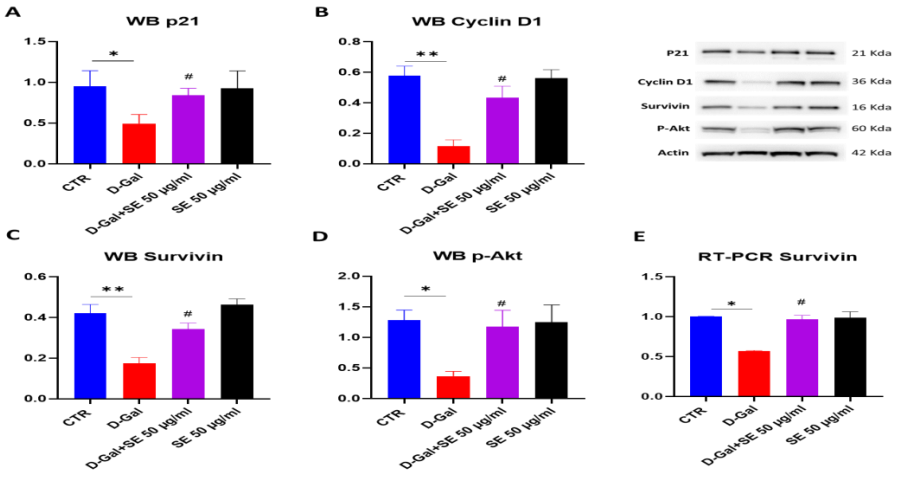


Figure 9: *Cell cycle pathway on D-Gal-induced senescence upon SE treatment.* ***A****) p21 protein levels and representative image.* ***B****) Cyclin D1 protein levels and representative image.* ***C****) Survivin protein level and representative image.* ***D****) p-Akt protein levels and representative image.* ***E****) RT-PCR of Survivin. ANOVA one-way: ***, P ≤ 0.001 vs CTR; ###, P ≤ 0.001 vs D-Gal. N=3.*

6.7 Effects on Endoplasmic Reticulum stress

Recent studies have showed how stress on the ER might be a trigger to the conditions of aging and aging-associated diseases [268]. In case of chronic ER stress, apoptosis is mediated using various methods and approaches which include the upregulation of CHOP, a protein responsible for regulating gene expression especially in response to ER stress. Glucose regulated protein (GRP78) is among the proteins useful in ER stress signaling and its expression decreases in time [269,270]. During our study, a notable increase of CHOP was observed in D-GAL induced senescence, while the pretreatment of the cells with SE revealed a similar condition to control group (**Figure 10A**). On the contrary, the levels of GRP78 were reduced after D-GAL cells exposure (**Figure 10B**). As a result, pretreatment of cells with SE contrasts ER stress leading a boosted cell survival [262].

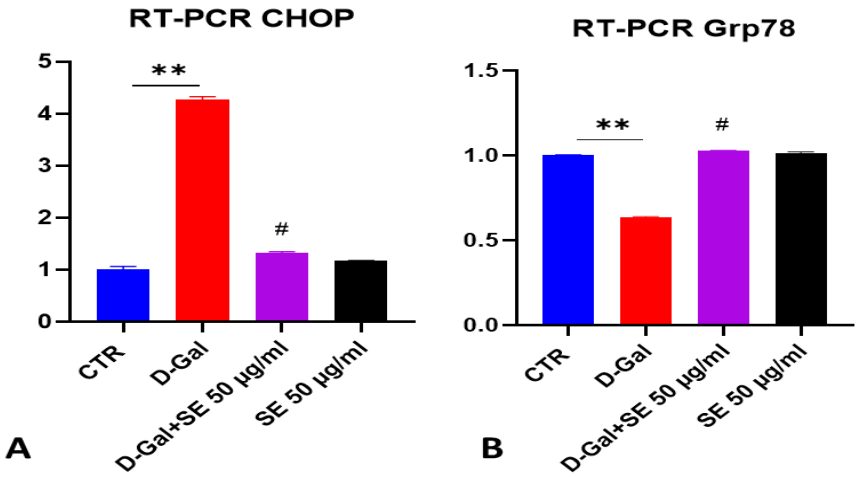


Figure 10: *RT-PCR of CHOP and Grp78 on D-Gal-induced senescence upon SE treatment. ANOVA one-way: **, P ≤ 0.005 vs CTR; #, P ≤ 0.05 vs D-Gal. N=3.*

Chapter VII: Discussion and conclusions

Neurological disorders have been a highly increasing issue in the last years. From among them, PD and AD have shown to be most

frequent. These types of disorders can alter the cognitive motor functions, injure the BBB and interrupt the nerve conduction. Since nowadays the population tends to reach a certain age, with aging more neurodegenerative disorders are being displayed.

Aging is a biological process that comes alongside with changes to all molecules, tissues and organs. From among all cells, neurons are more likely to alter their function and metabolism or morphologically change their structure if exposed to insults or oxidative stress during brain senescence. The age-related alterations in the aging brain might cause modifications in synaptic transmission. Aging is mostly connected to neuronal loss and impairment of the CNS. Senescent cells are recognized by elevated levels of inflammation and oxidative stress, DNA damage and apoptosis. While we find the senile plaques and neurofibrillary tangles in physiological and pathological conditions, we observe the presence of the Aβ protein and α-synuclein mostly during aging.

Among the main causes of mitochondrial dysfunction during aging is the generation of high quantity of ROS causing damage to cellular components and mutating mtDNA. Altering the function of the respiratory mitochondrial chain can be the cause of cell death. In senescent cells, we observe that there is also a decline of MMP. Mitochondria tend to create an inflammation chronic process. The gathering of many senescent cells causes morbidity and mortality, and these cells release proinflammatory cytokines. One of the most studied theories indicates that elderly people present more oxidized products compared to the younger generations and more ROS there are in the mitochondria, a reduced lifespan the individual will present. This redox imbalance is mainly caused by a reduced antioxidant ability of enzymes like SOD to counteract the production of ROS. Consequently, finding molecules that can counteract the production of high quantity of ROS and delay

therefore brain senescence is highly important for the lifespan of individuals.

Our study indicated that the pretreatment with SE had an anti-aging effect. The results implicated a regulation of lysosomal enzyme SA β-gal, reduction of neuronal loss and counteracted oxidative stress in ER in this D-GAL induced brain aging in SH-SY5Y cells model that we used.

Saffron is a herb which derives from the flower of *Crocus Sativus,* and it is composed of many biologically active compounds like crocin, crocetin, safranal and picrocin, each responsible for its characteristic smell, color or bitter taste. These molecules show neuroprotective effects in preclinical and clinical studies. The latest studies illustrated how SE can counteract cognitive impairment while reducing oxidative stress, which was supported also by our findings that suggest that SE can reduce ER stress.

Our model of D-GAL induced senescence in SH-SY5Y cells is a widespread helpful model in identifying the processes of neurodegeneration in aging. What we observed in our senescent model is elevated activity of the lysosomal enzyme SA-β-gal, which can be treated as a biomarker of senescence and also high levels of oxidative stress (high production of ROS alongside with reduced SOD and CAT activity) and ER stress (decreased GRP78 while increased CHOP expression). Preliminary treatment process with SE was not only able to reduce the oxidative stress condition and ER stress but also it was able to modulate and control the proteins that are participate in cell death/survival like P21, surviving, Akt and cyclin D1.

Crocin finds it hard to pass the BBB therefore it can be transformed in crocetin which crosses more easily and exert its neuroprotective effects. Nevertheless, the detailed mechanism of crossing remains non clear, and it needs further investigation. Not only so, but the future purposes might be to examine also other inflammatory

changes and conditions in the model that might influence brain aging. The used saffron in this study was collected based on protocol in Abruzzo. Further studies should probably compare the effects of different kinds of saffron, cultivated elsewhere to see the differences or the similarities in neural senescence and the disorders related to it.

Our findings suggest that saffron presents therapeutic benefits in preventing or delaying neural senescence. Nonetheless, future studies *in vivo* should be conducted in order to confirm if saffron is safe and efficient in humans. The present study is based on a single *in vitro* cellular model which may limit the applicability of the findings to more complex physiological contexts. The aim of this work was to provide a first mechanistic insight into the effects of SE under controlled experimental conditions. The use of complementary experimental systems would further strength the translational relevance of the results. For this reason, future studies in our laboratory will focus on examining the effect of the SE on SAMP8 mice model considering the accelerated senescence and also on 3D aging models such as, for example cerebral organoids.

References

1. Dumurgier J, Tzourio C. Epidemiology of neurological diseases in older adults. Rev Neurol (Paris). 2020;176: 642–648. doi:10.1016/j.neurol.2020.01.356

2. Ding C, Wu Y, Chen X, Chen Y, Wu Z, Lin Z, et al. Global, regional, and national burden and attributable risk factors of neurological

disorders: The Global Burden of Disease study 1990–2019. Front Public Health. 2022;10: 952161. doi:10.3389/fpubh.2022.952161

3. Feigin VL, Nichols E, Alam T, Bannick MS, Beghi E, Blake N, et al. Global, regional, and national burden of neurological disorders, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2019;18: 459–480. doi:10.1016/S1474-4422(18)30499-X

4. Dorsey ER, Elbaz A, Nichols E, Abbasi N, Abd-Allah F, Abdelalim A, et al. Global, regional, and national burden of Parkinson's disease, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2018;17: 939–953. doi:10.1016/S1474-4422(18)30295-3

5. Thakur KT, Albanese E, Giannakopoulos P, Jette N, Linde M, Prince MJ, et al. Neurological Disorders. In: Patel V, Chisholm D, Dua T, Laxminarayan R, Medina-Mora ME, editors. Mental, Neurological, and Substance Use Disorders: Disease Control Priorities, Third Edition (Volume 4). Washington (DC): The International Bank for Reconstruction and Development / The World Bank; 2016. Available: http://www.ncbi.nlm.nih.gov/books/NBK361950/

6. Qadir MI, Anwar S. Sirtuins in Brain Aging and Neurological Disorders. Crit Rev Eukaryot Gene Expr. 2017;27: 321–329. doi:10.1615/CritRevEukaryotGeneExpr.2017019532

7. Reuter-Lorenz PA, Lustig C. Brain aging: reorganizing discoveries about the aging mind. Curr Opin Neurobiol. 2005;15: 245–251. doi:10.1016/j.conb.2005.03.016

8. Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, Zhao Z, et al. Blood-Brain Barrier Breakdown in the Aging Human Hippocampus. Neuron. 2015;85: 296–302. doi:10.1016/j.neuron.2014.12.032

9. Lockhart SN, DeCarli C. Structural Imaging Measures of Brain Aging. Neuropsychol Rev. 2014;24: 271–289. doi:10.1007/s11065-014-9268-3

10. Swerdlow RH. Is aging part of Alzheimer's disease, or is Alzheimer's disease part of aging? Neurobiol Aging. 2007;28: 1465–1480. doi:10.1016/j.neurobiolaging.2006.06.021

11. Borson S. Cognition, Aging, and Disabilities: Conceptual Issues. Phys Med Rehabil Clin N Am. 2010;21: 375–382. doi:10.1016/j.pmr.2010.01.001

12. Folstein M, Folstein S. Functional expressions of the aging brain: Nutrition Reviews©, Vol. 68, No. s2. Nutr Rev. 2010;68: S70–S73. doi:10.1111/j.1753-4887.2010.00351.x

13. Poon HF, Calabrese V, Scapagnini G, Butterfield DA. Free radicals and brain aging. Clin Geriatr Med. 2004;20: 329–359. doi:10.1016/j.cger.2004.02.005

14. López-Otín C, Galluzzi L, Freije JMP, Madeo F, Kroemer G. Metabolic Control of Longevity. Cell. 2016;166: 802–821. doi:10.1016/j.cell.2016.07.031

15. Ziada AS, Smith M-SR, Côté HCF. Updating the Free Radical Theory of Aging. Front Cell Dev Biol. 2020;8. doi:10.3389/fcell.2020.575645

16. Payne BAI, Wilson IJ, Hateley CA, Horvath R, Santibanez-Koref M, Samuels DC, et al. Mitochondrial aging is accelerated by anti-retroviral therapy through the clonal expansion of mtDNA mutations. Nat Genet. 2011;43: 806–810. doi:10.1038/ng.863

17. Scialò F, Sriram A, Fernández-Ayala D, Gubina N, Lõhmus M, Nelson G, et al. Mitochondrial ROS Produced via Reverse Electron

Transport Extend Animal Lifespan. Cell Metab. 2016;23: 725–734. doi:10.1016/j.cmet.2016.03.009

18. Palikaras K, Lionaki E, Tavernarakis N. Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. Nature. 2015;521: 525–528. doi:10.1038/nature14300

19. Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, 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. Alzheimers Dement. 2011;7: 280–292. doi:10.1016/j.jalz.2011.03.003

20. Polvikoski T, Sulkava R, Rastas S, Sutela A, Niinistö L, Notkola I-L, et al. Incidence of Dementia in Very Elderly Individuals: A Clinical, Neuropathological and Molecular Genetic Study. Neuroepidemiology. 2006;26: 76–82. doi:10.1159/000090252

21. Sahin E, Colla S, Liesa M, Moslehi J, Müller FL, Guo M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. Nature. 2011;470: 359–365. doi:10.1038/nature09787

22. Van Deursen JM. The role of senescent cells in ageing. Nature. 2014;509: 439–446. doi:10.1038/nature13193

23. Childs BG, Gluscevic M, Baker DJ, Laberge R-M, Marquess D, Dananberg J, et al. Senescent cells: an emerging target for diseases of ageing. Nat Rev Drug Discov. 2017;16: 718–735. doi:10.1038/nrd.2017.116

24. Coppé J-P, Patil CK, Rodier F, Krtolica A, Beauséjour CM, Parrinello S, et al. A Human-Like Senescence-Associated Secretory Phenotype Is Conserved in Mouse Cells Dependent on

Physiological Oxygen. Blagosklonny MV, editor. PLoS ONE. 2010;5: e9188. doi:10.1371/journal.pone.0009188

25. Baker DJ, Petersen RC. Cellular senescence in brain aging and neurodegenerative diseases: evidence and perspectives. J Clin Invest. 2018;128: 1208–1216. doi:10.1172/jci95145

26. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. Nat Cell Biol. 2004;6: 168–170. doi:10.1038/ncb1095

27. Baker DJ, Perez-Terzic C, Jin F, Pitel KS, Niederländer NJ, Jeganathan K, et al. Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency. Nat Cell Biol. 2008;10: 825–836. doi:10.1038/ncb1744

28. Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 2006;38: 515–517. doi:10.1038/ng1769

29. Borgonetti V, Galeotti N. Microglia senescence is related to neuropathic pain–associated comorbidities in the spared nerve injury model. Pain. 2023;164: 1106–1117. doi:10.1097/j.pain.0000000000002807

30. Melo Dos Santos L, Trombetta-Lima M, Eggen B, Demaria M. Cellular senescence in brain aging and neurodegeneration. Ageing Res Rev. 2024;93: 102141. doi:10.1016/j.arr.2023.102141

31. Martini H, Passos JF. Cellular senescence: all roads lead to mitochondria. FEBS J. 2023;290: 1186–1202. doi:10.1111/febs.16361

32. Frank-Cannon TC, Alto LT, McAlpine FE, Tansey MG. Does neuroinflammation fan the flame in neurodegenerative diseases? Mol Neurodegener. 2009;4. doi:10.1186/1750-1326-4-47

33. Sierra A, Gottfried-Blackmore AC, McEwen BS, Bulloch K. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia. 2007;55: 412–424. doi:10.1002/glia.20468

34. Bachstetter AD, Xing B, De Almeida L, Dimayuga ER, Watterson DM, Van Eldik LJ. Microglial p38α MAPK is a key regulator of proinflammatory cytokine up-regulation induced by toll-like receptor (TLR) ligands or beta-amyloid (Aβ). J Neuroinflammation. 2011;8. doi:10.1186/1742-2094-8-79

35. Yu H-M, Zhao Y-M, Luo X-G, Feng Y, Ren Y, Shang H, et al. Repeated Lipopolysaccharide Stimulation Induces Cellular Senescence in BV2 Cells. Neuroimmunomodulation. 2012;19: 131–136. doi:10.1159/000330254

36. Sidoryk-Wegrzynowicz M, Wegrzynowicz M, Lee E, Bowman AB, Aschner M. Role of Astrocytes in Brain Function and Disease. Toxicol Pathol. 2011;39: 115–123. doi:10.1177/0192623310385254

37. Bitto A, Sell C, Crowe E, Lorenzini A, Malaguti M, Hrelia S, et al. Stress-induced senescence in human and rodent astrocytes. Exp Cell Res. 2010;316: 2961–2968. doi:10.1016/j.yexcr.2010.06.021

38. Zou Y, Zhang N, Ellerby LM, Davalos AR, Zeng X, Campisi J, et al. Responses of human embryonic stem cells and their differentiated progeny to ionizing radiation. Biochem Biophys Res Commun. 2012;426: 100–105. doi:10.1016/j.bbrc.2012.08.043

39. Chaib S, Tchkonia T, Kirkland JL. Cellular senescence and senolytics: the path to the clinic. Nat Med. 2022;28: 1556–1568. doi:10.1038/s41591-022-01923-y

40. Ishikawa S, Ishikawa F. Proteostasis failure and cellular senescence in long-term cultured postmitotic rat neurons. Aging Cell. 2020;19. doi:10.1111/acel.13071

41. Shamoto-Nagai M, Maruyama W, Yi H, Akao Y, Tribl F, Gerlach M, et al. Neuromelanin induces oxidative stress in mitochondria through release of iron: mechanism behind the inhibition of 26S proteasome. J Neural Transm. 2006;113: 633–644. doi:10.1007/s00702-005-0410-5

42. Zhang W. Human neuromelanin an endogenous microglial activator for dopaminergic neuron death. Front Biosci. 2013;E5: 1–11. doi:10.2741/e591

43. Chen D, Huang Y, Shi Z, Li J, Zhang Y, Wang K, et al. Demyelinating processes in aging and stroke in the central nervous system and the prospect of treatment strategy. CNS Neurosci Ther. 2020;26: 1219–1229. doi:10.1111/cns.13497

44. Hou Y, Dan X, Babbar M, Wei Y, Hasselbalch SG, Croteau DL, et al. Ageing as a risk factor for neurodegenerative disease. Nat Rev Neurol. 2019;15: 565–581. doi:10.1038/s41582-019-0244-7

45. Musi N, Valentine JM, Sickora KR, Baeuerle E, Thompson CS, Shen Q, et al. Tau protein aggregation is associated with cellular senescence in the brain. Aging Cell. 2018;17. doi:10.1111/acel.12840

46. Donner MW, Jones B. Aging and Neurological Disease. In: Jones B, Donner MW, editors. Normal and Abnormal Swallowing. New

York, NY: Springer New York; 1991. pp. 189–202. doi:10.1007/978-1-4757-4150-6_12

47. Zia A, Pourbagher-Shahri AM, Farkhondeh T, Samarghandian S. Molecular and cellular pathways contributing to brain aging. Behav Brain Funct BBF. 2021;17: 6. doi:10.1186/s12993-021-00179-9

48. Bej E, Cesare P, d'Angelo M, Volpe AR, Castelli V. Neuronal Cell Rearrangement During Aging: Antioxidant Compounds as a Potential Therapeutic Approach. Cells. 2024;13: 1945. doi:10.3390/cells13231945

49. Wang B, Huang M, Shang D, Yan X, Zhao B, Zhang X. Mitochondrial Behavior in Axon Degeneration and Regeneration. Front Aging Neurosci. 2021;13. doi:10.3389/fnagi.2021.650038

50. Smith GM, Gallo G. The role of mitochondria in axon development and regeneration. Dev Neurobiol. 2018;78: 221–237. doi:10.1002/dneu.22546

51. Neukomm LJ, Freeman MR. Diverse cellular and molecular modes of axon degeneration. Trends Cell Biol. 2014;24: 515–523. doi:10.1016/j.tcb.2014.04.003

52. Barrientos SA, Martinez NW, Yoo S, Jara JS, Zamorano S, Hetz C, et al. Axonal Degeneration Is Mediated by the Mitochondrial Permeability Transition Pore. J Neurosci. 2011;31: 966–978. doi:10.1523/JNEUROSCI.4065-10.2011

53. Blinkouskaya Y, Caçoilo A, Gollamudi T, Jalalian S, Weickenmeier J. Brain aging mechanisms with mechanical manifestations. Mech Ageing Dev. 2021;200: 111575. doi:10.1016/j.mad.2021.111575

54. Lupo G, Gaetani S, Cacci E, Biagioni S, Negri R. Molecular Signatures of the Aging Brain: Finding the Links Between Genes

and Phenotypes. Neurotherapeutics. 2019;16: 543–553. doi:10.1007/s13311-019-00743-2

55. Mattson MP. Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol. 2000;1: 120–130. doi:10.1038/35040009

56. Dickstein DL, Kabaso D, Rocher AB, Luebke JI, Wearne SL, Hof PR. Changes in the structural complexity of the aged brain. Aging Cell. 2007;6: 275–284. doi:10.1111/j.1474-9726.2007.00289.x

57. Ibáñez V, Pietrini P, Furey ML, Alexander GE, Millet P, Bokde ALW, et al. Resting state brain glucose metabolism is not reduced in normotensive healthy men during aging, after correction for brain atrophy. Brain Res Bull. 2004;63: 147–154. doi:10.1016/j.brainresbull.2004.02.003

58. Yankner BA, Lu T, Loerch P. The Aging Brain. Annu Rev Pathol Mech Dis. 2008;3: 41–66. doi:10.1146/annurev.pathmechdis.2.010506.092044

59. Sikora E, Bielak-Zmijewska A, Dudkowska M, Krzystyniak A, Mosieniak G, Wesierska M, et al. Cellular Senescence in Brain Aging. Front Aging Neurosci. 2021;13: 646924. doi:10.3389/fnagi.2021.646924

60. Shafqat A, Khan S, Omer MH, Niaz M, Albalkhi I, AlKattan K, et al. Cellular senescence in brain aging and cognitive decline. Front Aging Neurosci. 2023;15: 1281581. doi:10.3389/fnagi.2023.1281581

61. Fielder E, Tweedy C, Wilson C, Oakley F, LeBeau FEN, Passos JF, et al. Anti-inflammatory treatment rescues memory deficits during aging in *nfkb1*$^{-/-}$ mice. Aging Cell. 2020;19: e13188. doi:10.1111/acel.13188

62. Apostolova LG, Green AE, Babakchanian S, Hwang KS, Chou Y-Y, Toga AW, et al. Hippocampal Atrophy and Ventricular Enlargement in Normal Aging, Mild Cognitive Impairment (MCI), and Alzheimer Disease. Alzheimer Dis Assoc Disord. 2012;26: 17–27. doi:10.1097/WAD.0b013e3182163b62

63. Schmitt HP. epsilon-Glycation, APP and Abeta in ageing and Alzheimer disease: a hypothesis. Med Hypotheses. 2006;66: 898–906. doi:10.1016/j.mehy.2005.11.016

64. Van Noort J. Stress proteins in CNS inflammation. J Pathol. 2008;214: 267–275. doi:10.1002/path.2273

65. Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting Proteostasis for Disease Intervention. Science. 2008;319: 916–919. doi:10.1126/science.1141448

66. Calamini B, Silva MC, Madoux F, Hutt DM, Khanna S, Chalfant MA, et al. Small-molecule proteostasis regulators for protein conformational diseases. Nat Chem Biol. 2012;8: 185–196. doi:10.1038/nchembio.763

67. Rai M, Curley M, Coleman Z, Demontis F. Contribution of proteases to the hallmarks of aging and to age-related neurodegeneration. Aging Cell. 2022;21: e13603. doi:10.1111/acel.13603

68. Calderwood SK, Murshid A, Prince T. The Shock of Aging: Molecular Chaperones and the Heat Shock Response in Longevity and Aging – A Mini-Review. Gerontology. 2009;55: 550–558. doi:10.1159/000225957

69. Powers ET, Morimoto RI, Dillin A, Kelly JW, Balch WE. Biological and Chemical Approaches to Diseases of Proteostasis Deficiency.

Annu Rev Biochem. 2009;78: 959–991. doi:10.1146/annurev.biochem.052308.114844

70. Coppotelli G, Ross J. Mitochondria in Ageing and Diseases: The Super Trouper of the Cell. Int J Mol Sci. 2016;17: 711. doi:10.3390/ijms17050711

71. Balaban RS, Nemoto S, Finkel T. Mitochondria, Oxidants, and Aging. Cell. 2005;120: 483–495. doi:10.1016/j.cell.2005.02.001

72. Navarro A, Boveris A. The mitochondrial energy transduction system and the aging process. Am J Physiol-Cell Physiol. 2007;292: C670–C686. doi:10.1152/ajpcell.00213.2006

73. Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, et al. Mitochondrial DNA Mutations, Oxidative Stress, and Apoptosis in Mammalian Aging. Science. 2005;309: 481–484. doi:10.1126/science.1112125

74. Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, et al. Mitochondrial Abnormalities in Alzheimer's Disease. J Neurosci. 2001;21: 3017–3023. doi:10.1523/jneurosci.21-09-03017.2001

75. Gibson SB. A matter of balance between life and death: Targeting reactive oxygen species (ROS)-induced autophagy for cancer therapy. Autophagy. 2010;6: 835–837. doi:10.4161/auto.6.7.13335

76. Li L, Tan J, Miao Y, Lei P, Zhang Q. ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. Cell Mol Neurobiol. 2015;35: 615–621. doi:10.1007/s10571-015-0166-x

77. Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ. 2009;16: 1040–1052. doi:10.1038/cdd.2009.49

78. Zhou J, Li X-Y, Liu Y-J, Feng J, Wu Y, Shen H-M, et al. Full-coverage regulations of autophagy by ROS: from induction to maturation. Autophagy. 2022;18: 1240–1255. doi:10.1080/15548627.2021.1984656

79. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451: 1069–1075. doi:10.1038/nature06639

80. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. Nat Rev Mol Cell Biol. 2018;19: 349–364. doi:10.1038/s41580-018-0003-4

81. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. Trends Biochem Sci. 2011;36: 30–38. doi:10.1016/j.tibs.2010.07.007

82. Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic Biol Med. 1999;27: 1151–1163. doi:10.1016/S0891-5849(99)00206-3

83. Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans. 2007;35: 1147–1150. doi:10.1042/BST0351147

84. Simon H-U, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis. 2000;5: 415–418. doi:10.1023/a:1009616228304

85. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol-Lung Cell Mol Physiol. 2000;279: L1005–L1028. doi:10.1152/ajplung.2000.279.6.l1005

86. Singh KK. Mitochondria Damage Checkpoint, Aging, and Cancer. Ann N Y Acad Sci. 2006;1067: 182–190. doi:10.1196/annals.1354.022

87. Kim BM, Rode AB, Han EJ, Hong IS, Hong SH. 5-Phenylselenyl- and 5-methylselenyl-methyl-2'-deoxyuridine induce oxidative stress, DNA damage, and caspase-2-dependent apoptosis in cancer cells. Apoptosis. 2012;17: 200–216. doi:10.1007/s10495-011-0665-2

88. Zhang M, Harashima N, Moritani T, Huang W, Harada M. The Roles of ROS and Caspases in TRAIL-Induced Apoptosis and Necroptosis in Human Pancreatic Cancer Cells. Singh AP, editor. PLOS ONE. 2015;10: e0127386. doi:10.1371/journal.pone.0127386

89. Gómez-Lechón MJ, Ponsoda X, O'Connor E, Donato T, Castell JV, Jover R. Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. Biochem Pharmacol. 2003;66: 2155–2167. doi:10.1016/j.bcp.2003.08.003

90. Boveris A, Navarro A. Brain mitochondrial dysfunction in aging. IUBMB Life. 2008;60: 308–314. doi:10.1002/iub.46

91. Houtkooper RH, Argmann C, Houten SM, Cantó C, Jeninga EH, Andreux PA, et al. The metabolic footprint of aging in mice. Sci Rep. 2011;1. doi:10.1038/srep00134

92. Fortini P, Pascucci B. Special Issue "Mitochondrial Dysfunction: A Common Trigger in Neurodegenerative and Metabolic Non-Communicable Diseases." Int J Mol Sci. 2024;25: 4004. doi:10.3390/ijms25074004

93. Krishnan KJ, Reeve AK, Samuels DC, Chinnery PF, Blackwood JK, Taylor RW, et al. What causes mitochondrial DNA deletions in human cells? Nat Genet. 2008;40: 275–279. doi:10.1038/ng.f.94

94. Payne BAI, Chinnery PF. Mitochondrial dysfunction in aging: Much progress but many unresolved questions. Biochim Biophys Acta BBA - Bioenerg. 2015;1847: 1347–1353. doi:10.1016/j.bbabio.2015.05.022

95. Chomyn A, Attardi G. MtDNA mutations in aging and apoptosis. Biochem Biophys Res Commun. 2003;304: 519–529. doi:10.1016/S0006-291X(03)00625-9

96. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, et al. Mitochondrial Dysfunction in the Elderly: Possible Role in Insulin Resistance. Science. 2003;300: 1140–1142. doi:10.1126/science.1082889

97. Liu L, Su X, Quinn WJ, Hui S, Krukenberg K, Frederick DW, et al. Quantitative Analysis of NAD Synthesis-Breakdown Fluxes. Cell Metab. 2018;27: 1067-1080.e5. doi:10.1016/j.cmet.2018.03.018

98. Alano CC, Ying W, Swanson RA. Poly(ADP-ribose) Polymerase-1-mediated Cell Death in Astrocytes Requires NAD+ Depletion and Mitochondrial Permeability Transition. J Biol Chem. 2004;279: 18895–18902. doi:10.1074/jbc.m313329200

99. Bennett JP, Onyango IG. Energy, Entropy and Quantum Tunneling of Protons and Electrons in Brain Mitochondria: Relation to Mitochondrial Impairment in Aging-Related Human Brain Diseases and Therapeutic Measures. Biomedicines. 2021;9: 225. doi:10.3390/biomedicines9020225

100. Adam-Vizi V. Production of Reactive Oxygen Species in Brain Mitochondria: Contribution by Electron Transport Chain and Non–Electron Transport Chain Sources. Antioxid Redox Signal. 2005;7: 1140–1149. doi:10.1089/ars.2005.7.1140

101. Picca A, Lezza A, Leeuwenburgh C, Pesce V, Calvani R, Landi F, et al. Fueling Inflamm-Aging through Mitochondrial Dysfunction: Mechanisms and Molecular Targets. Int J Mol Sci. 2017;18: 933. doi:10.3390/ijms18050933

102. Montava-Garriga L, Ganley IG. Outstanding Questions in Mitophagy: What We Do and Do Not Know. J Mol Biol. 2020;432: 206–230. doi:10.1016/j.jmb.2019.06.032

103. Rowland AA, Voeltz GK. Endoplasmic reticulum–mitochondria contacts: function of the junction. Nat Rev Mol Cell Biol. 2012;13: 607–615. doi:10.1038/nrm3440

104. Gant JC, Chen K-C, Kadish I, Blalock EM, Thibault O, Porter NM, et al. Reversal of Aging-Related Neuronal Ca2+ Dysregulation and Cognitive Impairment by Delivery of a Transgene Encoding FK506-Binding Protein 12.6/1b to the Hippocampus. J Neurosci. 2015;35: 10878–10887. doi:10.1523/JNEUROSCI.1248-15.2015

105. Deshwal S, Fiedler KU, Langer T. Mitochondrial Proteases: Multifaceted Regulators of Mitochondrial Plasticity. Annu Rev Biochem. 2020;89: 501–528. doi:10.1146/annurev-biochem-062917-012739

106. Kirkland JL, Tchkonia T. Senolytic drugs: from discovery to translation. J Intern Med. 2020;288: 518–536. doi:10.1111/joim.13141

107. Keller JN, Dimayuga E, Chen Q, Thorpe J, Gee J, Ding Q. Autophagy, proteasomes, lipofuscin, and oxidative stress in the aging brain. Int J Biochem Cell Biol. 2004;36: 2376–2391. doi:10.1016/j.biocel.2004.05.003

108. Fonseca DB, Sheehy MRJ, Blackman N, Shelton PMJ, Prior AE. Reversal of a hallmark of brain ageing: lipofuscin accumulation. Neurobiol Aging. 2005;26: 69–76. doi:10.1016/j.neurobiolaging.2004.02.013

109. Brunk UT, Terman A. Lipofuscin: mechanisms of age-related accumulation and influence on cell function12 1Guest Editor:

Rajindar S. Sohal 2This article is part of a series of reviews on "Oxidative Stress and Aging." The full list of papers may be found on the homepage of the journal. Free Radic Biol Med. 2002;33: 611–619. doi:10.1016/S0891-5849(02)00959-0

110. Braak H, Thal DR, Ghebremedhin E, Del Tredici K. Stages of the Pathologic Process in Alzheimer Disease: Age Categories From 1 to 100 Years. J Neuropathol Exp Neurol. 2011;70: 960–969. doi:10.1097/NEN.0b013e318232a379

111. Sandell JH, Peters A. Disrupted myelin and axon loss in the anterior commissure of the aged rhesus monkey. J Comp Neurol. 2003;466: 14–30. doi:10.1002/cne.10859

112. Bowley MP, Cabral H, Rosene DL, Peters A. Age changes in myelinated nerve fibers of the cingulate bundle and corpus callosum in the rhesus monkey. J Comp Neurol. 2010;518: 3046–3064. doi:10.1002/cne.22379

113. Santoro A, Martucci M, Conte M, Capri M, Franceschi C, Salvioli S. Inflammaging, hormesis and the rationale for anti-aging strategies. Ageing Res Rev. 2020;64: 101142. doi:10.1016/j.arr.2020.101142

114. Fulop T, Larbi A, Dupuis G, Le Page A, Frost EH, Cohen AA, et al. Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes? Front Immunol. 2018;8: 1960. doi:10.3389/fimmu.2017.01960

115. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. Cell. 2023;186: 243–278. doi:10.1016/j.cell.2022.11.001

116. Tchkonia T, Zhu Y, Van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype:

therapeutic opportunities. J Clin Invest. 2013;123: 966–972. doi:10.1172/JCI64098

117. Miyazawa T. Lipid hydroperoxides in nutrition, health, and diseases. Proc Jpn Acad Ser B. 2021;97: 161–196. doi:10.2183/pjab.97.010

118. Biesalski HK. Free radical theory of aging. Curr Opin Clin Nutr Metab Care. 2002;5: 5–10. doi:10.1097/00075197-200201000-00002

119. Viña J, Borras C, Abdelaziz KM, Garcia-Valles R, Gomez-Cabrera MC. The Free Radical Theory of Aging Revisited: The Cell Signaling Disruption Theory of Aging. Antioxid Redox Signal. 2013;19: 779–787. doi:10.1089/ars.2012.5111

120. Cadenas E, Davies KJA. Mitochondrial free radical generation, oxidative stress, and aging11This article is dedicated to the memory of our dear friend, colleague, and mentor Lars Ernster (1920–1998), in gratitude for all he gave to us. Free Radic Biol Med. 2000;29: 222–230. doi:10.1016/S0891-5849(00)00317-8

121. Brieger K, Schiavone S, Miller FJ, Krause K-H. Reactive oxygen species: from health to disease. Swiss Med Wkly. 2012;142: w13659. doi:10.4414/smw.2012.13659

122. Van Horssen J, Van Schaik P, Witte M. Inflammation and mitochondrial dysfunction: A vicious circle in neurodegenerative disorders? Neurosci Lett. 2019;710: 132931. doi:10.1016/j.neulet.2017.06.050

123. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature. 2011;469: 221–225. doi:10.1038/nature09663

124. Barcia C, Bahillo AS, Fernández-Villalba E, Bautista V, Poza Y Poza M, Fernández-Barreiro A, et al. Evidence of active microglia in substantia nigra pars compacta of parkinsonian monkeys 1 year after MPTP exposure. Glia. 2004;46: 402–409. doi:10.1002/glia.20015

125. Joglar B, Rodriguez-Pallares J, Rodriguez-Perez AI, Rey P, Guerra MJ, Labandeira-Garcia JL. The inflammatory response in the MPTP model of Parkinson's disease is mediated by brain angiotensin: relevance to progression of the disease. J Neurochem. 2009;109: 656–669. doi:10.1111/j.1471-4159.2009.05999.x

126. Garrido-Gil P, Rodriguez-Pallares J, Dominguez-Meijide A, Guerra MJ, Labandeira-Garcia JL. Brain angiotensin regulates iron homeostasis in dopaminergic neurons and microglial cells. Exp Neurol. 2013;250: 384–396. doi:10.1016/j.expneurol.2013.10.013

127. Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, et al. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med. 2004;37: 755–767. doi:10.1016/j.freeradbiomed.2004.05.034

128. Weidinger A, Kozlov AV. Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. Biomolecules. 2015;5: 472–484. doi:10.3390/biom5020472

129. Ruszkiewicz J, Albrecht J. Changes in the mitochondrial antioxidant systems in neurodegenerative diseases and acute brain disorders. Neurochem Int. 2015;88: 66–72. doi:10.1016/j.neuint.2014.12.012

130. Morris G, Walker AJ, Berk M, Maes M, Puri BK. Cell Death Pathways: a Novel Therapeutic Approach for Neuroscientists. Mol Neurobiol. 2018;55: 5767–5786. doi:10.1007/s12035-017-0793-y

131. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta BBA - Mol Cell Res. 2016;1863: 2977–2992. doi:10.1016/j.bbamcr.2016.09.012

132. Bej E, Cesare P, Volpe AR, d'Angelo M, Castelli V. Oxidative Stress and Neurodegeneration: Insights and Therapeutic Strategies for Parkinson's Disease. Neurol Int. 2024;16: 502–517. doi:10.3390/neurolint16030037

133. Perier C, Vila M. Mitochondrial Biology and Parkinson's Disease. Cold Spring Harb Perspect Med. 2012;2: a009332–a009332. doi:10.1101/cshperspect.a009332

134. Sugrue MM, Tatton WG. Mitochondrial Membrane Potential in Aging Cells. Neurosignals. 2001;10: 176–188. doi:10.1159/000046886

135. Bernardi P, Krauskopf A, Basso E, Petronilli V, Blalchy-Dyson E, Di Lisa F, et al. The mitochondrial permeability transition from*in vitro*artifact to disease target. FEBS J. 2006;273: 2077–2099. doi:10.1111/j.1742-4658.2006.05213.x

136. Carraro M, Carrer A, Urbani A, Bernardi P. Molecular nature and regulation of the mitochondrial permeability transition pore(s), drug target(s) in cardioprotection. J Mol Cell Cardiol. 2020;144: 76–86. doi:10.1016/j.yjmcc.2020.05.014

137. Rottenberg H. The Reduction in the Mitochondrial Membrane Potential in Aging: The Role of the Mitochondrial Permeability

Transition Pore. Int J Mol Sci. 2023;24: 12295. doi:10.3390/ijms241512295

138. Rottenberg H, Hoek JB. The Mitochondrial Permeability Transition: Nexus of Aging, Disease and Longevity. Cells. 2021;10: 79. doi:10.3390/cells10010079

139. Di Lisa F, Menabò R, Canton M, Barile M, Bernardi P. Opening of the Mitochondrial Permeability Transition Pore Causes Depletion of Mitochondrial and Cytosolic NAD+and Is a Causative Event in the Death of Myocytes in Postischemic Reperfusion of the Heart. J Biol Chem. 2001;276: 2571–2575. doi:10.1074/jbc.m006825200

140. Covarrubias AJ, Perrone R, Grozio A, Verdin E. NAD+ metabolism and its roles in cellular processes during ageing. Nat Rev Mol Cell Biol. 2021;22: 119–141. doi:10.1038/s41580-020-00313-x

141. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of Cellular Senescence. Trends Cell Biol. 2018;28: 436–453. doi:10.1016/j.tcb.2018.02.001

142. Antelo-Iglesias L, Picallos-Rabina P, Estévez-Souto V, Da Silva-Álvarez S, Collado M. The role of cellular senescence in tissue repair and regeneration. Mech Ageing Dev. 2021;198: 111528. doi:10.1016/j.mad.2021.111528

143. Rodier F, Campisi J. Four faces of cellular senescence. J Cell Biol. 2011;192: 547–556. doi:10.1083/jcb.201009094

144. Guerrero A, De Strooper B, Arancibia-Cárcamo IL. Cellular senescence at the crossroads of inflammation and Alzheimer's disease. Trends Neurosci. 2021;44: 714–727. doi:10.1016/j.tins.2021.06.007

145. Morales-Rosales SL, Santín-Márquez R, Posadas-Rodriguez P, Rincon-Heredia R, Montiel T, Librado-Osorio R, et al. Senescence in Primary Rat Astrocytes Induces Loss of the Mitochondrial Membrane Potential and Alters Mitochondrial Dynamics in Cortical Neurons. Front Aging Neurosci. 2021;13. doi:10.3389/fnagi.2021.766306

146. Bekaert S, De Meyer T, Van Oostveldt P. Telomere attrition as ageing biomarker. Anticancer Res. 2005;25: 3011–3021.

147. Blackburn EH, Epel ES, Lin J. Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. Science. 2015;350: 1193–1198. doi:10.1126/science.aab3389

148. Iskandar M, Xiao Barbero M, Jaber M, Chen R, Gomez-Guevara R, Cruz E, et al. A Review of Telomere Attrition in Cancer and Aging: Current Molecular Insights and Future Therapeutic Approaches. Cancers. 2025;17: 257. doi:10.3390/cancers17020257

149. Shay JW. Telomeres and aging. Curr Opin Cell Biol. 2018;52: 1–7. doi:10.1016/j.ceb.2017.12.001

150. Liu L, Trimarchi JR, Smith PJS, Keefe DL. Mitochondrial dysfunction leads to telomere attrition and genomic instability. Aging Cell. 2002;1: 40–46. doi:10.1046/j.1474-9728.2002.00004.x

151. Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, et al. Accelerated telomere shortening in response to life stress. Proc Natl Acad Sci. 2004;101: 17312–17315. doi:10.1073/pnas.0407162101

152. Valdes AM, Deary IJ, Gardner J, Kimura M, Lu X, Spector TD, et al. Leukocyte telomere length is associated with cognitive

performance in healthy women. Neurobiol Aging. 2010;31: 986–992. doi:10.1016/j.neurobiolaging.2008.07.012

153. Srinivas N, Rachakonda S, Kumar R. Telomeres and Telomere Length: A General Overview. Cancers. 2020;12: 558. doi:10.3390/cancers12030558

154. Vaiserman A, Krasnienkov D. Telomere Length as a Marker of Biological Age: State-of-the-Art, Open Issues, and Future Perspectives. Front Genet. 2021;11: 630186. doi:10.3389/fgene.2020.630186

155. Daios S, Anogeianaki A, Kaiafa G, Kontana A, Veneti S, Gogou C, et al. Telomere Length as a Marker of Biological Aging: A Critical Reviewof Recent Literature. Curr Med Chem. 2022;29: 5478–5495. doi:10.2174/0929867329666220713123750

156. Jinesh S, Özüpek B, Aditi P. Premature aging and metabolic diseases: the impact of telomere attrition. Front Aging. 2025;6: 1541127. doi:10.3389/fragi.2025.1541127

157. Rossiello F, Jurk D, Passos JF, d'Adda Di Fagagna F. Telomere dysfunction in ageing and age-related diseases. Nat Cell Biol. 2022;24: 135–147. doi:10.1038/s41556-022-00842-x

158. Lapham K, Kvale MN, Lin J, Connell S, Croen LA, Dispensa BP, et al. Automated Assay of Telomere Length Measurement and Informatics for 100,000 Subjects in the Genetic Epidemiology Research on Adult Health and Aging (GERA) Cohort. Genetics. 2015;200: 1061–1072. doi:10.1534/genetics.115.178624

159. Assari S, Dezfuli M, Peyrovinasab A, Zare H. Adulthood Socioeconomic Status Fails to Predict Subsequent Telomere Length in Racially and Ethnically Diverse Women. J Biomed Life Sci. 2024;4: 47–59. doi:10.31586/jbls.2024.1023

160. Chen M, Wang Z, Xu H, Teng P, Li W, Ma L. Association between modifiable lifestyle factors and telomere length: a univariable and multivariable Mendelian randomization study. J Transl Med. 2024;22: 160. doi:10.1186/s12967-024-04956-8

161. Vyas CM, Ogata S, Reynolds CF, Mischoulon D, Chang G, Cook NR, et al. Telomere length and its relationships with lifestyle and behavioural factors: variations by sex and race/ethnicity. Age Ageing. 2021;50: 838–846. doi:10.1093/ageing/afaa186

162. Hughes AL, Gottschling DE. An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. Nature. 2012;492: 261–265. doi:10.1038/nature11654

163. Hughes CE, Coody TK, Jeong M-Y, Berg JA, Winge DR, Hughes AL. Cysteine Toxicity Drives Age-Related Mitochondrial Decline by Altering Iron Homeostasis. Cell. 2020;180: 296-310.e18. doi:10.1016/j.cell.2019.12.035

164. Higuchi-Sanabria R, Shen K, Kelet N, Frankino PA, Durieux J, Bar-Ziv R, et al. Lysosomal recycling of amino acids affects ER quality control. Sci Adv. 2020;6: eaaz9805. doi:10.1126/sciadv.aaz9805

165. Huang J, Meng P, Wang C, Zhang Y, Zhou L. The relevance of organelle interactions in cellular senescence. Theranostics. 2022;12: 2445–2464. doi:10.7150/thno.70588

166. Chernorudskiy AL, Zito E. Regulation of Calcium Homeostasis by ER Redox: A Close-Up of the ER/Mitochondria Connection. J Mol Biol. 2017;429: 620–632. doi:10.1016/j.jmb.2017.01.017

167. Martin N, Bernard D. Calcium signaling and cellular senescence. Cell Calcium. 2018;70: 16–23. doi:10.1016/j.ceca.2017.04.001

168. Wong YC, Kim S, Peng W, Krainc D. Regulation and Function of Mitochondria–Lysosome Membrane Contact Sites in Cellular Homeostasis. Trends Cell Biol. 2019;29: 500–513. doi:10.1016/j.tcb.2019.02.004

169. Santoni G, Maggi F, Amantini C, Marinelli O, Nabissi M, Morelli MB. Pathophysiological Role of Transient Receptor Potential Mucolipin Channel 1 in Calcium-Mediated Stress-Induced Neurodegenerative Diseases. Front Physiol. 2020;11: 251. doi:10.3389/fphys.2020.00251

170. Marchi S, Patergnani S, Missiroli S, Morciano G, Rimessi A, Wieckowski MR, et al. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell Calcium. 2018;69: 62–72. doi:10.1016/j.ceca.2017.05.003

171. Cavinato M, Madreiter-Sokolowski CT, Büttner S, Schosserer M, Zwerschke W, Wedel S, et al. Targeting cellular senescence based on interorganelle communication, multilevel proteostasis, and metabolic control. FEBS J. 2021;288: 3834–3854. doi:10.1111/febs.15631

172. Atakpa P, Thillaiappan NB, Mataragka S, Prole DL, Taylor CW. IP3 Receptors Preferentially Associate with ER-Lysosome Contact Sites and Selectively Deliver Ca2+ to Lysosomes. Cell Rep. 2018;25: 3180-3193.e7. doi:10.1016/j.celrep.2018.11.064

173. Giorgi C, Marchi S, Simoes ICM, Ren Z, Morciano G, Perrone M, et al. Mitochondria and Reactive Oxygen Species in Aging and Age-Related Diseases. International Review of Cell and Molecular Biology. Elsevier; 2018. pp. 209–344. doi:10.1016/bs.ircmb.2018.05.006

174. Janikiewicz J, Szymański J, Malinska D, Patalas-Krawczyk P, Michalska B, Duszyński J, et al. Mitochondria-associated

membranes in aging and senescence: structure, function, and dynamics. Cell Death Dis. 2018;9: 332. doi:10.1038/s41419-017-0105-5

175. Zhou C, Slaughter BD, Unruh JR, Guo F, Yu Z, Mickey K, et al. Organelle-Based Aggregation and Retention of Damaged Proteins in Asymmetrically Dividing Cells. Cell. 2014;159: 530–542. doi:10.1016/j.cell.2014.09.026

176. Calì T, Ottolini D, Negro A, Brini M. Enhanced parkin levels favor ER-mitochondria crosstalk and guarantee Ca2+ transfer to sustain cell bioenergetics. Biochim Biophys Acta BBA - Mol Basis Dis. 2013;1832: 495–508. doi:10.1016/j.bbadis.2013.01.004

177. Picca A, Calvani R, Coelho-Junior HJ, Landi F, Bernabei R, Marzetti E. Inter-Organelle Membrane Contact Sites and Mitochondrial Quality Control during Aging: A Geroscience View. Cells. 2020;9: 598. doi:10.3390/cells9030598

178. Bonomini F, Rodella LF, Rezzani R. Metabolic syndrome, aging and involvement of oxidative stress. Aging Dis. 2015;6: 109–120. doi:10.14336/AD.2014.0305

179. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005;115: 1111–1119. doi:10.1172/JCI25102

180. Stocker R, Keaney JF. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004;84: 1381–1478. doi:10.1152/physrev.00047.2003

181. Sahaf B, Heydari K, Herzenberg LA, Herzenberg LA. The extracellular microenvironment plays a key role in regulating the redox status of cell surface proteins in HIV-infected subjects. Arch

Biochem Biophys. 2005;434: 26–32. doi:10.1016/j.abb.2004.11.015

182. Guarner-Lans V, Rubio-Ruiz ME, Pérez-Torres I, Baños de MacCarthy G. Relation of aging and sex hormones to metabolic syndrome and cardiovascular disease. Exp Gerontol. 2011;46: 517–523. doi:10.1016/j.exger.2011.02.007

183. Liesa M, Palacín M, Zorzano A. Mitochondrial dynamics in mammalian health and disease. Physiol Rev. 2009;89: 799–845. doi:10.1152/physrev.00030.2008

184. Chen H, Chan DC. Mitochondrial dynamics--fusion, fission, movement, and mitophagy--in neurodegenerative diseases. Hum Mol Genet. 2009;18: R169-176. doi:10.1093/hmg/ddp326

185. Yamaguchi R, Perkins G. Dynamics of mitochondrial structure during apoptosis and the enigma of Opa1. Biochim Biophys Acta. 2009;1787: 963–972. doi:10.1016/j.bbabio.2009.02.005

186. Seo AY, Joseph A-M, Dutta D, Hwang JCY, Aris JP, Leeuwenburgh C. New insights into the role of mitochondria in aging: mitochondrial dynamics and more. J Cell Sci. 2010;123: 2533–2542. doi:10.1242/jcs.070490

187. Karbowski M, Lee Y-J, Gaume B, Jeong S-Y, Frank S, Nechushtan A, et al. Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J Cell Biol. 2002;159: 931–938. doi:10.1083/jcb.200209124

188. Breckenridge DG, Stojanovic M, Marcellus RC, Shore GC. Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. J Cell Biol. 2003;160: 1115–1127. doi:10.1083/jcb.200212059

189. Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 2008;27: 433–446. doi:10.1038/sj.emboj.7601963

190. Suen D-F, Norris KL, Youle RJ. Mitochondrial dynamics and apoptosis. Genes Dev. 2008;22: 1577–1590. doi:10.1101/gad.1658508

191. Fang EF, Scheibye-Knudsen M, Chua KF, Mattson MP, Croteau DL, Bohr VA. Nuclear DNA damage signalling to mitochondria in ageing. Nat Rev Mol Cell Biol. 2016;17: 308–321. doi:10.1038/nrm.2016.14

192. Zhu D, Li X, Tian Y. Mitochondrial-to-nuclear communication in aging: an epigenetic perspective. Trends Biochem Sci. 2022;47: 645–659. doi:10.1016/j.tibs.2022.03.008

193. Srivastava S. The Mitochondrial Basis of Aging and Age-Related Disorders. Genes. 2017;8: 398. doi:10.3390/genes8120398

194. Jazwinski SM, Kriete A. The yeast retrograde response as a model of intracellular signaling of mitochondrial dysfunction. Front Physiol. 2012;3: 139. doi:10.3389/fphys.2012.00139

195. Jovaisaite V, Auwerx J. The mitochondrial unfolded protein response—synchronizing genomes. Curr Opin Cell Biol. 2015;33: 74–81. doi:10.1016/j.ceb.2014.12.003

196. Van Cauwenberghe C, Vandendriessche C, Libert C, Vandenbroucke RE. Caloric restriction: beneficial effects on brain aging and Alzheimer's disease. Mamm Genome. 2016;27: 300–319. doi:10.1007/s00335-016-9647-6

197. Martin B, Mattson MP, Maudsley S. Caloric restriction and intermittent fasting: Two potential diets for successful brain aging. Ageing Res Rev. 2006;5: 332–353. doi:10.1016/j.arr.2006.04.002

198. Zhang L, Xu H, Ding N, Li X, Chen X, Chen Z. Beneficial Effects on Brain Micro-Environment by Caloric Restriction in Alleviating Neurodegenerative Diseases and Brain Aging. Front Physiol. 2021;12: 715443. doi:10.3389/fphys.2021.715443

199. Wahl D, Cogger VC, Solon-Biet SM, Waern RVR, Gokarn R, Pulpitel T, et al. Nutritional strategies to optimise cognitive function in the aging brain. Ageing Res Rev. 2016;31: 80–92. doi:10.1016/j.arr.2016.06.006

200. Wahl D, Coogan S, Solon-Biet S, De Cabo R, Haran J, Raubenheimer D, et al. Cognitive and behavioral evaluation of nutritional interventions in rodent models of brain aging and dementia. Clin Interv Aging. 2017;Volume 12: 1419–1428. doi:10.2147/CIA.S145247

201. Wahl D, Solon-Biet SM, Wang Q-P, Wali JA, Pulpitel T, Clark X, et al. Comparing the Effects of Low-Protein and High-Carbohydrate Diets and Caloric Restriction on Brain Aging in Mice. Cell Rep. 2018;25: 2234-2243.e6. doi:10.1016/j.celrep.2018.10.070

202. Gillette-Guyonnet S, Vellas B. Caloric restriction and brain function: Curr Opin Clin Nutr Metab Care. 2008;11: 686–692. doi:10.1097/MCO.0b013e328313968f

203. Weindruch R. Caloric restriction: life span extension and retardation of brain aging. Clin Neurosci Res. 2003;2: 279–284. doi:10.1016/S1566-2772(03)00004-5

204. Erbaba B, Arslan-Ergul A, Adams MM. Effects of caloric restriction on the antagonistic and integrative hallmarks of aging. Ageing Res Rev. 2021;66: 101228. doi:10.1016/j.arr.2020.101228

205. Qiu G, Liu S, So K-F. Dietary restriction and brain health. Neurosci Bull. 2010;26: 55–65. doi:10.1007/s12264-010-0716-7

206. Vasconcelos AR, Yshii LM, Viel TA, Buck HS, Mattson MP, Scavone C, et al. Intermittent fasting attenuates lipopolysaccharide-induced neuroinflammation and memory impairment. J Neuroinflammation. 2014;11: 85. doi:10.1186/1742-2094-11-85

207. Contestabile A. Benefits of Caloric Restriction on Brain Aging and Related Pathological States: Understanding Mechanisms to Devise Novel Therapies. Curr Med Chem. 2009;16: 350–361. doi:10.2174/092986709787002637

208. De Cabo R, Mattson MP. Effects of Intermittent Fasting on Health, Aging, and Disease. Longo DL, editor. N Engl J Med. 2019;381: 2541–2551. doi:10.1056/NEJMra1905136

209. Kawakami R, Sunaga H, Iso T, Kaneko R, Koitabashi N, Obokata M, et al. Ketone body and FGF21 coordinately regulate fasting-induced oxidative stress response in the heart. Sci Rep. 2022;12: 7338. doi:10.1038/s41598-022-10993-4

210. Mattson MP, Moehl K, Ghena N, Schmaedick M, Cheng A. Intermittent metabolic switching, neuroplasticity and brain health. Nat Rev Neurosci. 2018;19: 81–94. doi:10.1038/nrn.2017.156

211. Menzies FM, Fleming A, Caricasole A, Bento CF, Andrews SP, Ashkenazi A, et al. Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. Neuron. 2017;93: 1015–1034. doi:10.1016/j.neuron.2017.01.022

212. Ooi TC, Meramat A, Rajab NF, Shahar S, Ismail IS, Azam AA, et al. Intermittent Fasting Enhanced the Cognitive Function in Older Adults with Mild Cognitive Impairment by Inducing Biochemical and Metabolic changes: A 3-Year Progressive Study. Nutrients. 2020;12: 2644. doi:10.3390/nu12092644

213. Honjoh S, Yamamoto T, Uno M, Nishida E. Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. Nature. 2009;457: 726–730. doi:10.1038/nature07583

214. Uno M, Honjoh S, Matsuda M, Hoshikawa H, Kishimoto S, Yamamoto T, et al. A Fasting-Responsive Signaling Pathway that Extends Life Span in C. elegans. Cell Rep. 2013;3: 79–91. doi:10.1016/j.celrep.2012.12.018

215. Wallace MA, Aguirre NW, Marcotte GR, Marshall AG, Baehr LM, Hughes DC, et al. The ketogenic diet preserves skeletal muscle with aging in mice. Aging Cell. 2021;20: e13322. doi:10.1111/acel.13322

216. Roberts MN, Wallace MA, Tomilov AA, Zhou Z, Marcotte GR, Tran D, et al. A Ketogenic Diet Extends Longevity and Healthspan in Adult Mice. Cell Metab. 2018;27: 1156. doi:10.1016/j.cmet.2018.04.005

217. Jiang Z, Yin X, Wang M, Chen T, Wang Y, Gao Z, et al. Effects of Ketogenic Diet on Neuroinflammation in Neurodegenerative Diseases. Aging Dis. 2022;13: 1146–1165. doi:10.14336/AD.2021.1217

218. Surugiu R, Iancu MA, Vintilescu Ștefăniță B, Stepan MD, Burdusel D, Genunche-Dumitrescu AV, et al. Molecular Mechanisms of Healthy Aging: The Role of Caloric Restriction, Intermittent Fasting, Mediterranean Diet, and Ketogenic Diet—A

Scoping Review. Nutrients. 2024;16: 2878. doi:10.3390/nu16172878

219. Wang B-H, Hou Q, Lu Y-Q, Jia M-M, Qiu T, Wang X-H, et al. Ketogenic diet attenuates neuronal injury via autophagy and mitochondrial pathways in pentylenetetrazol-kindled seizures. Brain Res. 2018;1678: 106–115. doi:10.1016/j.brainres.2017.10.009

220. Gershuni VM, Yan SL, Medici V. Nutritional Ketosis for Weight Management and Reversal of Metabolic Syndrome. Curr Nutr Rep. 2018;7: 97–106. doi:10.1007/s13668-018-0235-0

221. Grammatikopoulou MG, Goulis DG, Gkiouras K, Theodoridis X, Gkouskou KK, Evangeliou A, et al. To Keto or Not to Keto? A Systematic Review of Randomized Controlled Trials Assessing the Effects of Ketogenic Therapy on Alzheimer Disease. Adv Nutr. 2020;11: 1583–1602. doi:10.1093/advances/nmaa073

222. Yu Y, Wang F, Wang J, Zhang D, Zhao X. Ketogenic diet attenuates aging-associated myocardial remodeling and dysfunction in mice. Exp Gerontol. 2020;140: 111058. doi:10.1016/j.exger.2020.111058

223. Fekete M, Lehoczki A, Tarantini S, Fazekas-Pongor V, Csípő T, Csizmadia Z, et al. Improving Cognitive Function with Nutritional Supplements in Aging: A Comprehensive Narrative Review of Clinical Studies Investigating the Effects of Vitamins, Minerals, Antioxidants, and Other Dietary Supplements. Nutrients. 2023;15: 5116. doi:10.3390/nu15245116

224. Sadowska-Bartosz I, Bartosz G. Effect of Antioxidants Supplementation on Aging and Longevity. BioMed Res Int. 2014;2014: 1–17. doi:10.1155/2014/404680

225. Kirkwood TBL, Kowald A. The free-radical theory of ageing – older, wiser and still alive: Modelling positional effects of the primary targets of ROS reveals new support. BioEssays. 2012;34: 692–700. doi:10.1002/bies.201200014

226. Conti V, Izzo V, Corbi G, Russomanno G, Manzo V, De Lise F, et al. Antioxidant Supplementation in the Treatment of Aging-Associated Diseases. Front Pharmacol. 2016;7. doi:10.3389/fphar.2016.00024

227. Kim J, Takahashi M, Shimizu T, Shirasawa T, Kajita M, Kanayama A, et al. Effects of a potent antioxidant, platinum nanoparticle, on the lifespan of Caenorhabditis elegans. Mech Ageing Dev. 2008;129: 322–331. doi:10.1016/j.mad.2008.02.011

228. Kitani K, Osawa T, Yokozawa T. The effects of tetrahydrocurcumin and green tea polyphenol on the survival of male C57BL/6 mice. Biogerontology. 2007;8: 567–573. doi:10.1007/s10522-007-9100-z

229. Hernández-Camacho JD, Bernier M, López-Lluch G, Navas P. Coenzyme Q10 Supplementation in Aging and Disease. Front Physiol. 2018;9: 44. doi:10.3389/fphys.2018.00044

230. Fan L, Feng Y, Chen G-C, Qin L-Q, Fu C, Chen L-H. Effects of coenzyme Q10 supplementation on inflammatory markers: A systematic review and meta-analysis of randomized controlled trials. Pharmacol Res. 2017;119: 128–136. doi:10.1016/j.phrs.2017.01.032

231. Upritchard JE, Schuurman CR, Wiersma A, Tijburg LB, Coolen SA, Rijken PJ, et al. Spread supplemented with moderate doses of vitamin E and carotenoids reduces lipid peroxidation in healthy, nonsmoking adults. Am J Clin Nutr. 2003;78: 985–992. doi:10.1093/ajcn/78.5.985

232. O'Leary KA, Pascual-Tereasa SD, Needs PW, Bao Y-P, O'Brien NM, Williamson G. Effect of flavonoids and Vitamin E on cyclooxygenase-2 (COX-2) transcription. Mutat Res Mol Mech Mutagen. 2004;551: 245–254. doi:10.1016/j.mrfmmm.2004.01.015

233. Aydogan S, Yerer MB, Goktas A. Melatonin and nitric oxide. J Endocrinol Invest. 2006;29: 281–287. doi:10.1007/BF03345555

234. Xu X, Shen X, Feng W, Yang D, Jin L, Wang J, et al. D-galactose induces senescence of glioblastoma cells through YAP-CDK6 pathway. Aging. 2020;12: 18501–18521. doi:10.18632/aging.103819

235. Baluchnejadmojarad T, Mohamadi-Zarch S-M, Roghani M. Safranal, an active ingredient of saffron, attenuates cognitive deficits in amyloid β-induced rat model of Alzheimer's disease: underlying mechanisms. Metab Brain Dis. 2019;34: 1747–1759. doi:10.1007/s11011-019-00481-6

236. Bian Y, Zhao C, Lee SM-Y. Neuroprotective Potency of Saffron Against Neuropsychiatric Diseases, Neurodegenerative Diseases, and Other Brain Disorders: From Bench to Bedside. Front Pharmacol. 2020;11: 579052. doi:10.3389/fphar.2020.579052

237. Assimopoulou AN, Sinakos Z, Papageorgiou VP. Radical scavenging activity of Crocus sativus L. extract and its bioactive constituents. Phytother Res PTR. 2005;19: 997–1000. doi:10.1002/ptr.1749

238. Bej E, Volpe AR, Cesare P, Cimini A, d'Angelo M, Castelli V. Therapeutic potential of saffron in brain disorders: From bench to bedside. Phytother Res PTR. 2024;38: 2482–2495. doi:10.1002/ptr.8169

239. Maggi MA, Bisti S, Picco C. Saffron: Chemical Composition and Neuroprotective Activity. Mol Basel Switz. 2020;25: 5618. doi:10.3390/molecules25235618

240. Asai A, Nakano T, Takahashi M, Nagao A. Orally administered crocetin and crocins are absorbed into blood plasma as crocetin and its glucuronide conjugates in mice. J Agric Food Chem. 2005;53: 7302–7306. doi:10.1021/jf0509355

241. Inoue E, Shimizu Y, Masui R, Hayakawa T, Tsubonoya T, Hori S, et al. Effects of saffron and its constituents, crocin-1, crocin-2, and crocetin on α-synuclein fibrils. J Nat Med. 2018;72: 274–279. doi:10.1007/s11418-017-1150-1

242. Sekler A, Jiménez JM, Rojo L, Pastene E, Fuentes P, Slachevsky A, et al. Cognitive impairment and Alzheimer's disease: Links with oxidative stress and cholesterol metabolism. Neuropsychiatr Dis Treat. 2008;4: 715–722. doi:10.2147/ndt.s3268

243. Khalili M, Hamzeh F. Effects of active constituents of Crocus sativus L., crocin on streptozocin-induced model of sporadic Alzheimer's disease in male rats. Iran Biomed J. 2010;14: 59–65.

244. Karakani AM, Riazi G, Mahmood Ghaffari S, Ahmadian S, Mokhtari F, Jalili Firuzi M, et al. Inhibitory effect of corcin on aggregation of 1N/4R human tau protein in vitro. Iran J Basic Med Sci. 2015;18: 485–492.

245. Akhondzadeh S, Shafiee Sabet M, Harirchian MH, Togha M, Cheraghmakani H, Razeghi S, et al. A 22-week, multicenter, randomized, double-blind controlled trial of Crocus sativus in the treatment of mild-to-moderate Alzheimer's disease. Psychopharmacology (Berl). 2010;207: 637–643. doi:10.1007/s00213-009-1706-1

246. Akhondzadeh S, Sabet MS, Harirchian MH, Togha M, Cheraghmakani H, Razeghi S, et al. Saffron in the treatment of patients with mild to moderate Alzheimer's disease: a 16-week, randomized and placebo-controlled trial. J Clin Pharm Ther. 2010;35: 581–588. doi:10.1111/j.1365-2710.2009.01133.x

247. Kanakis CD, Tarantilis PA, Tajmir-Riahi HA, Polissiou MG. Crocetin, dimethylcrocetin, and safranal bind human serum albumin: stability and antioxidative properties. J Agric Food Chem. 2007;55: 970–977. doi:10.1021/jf062638l

248. Rafieipour F, Hadipour E, Emami SA, Asili J, Tayarani-Najaran Z. Safranal protects against beta-amyloid peptide-induced cell toxicity in PC12 cells via MAPK and PI3 K pathways. Metab Brain Dis. 2019;34: 165–172. doi:10.1007/s11011-018-0329-9

249. Cerdá-Bernad D, Costa L, Serra AT, Bronze MR, Valero-Cases E, Pérez-Llamas F, et al. Saffron against Neuro-Cognitive Disorders: An Overview of Its Main Bioactive Compounds, Their Metabolic Fate and Potential Mechanisms of Neurological Protection. Nutrients. 2022;14: 5368. doi:10.3390/nu14245368

250. Ghasemzadeh Rahbardar M, Hosseinzadeh H. A review of how the saffron (Crocus sativus) petal and its main constituents interact with the Nrf2 and NF-κB signaling pathways. Naunyn Schmiedebergs Arch Pharmacol. 2023;396: 1879–1909. doi:10.1007/s00210-023-02487-5

251. Shaheen MJ, Bekdash AM, Itani HA, Borjac JM. Saffron extract attenuates neuroinflammation in rmTBI mouse model by suppressing NLRP3 inflammasome activation via SIRT1. PloS One. 2021;16: e0257211. doi:10.1371/journal.pone.0257211

252. Reeve AK, Grady JP, Cosgrave EM, Bennison E, Chen C, Hepplewhite PD, et al. Mitochondrial dysfunction within the

synapses of substantia nigra neurons in Parkinson's disease. NPJ Park Dis. 2018;4: 9. doi:10.1038/s41531-018-0044-6

253. Misrani A, Tabassum S, Yang L. Mitochondrial Dysfunction and Oxidative Stress in Alzheimer's Disease. Front Aging Neurosci. 2021;13: 617588. doi:10.3389/fnagi.2021.617588

254. Dasuri K, Zhang L, Keller JN. Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis. Free Radic Biol Med. 2013;62: 170–185. doi:10.1016/j.freeradbiomed.2012.09.016

255. Khazdair MR, Boskabady MH, Hosseini M, Rezaee R, M Tsatsakis A. The effects of Crocus sativus (saffron) and its constituents on nervous system: A review. Avicenna J Phytomedicine. 2015;5: 376–391.

256. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol. 2009;7: 65–74. doi:10.2174/157015909787602823

257. Naghizadeh B, Mansouri MT, Ghorbanzadeh B, Farbood Y, Sarkaki A. Protective effects of oral crocin against intracerebroventricular streptozotocin-induced spatial memory deficit and oxidative stress in rats. Phytomedicine Int J Phytother Phytopharm. 2013;20: 537–542. doi:10.1016/j.phymed.2012.12.019

258. D'Archivio AA, Di Donato F, Foschi M, Maggi MA, Ruggieri F. UHPLC Analysis of Saffron (Crocus sativus L.): Optimization of Separation Using Chemometrics and Detection of Minor Crocetin Esters. Molecules. 2018;23: 1851. doi:10.3390/molecules23081851

259. D'Archivio AA, Maggi MA. Geographical identification of saffron (Crocus sativus L.) by linear discriminant analysis applied to the UV–visible spectra of aqueous extracts. Food Chem. 2017;219: 408–413. doi:10.1016/j.foodchem.2016.09.169

260. Heidari S, Mehri S, Shariaty V, Hosseinzadeh H. Preventive effects of crocin on neuronal damages induced by D-galactose through AGEs and oxidative stress in human neuroblastoma cells (SH-SY5Y). J Pharmacopuncture. 2018;21: 18–25. doi:10.3831/KPI.2018.21.003

261. Rahimi VB, Askari VR, Mousavi SH. Ellagic acid reveals promising anti-aging effects against d-galactose-induced aging on human neuroblastoma cell line, SH-SY5Y: A mechanistic study. Biomed Pharmacother. 2018;108: 1712–1724. doi:10.1016/j.biopha.2018.10.024

262. Castelli V, Cesare P, Bej E, d'Angelo M, Volpe AR. Mechanistic insights into the anti-aging effects of Crocus sativus in a D-Gal-induced in vitro neural senescence model. Lorigooini, Z, editor. PLOS One. 2025;20: e0320572. doi:10.1371/journal.pone.0320572

263. ISO 3632-1 Saffron (Crocus sativus L.), Part 1 (Specification); ISO: Geneva, Switzerland, 2011.

264. ISO 3632-2 Saffron (Crocus sativus L.), Part 2 (Test Methods); ISO: Geneva, Switzerland, 2010.

265. Carmona M, Zalacain A, Sánchez AM, Novella JL, Alonso GL. Crocetin Esters, Picrocrocin and Its Related Compounds Present in *Crocus sativus* Stigmas and *Gardenia jasminoides* Fruits. Tentative Identification of Seven New Compounds by LC-ESI-MS. J Agric Food Chem. 2006;54: 973–979. doi:10.1021/jf052297w

266. Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC. Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc Natl Acad Sci. 1996;93: 13742–13747. doi:10.1073/pnas.93.24.13742

267. Shtutman M, Chang B-D, Schools GP, Broude EV. Cellular Model of p21-Induced Senescence. In: Nikiforov MA, editor. Oncogene-Induced Senescence. New York, NY: Springer New York; 2017. pp. 31–39. doi:10.1007/978-1-4939-6670-7_3

268. Martínez G, Duran-Aniotz C, Cabral-Miranda F, Vivar JP, Hetz C. Endoplasmic reticulum proteostasis impairment in aging. Aging Cell. 2017;16: 615–623. doi:10.1111/acel.12599

269. Gorbatyuk MS, Gorbatyuk OS. The Molecular Chaperone GRP78/BiP as a Therapeutic Target for Neurodegenerative Disorders: A Mini Review. J Genet Syndr Gene Ther. 2013;04. doi:10.4172/2157-7412.1000128

270. Salganik M, Sergeyev VG, Shinde V, Meyers CA, Gorbatyuk MS, Lin JH, et al. The loss of glucose-regulated protein 78 (GRP78) during normal aging or from siRNA knockdown augments human alpha-synuclein (α-syn) toxicity to rat nigral neurons. Neurobiol Aging. 2015;36: 2213–2223. doi:10.1016/j.neurobiolaging.2015.02.018