Universidade de Aveiro

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Departamento de Biologia

ANA VIRGÍNIA ALVES CARVALHO

The autophagic biomarker p62 as a tool for monitoring age-dependent cellular responses

O biomarcador autofágico p62 como uma ferramenta para monitorização de respostas celulares dependentes da idade

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Associada com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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o júri

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palavras-chave Envelhecimento, autofagia, proteassoma, *western blot*, mitocôndria, antioxidante, restrição calórica, stress oxidativo

resumo

O processo de síntese proteica é propenso a erros, por isso, existe um sistema de controlo de qualidade celular preparado para detetar estes erros e degradar proteínas danificadas. Quando este sistema falha, pode haver acumulação de proteínas agregadas, que é um evento associado a doenças neurodegenerativas, que estão também associadas ao envelhecimento. Células envelhecidas têm uma capacidade reduzida de eliminar proteínas *misfolded* e agregadas; além disso, células em envelhecimento estão expostas a stress oxidativo cumulativo, levando a um aumento nos erros de transcrição e tradução. O proteassoma executa a maioria da degradação de proteínas intracelulares, portanto, este processo tem um papel importante na regulação do ciclo celular. A autofagia é um processo de degradação lisossomal, induzido por restrição calórica ou por *stressors* extracelulares, que consiste na formação de um autofagossoma que sequestra o citoplasma e o conteúdo celular a ser degradado. A proteína p62/SQSTM1 é um recetor autofágico, que se transloca e liga aos substratos de autofagia, facilitando o seu sequestro e degradação por autofagia, sendo por isso utilizada como marcador de autofagia.

O objetivo desta dissertação foi entender como os insultos extracelulares (inibição do proteassoma, restrição calórica, despolarização da membrana mitocondrial, agentes antioxidantes) modulam a resposta de autofagia em fibroblastos de indivíduos de diferentes faixas etárias (21, 41, 69, e 80 anos), assim como para perceber a influencia do envelhecimento na resposta autofágica a estes mesmos distúrbios. Uma análise de *western blot* demonstrou que restrição calórica causou aumento de p62 nos 41 anos de idade. Despolarização da membrana mitocondrial causou um aumento da autofagia em todas as idades, exceto nos 21 anos; combinação da despolarização da membrana com inibição do proteassoma causou diminuição de p62 em todos os grupos. Na presença do antioxidante catequina, houve um aumento geral da autofagia, pois verificou-se menos concentração de p62 em todas as idades. Inibição do proteassoma juntamente com catequina causou um aumento significativo de autofagia nos 41 anos.

Este é um ramo que merece mais estudos, possivelmente complementando os presentes resultados com técnicas diferentes. No entanto, a hipótese apresentada nesta dissertação foi comprovada, pois, de facto, p62 provou ser um bom biomarcador de autofagia nos fibroblastos de diferentes idades, em resposta a stresses extracelulares.

keywords Aging, autophagy, proteasome, western blot, mitochondria, antioxidation, starvation, oxidative stress

abstract

The process of protein synthesis is error prone, thus, there is a cellular quality control system which is prepared to detect these errors and to degrade these damaged proteins. Upon failure from the cellular quality control system, there can be an accumulation of protein aggregates, which is associated with neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, which are aging-associated disorders. Aging cells are known to have a reduced ability to eliminate misfolded and aggregated proteins; furthermore, aged cells experience extended cumulative oxidative stress, leading to an increase in transcriptional and translational errors. Much of intracellular protein degradation is performed by the proteasome, which plays an important role in the regulation of the cell cycle. Another process, autophagy, is an intracellular lysosomal degradation process, which is induced by starvation or extracellular stressors, and consists in the formation of an autophagosome that sequesters cytoplasm and the cellular content to be degraded. p62/SQSTM1 protein is an autophagy receptor, which is translocated to autophagy substrates and binds to them, facilitating their sequestration and degradation by autophagy.

The objective of this thesis was to understand how extracellular stressors (proteasome inhibition, starvation, mitochondrial membrane depolarization, antioxidant agents) modulate the autophagy response on fibroblasts of different age groups (21, 41, 69, and 80 years old), as well as to understand the influence of aging on the autophagic response to stressors. A western blot analysis showed that starvation caused accumulation of p62 in 41-year-olds. Upon mitochondrial membrane depolarization, there was a general increase in autophagy, except on young cells; combined membrane depolarization and proteasome inhibition caused a decline in p62 in all age groups. Catechin-induced antioxidation caused an increase in autophagy, as p62 concentration reduced in all age groups. Proteasome inhibition together with antioxidation caused a significant increase in autophagy on 41-year-olds.

It would be relevant to continue to pursue this area, possibly complementing these results with further studies and more techniques. Hence, the underlying hypothesis of this study was proved true, as p62 is a good biomarker for autophagy monitoring on cells of different ages, upon extracellular stress.

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III. Abbreviations

AD: Alzheimer's disease

ALIS: Aggresome-like inducible structures

ALS: Amyotrophic lateral sclerosis

APP: Amyloid precursor protein

APS: Ammonium persulfate

Aβ: Amyloid beta

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

CMA: Chaperone-mediated autophagy

DMEM: Dulbecco's modified eagle's medium

EGCG: Epigallocatechin-3-gallate

ER: Endoplasmic reticulum

FBS: Fetal bovine serum

HD: Huntington's disease

HDAC6: Histone deacetylase 6

HSC: Heat shock cognate

HSP: Heat shock protein

LIR: LC3-interacting region

MAP1LC3/LC3: Microtubule-associated protein light chain 3

NBR1: Neighbor of BRCA1 gene 1

NDP52: Nuclear domain 10 protein

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NFTs: Neurofibrillary tangles

Nrf2: Nuclear factor erythroid 2-relatd factor 2

62/SQSTM1: Ubiquitin-binding protein p62/Sequestosome 1

PBS: Phosphate-buffered saline

PD: Parkinson’s disease

RIPA: Radioimmunoprecipitation assay

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulfate

TBS-T: Tris-buffered saline tween

TEMED: Tetramethylethylenediamine

UBA: Ubiquitin-associated

UPR: Unfolded protein response

UPS: Ubiquitin Proteasome System

1. Introduction

1. Introduction

1.1. Protein Aggregation

Proteins are, second only to water, the most abundant molecules in biology. There are thousands of different types of proteins in cells, which control almost all chemical processes and biological functions (Balchin, Hayer-Hartl and Hartl, 2016; Alam *et al.*, 2017). Proteins are synthesized by ribosomes using information coded in the cellular DNA, and the protein-coding sequence determines not only the protein's function and structure, but also how its structure is acquired (Stefani and Dobson, 2003). Most proteins, in order to be fully functional, are converted into compactly folded three-dimensional structures, many of which are very complex; termed the native fold. Some proteins start this folding process simultaneous to translation, while the nascent polypeptide chain is still attached to the ribosome; other proteins undergo folding in the cytoplasm after release from the ribosome; and yet others fold in specific cellular compartments, such as the endoplasmic reticulum (ER), following translocation (Alam *et al.*, 2017; Biza *et al.*, 2017).

The native fold structure is formed by efficient, high-fidelity post-translational modifications (among them phosphorylation, glycosylation and lipidation), and the failure to adopt proper protein structure is a major threat to cellular function and viability (Balchin, Hayer-Hartl and Hartl, 2016; Alam *et al.*, 2017).

Factors that stabilize the native state decelerate the process of aggregation, and vice-versa (Chaturvedi *et al.*, 2016). During the folding process, folding intermediates (which may be unfolded, partially folded, or misfolded), expose hydrophobic residues that would normally be protected in the native structure. This can trigger protein aggregation; a process distinct from protein complex formation (Gidalevitz *et al.*, 2006; Trigo, Nadais and da Cruz e Silva, 2019). Information on the structure of partially unfolded monomers

(which could aggregate), is scarcely available, given the difficulties in isolating these intermediates (Chaturvedi *et al.*, 2016).

The cellular quality control system can adjust to the severity of protein damage. These include inducing stress responses that modulate levels of chaperones (proteins that interact with, stabilize, or help other proteins acquire their functional conformation, without being present in the final structure) and proteases (enzymes that catalyze protein breakdown) (Tyedmers, Mogk and Bukau, 2010; Balchin, Hayer-Hartl and Hartl, 2016). However, as efficient as the protein folding process may be, it is still error-prone, as amino acid sequences can have many possible conformations (Brockwell and Radford, 2007).

When the amount of misfolded proteins exceeds the cellular capacity for refolding and degradation, and as the quality control system becomes overwhelmed by the simultaneous presence of otherwise non-pathogenic protein-folding errors, these proteins accumulate as aggregates (Figure 1) (Gidalevitz *et al.*, 2006). Increasing evidence suggests that aggregation is not just an unspecific and uncontrolled pathway, but it is a

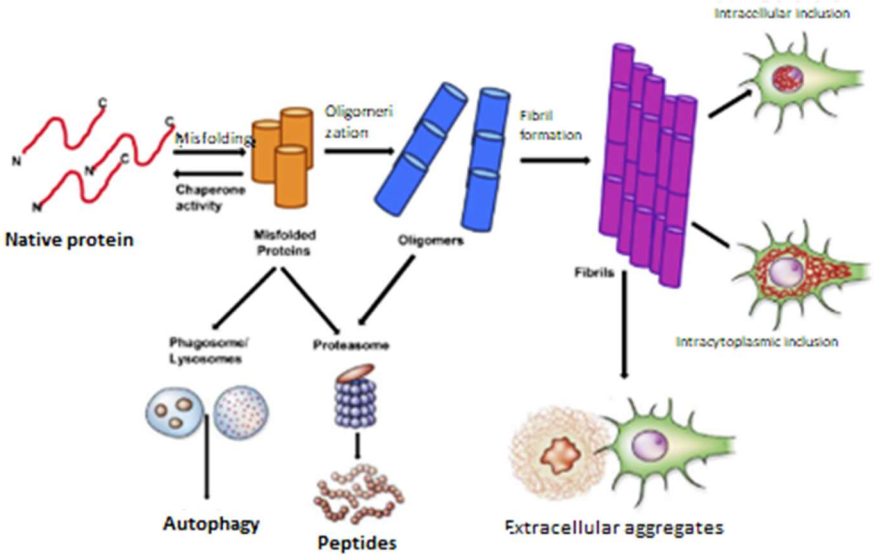

Figure 1 - **Representation of protein misfolding and aggregation**. Soluble native protein is misfolded and forms oligomers, protofibrils, and amyloid fibrils, which are deposited in the nucleus, cytoplasm, and extracellular space. Genetic and environmental factors may enhance the misfolding and aggregation processes. Cellular quality control mechanisms, including molecular chaperones, ubiquitin proteasome system, and the phagosome-lysosome system limit the accumulation of misfolded proteins. Taken from Forman, Trojanowski and Lee, 2004 and Kumar *et al.*, 2016.

controlled process. Namely as part of the cellular response to protein homeostasis (proteostasis), with deposition of aggregates at specific sites being viewed as a line of cellular defense, which is evolutionarily conserved, from bacteria to humans. Protein aggregation appears to occur as a consequence of the exhaustion of the protein control system (Tyedmers, Mogk and Bukau, 2010).

Oxidative stress, defined as the imbalance between the production and the elimination of reactive oxygen species (ROS), often leading to irreversible protein modifications through excess free radicals (Stadman and Levine, 2006; Tyedmers, Mogk and Bukau, 2010), and together with high temperature and pH deregulation, causes the bulk of protein unfolding (Tyedmers, Mogk and Bukau, 2010). Particularly relevant, aging cells have a reduced ability to eliminate misfolded protein species, which is thought to be the reason for the late-onset of Huntington's Disease (HD), and some forms of Alzheimer's Disease (AD) and Parkinson's Disease (PD) (Morley *et al.*, 2002). Thus, aging is a major risk factor for the development of the above-mentioned neurodegenerative disorders.

1.2. Aggregate Formation

Typically, three types of protein aggregates are considered, amyloid fibrils, oligomers, and amorphous aggregates. Most proteins are able to form amorphous aggregates, which are characterized by disordered intermolecular interactions, lacking defined shape and structure, and are also associated with protein denaturation. Due to exposed hydrophobic surfaces, these unfolded conformations have a greater propensity to aggregate, thus creating amorphous aggregates (Michaels *et al.*, 2015; Kumar *et al.*, 2016).

Although most aggregates are of the amorphous type, some form extracellular insoluble deposits – amyloid fibrils. The biological role of these fibrils is unknown, but they have been associated with a series of pathological conditions, often referred to as "amyloidosis" (Pepys *et al.*, 2001). These fibrils are formed when a hypersaturated amyloidogenic protein assembles itself into a water-excluded structure with an ordered

cross-β arrangement perpendicular to the fibril axis (Michaels *et al.*, 2015). This minimizes the energy of the aggregated proteins, resulting in a more stable form, harder to solubilize structure (Hirota-Nakaoka *et al.*, 2003; Trigo, Nadais and da Cruz e Silva, 2019).

Native monomers may have the tendency to self-associate, in a reversible dynamic equilibrium (Chaturvedi *et al.*, 2016). Thus, aggregates may form from the assembly of monomeric proteins with self-complementary motifs, which favor intramolecular interactions, such as electrostatic or van der Waals interactions, resulting in oligomerization, and subsequent aggregation (Figure 2AI) (Trigo, Nadais and da Cruz e Silva, 2019). As these oligomers increase in size, they become less soluble, due to the formation of covalent bonds, among them disulphide bonds, resulting in irreversible aggregates (Chaturvedi *et al.*, 2016).

In some cases, the non-native state experiences conformational changes due to external stressors, such as heat or shearing forces, making the native monomer more prone to aggregation (Figure 2AIII). In this case, monomers do not lose the tendency to associate reversibly, instead, a conformationally altered or partially unfolded monomer has a higher tendency to form aggregates (Chaturvedi *et al.*, 2016). Interferon-ϒ and granulocyte colony-stimulating factor undergo amyloidosis through this process (Kendrick *et al.*, 1998; Krishnan *et al.*, 2002).

The nucleation mechanism commonly forms visible aggregates or precipitates – while the monomer alone is unable to create fibrils, aggregates of critical size can promote the formation of larger aggregates (Figure 2AIII). This is referred as the “critical nucleus” (Chaturvedi *et al.*, 2016). There is usually a lag phase, the rate-limiting step, a long period of time with no visible precipitate formation, subsequently much larger species appears instantly. The intermediate species formed are often soluble oligomers or protofibrils that appear as small, thin, filamentous structures of at least 2.5 - 5.0 nanometers in diameter, with the tendency to further aggregate. Once a nucleus is formed, it grows exponentially by further association with monomers or oligomers (Kumar *et al.*, 2016). In cases when the critical nucleus itself is the aggregation product, it

is referred as “homogenous nucleus”; in “heterogenous nucleus”, the nucleus is formed from particles other than protein aggregates (Chi *et al.*, 2005).

Amyloid formation generally occurs by nucleation-dependent oligomerization: an ordered nucleus formed in the supersaturated protein solution exceeds the critical concentration of the amyloidogenic protein, and fibril growth occurs very quickly (Chaturvedi *et al.*, 2016).

In the mammalian cytoplasm, there is a different, specialized type of aggregation found in some human disorders, the ubiquitin-rich aggresome, composed of an accumulation of misfolded proteins overlooked by chaperones or ubiquitin proteasome system (UPS) degradation, overwhelming these systems (Johnston, Ward and Kopito, 1998; Kopito, 2000). The misfolded proteins are transported throughout the cytoskeleton, to a perinuclear location where other cellular components accumulate, such as

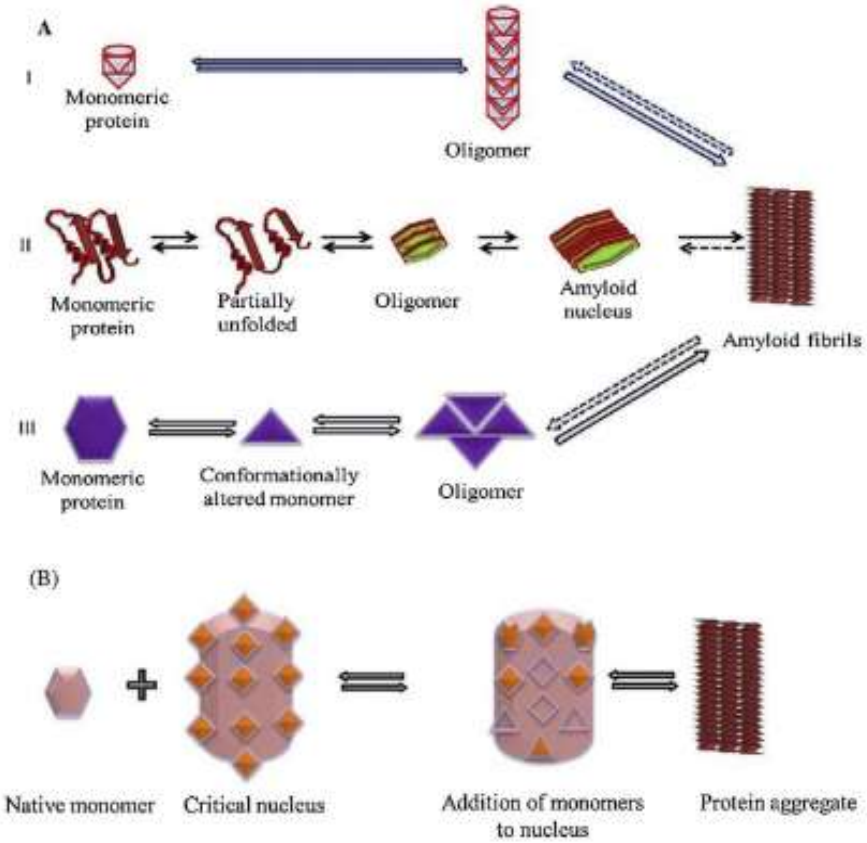

Figure 2 - Protein aggregation process. A(I) Oligomerisation of monomeric proteins into mature amyloid fibril; **(II)** Intermediate formation during protein aggregation; **(III)** Self-assembly of conformationally altered monomers into mature amyloid fibril; **(B)** Nucleation-dependent fibril formation. Taken from Chaturvedi et al., 2016.

chaperones, proteasome components, and motor proteins. Intracellular accumulation of ubiquitin-rich cytoplasmic aggregates is linked to the pathogenesis of many diseases (Zhang and Qian, 2011; Trigo, Nadais and da Cruz e Silva, 2019).

When proteostasis capacity is limited, for example, in the presence of an extracellular stressor or during aging, cells may reduce aggregate load by sequestering smaller aggregates into larger deposits (Hipp, Kasturi and Hartl, 2019). Inclusion bodies are amorphous or disordered aggregates formed *in vivo* (Chaturvedi *et al.*, 2016). In yeast, they form near the vacuole, as well as near and inside the nucleus (Miller *et al.*, 2015; Hipp, Kasturi and Hartl, 2019), while in mammalian cells inclusion bodies result from an accumulation of aggregated proteins, chaperones, ubiquitin-pathway molecules, cytoskeletal and centrosomal material, and nucleic acids (Kopito, 2000; Trigo, Nadais and da Cruz e Silva, 2019). Inclusion bodies may be used to lower the level of small diffusible oligomers, reducing the reactive surface and the amount of bound chaperones (Kim et al., 2016; Ramdzan et al., 2017). The presence of inclusion bodies correlates with improved cell survival in cellular models of neurodegenerative disease (Arrasate *et al.*, 2004).

Globular, thermophilic and prion proteins may follow different aggregation pathways (Chaturvedi *et al.*, 2016). There seems to be a preference for co-aggregation of the same protein type, but one aggregating protein species may influence the aggregation behavior of another, and different proteins are able to co-aggregate (Ben-Zvi and Goloubinoff, 2002). When the number of exposed hydrophobic surfaces increase, the solubility limit is reached, which, in normal physiological conditions, is irreversible (Trigo, Nadais and da Cruz e Silva, 2019).

1.3. Cellular Strategies Against Protein Aggregation

Proteostasis, or protein homeostasis, is the state of functional balance in a proteome, which is a tightly controlled process within individual cells, tissues, and organs (Balchin, Hayer-Hartl and Hartl, 2016).

Small proteins (up to 100 amino acids) usually fold within milliseconds, but, for larger proteins, folding can be inefficient, owing to off-pathway aggregation (Balchin, Hayer-Hartl and Hartl, 2016). Proteins to be secreted or sorted to other cell organelles fold in the ER lumen before being delivered to their target cellular location or exported (Walter and Ron, 2011). Since proteins larger than 100 amino acids make up for a significant portion of all proteomes, their folding *in vivo* is further compounded by macromolecular crowding, increasing the occurrence of misfolding, and, therefore, aggregation (Balchin, Hayer-Hartl and Hartl, 2016).

Protein aggregation has the potential for pathogenesis (further discussed below), and therefore, many organisms, from bacteria to humans, have developed mechanisms to prevent aggregate formation and accumulation (Johnston, Ward and Kopito, 1998; Alam *et al.*, 2017). The unfolded protein response (UPR) is a collection of conserved signaling pathways generated by phenomena such as accumulation of unfolded and damaged proteins in the ER, oxidative stress, damaged mitochondria, or mutations. The UPR regulates proteostasis by inducing the upregulation of a variety of proteins, including those involved in protein folding and degradation in the ER (Korolchuk, Menzies and Rubinsztein, 2010; Blasiak *et al.*, 2019), removing the accumulated protein load, and ensuring correct folding (Balchin, Hayer-Hartl and Hartl, 2016; Trigo, Nadais and da Cruz e Silva, 2019).

Molecular chaperones are proteins that interact with, stabilize, or help other proteins acquire their functionally active conformation, without being present in its final structure (Balchin, Hayer-Hartl and Hartl, 2016). Chaperone machineries are essential in allowing newly synthesized proteins to fold efficiently, promoting folding by a generic mechanism of kinetic partitioning of non-native states. When protein misfolding occurs, chaperonins, heat shock protein 70 (HSP70), and HSP90, recognize exposed hydrophobic residues (Trigo, Nadais and da Cruz e Silva, 2019). Chaperones assist folding and block aggregation, preventing abnormal intermolecular interactions that could have resulted in protein aggregation. Hence, chaperones block aggregation by sequestering misfolded or aggregation-prone folded intermediates from the cytosol, reducing the concentration of molecules prone to aggregation, thus reducing the rate of aggregation (Balchin, Hayer-

Hartl and Hartl, 2016). The subsequent protein release from the chaperone allows folding (Johnston, Ward and Kopito, 1998; Kim *et al.*, 2013; Balchin, Hayer-Hartl and Hartl, 2016).

The different chaperone classes and their cofactors cooperate with the protein synthesis and degradation machineries in a coordinated proteostasis network (Figure 3A), which must balance protein synthesis and turnover, misfolded and aggregated proteins, and respond to cellular stress (Balchin, Hayer-Hartl and Hartl, 2016), therefore, these molecules have a major role in protein quality control and proteostasis maintenance (Gamerdinger *et al.*, 2009).

The UPR is primarily adaptive and aims to restore proteostasis, but if the UPR system is overwhelmed, proteins can be irreversibly damaged, and are subsequently targeted by chaperones for degradation via the proteasomal and autophagy systems (Cuervo and Wong, 2014). These are cell-wide systems which are complemented by organelle-specific ones, found in the nucleus, ER, and mitochondria (Rosenbaum and Gardner, 2011). Misfolded proteins are transported along the cytoskeleton to the area surrounding the nucleus, where other cellular components such as chaperones, proteasome components, and motor proteins accumulate. While this is an important mechanism against accumulation of toxic aggregates, the aggresome can be toxic and cause apoptosis, depending on its contents (Trigo, Nadais and da Cruz e Silva, 2019).

1.3.1. Proteasome Degradation

Protein quality control is one of the ER's primary functions, and there are several signaling pathways associated to that control, thus, only properly folded proteins are packaged into cell exit vesicles and allowed to move to the cell surface (Walter and Ron, 2011). The proteasome pathway degrades misfolded cytoplasmic proteins, as well as membrane and secretory proteins unable to fold in the ER (Kopito, 1997; Johnston, Ward and Kopito, 1998). Many proteins termed 'misfolded beyond repair' are first removed from the ER via delivery to a transmembrane complex that coordinates their dislocation to the proteasome for degradation by the UPS, a process known as ER-associated

degradation (ERAD) pathway (Walter and Ron, 2011), which plays an important role in proteostasis maintenance (Figure 3A) (Balchin, Hayer-Hartl and Hartl, 2016).

Most intracellular protein degradation is performed by the proteasome (Lilienbaum, 2013). The proteasome is a protein complex that consists of a catalytic core, also known as the 20S proteasome, and one or two terminal 19S regulatory particles, that serve as

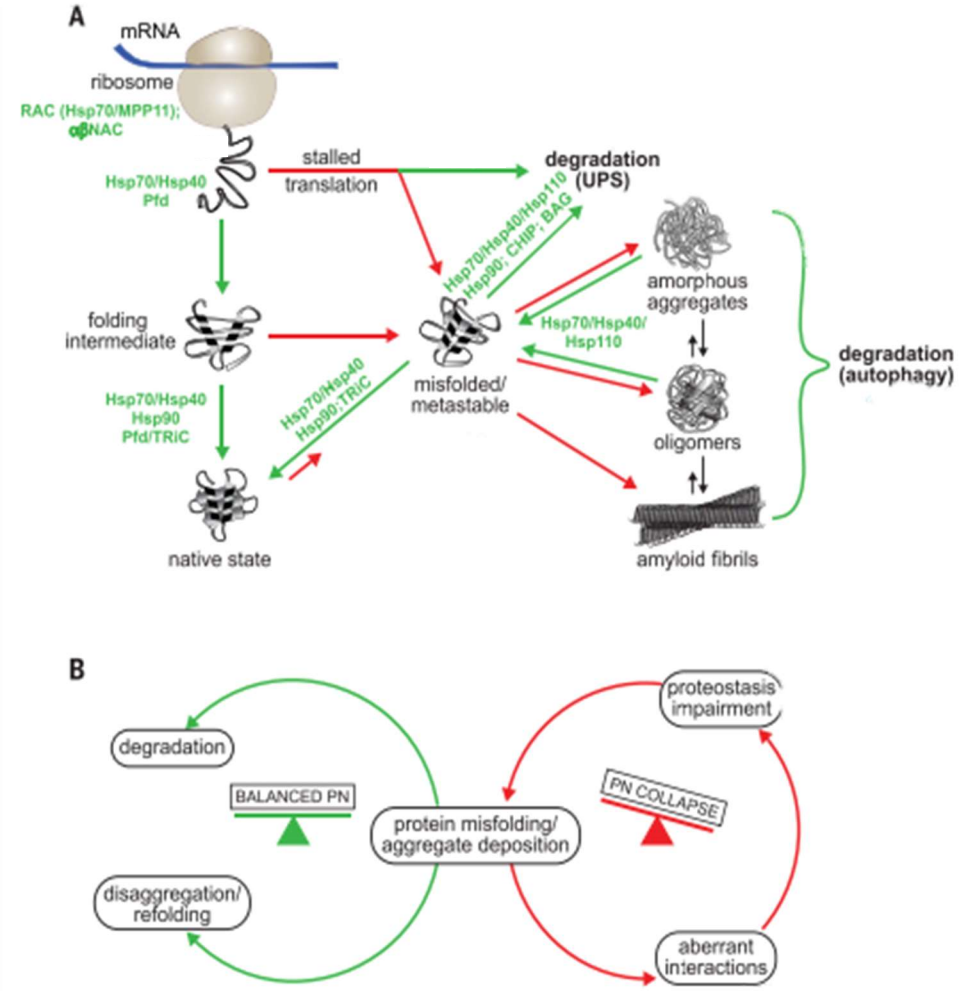

Figure 3 - The proteostasis network. (A) An extensive network of molecular chaperones and other factors are employed by cells to maintain proteostasis. The components listed participate in promoting the folding of newly synthesized proteins, destabilizing non-native protein conformations, and cooperating with degradation machineries, such as the ubiquitin proteasome system and autophagy. Pathways promoted by chaperones are represented by the green arrows, and off-pathway reactions with red arrows. **(B)** A balanced vs. a non-balanced proteostasis network. Protein misfolding and aggregate formation are shown as key processes prone to destabilizing the proteostasis network. Green circle: the proteostasis network at full capacity copes with misfolded proteins by refolding or degrading them. Red circle: the proteostasis network is overwhelmed through a positive feedback loop in which protein misfolding and aggregation lead to aberrant interactions with key proteins, resulting in a proteostasis impairment and eventual collapse. This is accelerated by age-dependent decline of proteostasis capacity and/or excessive production of aberrant proteins. PN: proteostasis network; UPS: ubiquitin proteasome system. Adapted from Balchin, Hayer-Hartl and Hartl, 2016.

proteasome activators (Korolchuk, Menzies and Rubinsztein, 2010). The 20S proteasome consists of 28 subunits arranged as four stacked rings; the two outer rings are formed by α subunits and the two inner rings by the β subunits (Bulteau *et al.*, 2007). The 19S regulatory particle binds to one or both ends of the latent 20S proteasome, forming an active 26S proteasome (Tanaka, 2009).

Prior to degradation by the UPS, proteins must be disagreggated; this task is performed by HSP70 in cooperation with HSP110 and a HSP40 complex. Protein aggregates that resist this process may be cleared for selective autophagy (Figure 3A) (Balchin, Hayer-Hartl and Hartl, 2016).

Aggregated proteins are marked for degradation by the UPS by covalent modification of a lysine residue with coordinated reactions of three enzymes: ubiquitin-activating enzyme, ubiquitin-conjugated enzyme, and ubiquitin ligases, known as E1, E2, and E3, respectively (Grumati and Dikic, 2018). E1 hydrolyzes ATP and adenylates a ubiquitin molecule, which is moved to E1's active site as another ubiquitin molecule is adenylated. The adenylated ubiquitin is relocated to a cysteine residue of E2, and then E3 recognizes the protein to be ubiquitinated and catalyzes ubiquitin transfer from E2 to the target protein (Foot, Henshall and Kumar, 2017). A protein must be tagged with at least four ubiquitin homoploymers to be recognized and transported to the proteasome (Korolchuk, Menzies and Rubinsztein, 2010).

The protein enters the 20S, into the proteolytic active site, and is deubiquitinated; the protein should be at least partially unfolded (around 20 amino acid residues) upon entering the proteasome core. Energy from ATP is necessary for subtrate unfolding, but not for translocation, and thus, the 26S proteasome can degrade unfolded proteins in the absence of ATP, but cannot degrade folded proteins (Smith *et al.*, 2005). The proteasome functions as an endoprotease, degrading proteins into oligopeptides, which are then released into the cytoplasm or nucleoplasm, where they are digested into amino acids by soluble peptidases (Korolchuk, Menzies and Rubinsztein, 2010).

The UPS mediates most intracellular proteolysis, playing an important role in regulating cellular processes such as the cell cycle, cell differentiation, and apoptosis. The

UPS regulates the levels of several cancer-related molecules, such as tumor inhibitors (i.e. P53), transcription factors (i.e. nuclear factor kappa-light-chain-enhancer of activated B cells; NF-kb), and cell cycle proteins (Guo and Peng, 2013).

Proteasome inhibition has been shown to induce apoptosis through ROS formation (Amanso, Debbas and Laurindo, 2011), as well as depletion of glutathione, a non-protein antioxidant, causing defects in mitochondrial function and leading to loss of cell viability (Han and Park, 2010). Furthermore, proteasome inhibitors cause polyubiquitinated misfolded protein accumulation, as they are recognized by histone deacetylase 6 (HDAC6) ubiquitin-binding domain. HDAC6 binds dynein motor proteins and transports the misfolded proteins along microtubules to the microtubule-organizing center, where the misfolded proteins form aggresomes. These aggresomes are sequestrated by autophagic vesicles, before lysosomal degradation (An and Statsyuk, 2015). This clearance of ubiquitinated proteins by autophagy represents an important component of the protective response to proteasome inhibition (Legesse-Miller *et al.*, 2012). At the same time, proteasome inhibition also represses UPR signaling, making cells more likely to die by apoptosis in the presence of stressors (Amanso, Debbas and Laurindo, 2011).

1.3.2. Autophagy

Autophagy is an intracellular lysosomal degradation process involved in cell growth, survival, development, and death, and has been implicated in cancers, neurodegenerative disorders, and myopathies (Kroemer, Mariño and Levine, 2010). Autophagy is induced by starvation, hypoxia, or stress (Mathew *et al.*, 2009), and is characterized by the formation of autophagosomes, which are double-membraned vesicles that sequester cytoplasm to be phagocytosed (Lilienbaum, 2013). This process sustains cell metabolism during starvation and eliminates stress-damaged proteins, as well as damaged organelles, cytosolic proteins, and invasive microbes (Mathew *et al.*, 2009; Kim *et al.*, 2018).

Autophagy can be selective or non-selective. Selective autophagy occurs to remove specific damaged or excessive organelles, or protein aggregates, while non-selective autophagy is triggered in response to starvation or nutrient deprivation, providing cells

with survival-essential amino acids and nutrients (Ding and Yin, 2012). There are three main types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Figure 4).

In macroautophagy, the main autophagy pathway, the autophagosome encloses expendable cytoplasmic components and organelles, along with a region of the cytosol, isolating them from the rest of the cell (Lilienbaum, 2013). The autophagosome later fuses with an available lysosome, where its contents are later degraded by acidic lysosomal hydrolase and recycled (Xie and Klionsky, 2007; Mizushima, Yoshimori and Ohsumi, 2011). During the process of microautophagy, however, the lysosomal membrane directly engulfs parts of the cytoplasmic material into the lysosome by invagination (Mizushima, Yoshimori and Ohsumi, 2011). Many facets of this process remain largely unknown.

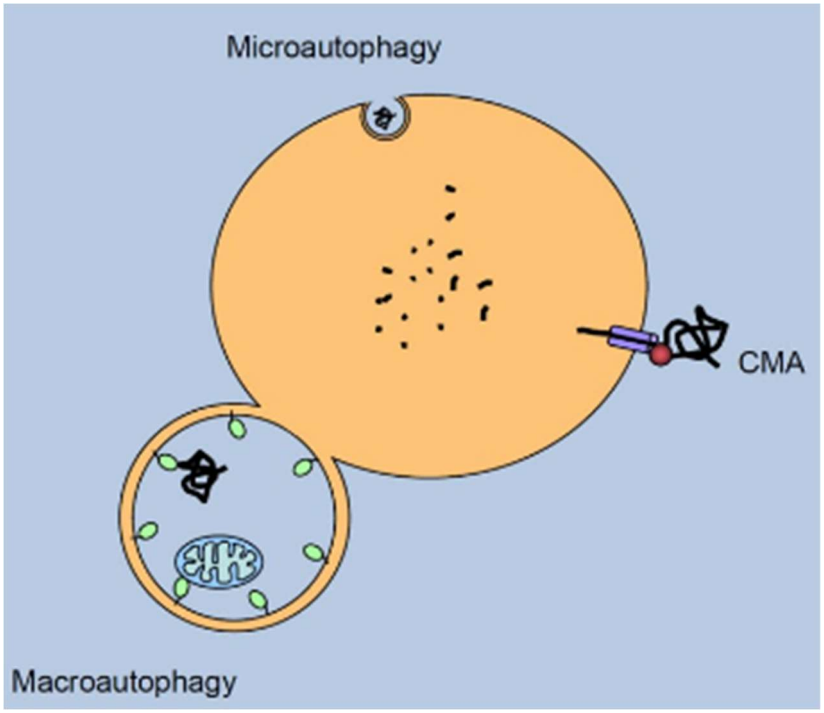

Figure 4 - The three types of autophagic pathways. In microautophagy, the lysosomal membrane invaginates a small portion of cytosol, along with its contents. In chaperone-mediated autophagy (CMA), a targeting motif in substrate proteins is recognized by a cytosolic chaperone (red circle), that delivers it to a lysosomal receptor, which multimerizes to form a translocation complex that mediates the translocation of the protein into the lysosome lumen. In macroautophagy, a double membrane vesicle sequesters cargo proteins and a region of the cytosol, then fusing with the lysosome for cargo delivery. Proteins, as well as other macromolecules, are rapidly degraded in the lysosomal lumen by enzymes. Taken from Lilienbaum, 2013.

CMA is a very complex and specific pathway, distinct from the others, as there is no vesicle formation or membrane invagination, rather, a single client molecule is directly transported across the lysosomal membrane for degradation (Kettern *et al.*, 2010). The molecules to be degraded by this pathway contain a lysosomal-targeting motif recognized directly by heat shock cognate 71 kDa protein (Hsc70), a member of the HSP70 protein family (Mayer and Bukau, 2005). The chaperone-client complex moves to the lysosomal membrane, where it binds to the membrane protein LAMP-2A (lysosome-associated membrane protein 2A), initiating the formation of a translocase within the membrane for molecule transfer (Kettern *et al.*, 2010). Translocation is facilitated by Hsc70 molecules present in the lysosomal lumen, but regulation of Hsc70 activity within the CMA process is not yet entirely described. CMA is activated during stress situations, such as prolonged starvation, oxidation, and any conditions that result in protein damage (Dice, 2007). CMA plays a major role in the degradation of oxidatively damaged proteins formed in aged cells, namely, CMA mediates the degradation of α-synuclein in neuronal cells, an amyloid aggregate associated to PD (Cuervo *et al.*, 1996).

In disease, autophagy is seen as an adaptive response to stress by promoting cellular survival; CMA activity decreases with age, which apparently due to the decrease in LAMP-2A receptor levels in older cells. The decreased cellular ability to remove damaged proteins through the CMA pathway may contribute significantly to the pathology of amyloid diseases, as well as contribute to the breakdown of proteostasis with age (Kettern *et al.*, 2010).

Autophagy can play a crucial role in the maintenance of intracellular ROS levels, as oxidative stress can induce autophagy. Free radicals, in the form of ROS or RNS (reactive nitrogen species) are a side product of healthy mitochondrial respiratory activity, and may also be formed in response to xenobiotics, cytokines, or bacterial invasion (Pajares *et al.*, 2018). There is a close relationship between signaling, mitochondria, and mitophagy, which consists in the selective removal of damaged mitochondria through autophagy (Ding and Yin, 2012). Redox signalling affects autophagy, due to free radical-induced modification of key autophagy regulators such as the Parkin protein, which acts as an E3-ubiquitin ligase, ubiquitinating cytosolic and outer mitochondrial membrane proteins

upon mitochondrial depolarization, mediating their autophagic and proteasomal degradation; meanwhile, chronic oxidative stress may lead to transcriptional activation of CMA via LAMP-2A (Pajares *et al.*, 2018). Moreover, oxidative stress triggers depolarization of the inner mitochondrial membrane, impairing oxidative phosphorylation. Damaged mitochondria produce ROS, especially superoxide anions (O^{2-}) and hydrogen peroxide (H_2O_2), which, in turn, further accelerate ROS generation (Park, Lee and Choi, 2011).

Prolonged autophagy may result in the degradation of essential proteins and organelles, as well as cell death. At the same time, although cell death is often accompanied by high autophagosome levels, indicating that dying cells often induce autophagy beforehand, there is no causal relationship between autophagy and apoptosis. When trying to survive a certain extracellular stressor, cells may increase their autophagy levels, and, if this effort fails, there is cell death (Denton, Nicolson and Kumar, 2012). Macromolecule autophagic degradation protects the cells from nutrient deprivation, providing the nutrients and energy needed to maintain the functions required for survival during starvation, thus, activation of the cell death pathway is not always required (Yonekawa and Thorburn, 2013).

1.3.2.1. p62/SQSTM1

Ubiquitin-binding protein p62, or sequestosome 1, referred to as p62 or SQSTM1, is a multifunctional protein conserved among metazoans, encoded by the SQSTM1 gene in humans (Clausen *et al.*, 2010; Katsuragi, Ichimura and Komatsu, 2015). p62 is an autophagy substrate that also acts as a cargo receptor for autophagic degradation of ubiquitinated target proteins (Jain *et al.*, 2010).

p62 is mainly located in the cytoplasm, but it is also found in the nucleus, autophagosomes and lysosomes; additionally, in response to stress, p62 is translocated to autophagy substrates, such as protein aggregates or damaged mitochondria (Katsuragi, Ichimura and Komatsu, 2015). p62 is particularly relevant to aggregation research, due to its role as a selective autophagy receptor for the degradation of ubiquitinated substrates

(Katsuragi, Ichimura and Komatsu, 2015), targeting autophagy substrates by binding to them for selective autophagy (Behl, 2016).

Autophagy selectivity is controlled by autophagy receptors, and the understanding of the autophagy process has developed due to the discovery of autophagy adaptors such as p62/SQSTM1, and their role as substrate receptors and functional interfaces for degradation, as well as their interaction with MAP1LC3/LC3 (microtubule-associated protein light chain 3), a protein present at the autophagosome membrane (Rogov *et al.*, 2014; Behl, 2016). Thus, p62 levels can be used to monitor autophagy (Kim *et al.*, 2018).

p62 is comprised of several domains (Figure 5), including a C-terminal ubiquitin-associated (UBA) domain, that binds specifically to Lys-63-linked polyubiquitin chains of polyubiquitinated substrates (Ciani *et al.*, 2003), as well as an N-terminal PB1 domain that interacts with atypical protein kinase Cs, and an LC3-interacting region (LIR) (Clausen *et al.*, 2010). Hence, p62 binds ubiquitin by its UBA domain, self-polymerizes via its PB1 domain, and binds to LC3 through the LIR motif acidic cluster and Trp338 and Leu341 residues, facilitating the sequestration of ubiquitinated protein aggregates into p62-bodies to be degraded by autophagy (Bjørkøy *et al.*, 2005; Clausen *et al.*, 2010). p62 self-

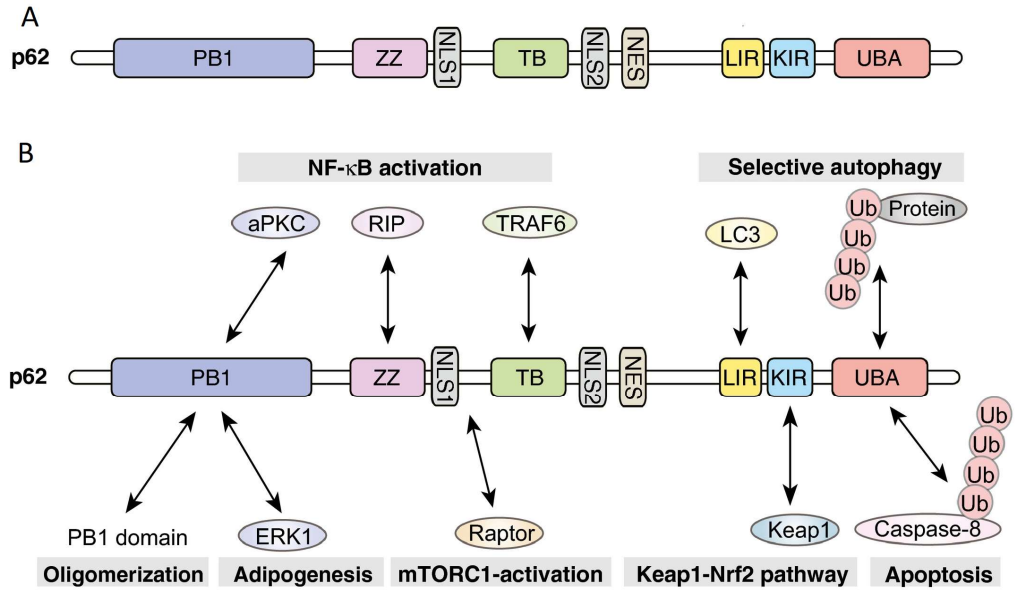

Figure 5 - p62 domain structure and interactions. (A) p62 protein domains; **(B)** p62 domain interactions with other proteins. The PB1 domain can self-oligomerize and form hetero-oligomers with other proteins with a PB1 domain, such as Nbr1. The ZZ domain, a Zinc-finger domain, and TRAF6-binding domain (TB) associate with RIP and TRAF6, respectively. LC3 recognizes p62 through the LIR domain. UBA domain can bind polyubiquitin chains. Adapted from Katsuragi, Ichimura and Komatsu, 2015.

oligomerizes via the PB1 domain in order to promote delivery of ubiquitinated cargo to the autophagy pathway (Katsuragi, Ichimura and Komatsu, 2015); PB1 is also able to form heteropolymers, by binding to other proteins containing this same domain (Bjørkøy *et al.*, 2005). Furthermore, p62 induces caspase-8 aggregation, which promotes its activation and triggers apoptosis (Yan *et al.*, 2019). The UBA domain, too, tends to form a stable intermolecular homodimer, consequently hindering the recognition and sorting of ubiquitinated conjugates for autophagic degradation (Peng *et al.*, 2017).

The LC3 protein plays several roles in the autophagy process, such as membrane fusion, cargo selection, and autophagosome transport (Slobodkin and Elazar, 2013). Thus, p62 binds to both ubiquitin and LC3, meaning that p62 is both a selective autophagy substrate and a cargo receptor for protein degradation by autophagy (Jain *et al.*, 2010). The interaction between LIR and LC3 is essential for the autophagic degradation of p62, although it does not contribute for p62 translocation on the autophagosome formation site, so upstream factors may be responsible for p62 assembly around the autophagosome formation site, prior to the formation of the isolation membrane (Itakura and Mizushima, 2011; Katsuragi, Ichimura and Komatsu, 2015).

Despite its important role in the recruitment of ubiquitinated proteins for autophagy, loss of p62 does not have a relevant effect on selective autophagy, which is explained by the presence of autophagy receptors, such as neighbor of BRCA1 gene 1 (NBR1), nuclear domain 10 protein (NDP52), and optineurin, which also possess the LIR motif that allows binding to LC3 (Rogov *et al.*, 2014; Peng *et al.*, 2017). Still, p62 plays an indispensable role in selective autophagy, not only during packaging of ubiquitinated aggregates, but also on the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates the expression of antioxidant proteins (Gold *et al.*, 2012). Although both p62 and NBR1 are receptors for selective autophagy, they also become autophagy substrates through their interaction with LC3 (Clausen *et al.*, 2010).

p62 is a stress response protein that is upregulated at the mRNA and protein levels upon exposure to oxidative stress. KEAP1 (kelch-like ECH-associated protein 1) is an E3 ligase adaptor protein which interacts with Nfr2 in one of the main cellular defense

mechanisms against oxidative stress (Jain *et al.*, 2010). Under normal conditions, Nrf2 binds to a conserved antioxidant response element of KEAP1, leading to Nrf1 ubiquitination and proteasomal degradation. However, under oxidative stress conditions, KEAP1 cysteine residues are modified by ROS and RNS, blocking Nrf2 ubiquitination and increasing Nrf2 levels; p62 sequesters KEAP1, thus, it can no longer bind to Nrf2, resulting in Nrf2 stabilization and relocation to the nucleus, inducing various cytoprotective genes through its heterodimerization with small Maf proteins (Bjørkøy *et al.*, 2005; Jiang *et al.*, 2015). This could be characteristic of pathological conditions, as constitutive activation of Nrf2 is known to promote carcinogenesis (Jain *et al.*, 2010; Komatsu *et al.*, 2010).

Autophagy is responsible for p62 degradation, therefore, autophagy impairment is followed by p62 accumulation and formation of aggregates positive for p62 and ubiquitin (Rogov *et al.*, 2014), thus, p62 is widely used as an autophagy marker (Lynch-Day *et al.*, 2012). Furthermore, ubiquitin and p62-positive aggregates, also named p62 bodies, or aggresome-like inducible structures (ALIS), may also form under cellular stress conditions, such as amino acid starvation, oxidative stress, proteasomal inhibition, or autophagy inhibition (Komatsu *et al.*, 2007; Clausen *et al.*, 2010; Peng *et al.*, 2017).

p62 upregulation and accumulation derived from stress-induced proteasome dysfunction implies that p62 may have a neuroprotective function (Kuusisto, Salminen and Alafuzoff, 2001). If so, overexpressed p62 may rescue cells from aggregate-induced cell death (Nagaoka *et al.*, 2004). Since the UBA domain binds noncovalently to ubiquitin, p62 could be recruited to ubiquitin-rich aggregates formed in neurodegenerative diseases, due to its ability to bind to polyubiquitin (Donaldson *et al.*, 2003). It has been speculated that, despite p62 upregulation in response to stress, the protein may lose its neuroprotective function, thus contributing to neurodegeneration. p62 accumulation in aggresomes or inclusions is one of the pathological processes behind some neurodegenerative disorders (Nagaoka *et al.*, 2004); p62 is often a component of protein aggregates found in protein aggregation diseases, such as Lewy bodies in PD, neurofibrillary tangles (NFTs) in AD, and huntingtin aggregates in HD (Kuusisto, Salminen and Alafuzoff, 2001; Zatloukal *et al.*, 2002). If p62-positive aggregates are not eliminated by autophagy and cell apoptosis does not occur, the high accumulation of p62 bodies may

also act as a tumor-promoting factors due to increased oxidative stress response, induction of inflammation and cytokine production (Mathew *et al.*, 2009).

If the cellular degradative systems fail to restore proteostasis, the UPR may trigger cell death, in a process associated with the pathogenesis of protein misfolding and aggregation-dependent diseases. The reason there is aggregate formation when cellular machineries are in place to recognize and degrade misfolded proteins and to deliver aggregates to cytoplasmic inclusions, is not yet known (Alam *et al.*, 2017; Trigo, Nadais and da Cruz e Silva, 2019). Although autophagy is seen as an adaptive response to nutrient deprivation, development, aging, or cell death, there is increasing evidence that this process is essential for neuronal survival, as a primary protective mechanism maintaining homeostasis in response to stress. Autophagy deregulation can potentially lead to the accumulation of abnormal proteins, commonly observed in neurodegenerative disorders (Lynch-Day *et al.*, 2012).

1.4. Aging

Protein folding errors may result from single, severe factors, or from the combination of various smaller conditions, which, on their own, would not be inherently overwhelming to the cell, such as mutations, errors in protein biogenesis (i.e. translational errors), environmental stress conditions, and, particularly, aging (Tyedmers, Mogk and Bukau, 2010).

Aging is a fundamental factor of life. Aging is generally understood as a time-dependent deterioration of physiological parameters of the organism, including diminished fitness, decreased reproduction rate, and an eventual exponential increase in mortality with time at the population level (Lindner and Demarez, 2009). The process of physiological decline leading to death of the individual is driven by deteriorated or altered functions at the tissue or cellular level, resulting in damage accumulation with age (Bitto *et al.*, 2014). Age-related impairments are mostly due to loss of cellular homeostasis, resulting in the accumulation of dysfunctional organelles and damaged macromolecules,

such as proteins, lipids, and nucleic acids. These physiological alterations are hallmarks of the aging process (Lindner and Demarez, 2009).

As cells and organisms age, respiratory chain efficiency tends to decrease, increasing electron leakage and reducing ATP generation. Oxidative phosphorylation in mitochondria encompasses electron transfer between the respiratory chain complexes I through IV, allowing the transport of protons across the mitochondrial membrane, creating a membrane potential. Proton flow across the gradient generated by electron transfer drives ATP synthase activity, which catalyzes ATP synthesis (Payne and Chinnery, 2015). Although oxidative phosphorylation is an efficient mechanism, there can be electron leakage from the electron transport chain during normal respiration, forming ROS (Liu, Fiskum and Schubert, 2002); while ROS are important signaling molecules, there is a threshold beyond which ROS become harmful (Handy and Loscalzo, 2012; Schieber and Chandel, 2014). Since the electron transport chain is the main producer of ROS, and 90% of all ROS production in mammal cells occurs in mitochondria, it has been suggested that mitochondria is a target for oxidative damage (Bratic and Trifunovic, 2010). Dysfunctional mitochondria can contribute to aging, regardless of ROS levels, by increased mitochondria permeabilization tendency in response to stress, affecting apoptotic signalling, as well as by triggering inflammatory reactions by favouring activation of inflammasomes (Green, Galluzzi and Kroemer, 2011; López-Otín *et al.*, 2013).

Mitochondrial loss of efficiency is both a cause and consequence of aging, resulting from several processes, such as reduced biogenesis of mitochondria, telomere attrition, accumulation of mtDNA mutations and deletions, oxidation of mitochondrial proteins, defective quality control by mitophagy, or an organelle-specific type of macroautophagy targeting mitochondria for proteolytic destruction (Boveris and Navarro, 2008; Wang and Klionsky, 2011). Moreover, due to the brain's elevated energy demands, this organ is especially susceptible to mitochondria dysfunction, making it one of the known mechanisms behind several neuropathologies (Trigo, Nadais and da Cruz e Silva, 2019).

Proteins have a dynamic structure, and protein homeostasis requires continuous surveillance. Cellular quality control mechanisms are in place to preserve their functionality and to stabilize folded proteins (predominantly done by proteins of the HSP family), as well as for misfolded protein restoration or degradation (by the proteasome and lysosome), preventing the accumulation and aggregation of damaged cellular components and assuring protein renewal (Miller *et al.*, 2015; Dikic, 2017). These mechanisms decline during aging, deregulating the proteostasis network, namely the UPR pathway (Brown and Naidoo, 2012), as well as cellular proteasome activity (Anselmi *et al.*, 1998; Keller, Hanni and Markesbery, 2001) and the CMA pathway (Cuervo and Dice, 2000).

Stressors, both endogenous and exogenous, cause protein unfolding and deteriorate proper folding during protein synthesis. Stress-induced synthesis of chaperones is significantly impaired in aging, and chaperone decline has been found to have a causative effect on longevity (López-Otín *et al.*, 2013). Furthermore, aged cells experience extended cumulative oxidative stress, leading to an increase in transcriptional and translational errors and impaired protein degradation (Gidalevitz, Kikis and Morimoto, 2010). Protein oxidation during aging has been theorized to prevent degradation, and increased debris and damaged proteins could overwork the phagocytic machinery (Brunk and Terman, 2002). The failure of all these processes may lead to an increase in misfolded protein formation and a decrease in their degradation, ultimately increasing their accumulation with age (Nowotny *et al.*, 2014).

Protein aggregation is very closely related to aging, and aging is the main risk factor for the development of neurodegenerative disorders such as AD, HD, and PD, mainly due to the age-related reduced capacity to deal with misfolded proteins (Trigo, Nadais and da Cruz e Silva, 2019). However, it is still debated whether protein aggregation is a major contributor for age-related disorders, or if they are normal physiological consequences of aging. The cause-consequence relationship between aggregation and aging is not yet clear.

1.5. Aggregation Connected to Pathogenesis

The autophagy and proteasome cellular control systems, which detect and destroy misfolded and partially folded proteins, are of extreme importance to the cell, but, at the same time, are not infallible. If these processes fail, there may be an increase in protein aggregation, possibly leading to protein-misfolding disorders (Chaturvedi *et al.*, 2016; Wechalekar, Gillmore and Hawkins, 2016; Alam *et al.*, 2017) (Table 1).Advanced age is characterized by a greater rate of several chronic diseases, harming the quality of life of these individuals, as well as their families, posing a problem for healthcare systems worldwide (Bitto *et al.*, 2014). Therefore, there has been growing interest in protein aggregation-associated disorders, since, these are some of the most currently prevailing and devastating medical conditions, due to their connection with aging (i.e. AD, PD) and lifestyle (i.e. type-II diabetes) (Trigo, Nadais and da Cruz e Silva, 2019).

Neurodegenerative diseases are the most typical pathologies associated with protein aggregation, and the number of cases is constantly increasing. For instance, 50 million people worldwide are living with dementia, two thirds of which suffer from AD, a number expected to triple by 2050 (Alzheimer's Disease International, 2018). In Europe, the population aged over 65 years old will increase from 15.4% to 22.4%, which will directly influence the prevalence of AD (Katzman, 1986; Kumar *et al.*, 2016). PD's worldwide prevalence has more than doubled over the last 26 years, from 2.5 million patients in 1990 to 6.1 million in 2016 (Ray Dorsey *et al.*, 2018), a natural consequence of the aging population resultant from prolonged life expectancy. This trend is expected to continue over the next decades, as studies predict the number of PD patients to increase to 12 million by 2050 (Rocca, 2018).Disease-associated proteins often accumulate toxic oligomers that expose hydrophobic amino acid residues and unpaired β-strands, which confer oligomers the ability to harmfully interact with multiple key proteins (Breydo and Uversky, 2015). These proteins are typically rich in unstructured regions and sequences of low complexity, which are also characteristic of many RNA-binding proteins (Olzscha *et al.*, 2011). In addition to protein degradation impairment, toxic protein aggregation interferes with interferes with nucleocytoplasmic transport of RNA and proteins and decreases RNA homeostasis (Balchin, Hayer-Hartl and Hartl, 2016). Age-dependent

Table 1 - List of main protein aggregation-associated disorders and their characteristic pathology, proteins involved, and tissues affected. SOD1: Superoxide dismutase 1; TTR: Transthyretin. Adapted from Trigo et al., 2019 and Kumar et al., 2016.

	Disease	*Characteristics*	*Protein/Peptide*	*Most affected tissues*
Neurodegenerative disorders	Alzheimer's disease	Senile plaques	Aβ 1-40 or 1-42 peptide	Cortex, hippocampus, basal forebrain, brain stem
		Neurofibrillary tangles	Tau	
		Lewy bodies	α-synuclein	
	Parkinson's disease	Lewy bodies and neurites	α-synuclein, Parkin (DJ-1, PINK1)	Substantia nigra, cortex matter, and brain stem
	Huntington's disease	Intranuclear inclusions, cytoplasmatic aggregates	Huntingtin with polyQ expansion	Cortex, hippocampus white matter, amygdala, thalamus
	Prion Diseases (Creutzfeldt–Jakob disease)	Spongiform degeneration, amyloid	Prion protein	Cortex, cerebellum, thalamus
	Tauopathies (fontotemporal dementia, frontotemporal lobar degeneration)	Pick bodies, stress granules	Tau	Frontotemporal cortex, hippocampus
	Amyotropic Lateral Sclerosis	Bunina bodies, axonal spheroids, stress granules	Superoxide dismutase (SOD1), ubiquitin	Spinal cord, cortical upper motor neurons, motor cortex
Nonneuropathic system amyloidosis	Senile Systemic amyloidosis	TTR deposits	Wild-type transthyretin	Atria, cardiac ventricules, lungs, etc
	Familial amyloid polyneuropathy			Heart, eyes, kidney
	Familial amyloid cardiomyopathy			Heart, atria

decline of proteostasis is associated to several neurodegenerative disorders which feature protein aggregation and misfolding, with intra- or extracellular accumulation of β-amyloid (Aβ) and tau in AD, α-synuclein in PD, superoxide dismutase in amyotrophic lateral sclerosis (ALS), and huntingtin in HD (Taylor, Hardy and Fischbeck, 2002; Ross and Poirier, 2004; Balchin, Hayer-Hartl and Hartl, 2016). Nevertheless, even though there is a clear correlation between aggregation and disease, causality has not yet been established (Trigo, Nadais and da Cruz e Silva, 2019).

Neurodegenerative disorders are generally characterized by selective neuronal loss, synaptic alterations, and neuroinflammation. Depending on the disorder, specific brain regions are affected (Table 1) (Martin, 1999; Kumar *et al.*, 2016). In ALS, there is degeneration of lower motor neurons in the spinal cord and brainstem, as well as loss of upper motor neurons in the motor cortex (Taylor *et al.*, 2016). HD is characterized by severe neuronal loss in the striatum and cerebral cortex (Myers *et al.*, 1991; Ross and Poirier, 2004).

Neuronal death in AD occurs mainly in the hippocampus, amygdala, and regions of the brain related to memory and thinking (Martin, 1999). PD is characterized by neuronal

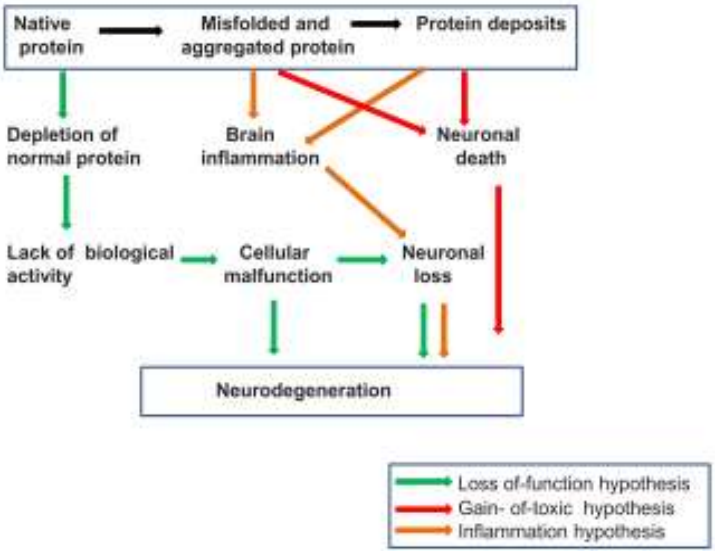

Figure 6 – Three hypotheses for the association of protein misfolding and aggregation with neurodegeneration. In the loss of function theory, the loss of normal protein activity is the focal point; in the gain of toxic function theory, the aggregated protein becomes toxic; in the inflammation model, neuronal death is indirectly mediated by activation of astroglia cells. In many diseases, these mechanisms might function together. Taken from Kumar et al., 2016.

loss by programmed cell death or apoptosis, mostly in the substantia nigra (Kumar *et al.*, 2016), along with depletion of dopamine in the striatum and cortex (Forno, 1996).

There are three suggested hypotheses to explain how protein misfolding and aggregation lead to neurodegeneration: loss of function, gain of function, and brain inflammation hypotheses (Figure 6). These mechanisms are not mutually exclusive and might even function together.

The loss of function hypothesis theorizes that neuronal death is caused by depleted activity of the misfolded and/or aggregated proteins, thus, leading to a lack of biological activity and cellular malfunction (Kumar *et al.*, 2016).

The gain of function hypothesis suggests that misfolding and aggregation of proteins results in the gain of neurotoxic function (Kumar *et al.*, 2016), and *in vitro* neuronal cell death caused by aggregates or misfolded proteins has been described in several studies (Loo *et al.*, 1993; El-Agnaf *et al.*, 1998; Lunkes and Mandel, 1998). In summary, aggregates produce ROS, causing oxidative stress, subsequently causing protein and lipid oxidation, increased intracellular calcium, and mitochondrial deterioration (Kumar *et al.*, 2016). Extracellular aggregates interact with specific cellular receptors to induce apoptosis (Yan *et al.*, 2000); while intracellular aggregates recruit cell viability factors into the fibrillar aggregates, damaging the cells (Cummings *et al.*, 1998).

According to the brain inflammation hypothesis, neurodegeneration is caused by an chronic inflammatory reaction in the brain triggered by protein aggregates (Wyss-Coray and Mucke, 2002). This has been observed experimentally, by widespread astrocytosis and microglial activation (Muhleisen, Gehrmann and Meyermann, 1995; Sapp *et al.*, 2001), as well as increased inflammatory proteins in the brain (Xia and Hyman, 1999), accumulation of inflammatory proteins in cerebral protein aggregates (McGeer and McGeer, 1995). Additionally, the experimental treatment with non-steroidal anti-inflammatory drugs resulted in a decreased incidence of AD in humans and animal models (Yates *et al.*, 2000; Vlad *et al.*, 2008; Benito-León *et al.*, 2019).

Furthermore, post-translational modifications such as hyperphosphorylation, acetylation, glycation, nitration, and truncation, have been associated with protein

aggregation, particularly with amyloidosis (Gandhi *et al.*, 2019). Disorders such as AD, PD, HD, and amyotrophic lateral sclerosis (ALS), develop at least partly due to specific misfolded or aggregated proteins (Table 1). Aggregation of misfolded proteins and their deposition in certain brain regions lead to amyloidosis of the central nervous system, during which highly soluble proteins are gradually converted into insoluble, filamentous polymers with a cross β-sheet structure. These structures accumulate as amyloid fibrils, which later deposit in the nucleus or cytoplasm of the affected brain cells or in the extracellular matrix (Kumar *et al.*, 2016).

1.6. Fibroblasts as Aging Study Models

In the present work, primary skin fibroblast cell cultures were used as a cell model for individuals at different life stages. Fibroblasts are the most common animal connective tissue cells, which secrete the components of the extracellular matrix and stroma, as well as collagen, glycoaminoglycans, and proteoglycans (Alberts, Johnson and Lewis, 2002). There are many different types of fibroblasts located in all tissues of the body, such as lungs, eyes, heart, liver, and, most notably, the skin (Chang *et al.*, 2002). Fibroblasts are morphologically heterogenous, depending on their location and activity, generally having a branched cytoplasm and an elliptical, speckled nucleus, as well as two or more nucleoli (Lo *et al.*, 2014).

Based on the work of López-Otín *et al* (2013) at establishing the hallmarks of aging, Tigges *et al.* (2014) adapted and created a set of hallmarks of fibroblast aging, among which are loss of proteostasis and mitochondrial damage and dysfunction. Loss of proteostasis manifests itself in the form of proteasome decline, decreased autophagy, induction of secreted matrix metalloproteinases; downregulation of hyaluronic acid synthases, while mitochondrial damage and dysfunction cause a decline in respiratory capacity, imbalanced ROS levels, and accumulation of mtDNA mutations, as well as altered mitochondrial fusion/fission equilibrium (Tigges *et al.*, 2014).

Loss of proteostasis, mitochondrial dysfunction, and increase in ROS levels are directly associated to protein aggregation and autophagy (López-Otín *et al.*, 2013; Hou *et*

al., 2019). There is an accumulation of oxidized proteins during skin aging, which need to be removed by the proteasome, and/or by autophagy (Kaushik and Cuervo, 2015). Proteasome activity decreases in fibroblasts during aging, which may be due to a decreased expression or inactivation of the proteasome subunits and the accumulation of inhibitors (Bulteau *et al.*, 2007). The age-related decline in proteasome activity may cause fibroblast senescence, as well as skin aging. Meanwhile, proteasome activity levels appear to be heterogenous in aged fibroblasts (Kozie *et al.*, 2011). On the other hand, CMA removal of defective macromolecules and macroautophagy-mediated removal of dysfunctional mitochondria are believed to be crucial in aging (Lemasters, 2005). Autophagy, like proteasome activity, decreases with age in many tissues, such as brain, heart, kidney, and muscle (Rubinsztein, Mariño and Kroemer, 2011), in large part due to post-translational modifications, such as acetylation/deacetylation of autophagy proteins (Morselli *et al.*, 2011).

Mitochondrial dysfunction, along with a declining respiratory chain activity, is a hallmark of aging (López-Otín *et al.*, 2013). The role of cell respiration in the aging process of dermal fibroblasts is not clear (Tigges *et al.*, 2014). ROS balance in aged tissues is affected by increased ROS leakage from dysfunctional electron transport chains within mitochondrial cell respiration, and by age-related reduction of antioxidative capacity (Balaban, Nemoto and Finkel, 2005).

In the present study, fibroblast cells from individuals of different age groups will be subjected to several extracellular stressors. These models will be used in order to demonstrate the effect of extracellular insults on the cellular autophagy response, and at the same time study the influence of age on this response.

2. Objectives

2. **Objectives**

Autophagy is central in cellular proteostasis and overall cell well-being. This study aims to use the p62 protein as a marker of autophagy under several environmental conditions, and the influence of aging on the cellular response to those same stressors. The underlying hypothesis is that p62 is a good biomarker for autophagy and can be used as a tool to monitor age-dependent cellular responses to extracellular stressors.

Thus, fibroblasts of several age groups were used, representing different aging stages, namely:

- 21 years old: young individuals;
- 41 years old: intermediate aging;
- 69 and 80 years old: aged individuals.

At the same time, cells of all the above age groups were subjected to several different treatments, representative of several environmental conditions:

- Proteasome inhibition (MG132);
- Starvation;
- Proteasome inhibition + starvation;
- Mitochondria membrane depolarization (oligomycin);
- Proteasome inhibition + mitochondria membrane depolarization;
- Antioxidation (catechin);
- Proteasome inhibition + antioxidation.

Thus, the main aims of this study were:

1. Understand how extracellular stressors (proteasome inhibition, starvation, mitochondrial membrane depolarization, or antioxidation) modulate the autophagy response on fibroblasts of different age groups;
2. Understand the influence of aging on the cellular response to stressors.

3. Material and Methods

3. Material and Methods

3.1. Cell Culture Maintenance

Fibroblast cultures were obtained from commercially available stocks. The cells were collected from the skin of the abdomen of human female donors of different age groups representative of different aging stages:

- 21, 24, and 25 years old: young adults;
- 41 years old: intermediate aging;
- 69 and 80 years old: aged individuals.

The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, cultured with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 15% fetal bovine serum (FBS), until reaching a maximum of 15 passages.

Cells were treated for 24 hours with MG132 (5 µM), starvation (DMEM without FBS supplementation), oligomycin (5 nM) and catechin (100 µM). Some experiments were performed by co-treating cells with MG132 together with either oligomycin or catechin.

3.2. Viability Assay

Cell viability was verified by the resazurin cell viability assay, using a commercially available kit (ab129732; Abcam). This assay can be used to assess mammalian cell toxicity, viability, migration, and invasion, while keeping cells intact, which allows other parallel analyses (Abcam, 2019).

Resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is often used as an indicator of cell viability. Since oxido-reductase reactions happen in live cells, the reduction of resazurin correlates with the number of live cells. The transference of electrons from NADPH to resazurin reduces the blue resazurin to a pink fluorescent counterpart, resorufin. Resazurin dye was added to the cells plated in a 96-well plate and

incubated at 37 °C for 4 hours and read by fluorescence of absorbance at 562 nm using a microplate reader (Infinite M200, TECAN)

MG132 was used as indicated by manufacturers. Oligomycin and catechin were tested in the concentrations used for cell treatments, namely, 5 nM oligomycin and 100 µM catechin.

3.3. Cell Lysis

Following treatment, cells were lysed (Abcam, 2016). In brief, cells were washed twice with ice-cold phosphate-buffered saline (PBS), following which ice cold radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) with protease inhibitor cocktail (Roche) was added to the cells and left at 4 °C for 5 minutes. RIPA contains sodium dodecyl sulfate (SDS), which is a strong ionic detergent used to solubilize lipids and proteins present in the cell membrane, creating pores, leading to full cell lysis. Cold plastic cell scrappers were used to remove adherent cells off the dish. Cells were then transferred into a pre-cooled microcentrifuge tube.

The cells were vortexed three times, ten minutes apart, always on ice. Then, they were centrifuged at 16.000 g for 20 minutes at 4 °C, to remove debris. The supernatant was placed in a new microtube, on ice, and the pellet was discarded.

3.4. Protein Quantification

After protein extraction, total protein concentration of the samples was determined, using the bicinchoninic acid (BCA) protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific), following the manufacturer's instructions. In alkaline medium, proteins reduce Cu^{2+} to Cu^{+}– the biuret reaction. This is a highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}), which reacts with two molecules of BCA, producing a purple reaction product, with strong absorbance at 562 nm that is nearly linear with increasing protein concentrations (Thermo Scientific, 2011).

Standard samples of increasing concentrations were prepared from bovine serum albumin (BSA) (Table 2), to create a protein standard curve. The assay was performed in 96-well plates. Cell samples were prepared by mixing 5 µL of lysate with 20 µL of deionized water and incubated with 200 µL of working reagent for 30 minutes, at 37 °C. After cooling, absorbance was measured at 562 nm using a microplate reader (Infinite M200, TECAN). A standard curve was calculated by plotting standard absorbance vs standard BSA concentration. All measurements were performed in duplicate.

Table 2 - Protein concentration standards used in BCA assay.

Standard	**BSA (µL)**	**H_2O (µL)**	**Protein Mass (µg)**
P0	0	25	0
P1	1	24	2
P2	2	23	4
P3	5	20	10
P4	10	15	20
P5	20	5	40

3.5. SDS-PAGE

Protein concentrations were mass-normalized and diluted in a loading buffer containing glycerol (to increase density and help the sample-loading into the wells), bromophenol blue (a dye that allows tracking of electrophoresis progress), SDS (a detergent that binds proteins to mask any inherent charge and denatures proteins to ensure they migrate by size), β-mercaptoethanol (a reducing agent that breaks inter- and intrachain disulfide bonds, linearizing the polypeptides and preventing the formation of tertiary or quaternary structures) (Morgan, 2011; Mahmood and Yang, 2012). Samples were heated for 10 minutes at 90-100 °C and spun down, in order to denature the proteins.

Protein samples were separated by molecular weight by polyacrylamide gel electrophoresis, using a resolving 5-20% acrylamide gel (lower gel), and a stacking gel with lower concentration of acrylamide (3.5%) (upper gel). Gradient resolving gels were prepared and allowed to polymerize for 1 hour at room temperature. Stacking gel was prepared and polymerized on top of the resolving gel, using a comb to shape the loading wells. The samples with loading buffer were loaded onto the gel alongside a molecular weight marker (Precision Plus Dual Color Standard, BioRad).

Protein separation was induced by an electric current, at 45 mA per gel, for 3-4 hours, using the bromophenol blue dye and molecular weight marker as visual indicators.

3.6. Protein Immunoblot

The protein immunoblot technique was performed to determine protein concentration of the cells in the different ages and treatments. In this experiment, at least three independent experiments were performed, in order to obtain an $n \geq 3$ in all experimental conditions.

This technique consists of three main steps: protein separation by molecular weight, protein transfer to a solid support, and incubation with a primary antibody specific to the target protein, followed by a secondary antibody to allow signal detection.

3.6.1. Protein Transfer

After separation in an SDS-PAGE gel, proteins were transferred by electrophoresis (200 mA) overnight (16 to 18h) from the gel to a nitrocellulose membrane

Gel loading and transfer efficiency were assessed using Ponceau S staining (Romero-Calvo *et al.*, 2010). Membranes were incubated with Ponceau stain for 5 minutes and washed with deionized water, following which they were imaged ChemiDoc Touch Imaging System (BioRad). Membranes were then washed with TBS-T 1x for 5 minutes to completely remove the stain.

3.6.2. ImmunoBlotting

Membranes were blocked with a solution of 5% bovine serum albumin (BSA) in TBS-T for 2 hours, to block non-specific binding to the primary antibody, and subsequently incubated overnight with a specific primary antibody at 4 °C, and then washed three times with TBS-T 1x for 10 minutes. Following, membranes were incubated with secondary antibody (horseradish peroxidase-linked anti-rabbit antibody) for 2 hours, and washed six times with TBS-T 1x for 5-10 minutes (Morgan, 2011; Mahmood and Yang, 2012).

3.6.2.1. Antibodies

The primary antibodies used in this experiment were polyclonal anti-p62/SQSTM1 rabbit antibody from Merck (P0067; 1:1000 dilution), and polyclonal anti-vinculin rabbit antibody from Abcam (ab91459;1:5000 dilution) was used as a loading control. Vinculin was used as loading control because of its high molecular weight, meaning that it does not interfere with p62 detection, (Johnson, 2012). The secondary antibody used was anti-rabbit IgG, horseradish peroxidase-linked antibody from Cell Signaling Technology (7074S; 1:10000 dilution).

3.6.3. Chemiluminescence Detection

The horseradish peroxidase conjugated onto the secondary antibody catalyzes the oxidation of luminol, emitting light (Thermo Scientific, 2013). Following washing, the membranes were incubated with either Immobilon Crescendo western HRP substrate (Merck) or an enhanced chemiluminescence (ECL) detection reagent (GE Healthcare Life Sciences). Protein detection is performed by chemiluminescence detection on the ChemiDoc System (BioRad). Band intensity was determined and used to correlate with the specific protein levels.

3.7. Data Analysis and Statistics

To analyze western blot data, the Fiji distribution (https://fiji.sc/) of ImageJ software (National Institutes of Health) was used to measure the amount of protein present in the membranes. Statistical analysis was carried out using the Student's t-test using Microsoft Excel 2019. Data is presented as mean ± SEM (standard error of the mean).

4. Results and Discussion

4. Results and Discussion

Autophagy is an evolutionarily conserved process that degrades protein aggregates, dysfunctional organelles, and intracellular pathogens, making it essential for cellular homeostasis (Chun and Kim, 2018). In this study, p62 concentration was used as a measure of autophagy.

A viability assay was performed to understand if these extracellular stressors had an apoptotic effect. The western blot technique was performed in order to evaluate p62 concentration in fibroblasts derived from individuals of several ages (21, 41, 69, and 80 years old), which were exposed to several different treatments, to help understand the effect of certain cell conditions on autophagy. The results are presented as fold increase comparative to control, allowing us to visualize whether there is indeed an increase or a decrease in p62 concentration levels.

4.1. Viability

Viability was verified by the resazurin cell viability assay which is often applied to assess mammalian cell toxicity, viability, migration, and invasion, while keeping cells intact (Abcam, 2019). The reduction of resazurin correlates with the number of live cells. The transference of electrons from NADPH to resazurin reduces the blue resazurin to a pink fluorescent counterpart, resorufin.

Resazurin dye was added to the cells plated in a 96-well plate and incubated at 37 °C for 4 hours and read by fluorescence of absorbance at 562 nm using a microplate reader (Infinite M200, TECAN). Results are summarized in Figure 7. In general, cell viability is high on all experimental conditions, with values ranging from 71 to 110%. With respect to starvation, this appears to be beneficial for the younger samples but less so in the fibroblasts derived from the older individuals. Oligomycin appeared not to affect viability in most cases, except for the 80-year-old fibroblasts. Whereas, catechin showed more variability, but viability values remained at around 100%.

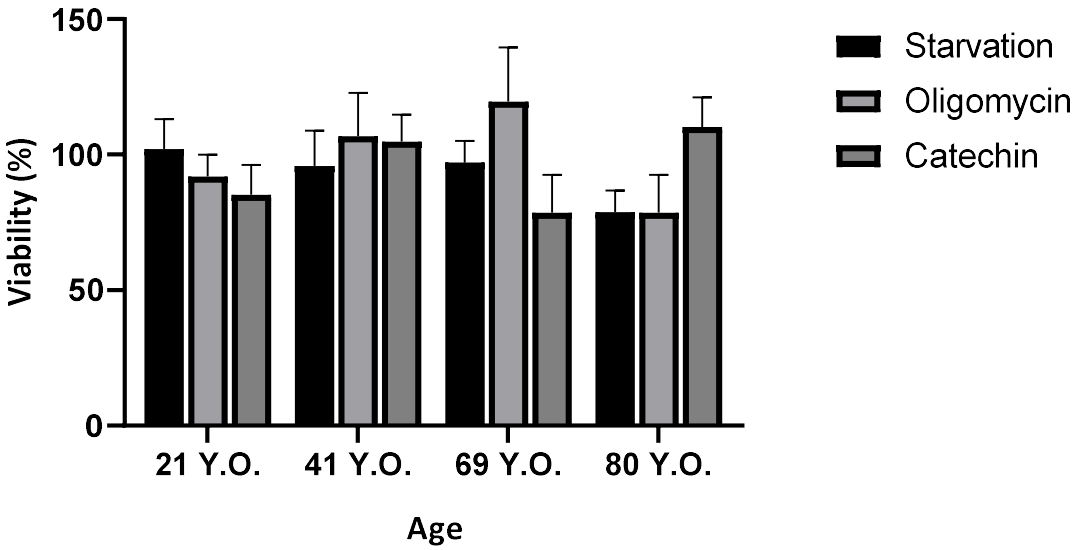

Figure 7 - Cell viability results. Expressed as percentage ± SD. Oligomycin and catechin were tested in the concentrations used for cell treatments, respectively, 5 nM and 100 µM.

4.2. Proteasome Inhibition

In order to represent a scenario of proteasome inhibition, similar to when the cell is disturbed by an extracellular insult or a ROS imbalance, cells were incubated with MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), a proteasome inhibitor, representing the positive control. MG132 is a peptide aldehyde able to inhibit the 20S proteasome, therefore, the catalytic core, by covalently binding to the active site of the β subunits, blocking the proteolytic activity of the entire 26S complex (Guo and Peng, 2013).

In the present study, fibroblasts cells of 21 (control), 41, 69, and 80 years of age were treated with MG132 for 24 hours and a western blot analysis was performed (Figure 8A).

The 41-year-old fibroblasts showed a significant increase in p62 concentration upon proteasomal inhibition ($p < 0.05$), which could be indicative of a decrease in autophagy (Figure 8B). Young individuals (21 years old) showed little to no change in p62 levels upon exposure to MG132, as did 69-year-olds. Results suggest that 80-year-olds exhibit a decrease in p62 concentration, which could mean an increase in autophagy.

Proteasome inhibition suppresses UPR signaling, making cells more likely to die by apoptosis in the presence of stressors (Amanso, Debbas and Laurindo, 2011). Proteasome inhibition causes accumulation of polyubiquitinated misfolded proteins, since these are recognized by HDAC6, which binds dynein and transports misfolded proteins to the microtubule-organizing center, where the misfolded proteins form aggresomes. These aggresomes are sequestrated by autophagic vesicles and degraded (An and Statsyuk, 2015).

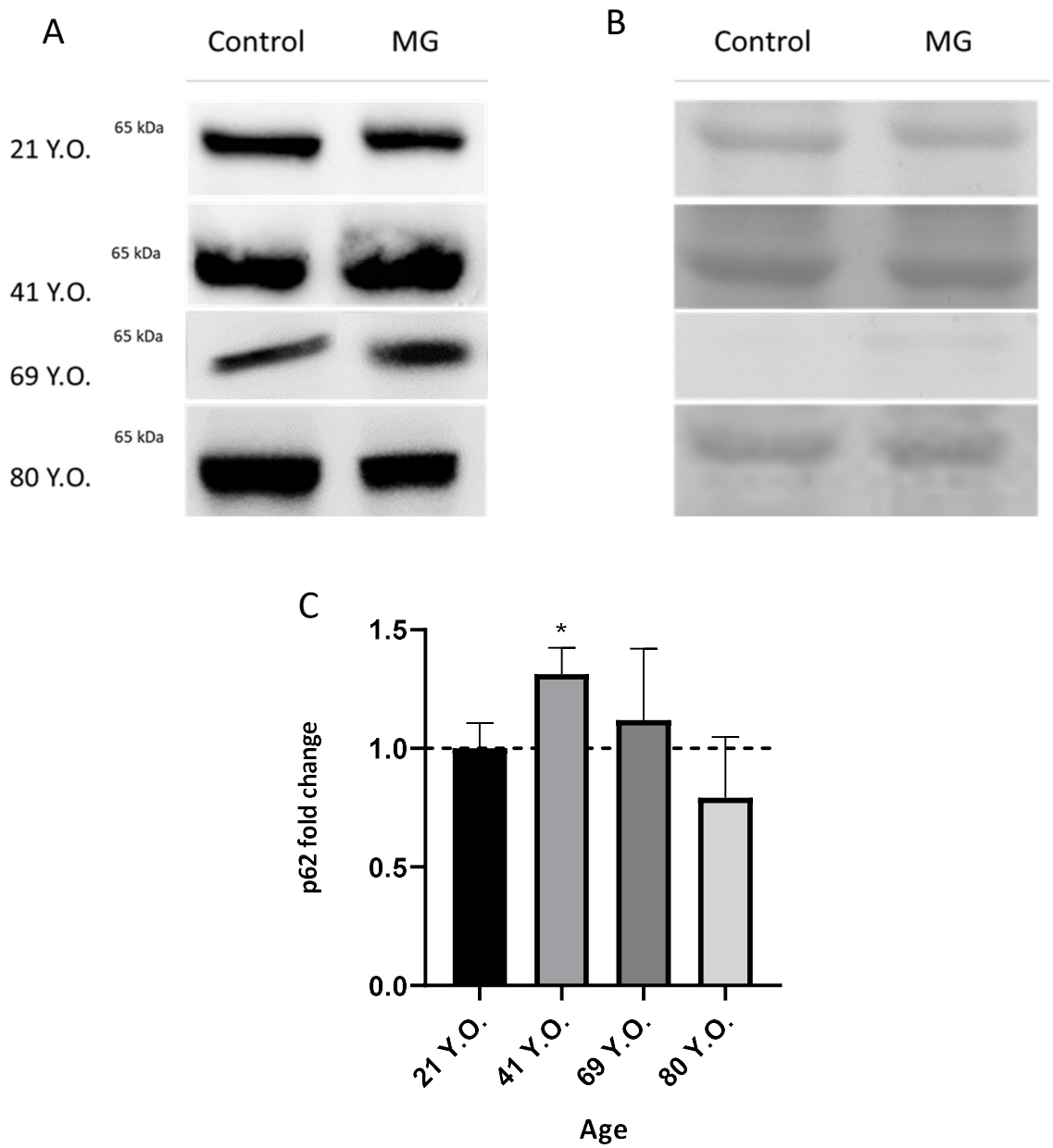

Figure 8 - Fibroblast exposure to proteasome inhibition by MG132. A) Western blot analysis comparing p62 expression in fibroblasts of different ages (21, 41, 69, and 80 years old). p62 is detected at 65 kDa. Control and MG groups are represented. **B)** Loading control, Ponceau staining. **C)** Relative expression of p62 upon exposure to MG. Results are expressed as fold increase relatively to control ± SEM. *: statistical significance comparative to control ($p < 0.05$).

The degradation of ubiquitinated proteins by autophagy enhances the protective response to proteasome inhibition (Legesse-Miller *et al.*, 2012). Additionally, proteasome inhibition induces ROS production, and, since p62 is a stress response protein induced by oxidative stress, this may signify an increase in autophagic response (Bjørkøy *et al.*, 2005).

Proteasomal inhibition has been shown to cause compensatory upregulation of autophagy in cardiomyocytes (Zheng *et al.*, 2011), cancer cell lines (Ding *et al.*, 2007; Zhu, Dunner and McConkey, 2010), and neuronal cell lines and cultures (Pan *et al.*, 2008; Du *et al.*, 2009). The same response occurs in the context of protein aggregation disorders, such as PD or HD, as the aggregated proteins interfere with the normal proteasome functions, causing autophagy upregulation. Autophagy facilitates the removal of these proteins as they form oligomers or aggregates, no longer removable by other proteolytic systems, enhancing cell survival (Du *et al.*, 2009; Zhu, Dunner and McConkey, 2010). It is not clear whether the activation of autophagy is a response to the presence of aggregates, or because of the dysfunctional proteasomal activity (Park and Cuervo, 2013).

4.3. Starvation

Under normal conditions, autophagy is kept at a basal level for housekeeping purposes (Shang *et al.*, 2011). However, under stress, most notably, caloric restriction, autophagy represents a cellular survival mechanism, degrading cellular constituents and recycling macromolecules to support cellular metabolism (Mathew *et al.*, 2009; Qi *et al.*, 2014). Thus, fibroblast cells of different ages (21, 41, 69, and 80 years old) were subjected to caloric restriction for 24 hours, to help understand if there are differences in cellular autophagy levels with aging (Figure 9A). Afterwards, cells were simultaneously subjected to starvation and proteasome inhibition by MG132, since these two stimuli are known to induce autophagy.

Levels of p62 concentration in young starved cells (21 years old) were similar to that of controls, indicating that starvation did not affect autophagy flux in these cells (Figure 9C). On the other hand, 41 and 69-year-old fibroblasts showed a statistically significant increase in p62 concentration ($p < 0.05$), thus demonstrating that starvation caused a decrease in autophagy and higher accumulation of p62 in cells (Rogov *et al.*, 2014).

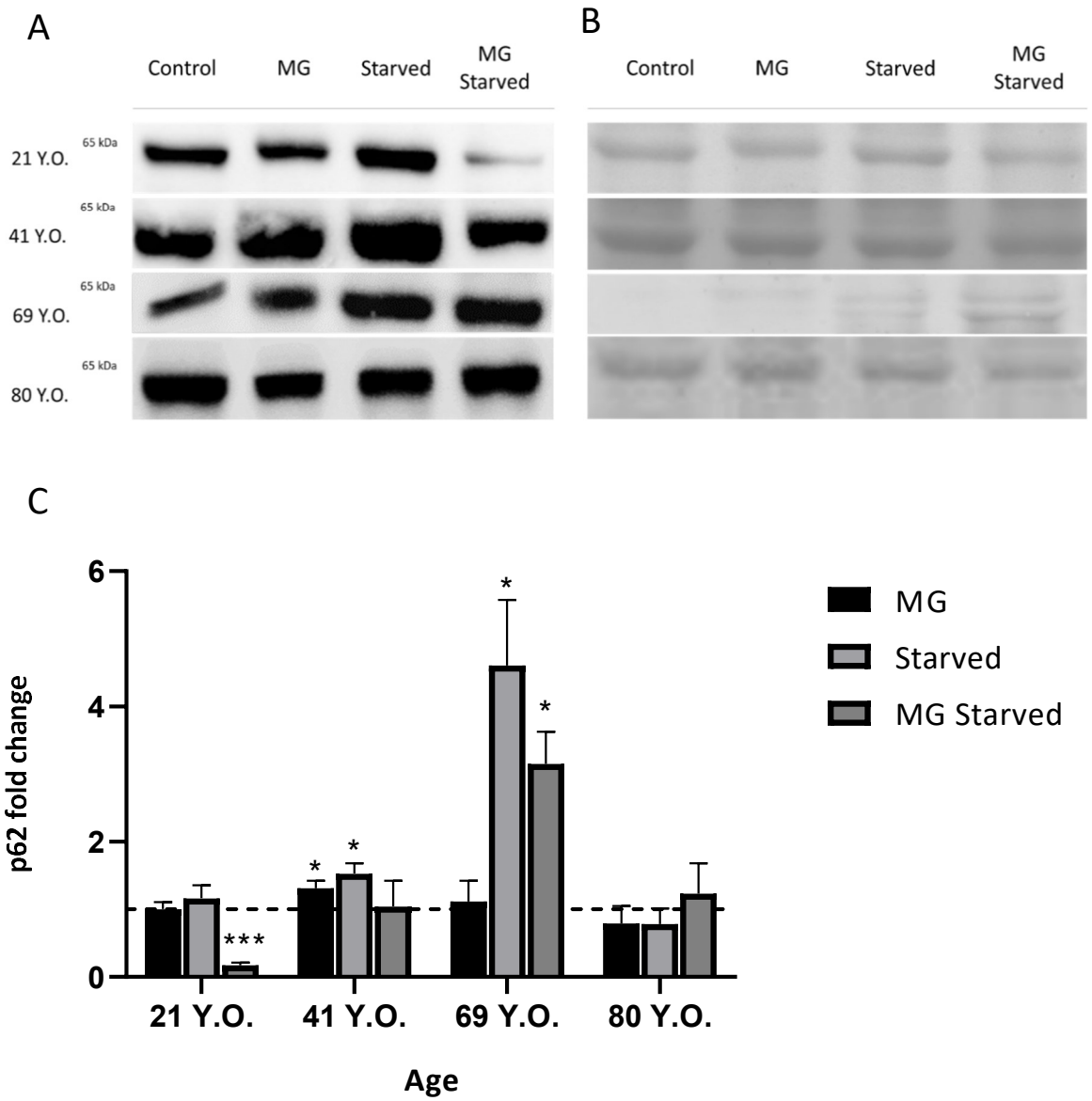

Figure 9 - Fibroblast exposure to proteasome inhibition, starvation, and proteasome inhibition together with starvation. A) Western blot analysis comparing p62 expression on fibroblasts of different ages (21, 41, 69, and 80 years old). The groups represented are MG, starvation, and MG + starvation. **B)** Loading control, Ponceau staining. **C)** Relative expression of p62 upon exposure to MG, starvation, and simultaneous proteasome inhibition and starvation, according to the legend. Results are expressed as fold increase comparatively to control ± SEM. *: statistical significance ($p < 0.05$); ***: statistical significance ($p < 0.001$).

However, 80-year-old cells showed a tendency towards decreased p62 concentration, which is indicative of an increased autophagic response and subsequent p62 degradation.

Upon combined starvation and proteasomal inhibition, the young adult models showed a significant decrease in p62 concentration ($p < 0.001$), representative of an increased autophagic response and, consequently, degradation of p62. In the meantime, 69-year-old fibroblasts showed the opposite response, with a 3-fold increase in p62 accumulation ($p < 0.05$), showing a significant decrease in autophagy in comparison to control conditions. Proteasomal inhibition and starvation caused no difference in p62 concentration in 41 and 80-year-olds. In general, young fibroblasts show a response to starvation by increasing autophagy, while 41 and 80-year-olds show no response, 69-year-olds show a decrease in autophagy, causing p62 accumulation in cells. Older cells were not able to respond to caloric restriction with the expected increase in autophagy, thus suggesting that older cells are more susceptible to stress, however, they still maintained viability.

Caloric restriction and proteasome inhibition are known to cause an increase in protein aggregation, such as ubiquitin and p62-positive aggregates (ALIS), which may accumulate to overcome degradation (Trigo, Nadais and da Cruz e Silva, 2019). A study by Kim *et al*. (2018) showed that, under starvation conditions induced by rapamycin, p62 decreased in both young and old fibroblasts in comparison to normal conditions (Kim *et al.*, 2018). In another study, p62 expression had an 80% decrease with starvation, showing a quick overall degradation of autophagy receptors by autophagy in response to shortage of extracellular amino acids – the first response to starvation appeared to be protein degradation by basal autophagy (Mejlvang *et al.*, 2018).

Another study reported that, upon starvation, p62 is degraded by autophagy within the first 2 hours, but it is restored to basal levels in mouse embryonic fibroblasts exposed to prolonged starvation (4 to 8 hours). The p62 restoration depends on its transcriptional upregulation, caused by amino acid starvation, and, at the same time, the amino acids derived from autophagic-lysosomal degradation are recycled for *de novo* synthesis of p62 under starvation conditions. This paper also theorized that p62 expression restoration in starved cells is determined by autophagic degradation, transcriptional upregulation, and

lysosomal-derived amino acid availability (Sahani, Itakura and Mizushima, 2014). In the present work, starvation was maintained for a period of 24 hours, which could help explain why this experimental condition did not result in diminished p62 levels; indeed, in control-aged cells, when starvation was performed together with proteasome inhibition, there was a marked 5-fold decrease in p62, indicating an increase in autophagy. Interestingly, this trend appears to be reversed with age, as 80-year-olds show a trend to increase in p62 levels, which is indeed consistent with the results obtained for the 69-year-olds, with an almost 4-fold increase in p62 levels following starvation in the presence of proteasome inhibitor. The fibroblasts derived from 69-year-old individuals appear to have some disruption, as they are sensitive to these stressors, but do not trigger the autophagy response in their presence, being actually decreased, resulting in p62 accumulation.

4.4. Mitochondria Membrane Depolarization

Respiratory chain efficiency tends to decrease with age, increasing electron leakage and reducing ATP generation (Hekimi, Lapointe and Wen, 2011). Dysfunctional mitochondria contribute to aging, due to an increased mitochondria permeabilization tendency in response to stress, which affects apoptotic signaling and inflammatory response (Green, Galluzzi and Kroemer, 2011). Mitochondria are an autophagy substrate, specifically mitophagy. p62 is translocated to damaged mitochondria in response to stress (Katsuragi, Ichimura and Komatsu, 2015) and mitochondrial membrane depolarization impairs oxidative phosphorylation, causing ubiquitination of mitochondrial membrane proteins, leading to their autophagic and proteasomal degradation (Pajares *et al.*, 2018).

Oligomycins are macrolides produced by *Streptomyces*, which have antibiotic properties and have been associated with antitumor and immunosuppressive effects (Yamazaki *et al.*, 1992). Oligomycin is often used in research to promote mitophagy (autophagic degradation of mitochondria), since it significantly decreases mitochondrial membrane potential and inhibits cellular respiration, thus inducing mitochondrial damage (López de Figueroa *et al.*, 2015; Hou *et al.*, 2019).

Fibroblast cells were exposed to oligomycin, and as a combination of oligomycin and MG132, for 24 hours, and a western blot analysis was performed to analyze the effect of mitochondrial dysfunction on the autophagy process (Figure 10A).

Young cells showed no apparent changes in p62 concentration following oligomycin treatment, while both intermediate and old age groups showed a fold decrease in p62, with 41-year-olds showing a significant 3-fold decrease ($p < 0.05$) (Figure 10C). Thus, young mitochondria might be more robust and able to deal with stress than older cells, since, if mitophagy is indeed being triggered by oligomycin, it is not activating the autophagy pathway. Upon combined proteasomal inhibition and mitochondrial membrane depolarization, all age groups responded to the combined stresses by increasing autophagy, resulting in a p62 concentration decrease comparative to control, with 21 and 41-year-olds showing a significant 2- and 5-fold decrease in p62, respectively (Figure 10C). Hence, when the proteasome is inhibited, this stimulus, together with mitochondrial membrane depolarization, appears to be too much for even young cells, thus triggering autophagy to ensure survival. This shows that the combined stressors had a notable effect on cellular response, particularly on 21 and 41-year-olds, as these stressors triggered autophagy, hence causing p62 degradation. Older cells (69 and 80 years old) were unable to activate the autophagy pathway as effectively as the remaining groups, nevertheless, overall cell viability was maintained.

Oligomycin A reduces cellular respiration by inhibiting the ATP synthase, which is necessary for oxidative phosphorylation of ADP into ATP. By blocking the ATP synthase proton channel, oligomycin significantly reduces electron flow through the electron transport chain and greatly decreases the rate of respiration, leading to mitochondrial defects (Jastroch *et al.*, 2010). Mitochondrial ATP production is mainly driven by an electrochemical proton gradient, which is established through energy harvested from the respiratory chain (Mitchell, 1961). Thus, when mitochondrial respiration is disrupted, ATP production is consequently hindered, causing an overall mitochondrial dysfunction. High concentrations of oligomycin may also have the indirect side effect of plasma membrane sodium-potassium ATPase inhibition, due to the lack of ATP production.

Oligomycin has been shown to cause early activation of autophagy in human chondrocytes after 24 hours of exposure, although, after 48 hours, there was a clear reduction of autophagy; it was theorized that oligomycin-induced mitochondrial dysfunction disrupts autophagy (López de Figueroa *et al.*, 2015).

Proteasome inhibition causes mitochondrial dysfunction, ROS accumulation and misfolded protein accumulation (Han and Park, 2010). Under normal conditions, Nrf2

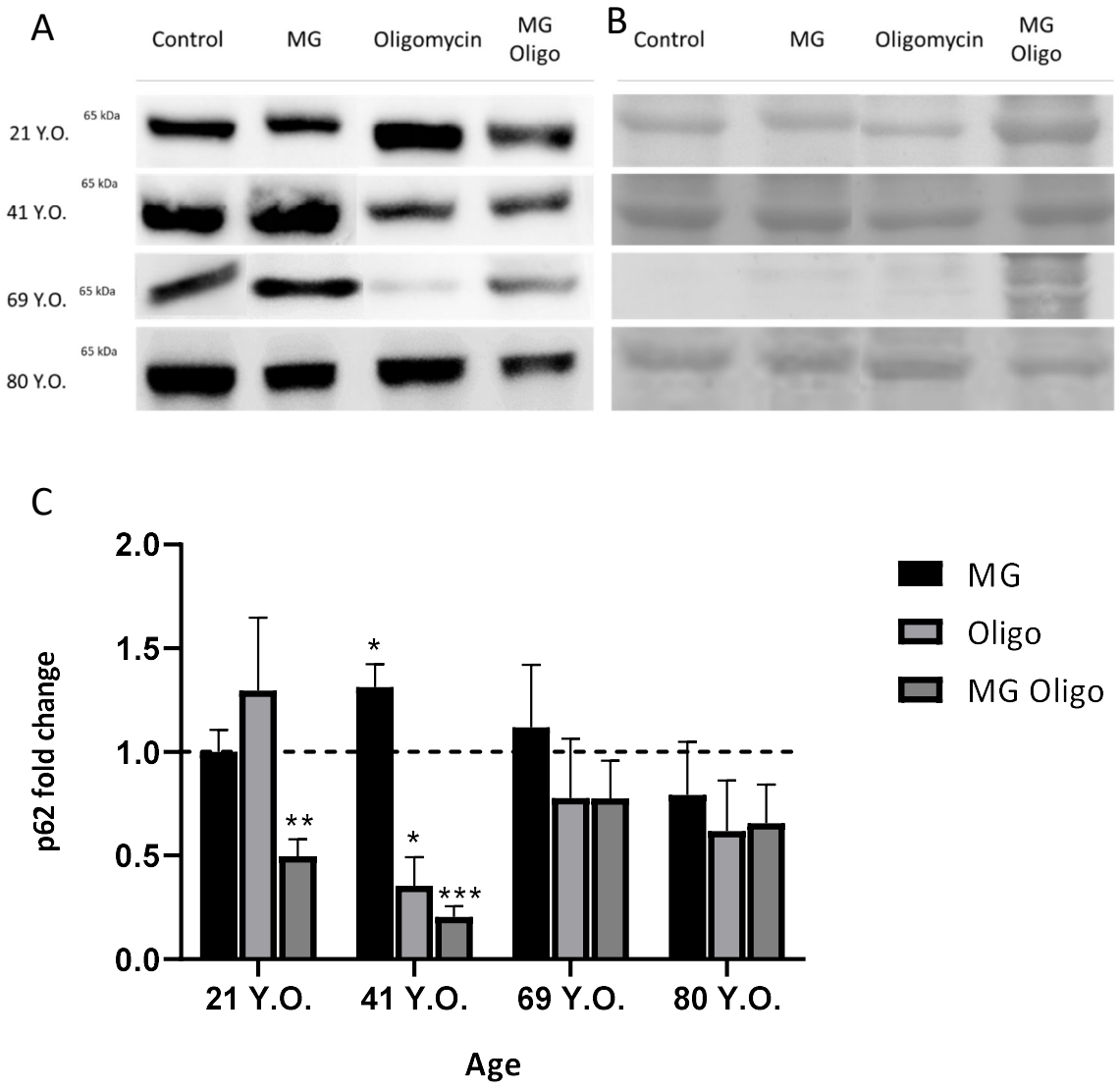

Figure 10 – Fibroblast exposure to proteasome inhibition, oligomycin, and proteasome inhibition together with oligomycin exposure. A) Western blot analysis comparing p62 expression on fibroblasts of different ages (21, 41, 69, and 80 years old). The groups represented are MG, oligomycin, and MG + oligomycin. **B)** Loading control, Ponceau staining. **C)** Relative expression of p62 upon exposure to MG, oligomycin (oligo), and simultaneous exposure to MG and oligomycin, according to the legend. Results are expressed as a fold increase of control ± SEM. *: statistical significance ($p < 0.05$); **: statistical significance ($p < 0.005$); ***: statistical significance ($p < 0.001$).

binds to KEAP1, leading to Nrf1 ubiquitination and proteasomal degradation. However, under oxidative stress, KEAP1 cysteine residues are modified by ROS and RNS, blocking Nrf2 ubiquitination, causing an increase in Nrf2 levels; p62 sequesters KEAP1, so it can no longer bind to Nrf2, resulting in Nrf2 stabilization and relocation to the nucleus, inducing various cytoprotective genes (Bjørkøy *et al.*, 2005; Jiang *et al.*, 2015). Thus, under proteasomal inhibition, Nrf2 degradation is also inhibited, which exacerbates the cellular damage caused by oligomycin. The combined effect of proteasome inhibition and mitochondrial dysfunction would be expected to increase autophagy.

4.5. Antioxidation

Considering the effects of mitochondria membrane depolarization, which induces mitochondrial damage, mitophagy, and autophagic degradation of mitochondrial proteins, and the previously described association between mitochondria dysfunction and ROS, the role of oxidative stress in autophagy was next evaluated. To do so, the antioxidative agent catechin was used.

Catechins are polyphenolic compounds, belonging to the flavanol group of flavonoids, being found in a variety of plants (Vogiatzoglou *et al.*, 2015). Catechin is a secondary metabolite found in commonly consumed foods and beverages, such as cocoa, green tea, and vinegar (Kwik-Uribe and Bektash, 2008). Catechins are ROS scavengers and metal ion chelators, and they possess antioxidant activity by induction of antioxidant enzymes (i.e. superoxide dismutase, catalase, glutathione peroxidase) and inhibition of pro-oxidant enzymes (nicotinamide adenine dinucleotide phosphate-oxidase; NADPH-oxidase) (Bernatoniene and Kopustinskiene, 2018).

Oxidative stress causes depolarization of the mitochondrial inner membrane, thus impairing mitochondrial oxidative phosphorylation. Damaged mitochondria produce ROS, especially superoxide anions (O^{2-}) and hydrogen peroxide (H_2O_2), further accelerating ROS generation (Park, Lee and Choi, 2011). Catechins have a protective effect on cells against oxidative stress and free radicals, which have been implicated in aging and aging-associated disorders such as cancer, cardiovascular and neurodegenerative disorders, and

diabetes (Bernatoniene and Kopustinskiene, 2018). Thus, the role of mitochondria in cellular ROS maintenance makes it a likely target for catechins (Bernatoniene and Kopustinskiene, 2018). Primary rat islets cultured with a catechin-rich cocoa flavanol fraction had an enhanced mitochondrial respiration and mitochondrial complexes III-V were upregulated (Rowley *et al.*, 2017). Epigallocatechin-3-gallate (EGCG) has been shown to increase mitochondrial respiration on rat liver (Mezera *et al.*, 2016); another study showed that this same catechin increased oxidative phosphorylation and ATP production in cultured human astrocytes and neurons, without toxicity (Castellano-González *et al.*, 2016), and yet another study found an increase in respiratory capacity of neuronal mitochondria (Nichols *et al.*, 2015).

Cells were treated for 24 hours with catechin, as well as catechin together with MG132, and a western blot analysis was performed to assess p62 concentration (Figure 11A).

Overall, all age groups showed an increase in autophagy upon exposure to catechin (Figure 11C). p62 concentration decreased significantly in fibroblasts from the control group (21-year-olds) ($p < 0.05$); similarly, 41-year-old cells also exhibited a decrease in p62 concentration ($p < 0.005$). These results show that antioxidation leads to increased autophagy. Combined proteasomal inhibition and exposure to catechin was performed, thus subjecting the cell to proteasome inhibition and antioxidation, which did not alter p62 concentration in 21 and 80-year-olds, and showed a tendency to decline on 69-year-olds, and declined significantly on the 41-year-old fibroblast cell models, thus, young and old fibroblasts showed no changes in autophagy patterns, while 41 and 69-year-old fibroblasts had an increase in autophagy.

It has previously been showed that HEK293T cells treated with EGCG, the main catechin present in green tea, had extended cell viability, by autophagy induction, suggesting that EGCG-dependent autophagy occurs via mammalian target of rapamycin (mTOR), a protein kinase that negatively regulates autophagy, and serine/threonine-protein kinase ULK1, which acts as a downstream effector and negative regulator of mTOR (Holczer *et al.*, 2018). Other studies have found that EGCG also stimulates

autophagy in vascular endothelial cells and in human hepatoma cells (Kim *et al.*, 2013; Zhong *et al.*, 2015). At the same time, antioxidants have been shown to inhibit both basal and induced autophagy, consequently increasing protein aggregation (Underwood *et al.*, 2010). Since antioxidants reduce oxidative stress, which could reasonably result in decreased autophagy. The same study found that Vitamin E enhanced the activity of mTOR, decreasing autophagy, but N-acetilcystein caused mTOR decrease, increasing autophagy, thus showing a contradictory effect of antioxidants on autophagy.

LLobet *et al*. (2008) found that antioxidants block proteasome inhibition in endometrial carcinoma cell lines. As ROS are mainly responsible for proteasome inhibitor-induced cell death, antioxidants are able to block apoptosis, which would be triggered by proteasome inhibition (Llobet *et al.*, 2008).

Respiratory chain efficiency tends to decrease as cells age, increasing electron leakage and reducing ATP generation. Damaged mitochondria can contribute to aging, regardless of ROS levels, by increased tendency for mitochondria permeabilization in response to stress, affecting apoptotic signalling, as well as by triggering inflammation (Green, Galluzzi and Kroemer, 2011; López-Otín *et al.*, 2013). Loss of mitochondrial efficiency is both a cause and consequence of aging, resulting from events like reduced biogenesis of mitochondria, telomere attrition, accumulation of mtDNA mutations and deletions, oxidation of mitochondrial proteins, defective quality control by mitophagy, or an organelle-specific type of macroautophagy targeting mitochondria for proteolytic destruction (Wang and Klionsky, 2011).

It is also worth mentioning that, while the antioxidant mechanism of action of catechins involves, as mentioned above, ROS and metal ion quenching, these flavonoids are unstable and may undergo auto-oxidative reactions, resulting in the production of ROS, thus no longer having an antioxidant effect on cells, but a pro-oxidant effect (Sang *et al.*, 2005). EGCG stability has been found to depend on its concentration, presence of oxygen, as well as pH and temperature of the system it is in (Sang *et al.*, 2005). A study which used a higher concentration of EGCG, namely 200 μM, which was two times the concentration of catechin used in the present work, has shown pro-oxidant effects on

isolated mouse hepatocytes, resulting in time and dose-dependent cytotoxicity that correlated with ROS production (Galati *et al.*, 2006). It remains unclear how the pro-oxidant response affects the cell, and there is a lack of careful dose-dependent studies (Lambert and Elias, 2010). Thus, on the present work, we could be seeing a pro-oxidant effect of catechin on fibroblast cells, instead of the intended antioxidation, although further studies are needed to verify if that is indeed the case. It would be relevant to study the effect of increasing catechin concentrations and see their effect on cellular redox balance, and then explore how p62 concentration in cells is modulated throughout.

The present results provide new information that allows for researching opportunities. Starvation did not result in increased autophagy, being unaltered in young fibroblasts, while intermediate and old age groups, particularly 41 and 69-year-olds, showed an increase in p62, thus, decreased autophagy response. Proteasome inhibition generally did not alter p62 concentration, although 41-year-olds showed a significant decrease in autophagy. Proteasome inhibition represses UPR signaling, thus making cells more likely to die by apoptosis in the presence of stressors (Amanso, Debbas and Laurindo, 2011). Of note, high autophagosome levels often accompany cell death, which indicates that, before cell death, there can be a strong autophagic response. Cells may increase autophagy when trying to survive extracellular stressors, and, should this effort fail, cell death occurs (Denton, Nicolson and Kumar, 2012). Autophagic degradation of macromolecules protects cells from nutrient starvation, providing the nutrients and energy needed to maintain the functions required for survival during starvation, hence paradoxically avoiding cell death by autophagy activation (Yonekawa and Thorburn, 2013). With mitochondrial membrane depolarization, induced by oligomycin, there was a general increase in autophagy, as p62 levels decreased, except for young cells. Exposure to oligomycin and MG132 caused a decrease in p62 in all age groups, compared to control. Catechin-induced antioxidation caused a general increase in autophagy, as p62 concentration reduced in all age groups. Treatment with proteasome inhibition together with this antioxidant agent increased autophagy on 41 and 69-year-olds.

Proteins have a dynamic structure, and protein homeostasis requires continuous surveillance by cellular quality control mechanisms, that stabilize folded proteins and

restore or degrade misfolded proteins, preventing the accumulation and aggregation of damaged cellular components and assuring protein renewal (Miller *et al.*, 2015; Dikic, 2017). These mechanisms decline during aging, deregulating the proteostasis network, namely the UPR pathway (Brown and Naidoo, 2012), cellular proteasome activity (Anselmi *et al.*, 1998; Keller, Hanni and Markesbery, 2001) and the CMA pathway (Cuervo and Dice, 2000).

The upregulation and accumulation of p62 in aggresomes or inclusions is one of the pathological processes behind some neurodegenerative disorders (Nagaoka *et al.*, 2004), as p62 is often a component of protein aggregates such as Lewy bodies in PD, NFTs in AD, and huntingtin aggregates in HD (Kuusisto, Salminen and Alafuzoff, 2001; Zatloukal *et al.*, 2002). If p62 aggregates are not eliminated by autophagy and there is no cell death, the accumulation of p62 bodies may act as tumor-promoting factors, due to increased oxidative stress response, induction of inflammation and cytokine production (Mathew *et al.*, 2009). Even though autophagy is an adaptive response to nutrient deprivation, development, aging, and cell death, there is increasing evidence that this process is essential for neuronal survival as a primary protective mechanism maintaining homeostasis in response to stress (Lynch-Day *et al.*, 2012).

From the work here presented, p62 proved to be a good biomarker for cellular autophagy response, as the results show that extracellular stressors, which are known to induce cellular quality control systems, do indeed affect p62 concentration, showing modulation of the autophagy process in the presence of stressors. This is qualitatively summarized in Table 3.

Young fibroblast cells dealt well with proteasome inhibition, maintaining p62 levels similar to control levels; on the other hand, 21-year-olds showed a tendency to decrease autophagy and accumulate p62 upon starvation and mitochondrial membrane depolarization. Meanwhile, 21-year-olds had a significant decreased concentration of p62 upon exposure to catechin, as well as upon co-treatment of MG132 with starvation, and MG 132 with oligomycin. Proteasome inhibition together with antioxidants had inconclusive results.

Table 3 - Summary of the p62 concentration results for all age groups and all treatments. Values are represented as a comparison to control, obtained by relative fold increase data. =: concentration equal to control or with no apparent changes; ↑: increased concentration comparatively to control; ↓: decreased concentration comparatively to control. *: significantly different from control ($p < 0.05$); **: significantly different from control ($p < 0.005$); ***: significantly different from control ($p < 0.001$).

Age	MG	Starved	MG Starved	Oligo	MG Oligo	Catechin	MG Catechin
21	=	=	↓***	=	↓**	↓**	=
41	↑*	↑*	=	↓*	↓***	↓**	↓*
69	=	↑*	↑*	↓	↓	↓	↓
80	↓	↓	=	↓	↓	↓	=

Young fibroblast cells dealt well with proteasome inhibition, maintaining p62 levels similar to control levels; on the other hand, 21-year-olds showed a tendency to decrease autophagy and accumulate p62 upon starvation and mitochondrial membrane depolarization. Meanwhile, 21-year-olds had a significant decreased concentration of p62 upon exposure to catechin, as well as upon co-treatment of MG132 with starvation, and MG 132 with oligomycin. Proteasome inhibition together with antioxidants had inconclusive results.

For the 41-year-olds, the intermediate age model used in this study, showed a significant decrease of autophagy upon proteasome inhibition, as well as after starvation, while the corresponding co-treatment had no effect. The p62 concentration significantly decreased upon exposure to oligomycin, catechin, and both corresponding co-treatments with MG132, showed a cellular response to increase autophagy in the presence of extracellular stressors.

For the 69-year-old fibroblasts, particularly, these exhibited a 4.5-fold increase in p62 concentration upon starvation, and, similarly, upon proteasomal inhibition together with starvation, this age group exhibited a 3-fold increase in p62, showing a strong accumulation of this protein, which may indicate a failure of the cells to respond to these

stressors. These cells were mostly unaffected by proteasome inhibition. Meanwhile, there was a tendency for a decrease in p62 concentration in 69-year-olds, thus an increase in autophagy, when exposed to the antioxidant agent catechin and upon mitochondrial membrane depolarization by oligomycin, as well as on the respective combined exposures to MG132.

The oldest age group represented in this study, 80-year-olds, showed a tendency for autophagy increase upon proteasome inhibition, mitochondrial membrane depolarization, and starvation, as showed by a decrease in p62 concentration. At the same time, these cells tended to decrease autophagy, similarly to 69-year-olds, as mentioned above, upon exposure to oligomycin, on the combined exposure of oligomycin and MG132, as well as in the presence of catechin. Co-exposure to MG132 and catechin was inconclusive.

Undoubtedly, these results are interesting, but demand an increase in the number of experiments performed, as some of the results have confounding, sizeable error bars. Thus, it would be relevant to increase the number of replicates to reduce this error, and then produce more accurate results. While there does not appear to be an obvious pattern of autophagy increase or decrease with aging, there certainly is a difference in how different age groups react to the various stimuli, indeed strengthening the notion that age is a determining factor in p62 concentration and autophagy modulation.

Being so, further studies and experiments are necessary. It would be interesting to expand the present results using complementary techniques, such as immunofluorescence analysis of p62 (Bjørkøy *et al.*, 2009), and other autophagy related proteins, such as LC3. It would be particularly interesting to further study mitophagy, as it was evident from this thesis that changes in mitochondrial health influenced general autophagy patterns. It could also be interesting to study other autophagy receptors, such as NBR1, NDP52, or optineurin. Another option for further investigation would be to carry out this protocol with different cell types, such as neuronal in origin, which could help in the understanding the role of p62 in protein aggregation in aging-associated disorders.

5. Conclusion

5. **Conclusion**

This thesis aimed to evaluate whether aging has an effect on the cellular response to stressors, namely the autophagy system, as well as to verify the cellular responses of cells of different age groups to extracellular stressors (proteasome inhibition, starvation, mitochondria membrane depolarization, and antioxidation).

Results from the western blot analysis showed that p62 is a good tool to measure autophagy response in cells, as extracellular stressors known to induce a response from cellular quality control systems, did indeed influence p62 concentration. This data shows there was modulation of the autophagy process in response to stressing agents.

While starvation did not significantly alter autophagy levels in younger cells, this age-group reacted with a marked increase in autophagy upon proteasome inhibition and starvation, showing that, in young cells, while proteasome inhibition on its own is not sufficient to trigger autophagy, when occurring in a situation of caloric restriction, the cell activates this pathway. The 41-year-old fibroblasts showed a significant decrease in p62 concentration upon proteasomal inhibition and starvation but were unaffected by the combined stimuli. In contrast, there was p62 accumulation in 69-year-old fibroblasts upon starvation, with a 4.5-fold increase of p62, that was decreased to a 3-fold increase when treated together with proteasomal inhibition. While these conditions did not have the same result in similarly-aged cells, as 80-year-old fibroblasts showed a tendency to increase autophagy during starvation, as well as a tendency to decrease autophagy when co-treated with proteasome inhibition. This may indicate that in some individuals, cells fail to effectively respond to these stressors.

While young cells show no significant alteration in p62 levels in response to mitochondrial membrane depolarization by oligomycin, older and intermediately aged cells show an increase in autophagy. However, when exposure to oligomycin was accompanied by proteasome inhibition by MG132, all age groups, including young cells, showed a general decline in p62.

Antioxidation generally promoted autophagy increase, as p62 concentration declined in all age groups, significantly so on young and intermediate-aged cells. Proteasome inhibition and catechin combined caused a significant increase in autophagy on 41-year-olds, while 21 and 80-year-olds' p62 concentration was not altered.

To summarize the present thesis, one can propose a model for age-dependent proteostasis regulation, which autophagy is a fundamental part of (Figure 12). The cellular quality control system of young individuals is represented by the green arrows, showing that their cells are able to cope with stressors, be it through the UPR, the proteasome, or autophagy (macroautophagy or CMA). With aging, however, this ability becomes increasingly impaired, as aged cells experience more extended cumulative oxidative

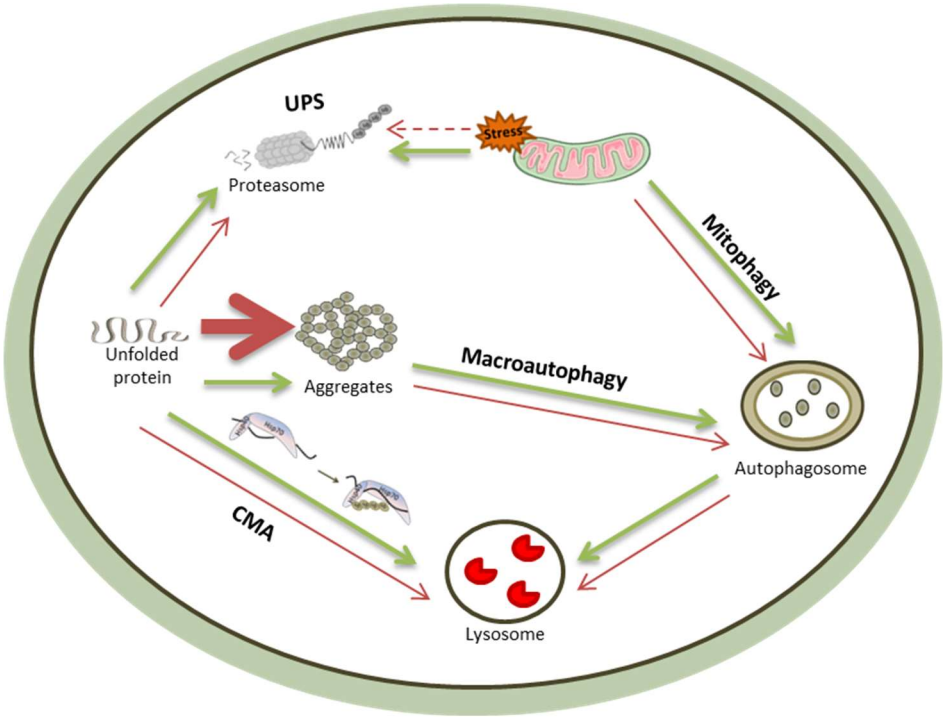

Figure 11 – Functional model of the celular quality control system, which maintains proteostasis in the cytosol and mitochondria. The green arrows represent basal state mechanisms of proteostasis on young individuals, where this network, composed of the unfolded protein response, proteasome, and autophagy, work efficiently. Thus, unfolded and misfolded proteins are degraded in the proteasome (upon ubiquitination) or by chaperone-mediated autophagy. Misfolded proteins can form insoluble aggregates, which are eliminated by macroautophagy. In the presence of extracelular stressors, ER and mitochondria activate the UPR, and, if this response is not sufficient, unfolded proteins are degraded by the proteasome. The red arrows represent old age, where these processes are increasingly impaired (thin red arrows), disrupted (red dashed arrows), or increased (thick red arrows). In old age, there is an increased aggregate production, which, in turn, is more likely to overload the ER and mitochondria and compromisse the proteostasis network in general. This causes a decrease in protein aggregate degradation, and thus, an increase in protein aggregate accumulation. CMA: chaperone-mediated autophagy; ERAD: endoplasmatic reticulum associated degradation; UPR: unfolded protein response; UPS: ubiquitin proteasome system.

stress, which in turn leads to protein folding errors and protein degradation is also impaired. Thus, the various components of the quality control system in old cells become more prone to being overwhelmed by stress, which leads to accumulation of aggregated proteins and dysfunctional organelles.

While the results obtained are interesting, it would be relevant to continue to pursue this area, possibly complementing these results with further studies and more techniques. Hence, the underlying hypothesis of this study was proved to be correct, and indeed p62 appears to be a good biomarker for autophagy monitoring on cells of different ages, upon extracellular stress.

6. References

6. **References**

Abcam (2016) *Sample preparation for western blot Lysis buffers*. Available at: https://www.abcam.com/protocols/sample-preparation-for-western-blot (Accessed: 12 November 2018).

Abcam (2019) *Resazurin Cell Viability Assay*. Available at: www.abcam.com/ab129732 (Accessed: 31 October 2019).

Alam, P. *et al.* (2017) 'Protein aggregation: From background to inhibition strategies', *International Journal of Biological Macromolecules*. Elsevier, pp. 208–219. doi: 10.1016/j.ijbiomac.2017.05.048.

Alberts, B., Johnson, A. and Lewis, J. (2002) 'Molecular Biology of the Cell: Fibroblasts and Their Transformations 4th edition: The Connective-Tissue Cell Family', *Garland Science*. Garland Science. doi: 10.1016/j.stem.2010.07.007.

Alzheimer's Disease International (2018) *World Alzheimer Report 2018: The state of the art of dementia research: New frontiers*, *Alzheimer's Disease International*. doi: 10.1111/j.0033-0124.1950.24_14.x.

Amanso, A. M., Debbas, V. and Laurindo, F. R. M. (2011) 'Proteasome inhibition represses unfolded protein response and Nox4, sensitizing vascular cells to endoplasmic reticulum stress-induced death.', *PloS one*. Public Library of Science, 6(1), p. e14591. doi: 10.1371/journal.pone.0014591.

An, H. and Statsyuk, A. V. (2015) 'An inhibitor of ubiquitin conjugation and aggresome formation', *Chemical Science*. Royal Society of Chemistry, 6(9), pp. 5235–5245. doi: 10.1039/c5sc01351h.

Anselmi, B. *et al.* (1998) 'Dietary self-selection can compensate and age-related decrease of rat liver 20 S proteasome activity observed with standard diet', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 53(3), pp. 173–179. doi: 10.1093/gerona/53A.3.B173.

Arrasate, M. *et al.* (2004) 'Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death', *Nature*, 431(7010), pp. 805–810. doi: 10.1038/nature02998.

Balaban, R. S., Nemoto, S. and Finkel, T. (2005) 'Mitochondria, Oxidants, and Aging', *Cell*. Cell Press, 120(4), pp. 483–495. doi: 10.1016/J.CELL.2005.02.001.

Balchin, D., Hayer-Hartl, M. and Hartl, F. U. (2016) 'In vivo aspects of protein folding and quality control', *Science*, 353(6294). doi: 10.1126/science.aac4354.

Behl, C. (2016) 'Breaking BAG: The Co-Chaperone BAG3 in Health and Disease', *Trends in Pharmacological Sciences*. Elsevier Current Trends, pp. 672–688. doi: 10.1016/j.tips.2016.04.007.

Ben-Zvi, A. P. and Goloubinoff, P. (2002) 'Proteinaceous infectious behavior in non-pathogenic proteins is controlled by molecular chaperones', *Journal of Biological Chemistry*, 277(51), pp. 49422–49427. doi: 10.1074/jbc.M209163200.

Benito-León, J. *et al.* (2019) 'Non-steroidal anti-inflammatory drugs use in older adults decreases risk of Alzheimer's disease mortality', *PLOS ONE*. Edited by M. P. Tan, 14(9), p. e0222505. doi: 10.1371/journal.pone.0222505.

Bernatoniene, J. and Kopustinskiene, D. M. (2018) 'The Role of Catechins in Cellular Responses to Oxidative Stress', *Molecules*. MDPI AG. doi: 10.3390/molecules23040965.

Bitto, A. *et al.* (2014) 'P62/SQSTM1 at the interface of aging, autophagy, and disease.', *Age (Dordrecht, Netherlands)*. Springer, 36(3), p. 9626. doi: 10.1007/s11357-014-9626-3.

Biza, K. V *et al.* (2017) 'The amyloid interactome: Exploring protein aggregation', *PLoS ONE*, 12(3). doi: 10.1371/journal.pone.0173163.

Bjørkøy, G. *et al.* (2005) 'p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death', *Journal of Cell Biology*. The Rockefeller University Press, 171(4), pp. 603–614. doi: 10.1083/jcb.200507002.

Bjørkøy, G. *et al.* (2009) 'Monitoring Autophagic Degradation of p62/SQSTM1', in *Methods in Enzymology*. Academic Press, pp. 181–197. doi: 10.1016/S0076-6879(08)03612-4.

Blasiak, J. *et al.* (2019) 'Interplay between Autophagy and the Ubiquitin-Proteasome System and Its Role in the Pathogenesis of Age-Related Macular Degeneration', *International journal of molecular sciences*. Multidisciplinary Digital Publishing Institute, p. 210. doi: 10.3390/ijms20010210.

Boveris, A. and Navarro, A. (2008) 'Brain mitochondrial dysfunction in aging', *IUBMB Life*, pp. 308–314. doi: 10.1002/iub.46.

Bratic, I. and Trifunovic, A. (2010) 'Mitochondrial energy metabolism and ageing', *Biochimica et Biophysica Acta - Bioenergetics*, pp. 961–967. doi: 10.1016/j.bbabio.2010.01.004.

Breydo, L. and Uversky, V. N. (2015) 'Structural, morphological, and functional diversity of amyloid oligomers', *FEBS Letters*. John Wiley & Sons, Ltd, 589(19PartA), pp. 2640–2648. doi: 10.1016/j.febslet.2015.07.013.

Brockwell, D. J. and Radford, S. E. (2007) 'Intermediates: ubiquitous species on folding energy landscapes?', *Current Opinion in Structural Biology*. Elsevier Current Trends, 17(1), pp. 30–37. doi: 10.1016/J.SBI.2007.01.003.

Brown, M. K. and Naidoo, N. (2012) 'The endoplasmic reticulum stress response in aging and age-related diseases', *Frontiers in Physiology*. Frontiers, 3, p. 263. doi: 10.3389/fphys.2012.00263.

Brunk, U. T. and Terman, A. (2002) 'The mitochondrial-lysosomal axis theory of aging', *European Journal of Biochemistry*. John Wiley & Sons, Ltd (10.1111), 269(8), pp. 1996–2002. doi: 10.1046/j.1432-1033.2002.02869.x.

Bulteau, A. L. *et al.* (2007) 'Proteasome and photoaging: The effects of UV irradiation', in *Annals of the New York Academy of Sciences*. John Wiley & Sons, Ltd (10.1111), pp. 280–290. doi: 10.1196/annals.1395.029.

Castellano-González, G. *et al.* (2016) 'Epigallocatechin-3-gallate induces oxidative phosphorylation by activating cytochrome c oxidase in human cultured neurons and astrocytes', *Oncotarget*. Impact Journals LLC, 7(7), pp. 7426–7440. doi: 10.18632/oncotarget.6863.

Chang, H. Y. *et al.* (2002) 'Diversity, topographic differentiation, and positional memory in human fibroblasts', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 99(20), p. 12877. doi: 10.1073/PNAS.162488599.

Chaturvedi, S. K. *et al.* (2016) 'Protein misfolding and aggregation: Mechanism, factors and detection', *Process Biochemistry*, pp. 1183–1192. doi: 10.1016/j.procbio.2016.05.015.

Chi, E. Y. *et al.* (2005) 'Heterogeneous Nucleation-Controlled Particulate Formation of Recombinant Human Platelet-Activating Factor Acetylhydrolase in Pharmaceutical Formulation', *Journal of Pharmaceutical Sciences*. Elsevier, 94(2), pp. 256–274. doi: 10.1002/JPS.20237.

Chun, Y. and Kim, J. (2018) 'Autophagy: An Essential Degradation Program for Cellular Homeostasis and Life', *Cells*. MDPI AG, 7(12), p. 278. doi: 10.3390/cells7120278.

Ciani, B. *et al.* (2003) 'Structure of the ubiquitin-associated domain of p62 (SQSTM1) and implications for mutations that cause Paget's disease of bone', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 278(39), pp. 37409–37412. doi: 10.1074/jbc.M307416200.

Clausen, T. H. *et al.* (2010) 'p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy', *Autophagy*, pp. 330–344. doi: 10.4161/auto.6.3.11226.

Cuervo, A. M. *et al.* (1996) 'A receptor for the selective uptake and degradation of proteins by lysosomes.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 273(5274), pp. 501–3. doi: 10.1126/science.273.5274.501.

Cuervo, A. M. and Dice, J. F. (2000) 'Age-related decline in chaperone-mediated autophagy.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 275(40), pp. 31505–13. doi: 10.1074/jbc.M002102200.

Cuervo, A. M. and Wong, E. (2014) 'Chaperone-mediated autophagy: Roles in disease and aging', *Cell Research*. Nature Publishing Group, pp. 92–104. doi: 10.1038/cr.2013.153.

Cummings, C. J. *et al.* (1998) 'Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1', *Nature Genetics*. Nature Publishing Group, 19(2), pp. 148–154. doi: 10.1038/502.

Denton, D., Nicolson, S. and Kumar, S. (2012) 'Cell death by autophagy: facts and apparent artefacts.', *Cell death and differentiation*. Nature Publishing Group, 19(1), pp. 87–95. doi: 10.1038/cdd.2011.146.

Dice, J. F. (2007) 'Chaperone-Mediated Autophagy', *Autophagy*. Taylor & Francis, 3(4), pp. 295–299. doi: 10.4161/auto.4144.

Dikic, I. (2017) 'Proteasomal and Autophagic Degradation Systems', *Annual Review of Biochemistry*. Annual Reviews , 86(1), pp. 193–224. doi: 10.1146/annurev-biochem-061516-044908.

Ding, W.-X. and Yin, X.-M. (2012) 'Mitophagy: mechanisms, pathophysiological roles, and analysis.', *Biological chemistry*. NIH Public Access, 393(7), pp. 547–64. doi: 10.1515/hsz-2012-0119.

Ding, W. X. *et al.* (2007) 'Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability', *American Journal of Pathology*. American Society for Investigative Pathology Inc., 171(2), pp. 513–524. doi: 10.2353/ajpath.2007.070188.

Donaldson, K. M. *et al.* (2003) 'Ubiquitin-mediated sequestration of normal cellular proteins into polyglutamine aggregates', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(15), pp. 8892–8897. doi: 10.1073/pnas.1530212100.

Du, Y. *et al.* (2009) 'An insight into the mechanistic role of p53-mediated autophagy induction in response to proteasomal inhibition-induced neurotoxicity', *Autophagy*. Taylor and Francis Inc., 5(5), pp. 663–675. doi: 10.4161/auto.5.5.8377.

El-Agnaf, O. M. . *et al.* (1998) 'Aggregates from mutant and wild-type α-synuclein proteins and NAC peptide induce apoptotic cell death in human neuroblastoma cells by formation of β-sheet and amyloid-like filaments', *FEBS Letters*. No longer published by Elsevier, 440(1–2), pp. 71–75. doi: 10.1016/S0014-5793(98)01418-5.

Foot, N., Henshall, T. and Kumar, S. (2017) 'Ubiquitination and the Regulation of Membrane Proteins', *Physiological Reviews*. American Physiological Society Bethesda, MD, 97(1), pp. 253–281. doi: 10.1152/physrev.00012.2016.

Forman, M. S., Trojanowski, J. Q. and Lee, V. M.-Y. (2004) 'Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs', *Nature Medicine*. Nature Publishing Group, 10(10), pp. 1055–1063. doi: 10.1038/nm1113.

Forno, L. S. (1996) 'Neuropathology of Parkinson's Disease', *Journal of Neuropathology and Experimental Neurology*, 55(3), pp. 259–272. Available at: https://academic.oup.com/jnen/article-abstract/55/3/259/2610516 (Accessed: 2 September 2019).

Gamerdinger, M. *et al.* (2009) 'Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3.', *The EMBO journal*. European Molecular Biology Organization, 28(7), pp. 889–901. doi: 10.1038/emboj.2009.29.

Galati, G., Lin, A., Sultan, A. M., & O'Brien, P. J. (2006). Cellular and in vivo hepatotoxicity caused by green tea phenolic acids and catechins. Free Radical Biology and Medicine, 40(4), 570–580. https://doi.org/10.1016/j.freeradbiomed.2005.09.014

Gandhi, J. *et al.* (2019) 'Protein misfolding and aggregation in neurodegenerative diseases: A review of pathogeneses, novel detection strategies, and potential therapeutics', *Reviews in the Neurosciences*. De Gruyter, 30(4), pp. 339–358. doi: 10.1515/revneuro-2016-0035.

Gidalevitz, T. *et al.* (2006) 'Progressive disruption of cellular protein folding in models of polyglutamine diseases', *Science*, 311(5766), pp. 1471–1474. doi: 10.1126/science.1124514.

Gidalevitz, T., Kikis, E. A. and Morimoto, R. I. (2010) 'A cellular perspective on conformational disease: the role of genetic background and proteostasis networks', *Current Opinion in Structural Biology*. Elsevier Current Trends, 20(1), pp. 23–32. doi: 10.1016/J.SBI.2009.11.001.

Gold, R. *et al.* (2012) 'Placebo-Controlled Phase 3 Study of Oral BG-12 for Relapsing Multiple Sclerosis', *New England Journal of Medicine*. Massachusetts Medical Society , 367(12), pp. 1098–1107. doi: 10.1056/NEJMoa1114287.

Green, D. R., Galluzzi, L. and Kroemer, G. (2011) 'Mitochondria and the autophagy-inflammation-cell death axis in organismal aging.', *Science (New York, N.Y.)*. NIH Public Access, 333(6046), pp. 1109–12. doi: 10.1126/science.1201940.

Grumati, P. and Dikic, I. (2018) 'Ubiquitin signaling and autophagy.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 293(15), pp. 5404–5413. doi: 10.1074/jbc.TM117.000117.

Guo, N. and Peng, Z. (2013) 'MG132, a proteasome inhibitor, induces apoptosis in tumor cells', *Asia-Pacific Journal of Clinical Oncology*. John Wiley & Sons, Ltd (10.1111), pp. 6–11. doi: 10.1111/j.1743-7563.2012.01535.x.

Han, Y. H. and Park, W. H. (2010) 'MG132, a proteasome inhibitor decreased the growth of Calu-6 lung cancer cells via apoptosis and GSH depletion', *Toxicology in Vitro*. Pergamon, 24(4), pp. 1237–1242. doi: 10.1016/J.TIV.2010.02.005.

Handy, D. E. and Loscalzo, J. (2012) 'Redox regulation of mitochondrial function', *Antioxidants and Redox Signaling*, pp. 1323–1367. doi: 10.1089/ars.2011.4123.

Hekimi, S., Lapointe, J. and Wen, Y. (2011) 'Taking a "good" look at free radicals in the aging process.', *Trends in cell biology*. PMC Canada manuscript submission, 21(10), pp. 569–76. doi: 10.1016/j.tcb.2011.06.008.

Hipp, M. S., Kasturi, P. and Hartl, F. U. (2019) 'The proteostasis network and its decline in ageing', *Nature Reviews Molecular Cell Biology*, pp. 421–435. doi: 10.1038/s41580-019-0101-y.

Hirota-Nakaoka, N. *et al.* (2003) 'Dissolution of 2-Microglobulin Amyloid Fibrils by Dimethylsulfoxide', *Journal of Biochemistry*, 134(1), pp. 159–164. doi: 10.1093/jb/mvg124.

Holczer, M. *et al.* (2018) 'Epigallocatechin-3-Gallate (EGCG) promotes autophagy-dependent survival via influencing the balance of mTOR-AMPK pathways upon endoplasmic reticulum stress', *Oxidative Medicine and Cellular Longevity*. Hindawi Limited, 2018, p. 6721530. doi: 10.1155/2018/6721530.

Hou, Y. *et al.* (2019) 'Ageing as a risk factor for neurodegenerative disease', *Nature Reviews Neurology*. Springer US. doi: 10.1093/hmg/ddx247.

Itakura, E. and Mizushima, N. (2011) 'p62 targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding', *Journal of Cell Biology*. The Rockefeller University Press, 192(1), pp. 17–27. doi: 10.1083/jcb.201009067.

Jain, A. *et al.* (2010) 'p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a

positive feedback loop by inducing antioxidant response element-driven gene transcription', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 285(29), pp. 22576–22591. doi: 10.1074/jbc.M110.118976.

Jastroch, M. *et al.* (2010) 'Mitochondrial proton and electron leaks.', *Essays in biochemistry*. NIH Public Access, 47, pp. 53–67. doi: 10.1042/bse0470053.

Jiang, T. *et al.* (2015) 'p62 links autophagy and Nrf2 signaling.', *Free radical biology & medicine*. NIH Public Access, 88(Pt B), pp. 199–204. doi: 10.1016/j.freeradbiomed.2015.06.014.

Johnson, M. (2012) 'Loading Controls for Western Blots', *Materials and Methods*, 2. doi: 10.13070/mm.en.2.114.

Johnston, J. A., Ward, C. L. and Kopito, R. R. (1998) 'Aggresomes: A cellular response to misfolded proteins', *Journal of Cell Biology*, 143(7), pp. 1883–1898. doi: 10.1083/jcb.143.7.1883.

Katsuragi, Y., Ichimura, Y. and Komatsu, M. (2015) 'P62/SQSTM1 functions as a signaling hub and an autophagy adaptor', *FEBS Journal*. John Wiley & Sons, Ltd (10.1111), pp. 4672–4678. doi: 10.1111/febs.13540.

Katzman, R. (1986) 'Alzheimer's Disease', *The New England Journal of Medicine*, (314), pp. 964–973. doi: 10.1056/NEJM198603063141003.

Kaushik, S. and Cuervo, A. M. (2015) 'Proteostasis and aging', *Nature Medicine*. Nature Publishing Group, pp. 1406–1415. doi: 10.1038/nm.4001.

Keller, J. N., Hanni, K. B. and Markesbery, W. R. (2001) 'Impaired Proteasome Function in Alzheimer's Disease', *Journal of Neurochemistry*. John Wiley & Sons, Ltd (10.1111), 75(1), pp. 436–439. doi: 10.1046/j.1471-4159.2000.0750436.x.

Kendrick, B. S. *et al.* (1998) 'A transient expansion of the native state precedes aggregation of recombinant human interferon-', *Proceedings of the National Academy of Sciences*, 95(24), pp. 14142–14146. doi: 10.1073/pnas.95.24.14142.

Kettern, N. *et al.* (2010) 'Chaperone-assisted degradation: multiple paths to destruction', *Biol. Chem*, 391, pp. 481–489. doi: 10.1515/BC.2010.058.

Kim, H. *et al.* (2013) 'Epigallocatechin gallate (EGCG) stimulates autophagy in vascular endothelial cells: A potential role for reducing lipid accumulation', *Journal of Biological Chemistry*, 288(31), pp. 22693–22705. doi: 10.1074/jbc.M113.477505.

Kim, H. S. *et al.* (2018) 'Autophagy in Human Skin Fibroblasts: Impact of Age.', *International journal of molecular sciences*. Multidisciplinary Digital Publishing Institute (MDPI), 19(8). doi: 10.3390/ijms19082254.

Kim, Y. *et al.* (2013) 'Molecular Chaperone Functions in Protein Folding and Proteostasis', *Annual Review of Biochemistry*. Annual Reviews, 82(1), pp. 323–355. doi: 10.1146/annurev-biochem-060208-092442.

Kim, Y. E. *et al.* (2016) 'Soluble Oligomers of PolyQ-Expanded Huntingtin Target a Multiplicity of Key Cellular Factors', *Molecular Cell*, 63(6), pp. 951–964. doi: 10.1016/j.molcel.2016.07.022.

Komatsu, M. *et al.* (2007) 'Homeostatic Levels of p62 Control Cytoplasmic Inclusion Body Formation in Autophagy-Deficient Mice', *Cell*. Elsevier, 131(6), pp. 1149–1163. doi: 10.1016/j.cell.2007.10.035.

Komatsu, M. *et al.* (2010) 'The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1', *Nature Cell Biology*, 12(3), pp. 213–223. doi: 10.1038/ncb2021.

Kopito, R. R. (1997) 'ER quality control: The cytoplasmic connection', *Cell*. Elsevier, pp. 427–430. doi:

10.1016/S0092-8674(00)81881-4.

Kopito, R. R. (2000) 'Aggresomes, inclusion bodies and protein aggregation', *Trends in Cell Biology*. Elsevier Current Trends, 10(12), pp. 524–530. doi: 10.1016/S0962-8924(00)01852-3.

Korolchuk, V. I., Menzies, F. M. and Rubinsztein, D. C. (2010) 'Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems', *FEBS Letters*. No longer published by Elsevier, pp. 1393–1398. doi: 10.1016/j.febslet.2009.12.047.

Kozie, R. *et al.* (2011) 'Functional interplay between mitochondrial and proteasome activity in skin aging', *Journal of Investigative Dermatology*, 131(3), pp. 594–603. doi: 10.1038/jid.2010.383.

Krishnan, S. *et al.* (2002) 'Aggregation of Granulocyte Colony Stimulating Factor under Physiological Conditions: Characterization and Thermodynamic Inhibition †', *Biochemistry*, 41(20), pp. 6422–6431. doi: 10.1021/bi012006m.

Kroemer, G., Mariño, G. and Levine, B. (2010) 'Autophagy and the integrated stress response.', *Molecular cell*. NIH Public Access, 40(2), pp. 280–93. doi: 10.1016/j.molcel.2010.09.023.

Kumar, V. *et al.* (2016) 'Protein aggregation and neurodegenerative diseases: From theory to therapy', *European Journal of Medicinal Chemistry*. Elsevier Masson, 124, pp. 1105–1120. doi: 10.1016/j.ejmech.2016.07.054.

Kuusisto, E., Salminen, A. and Alafuzoff, I. (2001) 'Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies', *NeuroReport*, 12(10), pp. 2085–2090. doi: 10.1097/00001756-200107200-00009.

Kwik-Uribe, C. and Bektash, R. M. (2008) *Cocoa flavanols: measurement, bioavailability and bioactivity*, *Asia Pac J Clin Nutr*.

Lambert, J. D., & Elias, R. J. (2010, September). The antioxidant and pro-oxidant activities of green tea polyphenols: A role in cancer prevention. Archives of Biochemistry and Biophysics, Vol. 501, pp. 65–72. https://doi.org/10.1016/j.abb.2010.06.013

Legesse-Miller, A. *et al.* (2012) 'Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity', *Molecular Biology of the Cell*. Edited by C.-H. Heldin, 23(18), pp. 3566–3581. doi: 10.1091/mbc.E12-03-0192.

Lemasters, J. J. (2005) 'Selective Mitochondrial Autophagy, or Mitophagy, as a Targeted Defense Against Oxidative Stress, Mitochondrial Dysfunction, and Aging', *Rejuvenation Research*, 8(1), pp. 3–5. doi: 10.1089/rej.2005.8.3.

Lilienbaum, A. (2013) 'Relationship between the proteasomal system and autophagy.', *International journal of biochemistry and molecular biology*. e-Century Publishing Corporation, 4(1), pp. 1–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23638318 (Accessed: 9 September 2019).

Lindner, A. B. and Demarez, A. (2009) 'Protein aggregation as a paradigm of aging', *Biochimica et Biophysica Acta - General Subjects*. Elsevier, pp. 980–996. doi: 10.1016/j.bbagen.2009.06.005.

Liu, Y., Fiskum, G. and Schubert, D. (2002) 'Generation of reactive oxygen species by the mitochondrial electron transport chain', *Journal of Neurochemistry*, 80(5), pp. 780–787. doi: 10.1046/j.0022-3042.2002.00744.x.

Llobet, D. *et al.* (2008) 'Antioxidants block proteasome inhibitor function in endometrial carcinoma cells', *Anti-Cancer Drugs*, 19(2), pp. 115–124. doi: 10.1097/CAD.0b013e3282f24031.

Lo, D. D. *et al.* (2014) 'Differences in Foetal, Adult Skin and Mucosal Repair', in *Stem Cell Biology and Tissue Engineering in Dental Sciences*. Academic Press, pp. 691–702. doi: 10.1016/B978-0-12-397157-9.00055-2.

Loo, D. T. *et al.* (1993) 'Apoptosis is induced by beta-amyloid in cultured central nervous system neurons.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 90(17), pp. 7951–5. doi: 10.1073/pnas.90.17.7951.

López-Otín, C. *et al.* (2013) 'The Hallmarks of Aging', *Cell*. Europe PMC Funders, 153(6), p. 1194. doi: 10.1016/J.CELL.2013.05.039.

López de Figueroa, P. *et al.* (2015) 'Autophagy Activation and Protection From Mitochondrial Dysfunction in Human Chondrocytes', *Arthritis & Rheumatology*. John Wiley and Sons Inc., 67(4), pp. 966–976. doi: 10.1002/art.39025.

Lunkes, A. and Mandel, J.-L. (1998) 'A cellular model that recapitulates major pathogenic steps of Huntington's disease', *Human Molecular Genetics*. Narnia, 7(9), pp. 1355–1361. doi: 10.1093/hmg/7.9.1355.

Lynch-Day, M. A. *et al.* (2012) 'The role of autophagy in Parkinson's disease.', *Cold Spring Harbor perspectives in medicine*. Cold Spring Harbor Laboratory Press, 2(4), p. a009357. doi: 10.1101/cshperspect.a009357.

Mahmood, T. and Yang, P.-C. C. (2012) 'Western blot: Technique, theory, and trouble shooting', *North American Journal of Medical Sciences*. Wolters Kluwer -- Medknow Publications, 4(9), pp. 429–434. doi: 10.4103/1947-2714.100998.

Martin, J. B. (1999) 'Molecular Basis of the Neurodegenerative Disorders', *New England Journal of Medicine*. Edited by F. H. Epstein. Massachusetts Medical Society , 340(25), pp. 1970–1980. doi: 10.1056/NEJM199906243402507.

Mathew, R. *et al.* (2009) 'Autophagy Suppresses Tumorigenesis Through Elimination of p62', *Cell*. NIH Public Access, 137(6), p. 1062. doi: 10.1016/J.CELL.2009.03.048.

Mayer, M. P. and Bukau, B. (2005) 'Hsp70 chaperones: cellular functions and molecular mechanism.', *Cellular and molecular life sciences : CMLS*. Springer, 62(6), pp. 670–84. doi: 10.1007/s00018-004-4464-6.

McGeer, P. L. and McGeer, E. G. (1995) 'The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases', *Brain Research Reviews*. Elsevier, 21(2), pp. 195–218. doi: 10.1016/0165-0173(95)00011-9.

Mejlvang, J. *et al.* (2018) 'Starvation induces rapid degradation of selective autophagy receptors by endosomal microautophagy'. doi: 10.1083/jcb.201711002.

Mezera, V. *et al.* (2016) 'Effects of Epigallocatechin Gallate on Tert-Butyl Hydroperoxide-Induced Mitochondrial Dysfunction in Rat Liver Mitochondria and Hepatocytes', *Oxidative Medicine and Cellular Longevity*. Hindawi Limited, 2016. doi: 10.1155/2016/7573131.

Michaels, T. C. T. *et al.* (2015) 'Dynamics of protein aggregation and oligomer formation governed by secondary nucleation', *The Journal of Chemical Physics*. AIP Publishing LLC, 143(5), p. 054901. doi: 10.1063/1.4927655.

Miller, S. B. *et al.* (2015) 'Compartment-specific aggregases direct distinct nuclear and cytoplasmic aggregate deposition', *The EMBO Journal*. John Wiley & Sons, Ltd, 34(6), pp. 778–797. doi: 10.15252/embj.201489524.

Mitchell, P. (1961) 'Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism', *Nature*, 191(4784), pp. 144–148. doi: 10.1038/191144a0.

Mizushima, N., Yoshimori, T. and Ohsumi, Y. (2011) 'The Role of Atg Proteins in Autophagosome Formation', *Annual Review of Cell and Developmental Biology*. Annual Reviews , 27(1), pp. 107–132. doi: 10.1146/annurev-cellbio-092910-154005.

Morgan, B. (2011) 'Western blotting: Principles and Methods', *Tropical gastroenterology official*

journal of the Digestive Diseases Foundation, 10(1), pp. 1–181. doi: 10.1016/j.ymeth.2005.11.007.

Morley, J. F. *et al.* (2002) 'The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 99(16), pp. 10417–22. doi: 10.1073/pnas.152161099.

Morselli, E. *et al.* (2011) 'Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome.', *The Journal of cell biology*. The Rockefeller University Press, 192(4), pp. 615–29. doi: 10.1083/jcb.201008167.

Muhleisen, H., Gehrmann, J. and Meyermann, R. (1995) 'Reactive microglia in Creutzfeldt-Jakob disease', *Neuropathology and Applied Neurobiology*. John Wiley & Sons, Ltd (10.1111), 21(6), pp. 505–517. doi: 10.1111/j.1365-2990.1995.tb01097.x.

Myers, R. H. *et al.* (1991) 'Decreased Neuronal and Increased Oligodendroglial Densities in Huntington's Disease Caudate Nucleus', *Journal of Neuropathology & Experimental Neurology*. Narnia, 50(6), pp. 729–742. doi: 10.1097/00005072-199111000-00005.

Nagaoka, U. *et al.* (2004) 'Increased expression of p62 in expanded polyglutamine-expressing cells and its association with polyglutamine inclusions', *Journal of Neurochemistry*. John Wiley & Sons, Ltd (10.1111), 91(1), pp. 57–68. doi: 10.1111/j.1471-4159.2004.02692.x.

Nicholls, D. G. and Budd, S. L. (2000) 'Mitochondria and Neuronal Survival', *Physiological Reviews*. American Physiological SocietyBethesda, MD , 80(1), pp. 315–360. doi: 10.1152/physrev.2000.80.1.315.

Nichols, M. *et al.* (2015) 'Synergistic neuroprotection by epicatechin and quercetin: Activation of convergent mitochondrial signaling pathways', *Neuroscience*. Elsevier Ltd, 308, pp. 75–94. doi: 10.1016/j.neuroscience.2015.09.012.

Nowotny, K. *et al.* (2014) 'Accumulation of modified proteins and aggregate formation in aging', *Experimental Gerontology*. Pergamon, 57, pp. 122–131. doi: 10.1016/J.EXGER.2014.05.016.

Olzscha, H. *et al.* (2011) 'Amyloid-like aggregates sequester numerous metastable proteins with essential cellular functions', *Cell*. Elsevier, 144(1), pp. 67–78. doi: 10.1016/j.cell.2010.11.050.

Pajares, M. *et al.* (2018) 'The Role of Free Radicals in Autophagy Regulation: Implications for Ageing.', *Oxidative medicine and cellular longevity*. Hindawi Limited, 2018, p. 2450748. doi: 10.1155/2018/2450748.

Pan, T. *et al.* (2008) 'Neuroprotection of rapamycin in lactacystin-induced neurodegeneration via autophagy enhancement', *Neurobiology of Disease*, 32(1), pp. 16–25. doi: 10.1016/j.nbd.2008.06.003.

Park, C. and Cuervo, A. M. (2013) 'Selective Autophagy: Talking with the UPS', *Cell Biochemistry and Biophysics*, 67(1), pp. 3–13. doi: 10.1007/s12013-013-9623-7.

Park, J., Lee, J. and Choi, C. (2011) 'Mitochondrial Network Determines Intracellular ROS Dynamics and Sensitivity to Oxidative Stress through Switching Inter-Mitochondrial Messengers', *PLoS ONE*. Edited by S. Pandey, 6(8), p. e23211. doi: 10.1371/journal.pone.0023211.

Payne, B. A. I. and Chinnery, P. F. (2015) 'Mitochondrial dysfunction in aging: Much progress but many unresolved questions', *Biochimica et Biophysica Acta - Bioenergetics*, 1847(11), pp. 1347–1353. doi: 10.1016/j.bbabio.2015.05.022.

Peng, H. *et al.* (2017) 'Ubiquitylation of p62/sequestosome1 activates its autophagy receptor function and controls selective autophagy upon ubiquitin stress', *Cell Research*. Nature Publishing Group, 27(5), pp. 657–674. doi: 10.1038/cr.2017.40.

Pepys, M. B. *et al.* (2001) 'Pathogenesis, diagnosis and treatment of systemic amyloidosis', in Dobson, C. M., Ellis, R. J., and Fersht, A. R. (eds) *Philosophical Transactions of the Royal Society B: Biological Sciences*. The Royal Society, pp. 203–211. doi: 10.1098/rstb.2000.0766.

Qi, Z. *et al.* (2014) 'Antioxidant supplement inhibits skeletal muscle constitutive autophagy rather than fasting-induced autophagy in mice', *Oxidative Medicine and Cellular Longevity*. Hindawi Publishing Corporation, 2014. doi: 10.1155/2014/315896.

Ramdzan, Y. M. *et al.* (2017) 'Huntingtin Inclusions Trigger Cellular Quiescence, Deactivate Apoptosis, and Lead to Delayed Necrosis', *Cell Reports*, 19(5), pp. 919–927. doi: 10.1016/j.celrep.2017.04.029.

Ray Dorsey, E. *et al.* (2018) 'Global, regional, and national burden of Parkinson's disease, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016', *The Lancet Neurology*, 17(11), pp. 939–953. doi: 10.1016/S1474-4422(18)30295-3.

Rocca, W. A. (2018) 'The burden of Parkinson's disease: a worldwide perspective', *The Lancet Neurology*. Elsevier, 17(11), pp. 928–929. doi: 10.1016/S1474-4422(18)30355-7.

Rogov, V. *et al.* (2014) 'Interactions between Autophagy Receptors and Ubiquitin-like Proteins Form the Molecular Basis for Selective Autophagy', *Molecular Cell*. Cell Press, 53(2), pp. 167–178. doi: 10.1016/J.MOLCEL.2013.12.014.

Romero-Calvo, I. *et al.* (2010) 'Reversible Ponceau staining as a loading control alternative to actin in Western blots', *Analytical Biochemistry*. Academic Press, 401(2), pp. 318–320. doi: 10.1016/j.ab.2010.02.036.

Rosenbaum, J. C. and Gardner, R. G. (2011) 'How a disordered ubiquitin ligase maintains order in nuclear protein homeostasis', *Nucleus*. Taylor & Francis, 2(4), pp. 264–270. doi: 10.4161/nucl.2.4.16118.

Ross, C. A. and Poirier, M. A. (2004) 'Protein aggregation and neurodegenerative disease', *Nature Medicine*, 10(7), p. S10. doi: 10.1038/nm1066.

Rowley, T. J. *et al.* (2017) 'Monomeric cocoa catechins enhance β-cell function by increasing mitochondrial respiration', *Journal of Nutritional Biochemistry*. Elsevier Inc., 49, pp. 30–41. doi: 10.1016/j.jnutbio.2017.07.015.

Rubinsztein, D. C., Mariño, G. and Kroemer, G. (2011) 'Autophagy and aging', *Cell*. Cell Press, pp. 682–695. doi: 10.1016/j.cell.2011.07.030.

Sahani, M. H., Itakura, E. and Mizushima, N. (2014) 'Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids', *Autophagy*. Taylor and Francis Inc., 10(3), pp. 431–441. doi: 10.4161/auto.27344.

Sang, S., Lee, M. J., Hou, Z., Ho, C. T., & Yang, C. S. (2005). Stability of tea polyphenol (-)-epigallocatechin-3-gallate and formation of dimers and epimers under common experimental conditions. Journal of Agricultural and Food Chemistry, 53(24), 9478–9484. https://doi.org/10.1021/jf0519055

Sapp, E. *et al.* (2001) 'Early and Progressive Accumulation of Reactive Microglia in the Huntington Disease Brain', *Journal of Neuropathology & Experimental Neurology*. Narnia, 60(2), pp. 161–172. doi: 10.1093/jnen/60.2.161.

Schieber, M. and Chandel, N. S. (2014) 'ROS function in redox signaling and oxidative stress', *Current Biology*. Cell Press. doi: 10.1016/j.cub.2014.03.034.

Shang, L. *et al.* (2011) 'Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK', *Proceedings of the National Academy of Sciences of the United States of America*, 108(12), pp. 4788–4793. doi: 10.1073/pnas.1100844108.

Slobodkin, M. R. and Elazar, Z. (2013) 'The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy', *Essays In Biochemistry*, 55, pp. 51–64. doi: 10.1042/bse0550051.

Smith, D. M. *et al.* (2005) 'ATP Binding to PAN or the 26S ATPases Causes Association with the 20S Proteasome, Gate Opening, and Translocation of Unfolded Proteins', *Molecular Cell*. Cell Press, 20(5), pp.

687–698. doi: 10.1016/J.MOLCEL.2005.10.019.

Stadman, E. R. and Levine, R. L. (2006) 'Protein oxidation', *Annals of the New York Academy of Sciences*. John Wiley & Sons, Ltd (10.1111), pp. 191–208. doi: 10.1007/978-1-4419-6382-6-3.

Stefani, M. and Dobson, C. M. (2003) 'Protein aggregation and aggregate toxicity: New insights into protein folding, misfolding diseases and biological evolution', *Journal of Molecular Medicine*. Springer-Verlag, pp. 678–699. doi: 10.1007/s00109-003-0464-5.

Tanaka, K. (2009) 'The proteasome: Overview of structure and functions', *Proceedings of the Japan Academy Series B: Physical and Biological Sciences*. The Japan Academy, pp. 12–36. doi: 10.2183/pjab.85.12.

Taylor, J. P. *et al.* (2016) 'Decoding ALS: from genes to mechanism.', *Nature*. NIH Public Access, 539(7628), pp. 197–206. doi: 10.1038/nature20413.

Taylor, J. P., Hardy, J. and Fischbeck, K. H. (2002) 'Toxic proteins in neurodegenerative disease', *Science*, pp. 1991–1995. doi: 10.1126/science.1067122.

Thermo Scientific (2011) 'User Guide: Pierce BCA Protein Assay Kit (MAN0011430 Rev. A)', *Pierce Biotechnology*, 0747(23225), pp. 1–7. doi: 10.1016/j.ijproman.2010.02.012.

Thermo Scientific (2013) *Chemiluminescent Western blotting technical guide and protocols*, *Thermo Scientific*. Available at: www.thermo.com/pierce (Accessed: 13 August 2019).

Tigges, J. *et al.* (2014) 'The hallmarks of fibroblast ageing', *Mechanisms of Ageing and Development*, 138(1), pp. 26–44. doi: 10.1016/j.mad.2014.03.004.

Trigo, D., Nadais, A. and da Cruz e Silva, O. A. B. (2019) 'Unravelling protein aggregation as an ageing related process or a neuropathological response', *Ageing Research Reviews*, pp. 67–77. doi: 10.1016/j.arr.2019.02.001.

Tyedmers, J., Mogk, A. and Bukau, B. (2010) 'Cellular strategies for controlling protein aggregation', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 11(11), pp. 777–788. doi: 10.1038/nrm2993.

Underwood, B. R. *et al.* (2010) 'Antioxidants can inhibit basal autophagy and enhance neurodegeneration in models of polyglutamine disease', *Human Molecular Genetics*, 19(17), pp. 3413–3429. doi: 10.1093/hmg/ddq253.

Vlad, S. C. *et al.* (2008) 'Protective effects of NSAIDs on the development of Alzheimer disease', *Neurology*. Lippincott Williams and Wilkins, 70(19 PART 1), pp. 1672–1677. doi: 10.1212/01.wnl.0000311269.57716.63.

Vogiatzoglou, A. *et al.* (2015) 'Flavonoid intake in European adults (18 to 64 Years)', *PLoS ONE*. Public Library of Science, 10(5). doi: 10.1371/journal.pone.0128132.

Walter, P. and Ron, D. (2011) 'The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation', *Science*, 334(November), pp. 1081–1087. Available at: http://science.sciencemag.org/ (Accessed: 26 August 2019).

Wang, K. and Klionsky, D. J. (2011) 'Mitochondria removal by autophagy', *Autophagy*. Taylor & Francis, 7(3), p. 297. doi: 10.4161/AUTO.7.3.14502.

Wechalekar, A. D., Gillmore, J. D. and Hawkins, P. N. (2016) 'Systemic amyloidosis', *The Lancet*. NIH Public Access, pp. 2641–2654. doi: 10.1016/S0140-6736(15)01274-X.

Wyss-Coray, T. and Mucke, L. (2002) 'Inflammation in Neurodegenerative Disease—A Double-Edged Sword', *Neuron*. Cell Press, 35(3), pp. 419–432. doi: 10.1016/S0896-6273(02)00794-8.

Xia, M. and Hyman, B. T. (1999) 'Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease', *Journal of Neurovirology*, 5(1), pp. 32–41. doi: 10.3109/13550289909029743.

Xie, Z. and Klionsky, D. J. (2007) 'Autophagosome formation: core machinery and adaptations', *Nature Cell Biology*. Nature Publishing Group, 9(10), pp. 1102–1109. doi: 10.1038/ncb1007-1102.

Yamazaki, M. *et al.* (1992) '44-homooligomycins a and b, new antitumor antibiotics from streptomyces bottropensis: Producing organism, fermentation, isolation, structure elucidation and biological properties', *The Journal of Antibiotics*, 45(2), pp. 171–179. doi: 10.7164/antibiotics.45.171.

Yan, S. Du *et al.* (2000) 'Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis', *Nature Medicine*. Nature Publishing Group, 6(6), pp. 643–651. doi: 10.1038/76216.

Yan, X. *et al.* (2019) 'p62 aggregates mediated Caspase 8 activation is responsible for progression of ovarian cancer', *Journal of Cellular and Molecular Medicine*. Wiley-Blackwell, 23(6), pp. 4030–4042. doi: 10.1111/jcmm.14288.

Yates, S. L. *et al.* (2000) 'Amyloid β and Amylin Fibrils Induce Increases in Proinflammatory Cytokine and Chemokine Production by THP-1 Cells and Murine Microglia', *Journal of Neurochemistry*. John Wiley & Sons, Ltd (10.1111), 74(3), pp. 1017–1025. doi: 10.1046/j.1471-4159.2000.0741017.x.

Yonekawa, T. and Thorburn, A. (2013) 'Autophagy and cell death.', *Essays in biochemistry*. NIH Public Access, 55, pp. 105–17. doi: 10.1042/bse0550105.

Zatloukal, K. *et al.* (2002) 'p62 is a common component of cytoplasmic inclusions in protein aggregation diseases', *American Journal of Pathology*. American Society for Investigative Pathology, 160(1), pp. 255–263. doi: 10.1016/S0002-9440(10)64369-6.

Zhang, X. and Qian, S.-B. (2011) 'Chaperone-mediated hierarchical control in targeting misfolded proteins to aggresomes', *Molecular Biology of the Cell*. Edited by J. L. Brodsky, 22(18), pp. 3277–3288. doi: 10.1091/mbc.e11-05-0388.

Zheng, Q. *et al.* (2011) 'Proteasome malfunction activates macroautophagy in the heart.', *American journal of cardiovascular disease*, 1(3), pp. 214–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22081794 (Accessed: 22 October 2019).

Zhong, L. *et al.* (2015) 'Epigallocatechin-3-gallate opposes HBV-induced incomplete autophagy by enhancing lysosomal acidification, which is unfavorable for HBV replication', *Cell Death and Disease*. Nature Publishing Group, 6(5). doi: 10.1038/cddis.2015.136.

Zhu, K., Dunner, K. and McConkey, D. J. (2010) 'Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells', *Oncogene*, 29(3), pp. 451–462. doi: 10.1038/onc.2009.343.

7. Appendix

7. **Appendix**

1) Cell Culture Reagents and Equipment

a) **Equipment**

i) Hera cell CO2 incubator (Heraeus)
ii) Safety cabinet Hera safe (Heraeus)
iii) Inverted optical microscope (LEICA)
iv) Hemacytometer (Sigma)
v) Sonicator (U200S (IKA))
vi) Bath SBB6 (Grant)

b) **Reagents and solutions**

i) Trypsin-EDTA 0,05% (Thermo)
ii) PBS (1x)

For a final volume of 500 mL, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H_2O. Final composition:

- 8 mM Sodium Phosphate
- mM Potassium Phosphate
- 140 mM Sodium Chloride
- 10 mM Potassim Chloride

Sterilize by filtering through a 0.2 µm filter and store at 4 °C.

iii) RIPA buffer

To 980 µL of RIPA buffer (Sigma-Aldrich) add:

- 20 µL Protease inhibitor cocktail 50x (Roche)

2) Protein Content Determination

a) **Equipment**

i) Tecan 5000

b) **Reagents**

i) BCA assay kit (Pierce, Rockfort, IL)
ii) Bovine Serum Albumin (BSA) (Pierce)
iii) Working Reagent (WB) (50 Reagent A : 1 Reagent B)
    - Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,2N sodium hydroxide

- Reagent B: 4% cupric sulfate

3) SDS-PAGE Equipment and Solutions

a) **Equipment**

i) Electrophoresis system (Hoefer SE600 vertical unit)
ii) PowerPac 3000 Electrophoresis Power Supply (BioRad)

b) **Reagents and Solutions**

i) Acrylamide: Bis-Acrylamide 29:1 solution 40%, Dnase, Rnase free (Fisher)
ii) 10% APS (ammonium persulfate)

In 10 mL of deionised H_2O dissolve 1 g of APS. Note: prepared fresh before use.

iii) 10% SDS (sodium dodecyl sulfate)

In 10 mL of deionised H_2O dissolve 1 g of SDS.

iv) LGB (Lower gel buffer) (4x)

Per 900 mL of deionised H_2O add:

- 181.65 g Tris
- 4 g SDS

Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with deionised H_2O.

ii) UGB (Upper gel buffer) (5x)

Per 900 mL of deionised H_2O add:

- 75.69 g Tris

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1 L with deionised H_2O.

iii) Loading Gel Buffer (LB) (4x)

- 2.5 mL 1 M Tris solution (pH 6.8) (250 mM)
- 0.8 g SDS (8%)
- 4 mL Glicerol (40%)
- mL Beta-Mercaptoethanol (2%)
- 1 mg Bromophenol blue (0.01%)

Adjust the volume to 10 mL with deionised H_2O. Store in darkness at room temperature.

iv) 1 M Tris (pH 6.8)

Per 150 mL of deionised H_2O add:

- 30.3 g Tris base

Adjust the pH to 6.8 and adjust the final volume to 250 mL.

v) 10x Running Buffer

Per 150 mL of deionised H_2O add:

- 30.3 g Tris (250 mM)
- 144.2 g Glycine (2.5 M)
- 10 g SDS (1%)

Dissolve in deionised H_2O, adjust the pH to 8.3 and adjust the volume to 1 L.

vi) **Resolving and Stacking gel solutions**

Per gel:

	Resolving Gel		Stacking Gel
	5%	20%	3.5%
H_2O	9.29 mL	3.67 mL	6.92 mL
LGB (4x)	3.75 mL	3.75 mL	-
UGB (5x)	-	-	4.0 mL
29:1 Bis-Acrylamide	1.875 mL	7.5 mL	0.88 mL
10% APS	75 µL	75 µL	100 µL
10% SDS	-	-	100 µL
TEMED	7.5 µL	7.5 µL	10 µL
	30 mL		10 mL

3) Western-Blotting Solutions and Equipment

a) **Equipment**

i) Transfer Electrophoresis unit (Hoefer™ TE 42)

ii) Electrophoresis power supply EPS 1000 (Amersham Pharmacie Biotec)

b) **Reagents**

i) 1x Transfer Buffer

- 3.03 g Tris (25 mM)

- 14.41 g Glycine (192 mM)

Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 mL with deionised H_2O. Just prior to use add 200 mL of methanol (20%).

4) ImmunoBlotting Solutions

a) **Equipment**

i) ChemiDoc Touch Imaging System (BioRad)

b) **Reagents**

i) 10x TBS (Tris buffered saline)

- 12.11 g Tris (10 mM)
- 87.66 g NaCl (150 mM)

Adjust the pH to 8.0 with HCl and adjust the volume to 1L with deionised H_2O.

ii) 10x TBS-T (TBS+Tween)

- 12.11 g Tris (10 mM)
- 87.66 g NaCl (150 mM)
- 5 mL Tween20 (0.05%)

Adjust the pH to 8.0 with HCl and adjust the volume to 1L with deionised H_2O.

iii) Blocking solution

5% of BSA (Bovine Serum Albumine, Sigma) in 1x TBS-T.

iv) Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences)

v) Immobilon Crescendo Western HRP substrate (Merck)