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Effects of 1.3-1.6 ß-glucans on aging in vivo and ex-vivo

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ABSTRACT

The short-lived annual fish *Nothobranchius furzeri (Nfu)* has an extremely short life span and accelerated expression of age markers. This animal model recapitulates the major features of mammalian brain aging, such as gliosis and lipofuscin. *Nfu* is a convenient model for investigating the effects of interventions on longevity and age-related pathologies in Vertebrates. In this study, I observed the effects of 1.3-1.6 ß-glucans (BGs) in aging. BGs, natural food ingredients derived from the cell wall of *Saccharomyces cerevisiae*, have immunomodulatory, antioxidant, anti-inflammatory and antineoplastic actions.

In the initial part of my thesis, I have used BGs as a chronic and acute dietary supplement in *Nfu*. Chronic administration of BGs reduces multiple aging hallmarks in several organs, including the brain. BGs act specifically in mechanism related to autophagy, lysosomal activity, peroxidation of proteins, and inflammation. Even acute as well as chronic diet increases autophagy in brain aging, although it is unable to reduce the accumulation of lipofuscin throughout life. Subsequently, I created an ex-vivo brain culture system for *Nfu* to investigate whether BGs could influence the brain directly. This system replicates age-dependent reduction of autophagy observed in vivo and confirms the induction of autophagy by BGs. Acute treatment with BGs restores impairment of autophagy, induces biogenesis of mitochondria and lysosomes in aged ex-vivo brains. Proteomic analyzes in ex-vivo adult and old brain confirmed the positive activity of BGs on mitochondrial respiration and autophagy. In detail, BGs act on V-ATPase proteins (i.e. ATP6V1A), involved in mitochondrial respiration and the acidification of autophagosomes. Furthermore, BGs restore the inflammation in the brain by acting on microglia. BGs reduce the inflamed microglia in the ex-vivo Tg(mpeg1.1: EGFP) zebrafish model treated by Bafilomycin A1. Moreover, BGs induce autophagy partially mediated by microglia. In fact, the depletion of microglia in the ex-vivo model treated with Plx5622 involves an increase in autophagy by BGs, although to a lesser extent the volume of autophagosomes. Finally, to investigate the direct effect of BGs on neurons, I cultured human iPSC-derived neurons up to 90 DIV. BGs are able to reduce mortality in neurons where autophagy is blocked for 72 hours by Bafilomycin A1. Also, BGs increase autophagy in neurons where autophagic mechanism has been blocked for 24 hours.

Overall, these results indicate that BGs can slow progression some age-related markers. BGs act directly on the brain, normalizing cellular processes that are impaired during the aging even in acute treatments. As BGs are part of our normal diet, our results advocate BGs-fortified diet to promote human longevity.

1. INTRODUCTION

1.1 Aging

1.1.1 Theories of aging

Aging has been at the centre of human thoughts ever since the dawn of human consciousness. However, the complexity of aging processes has made precise understanding and adequate definition of aging difficult. Currently, some authors define aging as “the process of accumulation of life consequences, such as molecular and cellular damage, leading to functional decline, chronic diseases and, ultimately, mortality” (Moqri et al., 2023). Aging affects all living beings, such as animals, plants and fungi. In addition, recent studies suggest that also bacteria can age under certain conditions to improve the survival chances of half the population (Florea et al., 2017). Over the years, a question has arisen: "Does aging have a biological meaning?" and experts’ opinions are divided and can be split into two main theory groups: theories of programmed or non-stochastic aging and theories of unprogrammed or stochastic aging (Tartiere et al., 2024). The main difference between these two interpretations of aging lies in the postulated existence of a specific genetic program for aging. Theories of unprogrammed aging state that aging is not driven by a specific genetic program and argue that aging is an intrinsic consequence of life, the result of the accumulation of damage over a lifetime. On the contrary, theories of programmed aging postulate that aging is the result of an evolutionary selection process because the demise of the parents confers a fitness gain to the parental alleles via the offspring that exceeds the loss generated by the death of the parents. A theory has recently been proposed that lies in the middle of these two concepts, postulating that aging arises because of "defects" in the genetic program that controls development that fails to be correctly terminated and remains active at a much smaller pace during adult life. This means that aging is not a stochastic process but also denies the existence of a specific genetic program for aging, arguing that it is the consequence of defects in the developmental program (Khokhlov, 2013; De Magalhães, 2023). A similar theory, called the Danaid theory of aging, presents aging as an inexorable consequence of taxa's biology, further modulated by the accumulation and selection of mutations (Wensink and Cohen, 2022). The Figure 1 summarizes the main theories of aging.

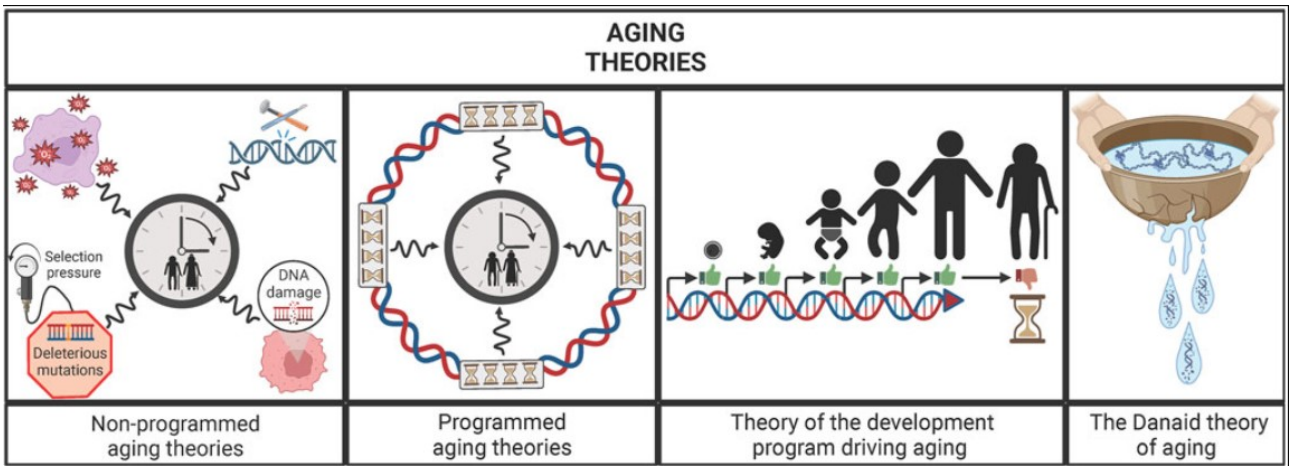


Figure 1: Aging theories. Theories of unprogrammed aging postulate that damage accumulated over a lifetime is the driver of the aging process. Programmed aging theories argue that a specific genetic program is what moves the "aging clock." The developmental program theory of aging postulates that defects in the developmental program are major factors determining aging. The Danaid theory states that aging is an intrinsic consequence of the biology of organisms because living beings are like the cracked vessels of the Danaids, that is, incapable of retaining life (water) for eternity. From Tartiere et al., 2024

1.1.2 Hallmarks of aging

The interest in understanding the mechanisms and causes of aging has gradually gained traction as it became increasingly clear that these mechanisms are in common with the major chronic diseases that represent the main causes of mortality in our society. To better understand the main causes of aging and the interactions between them, in 2013 an integrative vision of the aging process was proposed by identifying the so-called "hallmarks of aging", (López-Otín et al., 2013). Lopez-Otìn et al. named nine hallmarks that contribute to the aging process and together determine the aging phenotype. In "New hallmarks of aging" conference, in 2022 in Copenhagen (Denmark) the nine hallmarks of aging were recontextualized and other five further hallmarks were added to incorporate newer findings, as shown in Figure 2 (Schmauck-Medina et al., 2022).

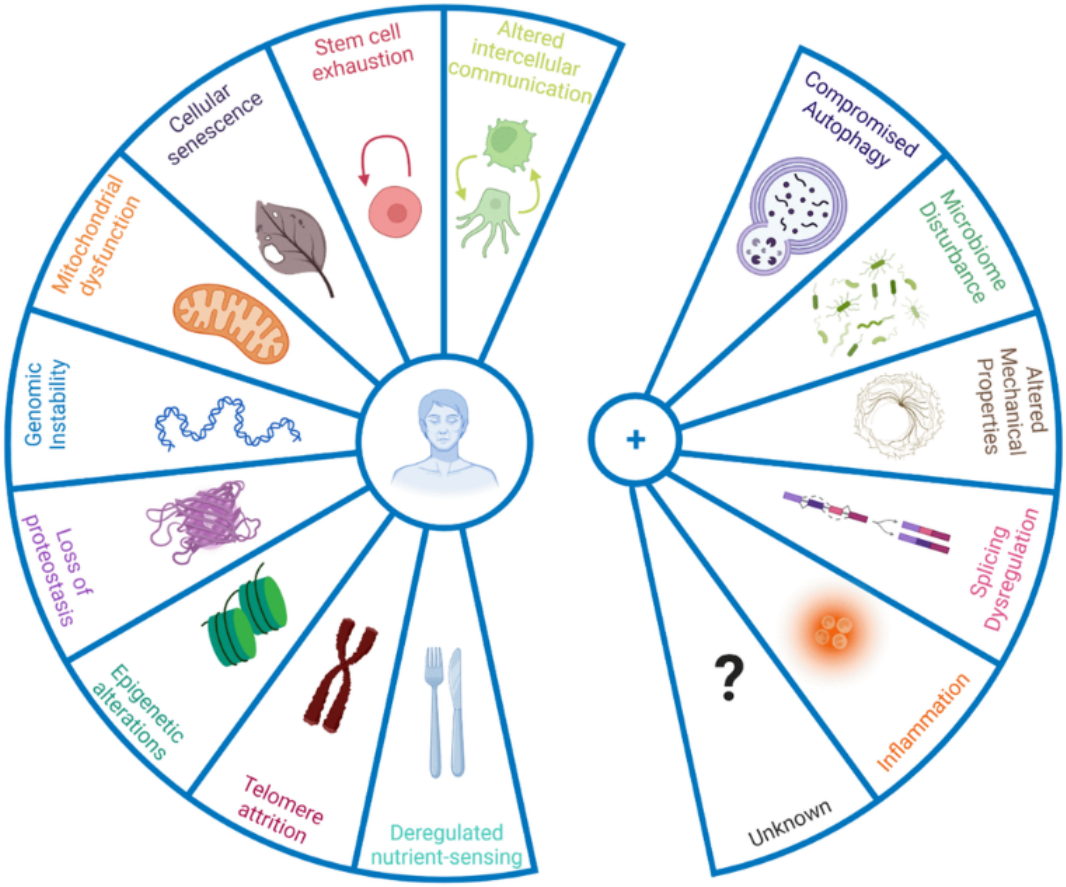


Figure 2: All hallmarks of aging. The figure lists the original hallmarks of aging plus the five proposed new hallmarks (Compromised autophagy, Microbiome Disturbance, Altered Mechaniscal Properties, Splicing Dysregulation and inflammation). From Schmauck-Medina et al., 2022

Next, the nine hallmarks of aging will be briefly described.

Genome instability:

During aging and premature aging diseases, a progressive accumulation of genetic damage has been observed (Moskalev et al., 2012). The integrity and stability of DNA are continually challenged by exogenous and endogenous physical, chemical and biological agents, including DNA replication errors, spontaneous hydrolytic reactions and reactive oxygen species (ROS) (Hoeijmakers, 2009). Genetic lesions resulting from extrinsic or intrinsic damage are very diverse and include mutations, translocations, chromosomal gains and losses, telomere shortening, and genetic disruption caused by the integration of viruses or transposons. To minimize these lesions, organisms have evolved a complex network of DNA repair mechanisms that are collectively capable of addressing most of the damage inflicted on nuclear DNA (Lord and Ashworth, 2012). DNA repair mechanisms are not perfect,

however, and DNA accumulates. In addition, the efficacy of these mechanisms is reduced during aging resulting in genome instability.

Of note, the rate of accumulation of somatic mutations scales with lifespan (Cagan et al., 2022) and long-living species are characterized by high expression of DNA repair genes (Toren et al., 2020) and more efficient double-strand repair (Tian et al., 2019)

Telomere attrition:

Telomeres are chromosomal regions that are particularly susceptible to the accumulation of damage with age (Blackburn et al., 2006). Replicative DNA polymerases lacks the ability to completely replicate the terminal ends of linear DNA molecules, a function that is specific for a specialized DNA polymerase known as telomerase. However, most mammalian somatic cells do not express telomerase, and this leads to the progressive and cumulative loss of telomere protective sequences from the ends of chromosomes in replicative tissues. Telomere attrition is mechanism responsible for the limited proliferative capacity of some cell types *in vitro*, the so-called replicative senescence (Hayflick and Moorhead, 1961; Olovnikov, 1996). Indeed, ectopic expression of telomerase is sufficient to confer unlimited replicative capacity in these cells without causing oncogenic transformation (Bodnar et al., 1998). Importantly, telomere attrition is observed during normal aging in both humans and mice (Blasco, 2007a). Telomeres are bound by a characteristic multiprotein complex known as "Shelterin" (Palm and de Lange, 2008). One of the main functions of riparian is to prevent DNA repair and chromosome fusions by preventing DNA repair proteins to access telomeres. Due to limited DNA repair, DNA damage at telomeres is remarkably persistent and highly efficient in inducing senescence and/or apoptosis (Fumagalli et al., 2012; Hewitt et al., 2012). In various loss-of-function models, the components of sheltering are characterized by a rapid decline in the regenerative capacity of tissues and accelerated aging in the absence of telomere attrition (Martínez and Blasco, 2010). Furthermore, a correlation between short telomeres and mortality risk has been observed in several models and in humans (Blasco et al., 1997; Herrera et al., 1999; Rudolph et al., 1999; Tomás-Loba et al., 2008; Armanios et al., 2009; Boonekamp et al., 2013). Recent evidence also indicates that aging can be reversed by activating telomerase. Indeed, normal physiological aging can be delayed without increasing the incidence of cancer in adult wild-type mice by systemic viral transduction of telomerase (Bernardes de Jesus et al., 2012).

Epigenetic alterations:

Aging is accompanied by epigenetic changes both at the level of DNA methylation (Johnson et al., 2012; Jung and Pfeifer, 2015; Xiao et al., 2019; Salameh et al., 2020) and post-translational modifications of histones (Wang et al., 2017). In particular, patterns of DNA methylation are strongly age-dependent and can be used even to predict age with great

accuracy (Seale et al., 2022). Specifically, loss of function of epigenetically relevant enzymes, such as SIRT6, reduces longevity and whose gain of function extends longevity in mice (Kanfi et al., 2012; Mostoslavsky et al., 2006). Understanding and manipulating the epigenome can extend healthy lifespan and improve age-related pathologies.

Loss proteostasis:

Aging and some age-related diseases are linked to altered proteostasis (Powers et al., 2009). Proteostasis involves mechanisms for the stabilization of correctly folded proteins and mechanisms for the degradation of spent proteins by the proteasome or lysosome (Hartl et al., 2011; Koga et al., 2011; Mizushima et al., 2008). Proteostasis and other alternative mechanisms refold misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged components and ensuring the continuous renewal of intracellular proteins. During aging, the alteration of both proteasome activity and lysosome function has been observed (Koga et al., 2011). Specifically, the chronic expression of unfolded, misfolded, or aggregated proteins contributes to the development of some age-related pathologies, such as Alzheimer's disease, Parkinson's disease (Powers et al., 2009).

Cellular senescence:

Cellular senescence can be defined as a stable cell cycle arrest coupled to stereotyped phenotypic changes (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007; Kuilman et al., 2010). The replicative senescence originally reported by Hayflick is caused by telomere attrition and the associated chronic activation of DNA damage response (Bodnar et al., 1998), but other stimuli associated with aging cant trigger senescence independently of telomeres. For example, non-telomeric DNA damage and derepression of the INK4/ARF locus are capable of inducing senescence (Collado et al., 2007). The accumulation of senescent cells in aged tissues has often been inferred using surrogate markers such as DNA damage or expression of the cell cycle inhibitor CDKN2A (p16). Furthermore, cellular senescence is not a generalized property of all tissues in aged organisms. Presumably, the accumulation of senescent cells with aging may reflect an increased rate of generation of senescent cells and/or a decrease in their rate of elimination, for example because of an attenuated immune response. Most importantly, due to their senesce-associated secretory phenotype (SASP) and the production of inflammatory molecules, senescent cells may exert detrimental effects of their neighbouring cells.

Deregulating nutrient Sensing:

The insulin and IGF-1 signalling pathway is a conserved aging control pathway in evolution. This pathway comprises multiple genes of key importance including the FOXO family of transcription factors and the mTOR complexes, which are also involved in aging (Fontana

et al., 2010; Kenyon, 2010; Barzilai et al., 2012). Genetic polymorphisms or mutations that reduce the functions of GH, the IGF-1 receptor, the insulin receptor, or downstream intracellular effectors such as AKT, mTOR, and FOXO have been linked to longevity. Therefore, anabolic signalling accelerates aging and decreased nutrient signalling prolongs longevity (Fontana et al., 2010).

Mitochondrial dysregulation:

During aging, the activity of the mitochondrial respiratory chain tends to decrease, thus increasing the loss of electrons and reducing the generation of ATP (Green et al., 2011). Furthermore, genetically-imposed mitochondrial dysfunction can accelerate aging in mammals (Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008). In particular, mitochondrial dysfunction during aging results from an increase in ROS production, which in turn causes further mitochondrial deterioration and global cellular damage (Harman, 1965).

Altered cellular communication:

Aging also involves changes at the level of intercellular communication (Laplante and Sabatini, 2012; Rando and Chang, 2012; Russell and Kahn, 2007; Zhang et al., 2013). For example, neurohormonal signalling tends to be deregulated during aging as inflammatory reactions increase, decrease immunosurveillance against pathogens and precancerous cells.

In addition to the nine hallmarks of aging, another five have been added, namely altered mechanical properties, microbiome disorders, dysregulation of splicing, inflammation, and impairment of autophagy.

Altered mechanical properties:

Altered mechanical properties affect both cells and the extracellular environment. Senescent cells show a major change of the mobilizable pool of actin that can be easily polymerized and depolymerized during cell motility (Tivey et al., 2013; Walters et al., 2016). Specifically, motility changes in the aging innate immune system have great relevance. The nucleoskeleton is also altered during aging, with destabilization of the nuclear lamina, with concomitant extrusion of chromatin into the cytoplasm as Cytoplasmic Chromatin Fragments (CCFs) activating the SASP in senescence (Vizioli et al., 2020). Finally, the extracellular matrix also changes with aging, which significantly alters cellular behaviour (Jaini et al., 2018). Increased stiffness and loss of elasticity, resulting for example from glycation cross-links between collagen molecules, can lead to multiple age-related disease states such as hypertension with concomitant renal and neurological defects, or can contribute to Accelerated aging observed in patients with diabetes (Bahouti et al., 2022).

Microbiome disorders:

Recent advances in next-generation sequencing technologies have enabled the identification of notable changes in the gut microbiome with age (Wilmanski et al., 2021), particularly indicating changes in microbial populations and loss of species diversity. Changing microbial populations during aging or in age-associated pathologies can drive inflammation.

Dysregulation of splicing

Dysregulation of splicing has been noted in studies of the aging human population (Holly et al., 2013). However, interventions that appear to reverse senescent phenotypes exert their effects at least in part by restoring juvenile patterns of splicing factor expression (Latorre et al., 2017). Alternative polyadenylation of mRNAs is also altered with aging and may contribute to senescence (Shen et al., 2019). Such changes in RNA processing add an additional layer of control of gene expression to those already known, such as genome integrity, transcriptional efficacy and epigenetic regulation.

Inflammaging:

Chronic age-dependent inflammation is implicated in a wide range of age-related diseases (Leonardi et al., 2018). Aging is associated to elevated levels of inflammatory mediators in the blood, such as IL-1, IL-6, C-reactive protein, IFNα, and many others (Ferrucci et al., 2013). Although inflammation was included in Hallmarl's "impaired intercellular communication," inflammation was later proposed as a separate hallmark due to its broad contribution to the aging process and its interaction with other hallmarks such as cellular senescence.

Impaired autophagy:

Impaired autophagy is observed in numerous aging conditions including neurodegeneration and immunosenescence (Wong et al., 2020; Aman et al., 2021). Activation of autophagy is associated to life-extending interventions such as dietary restriction, sperimidine and rapamycin and can increase the lifespan of mice (Fernandez et al., 2018) and even improve the immune response to vaccination in older humans by overcoming immunosenescence (Alsaleh et al., 2020).

1.2 Autophagy

Autophagy is a candidate pathway known to prevent both cellular aging and chronic low-grade inflammation (Klionsky et al., 2021). This evolutionary conserved process degrades and removes damaged cell material, such as protein aggregates and organelles, to maintain cellular and tissue homeostasis. It enables adaptation to cellular stress, including excessive

reactive oxygen species (ROS) production and metabolic stress, such as nutrient starvation. The core process of autophagy is instigated by inhibition of mechanistic target of rapamycin complex 1 (mTORC1) and/or activation of 5′ AMP-activated protein kinase (AMPK), both of which are canonical inducers of autophagy in response to metabolic stress (Fig. 3). The TOR pathway consists of two functional complexes: mTORC1 and mTORC2, each comprising several subunits (Laplante and Sabatin, 2012). Reduced mTORC1 activity by mutations or treatment with rapamycin induces autophagy leading to delayed aging and lifespan extension.

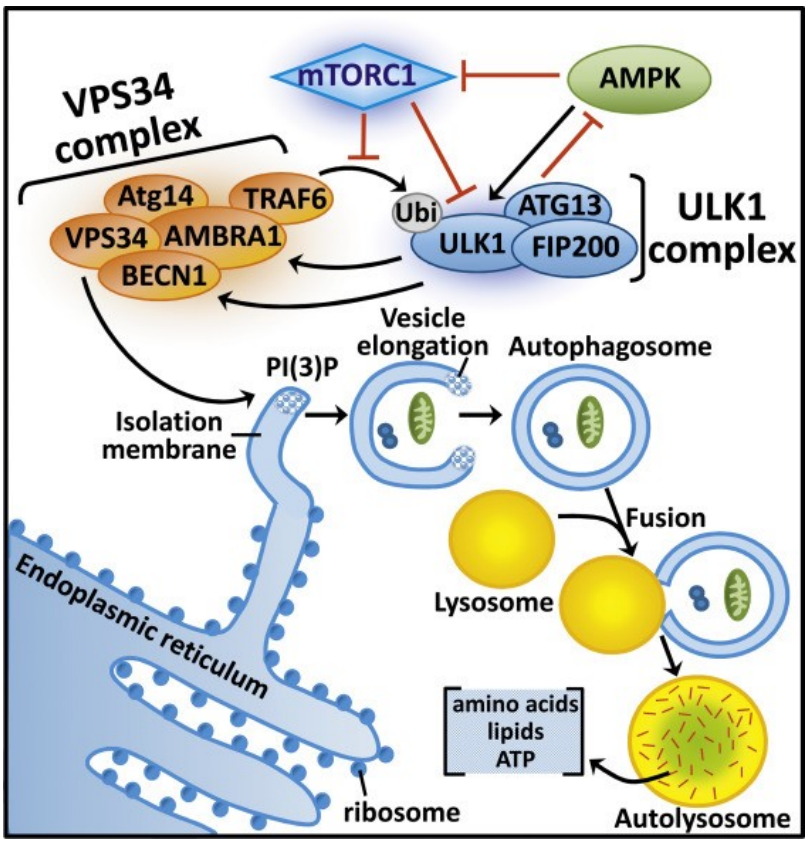


Figure 3: Signalling cross-talk between mTORC1, AMPK, ULK1 and VPS34 and induction of autophagy. When nutrients are available, mTORC1 negatively regulates ULK1 through direct phosphorylation and destabilization of ULK1 protein. In case of energy deficiency, AMPK activates ULK1 through protein phosphorylation. There is a negative feedback mechanism in which ULK1 inhibits AMPK through phosphorylation. After nutrient withdrawal (when mTORC1 is inactive) ULK1 initiates autophagy in part through phosphorylation of AMBRA1 and BECN1, which activates VPS34 to cause autophagosome maturation from the Endoplasmic reticulum. From Dunlop and Tee, 2014

There are canonical and non-canonical autophagy pathways. During non-canonical autophagy, the formation of the double-membrane-bound autophagosome does not require the hierarchical intervention of all the ATG proteins, whereas canonical autophagy does, Figure 4 (Durgan et al., 2021).

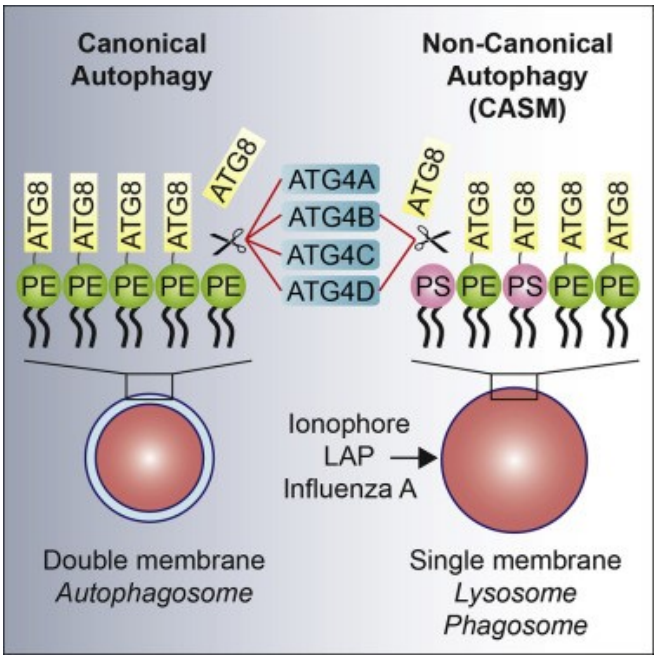
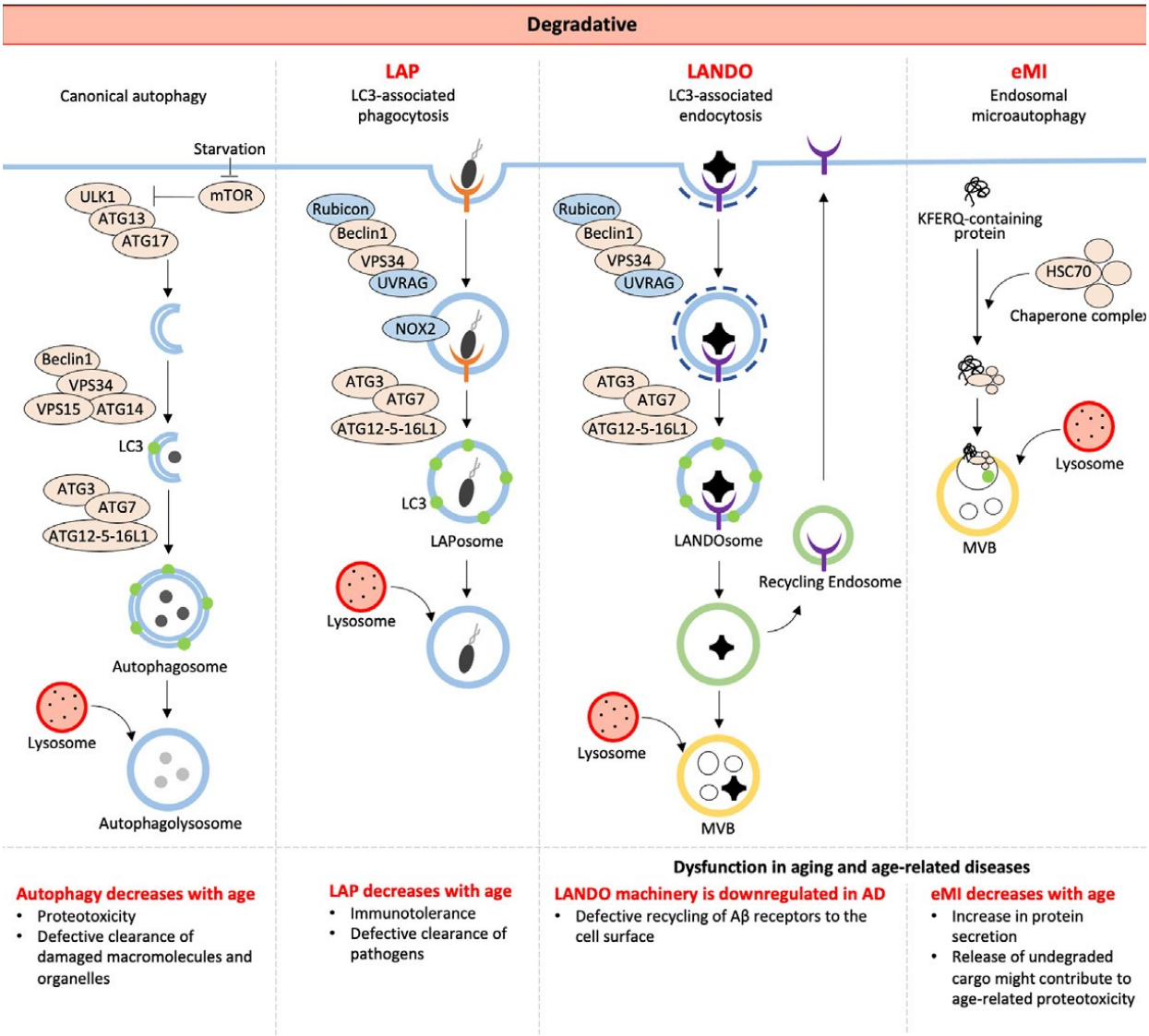


Figure 4: Canonical and non-canonical autophagy. Lipidation of ATG8 occurs during canonical and non-canonical autophagy. The hallmark of non-canonical autophagy is the alternative lipidation of ATG8 to PS. Non-canonical autophagy is a parallel pathway involving the conjugation of ATG8 to individual membranes (CASM) in endolysosomal compartments. From Durgan et al., 2021

Figure 5 shows that non-canonical autophagy can be subdivided into LAP (LC3-associated phagocytosis), LANDO (LC3-associated endocytosis), eMI (Endosomal microautophagy). The last two classified as secretory and non-degradative, are LDELS (LC3-dependent extracellular vesicle loading and secretion) and UPS (Unconventional protein secretion).


Degradative
Canonical autophagy
LAP
LC3-associated phagocytosis
LANDO
LC3-associated endocytosis
eMI
Endosomal microautophagy
Starvation
ULK1
mTOR
ATG13
ATG17
Beclin1
VPS34
VPS15
ATG14
LC3
ATG3
ATG7
ATG12-5-16L1
Autophagosome
Lysosome
Autophagolysosome
Rubicon
Beclin1
VPS34
UVRAG
NOX2
ATG3
ATG7
ATG12-5-16L1
LC3
LAPosome
Lysosome
Rubicon
Beclin1
VPS34
UVRAG
ATG3
ATG7
ATG12-5-16L1
LANDOsome
Recycling Endosome
Lysosome
MVB
KFERQ-containing protein
HSC70
Chaperone comple
Lysosome
MVB
Dysfunction in aging and age-related diseases
Autophagy decreases with age
• Proteotoxicity
• Defective clearance of damaged macromolecules and organelles
LAP decreases with age
• Immunotolerance
• Defective clearance of pathogens
LANDO machinery is downregulated in AD
• Defective recycling of Aβ receptors to the cell surface
eMI decreases with age
• Increase in protein secretion
• Release of undegraded cargo might contribute to age-related proteotoxicity

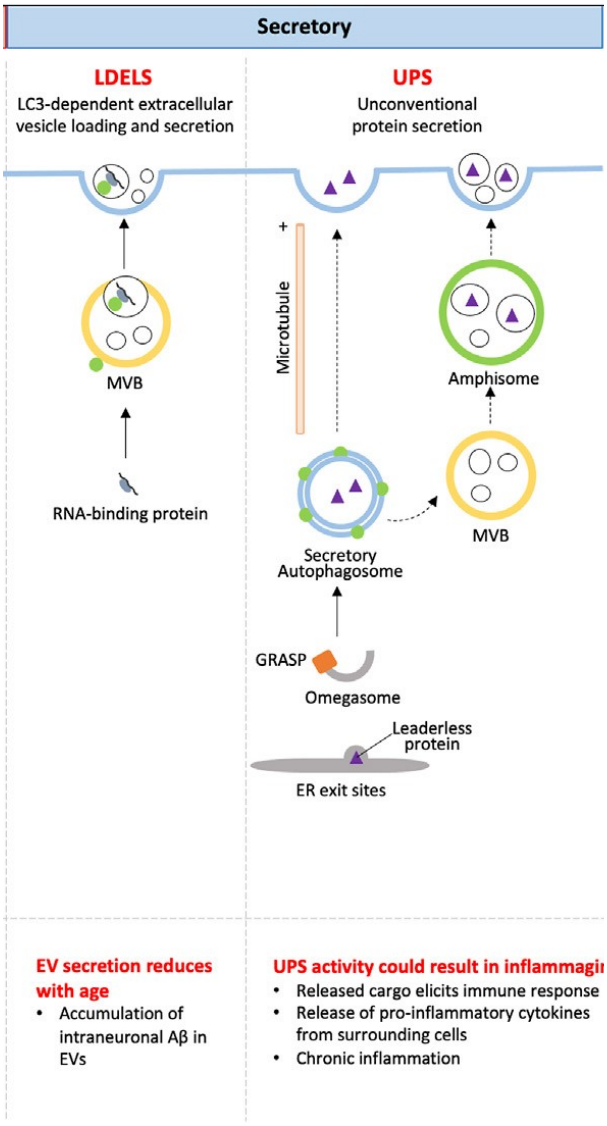


Figure 5: Different type of canonical and non-canonical autophagy. Autophagy is degradative and secretory. Degrative autophagy is divided into canonica, LAP (LC3-associated phagocytosis), LANDO (LC3-associated endocytosis), eMI (Endosomal microautophagy). Secretory autophagy is divided into LDELS (LC3-dependent extracellular vesicle loading and secretion) and UPS (Unconventional protein secretion). Figure above shows the types of degrative autophagy nd figure below shows type of secretive autophagy. From Kumar and Mills, 2023

In summary, cytoplasmic material is entrapped in membrane-delimited autophagosomes that then fuse with lysosomes in which their content is degraded and recycled. The three types of autophagy [Bejarano and Cuervo, 2010; (Fig. 6)] can be classified as macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy, generally referred to as autophagy, is mostly a highly selective, receptor-mediated pathway (selective autophagy) that targets, degrades and recycles specific cargos. This pathway enables adaptation to environmental challenges through the removal of pathogens or damaged organelles. In contrast, nonselective autophagy, as such microautophagy, occurs in response to starvation, providing energy and new anabolic

substrates. In detail, microautophagy involves direct engulfment of cytoplasmic cargo at a boundary membrane by autophagic tubes, which mediate both invagination and vesicle scission into the lumen (Li et al., 2011). CMA contributes to degradation of a specific subset of soluble proteins, and it doesn't require the formation of vacuoles for substrate delivery to lysosomes.

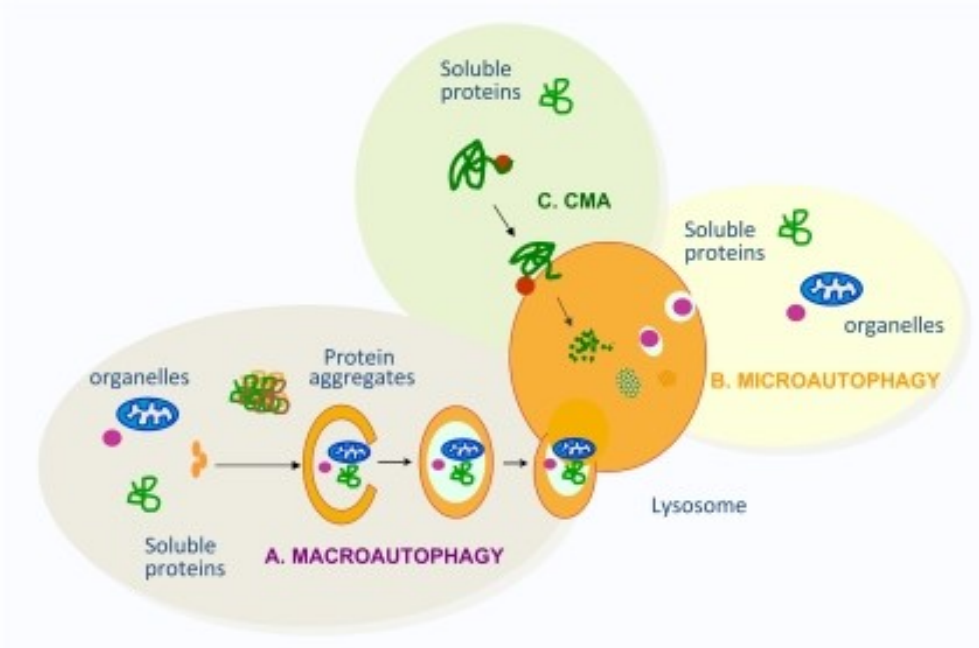


Figure 6: Three main types of autophagy in mammalian cells: Macroautophagy, microautophagy and Chaperone-mediated autophagy. Macroautophagy degrades soluble proteins,organelles and protein aggregates on them sequestration in a double-membrane vesicle which fuses with the lysosome to acquire hydrolases necessary for roteolysis. Microautophagy degrades soluble proteins and embedded organelles in lysosomes in small vesicles that form from invaginations of the lysosomal membrane. Chaperone-mediated autophagy (CMA) only contributes to the degradation of a specific subset of soluble proteins and does not require any type of membrane deformity for substrate delivery lysosomes. From Bejarano and Cuervo, 2010

Autophagy plays an important role in different physiological processes, as such Longevity, Tissue homeostasis, Differentiation, Development and Immunity. In fact, autophagy acts as an immune effector that mediates clearance of pathogens and pathogen-derived macromolecules. The role of autophagy bridge both innate and adaptive immune system. Moreover, the induction of autophagy reverses immune aging, senescence, and SASP phenotypes and rejuvenates the immune system.

1.3 Immunosenescence

A growing body of evidence suggests that aging of the immune system significantly contributes to multimorbidity and mortality of older subjects. Overall, older adults are not only prone to age-related conditions, but are also more susceptible to microbial infections

and they exhibit diminished vaccine responses due to decreased immune function (Desdlin-Mico et al., 2020; Fane and Weerartna et al., 2020; Haynes, 2020; Mueller et al., 2020). The age-related changes of immune functions were named “immunosenescence”. The systemic deterioration of immune function with age, which is accompanied by a chronic state of innate immune activation that causes low-grade systemic inflammation named “Inflamaging” (Franceschi, 2000; Furman et al., 2019). Immune aging, characterized by dysregulated and impaired immune function, involves changes to the number, function, and maturation of immune cells, as well as transcriptional and metabolic reprogramming in individual cells. To better understand immunosenescence, it is necessary to consider age-driven and immunity-related physiological changes. The body's physical barriers are the first line of defense against pathogens, and in older adults, the skin becomes thinner and drier, which in turn reduces the amount of fat-soluble defensins. Likewise, the mucosal barrier loses efficiency during aging because the ciliary function is compromised, which consequently facilitates the establishment of pathogens (Wlaschek et al., 2021). Age-related decline in coping ability and concomitant increase in proinflammatory state are hallmarks of immunosenescence. In addition, thymic involution, decreased numbers of T and B lymphocyte cells, reduced telomerase activity and a weak immune response have been the most dramatic and ubiquitous changes in aging (Franceschi, 2000; Jose et al., 2017; Thomas et al., 2020; Martinez et al., 2021; Tessier et al., 2022). It is worth mentioning that these deficiencies are aggravated by exposure to pathogens (Pawelec, 2018; Aiello et al., 2019). Furthermore, alterations in innate immune cells (neutrophils, macrophages, natural killer, dendritic and mast cells) were detected in elderly individuals. Neutrophils show a reduced killing capacity, although their production in the bone marrow remains unchanged and their number in the blood increases slightly with aging (Rosalec, 2018). Similarly, the function of natural killer cells is disturbed with aging, although their turnover in the bone marrow decreases and their baseline number increases (Bozzano et al., 2015). Macrophages and dendritic cells show similar phagocytic activity between the young and the elderly; however, both the total number of these cells in the peripheral blood and their ability to present antigens and stimulate T cells are defective in the elderly (Pinti et al., 2010; Oishi et al., 2016). Furthermore, macrophages show an increased inflammatory response (Rawji et al., 2016). Finally, the activation and function of mast cells are altered in elderly individuals (Kundu et al., 2020). Adaptive immune cells are affected by aging as well. The repertoire of naïve T cells and memory B cells is abundant during childhood, while in older age, a decline in B cell production in the bone marrow (Ma et al., 2019) and a reduction in the number of T cells due to thymic activity involution occurs (Pinti et al., 2010; Yang et al., 2010). Overall, these events result in an overall reduction in immunity in older individuals (Laidlaw et al., 2021). In summary, the hallmarks of immunosenescence are thymus involution, inflammaging, change in T-cells, metabolic change, such as increase of ROS (Liu et al., 2023). Thymic involution plays a vital role in the imbalance of immune cell proportions,

especially for T cells (Palmer, 2013). The atrophy of aged human thymus results in a decrease in naïve T cells, an increase in late-differentiated peripheral memory T cells, and a diminished migration of naïve T cells to the periphery. Evidence for the role of thymic involution in the aging of the immune system is provided by the observation that premature thymectomy result in immunosenescence (Sauce et al., 2009). Furthermore, one of the hallmarks of immunosenescence is "inflammaging," which refers to a systemic state of chronic low-grade inflammation characterized by upregulated blood inflammatory markers and is considered the central pillar of aging (Fulop et al., 2017; Accardi and Caruso, 2018). The accumulation of damaged macromolecules is responsible for inflammation. Endogenous host-derived cellular debris is the source of chronic tissue damage. Cellular senescence is central to the inflammation process. Senescent cells display a characteristic senescence-associated secretory phenotype (SASP) that implies secretion of a plethora of soluble factors, including interleukin-1 (IL-1), IL-6, IL-8, IL-13, IL-18 and tumor necrosis factor (TNF) and its receptors, which lead to the inflammatory phenotype (Ershler et a., 2000; Bruunsgaard et al., 2003; Ferrucci et al., 2018). Cellular senescence is a classic example of antagonistic pleiotropy, in which a specific genetic trait elicits a phenotype that is beneficial early in life, for example, by generating a well-recognized protective barrier to prevent tumorigenesis, which is essential for normal embryonic development and tissue repair. On the one hand, the chronic presence of senescent cells is harmful to surrounding cells and tissues owing to their secretion of cytokines, chemokines, proteases and bioactive lipids, and the attraction of inflammatory cells (Campisi, 2013; Calcinotto et al., 2019; Gorgoulis et al., 2019; Zhou et al., 2021). On the other hand, cellular aging is a consequence of the accumulation of damaged macromolecules (DNA, proteins and lipids) and organelles (e.g. mitochondria) and occurs in long-lived, post-mitotic cells (Campisi and di Fagagna, 2007; López- Otín et al., 2013). Moreover, as the immune system ages, metabolism undergoes changes resulting in increased glycolysis, mitochondrial dysfunction, and reactive oxygen species (ROS) (Henson et al., 2014; Patsoukis et al., 2015). The combination of persistent environmental insults, and the decline of repair and removal mechanisms leads to this increased damage (Desdín-Micó et al., 2020; Goronzy and Weyand, 2019). Then, these features of immunosenescence are strongly correlated with high morbidity and mortality from age-associated diseases such as cardiovascular diseases, neurodegenerative diseases, autoimmune diseases, metabolic diseases, and cancers in elderly subjects.

Immune aging accelerates the course of the organism's aging process. Immunosenescence and the chronic low-grade inflammation that characterizes aging have been correlated with altered autophagic flux in macrophages (Stranks et al., 2015; Zhang et al., 2016; Franceschi et al., 2017).

1.4 Autophagy in immune system

Autophagy helps in the elimination of pathogens, it also acts in the release of endogenous or exogenous molecules into intracellular compartments, in the modulation of inflammasome activity, the control of cytokine secretion and the regulation of phagocytosis (Bonilla et al., 2013). In particular, autophagy is key in modulating immunity and immune cell dynamics (Germic et al., 2019; Metur and Klionsky, 2021). In addition, autophagy is further involved in differentiation and proliferation of immune cells, although what exactly underlies molecular mechanisms remains partly elusive (Clarke and Simon, 2019). Evidence indicates that mitophagy, which encompasses selective degradation of damaged or excessive mitochondria, is an especially crucial regulator of innate immune cell function (Gkikas et al., 2018). Autophagy also prevents mitochondrial DNA escaping into the cytoplasm by maintaining mitochondrial homeostasis, which inhibits initiation of type I interferon signalling and, ultimately, inflammation (Riley and Tait, 2020). Furthermore, autophagy is essential for T cell immunity and its decline with age leads to immunosenescence (Phadwal et al., 2012; Puleston and Simon, 2014). Autophagy facilitates adaptive immune cell activation (Clarke and Simon, 2019; Hubbard et al., 2010; Pua et al., 2007), differentiation (Mortensen et al., 2011; Riffelmacher et al., 2017), and reverses systemic immunosenescence through modulation of T cell immunity (Macian, 2019). Autophagy participates in all aspects of immunity, influencing both innate and adaptive immune processes, with the term "immunophagy" referring to all these processes collectively (Deretic, 2006). In innate immunity, autophagy works downstream of pattern recognition receptors by activation of innate immune receptors, including TLRs and NLRs, where it facilitates a few effector responses, cytokine production, and phagocytosis. In adaptive immunity, autophagy provides a substantial source of antigens for loading onto MHC class II molecules, and it may be important in dendritic cells for cross-priming to CD8+ T cells. In detail, there are three main types of autophagic process that contribute to immune system function [Deretic, 2011; (Fig. 7)]. Type I: specialized autophagic immune processes that are executed by autophagic machinery at the cellular level. These include the recognition, capture, and elimination of intracellular pathogens through a process called "xenophagy" (Levine, 2005) These processes contribute to a recently defined specialized form of Autophagic Macrophage Activation [APMA; (Deretic and Levine, 2009)], to improved recognition of microbial products by innate immunity receptors such as intraluminal Toll-like receptors (TLRs; Lee et al., 2007), and to enhanced MHC-restricted presentation II of the cytoplasmic self - either foreign antigens (Munz, 2009) or phagocytosed antigens (Lee et al., 2010). Autophagy is also a specialized effector mechanism downstream of the stimulation of innate immunity receptors (Delgrado et al., 2009) such as TLRs (Xu et al., 2007; Delgrado et al., 2008; Sanjuan et al., 2007; Shi et al., 2010), Nod-like receptors (NLRs) (Yano et al., 2008; Cooney et al., 2010; Travassor et al., 2010), and RIG-I-like (RLR) or intracellular stimulation by damage-associated molecular patterns (DAMPs; also known as alarmins). Autophagy

doesn't only help induce, deliver, and execute innate immunity responses, but can also limit innate immunity responses and inflammation (Jounai et al., 2007; Sainton et al., 2008; Sainton et al., 2009). Overall, autophagy in its specialized immune functions contributes to immune activation, acts as an effector and regulator of innate and adaptive immune responses, and ultimately quenches inflammation. Type II: autophagy controls cellular homeostasis. In this capacity, autophagy affects immune cells in the same way it affects any other cell type. Autophagy type II maintains cell viability and intracellular organelles, such as mitochondria. Some immune cells are more dependent on autophagy, such as T cells (Li et al., 2006; Pua et al., 2007). T cells may require autophagy to survive when growth factors are withdrawn, and as T cells mature, autophagy is the process that carries out the programmed reduction in the number of mitochondria that occurs upon the exit of naïve T cells from the thymus (Pua et al., 2009; Mortensens et al., 2010). On the other hand, B cells are less sensitive to autophagy. Type III: non-autophagic role of Atg factors. This is based on a separate group of activities that reflect the non-autophagic participation of one or more Atg genes, when they exert their function other than autophagy (Virgin and Levin, 2009). These activities fall within the area of peripheral interactions and coordination between different functional systems.

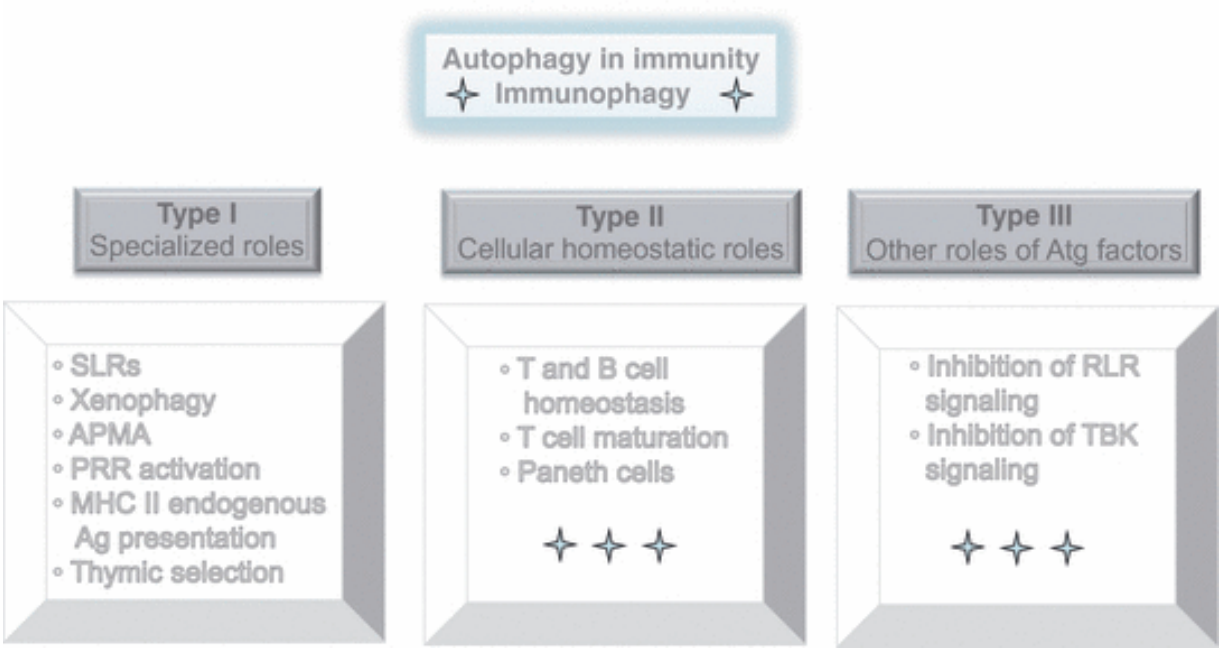


Figure 7: Three types of autophagy intersections with immune processes. Type I immunophagy specializes in autophagy capturing, processing, or delivering immunologically active microbes or microbial or endogenous molecules. Type II immunophagy has roles in controlling cell viability and general functionality of immune cells in ways that are not different from effects in all other cell types (e.g. neurons). Type III processes are those that are influenced by isolated Atg factors but do not depend on the execution of the entire autophagic pathway. From Deretic, 2011

1.5 Autophagy in aging

Compromised autophagy is a hallmark of aging (Aman et al., 2021). However, decreased autophagy contributes to senescence and the aging process itself (Rubinsztein et al., 2011; Aman et al., 2021). The Figure 8 summarises the cellular component reduced by impaired autophagy during aging.

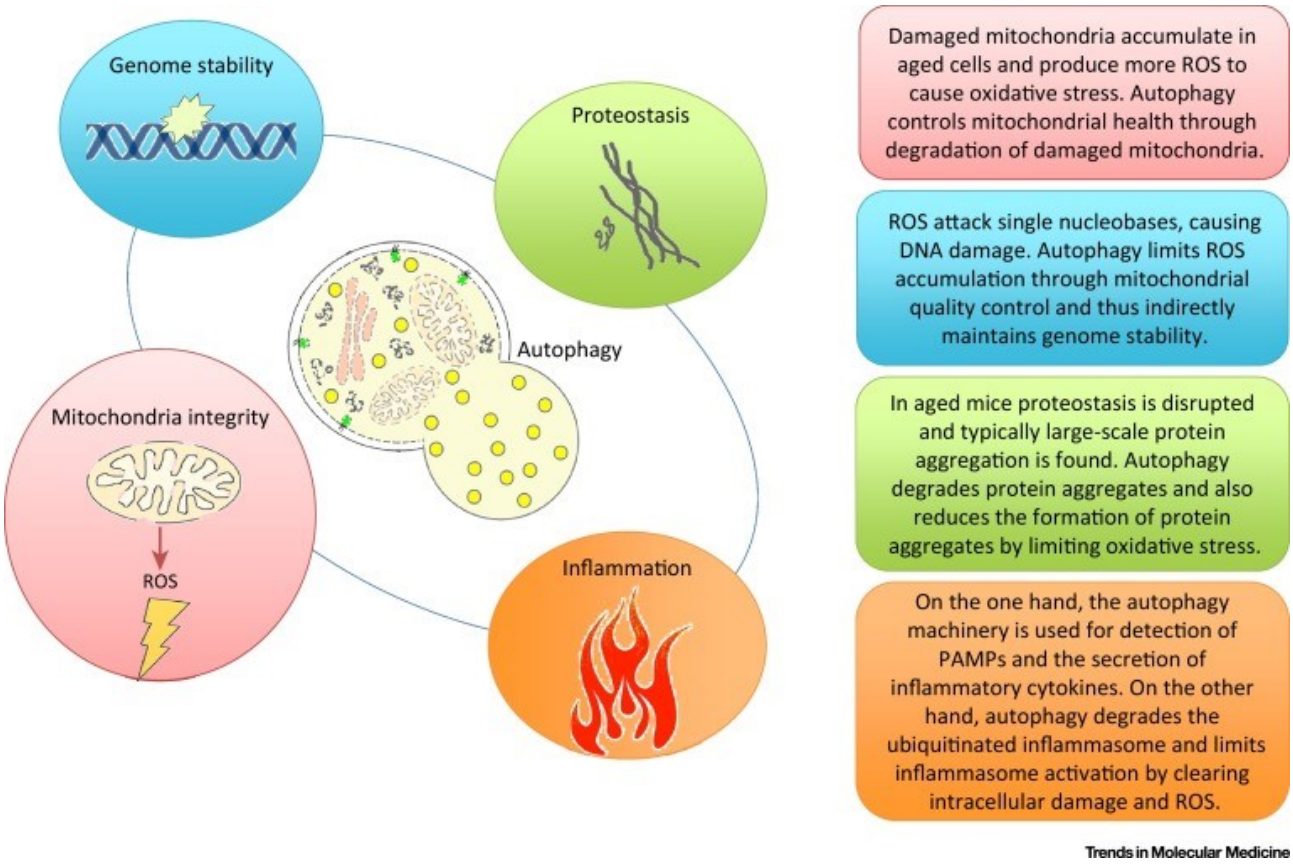


Figure 8: Decline of mechanisms in impaired autophagy during aging. The diagram describes biological processes, as inflammation, mitochondria integrity, genome instability and proteostasis, modulated by autophagy and inhibited during aging from Zhang et al., 2016

Autophagy has a crucial role in the regulation of animal lifespan. Activation of macroautophagy has been shown to be necessary for the lifespan extension observed in worms and flies after specific mutations or caloric restriction (Melendez et al., 2003; Hansen et al., 2008). Macroautophagy is also required to sustain a normal lifespan. In fact, mice with impaired autophagy exhibit shorter lifespans. On the other hand, mice with overexpression of the Atg proteins show a notable increase in lifespan (Pyo et al., 2013). Moreover, genetic correction of age-dependent Chaperon-Mediated Autophagy (CMA) decline in the liver has been shown to be sufficient to prevent functional decline of this organ in aged rodents (Zhang and Cuervo, 2014). Inhibition of mTOR is a known inductor of autophagy and doubled normal lifespan in nematodes and increases autophagy (Vellai et al.,

2003), increased lifespan in flies (Bjedov at al., 2010), and prolonged mice lifespan (Harrison et al., 2009; Bitto et al., 2016). Thus, defects in autophagy are linked to a variety of human diseases, including immune-associated and age-related diseases, both of which are often linked to increased inflammation (Aman et al., 2021; Klionsky et al., 2021). Recent studies have linked impairment of general autophagy to pathological states such as progeria and a series of accelerated aging diseases (Fang et al., 2016) neurodegenerative diseases (Menzies et al., 2015; Lou et al., 2019), and other disorders (Rubinsztein et al., 2011; Hansen et al., 2018). Therefore, the first changes in the regulation of macroautophagy may be secondary to age-related alterations in metabolism and/or in the hormonal response to fasting (Bergamini et al., 1990; Donati et al., 2001; Del Roso et al., 2003). This alteration in the regulation of macroautophagic proteolysis, which leads to the accumulation of organelles and altered membranes, may initiate a vicious cycle pro-aging (Bergamini et al., 2004). Specifically, defective macroautophagy in aged cells results in part from impaired autophagosome formation and reduced autophagosome clearance. The presence of undigested materials in lysosomes (aggregates, such as lipofuscin) could be responsible for their reduced ability to fuse and/or degrade autophagosome contents (Fig. 9A). Recent genetic evidence also supports this critical role of macroautophagy as an anti-aging mechanism. CMA activity also decreases with age (Cuervo et al., 2005). In detail, a decrease in lysosomal levels of the CMA receptor is responsible for the reduced activity of CMA during aging. CMA activity is initially maintained in adults, increasing the amount of luminal chaperone. On the opposite, receptor levels decrease during aging to the point that they are so low that compensation by the chaperons is no longer possible (Fig. 9B). The concomitant failure of both macroautophagy and CMA probably precipitates the accumulation of damaged cytosolic proteins with age.

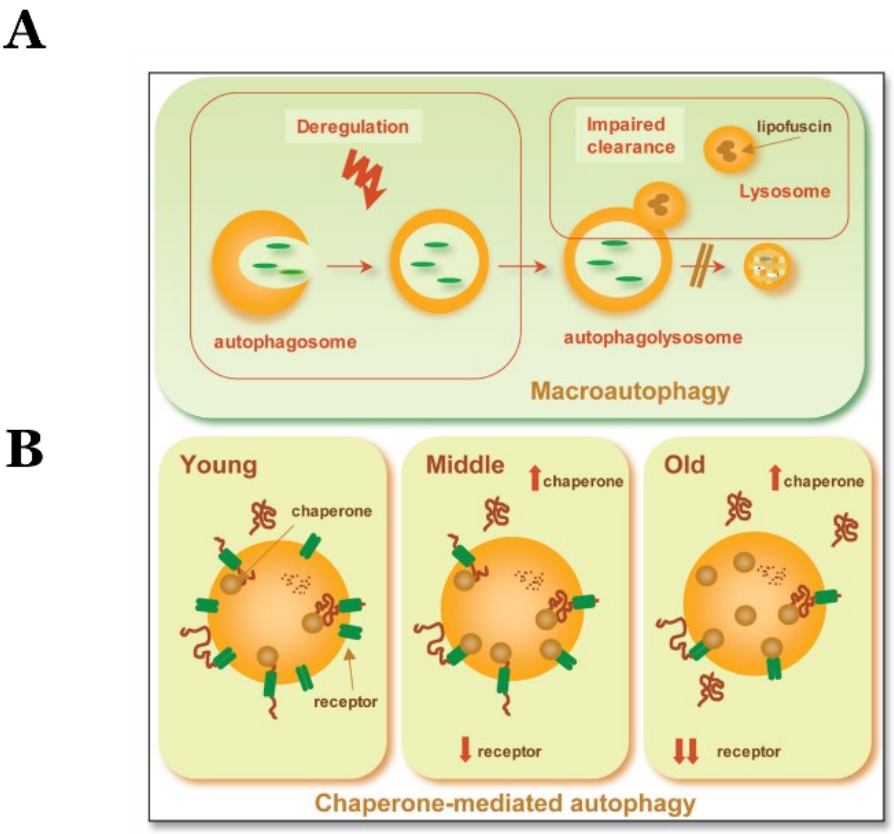


Figure 9: Impaired autophagy during aging. A) Impaired of macroautophagy during aging. Presence of undigested materials in lysosomes, i.e. lipofuscin, could be responsible for the reduced ability to fuse and degrade the contents of the autophagosome. B) Impaired of CMA in old cells in comparison with young and adult cells. Normal CMA activity is initially maintained (middle age) by increasing the amount of luminal chaperone. Conversely, in the elderly the levels of the receptor are very low. From Cuervo et al., 2005

The most imminent consequence of autophagic failure is probably the age-related accumulation of "waste" within long-lived post-mitotic cells like neurons that are characterized by a very low replacement rate. This so-called “biological waste” progressively accumulates within cells, suggesting failures in turned over of damaged biomolecules (Sheldrake, 1974; Terman and Brunk, 2004). Extra- and intralysosomal intracellular “waste” material accumulates, reflecting insufficient autophagic sequestration and degradation, respectively. During aging, the autophagic capacity decreases thus increasing the production of ROS, the aggregated proteins, "biological waste" that activate the inflammasomes. However, that inflammation inhibits autophagy and accelerates the aging process [Salminen et al., 2012; (Fig. 10A)]. In detail, the inflammation during aging involves a decline of the degradative autophagy are blocked. On the contrary, the secretory autophagy is activated. The secretory autophagy causes a release of inflammatory cytokines, which increase the hyperactivation of macrophages with increased inflammation, as Figure 10B

shows (Kumar and Mills, 2023).

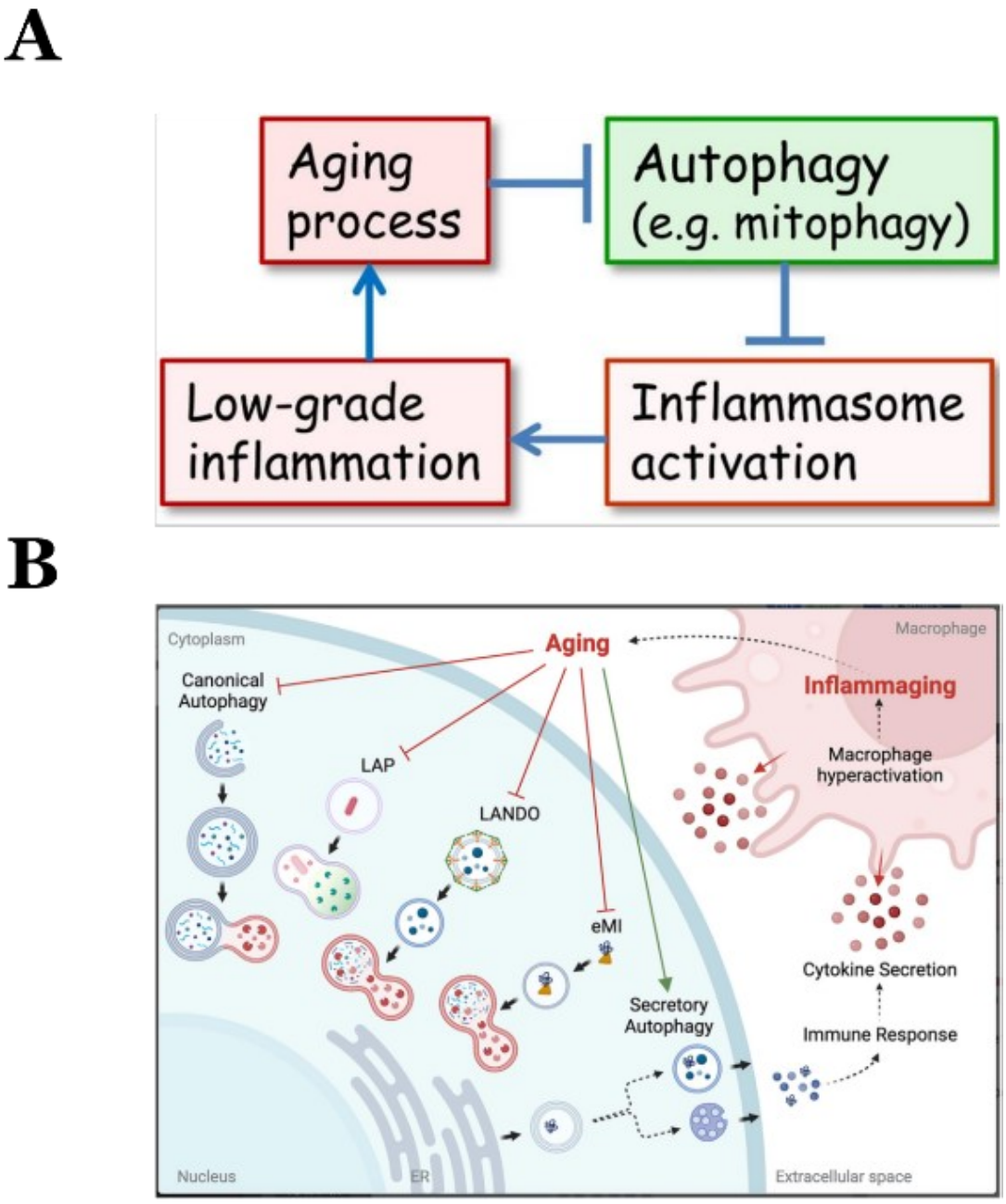


Figure 10: Decline of autophagy in aging. A) Link between autophagy and inflammation during aging. Autophagic uptake of dysfunctional mitochondria prevents excessive ROS production and thus the activation of inflammasomes. during aging, autophagic capacity decreases and increased production of ROS and aggregated proteins activate inflammasomes which cause low-grade inflammation in different tissues and thereby inhibit autophagy and accelerate the aging process. From Salminen et al., 2012. B) Block of degrative autophagy and activation of secretory autophagy cause inflammaging. Overactivation of macrophages will lead to increased phagocytosis of discarded cargo, bringing it back into the cell to attempt to be cleared by LAP or LANDO. If the limitation is in lysosomes, the effort is futile and will lead to the deposition of proteins aggregated both intracellularly and extracellularly space. From Kumar and Mills, 2023

1.5.1 Mitochondrial respiration and autophagy in aging

Impairment of autophagy during aging causes the accumulation of abnormal mitochondria, which increases oxidative stress, Figure 11 (Cuervo et al., 2005).

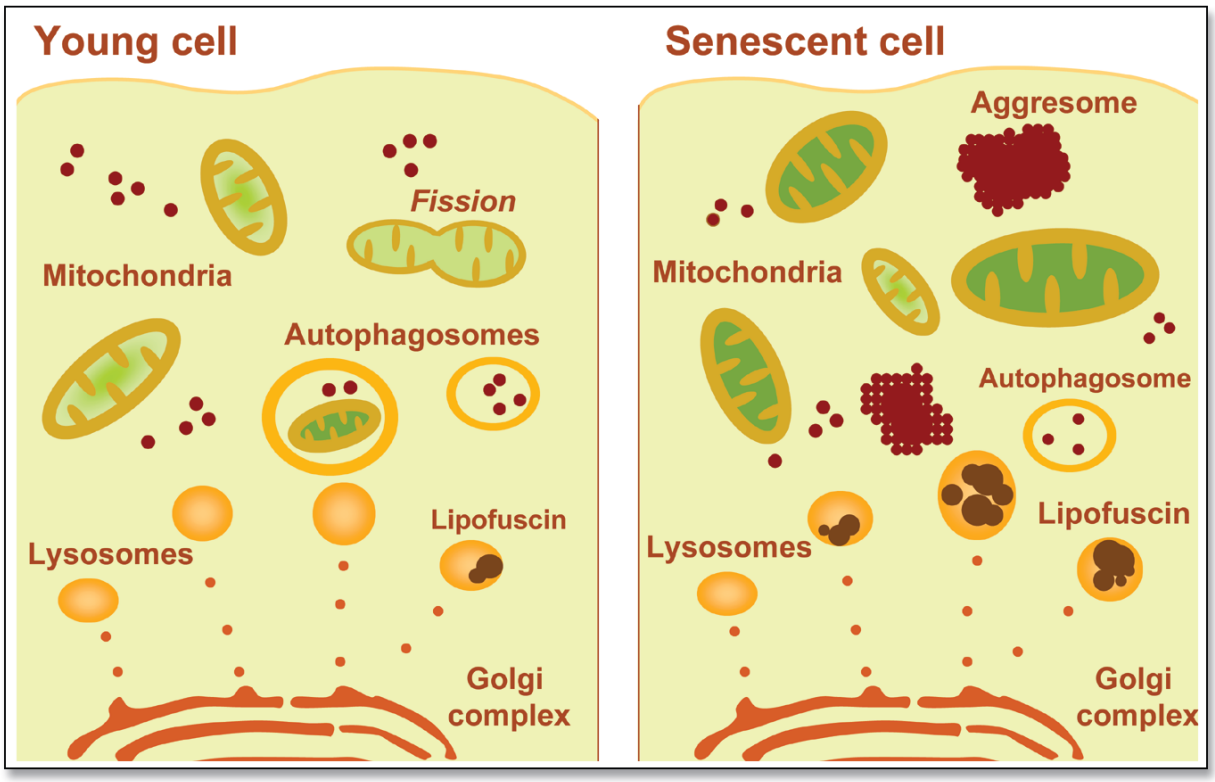


Figure 11: Age-related accumulation of damaged structure as a result of imperfect macroautophagy. The oxidative modification of macromolecules subjected to autophagic degradation determines the formation of a non-degradable intralysosomal pigment, lipofuscin. Over time, lipofuscin occupies a growing portion of the lysosomal compartment, which hinders macroautophagy. As a result, the number increases of ATP-deficient mitochondria with the increase in the amount of ROS increases progressively. Cytosolic proteins are oxidatively modified to form large indigestible aggregates (aggresomes). The dark red dots symbolize cytosolic proteins, while the dark green colour of the mitochondria indicates damage. From Cuervo et al., 2005

Mitochondria in aged post-mitotic cells are structurally impaired, exhibiting swelling and disruption of cristae, often resulting in the formation of amorphous material structures (Terman and Brunk., 2004). Senescent mitochondria are defective in ATP production and mitochondria respiration by increasing of reactive oxygen species (ROS) in mitochondria [Sohal and Sohal, 1991; Zorov et al., 2014; (Fig. 12)]. In detail, excessive ROS induces oxidative stress leading to mutations in mitochondrial DNA and oxidation of proteins. Production of abnormal proteins in the mitochondria reduce ATP production and increase the AMP/ATP ratio. AMPK, an energy sensor, upregulates mitochondrial biogenesis and

promote quality control under conditions of acute energy deprivation. However, persistently reduced ATP production induces abnormal energy metabolism, which causes impaired mitochondrial biogenesis, Mitochondria Quality Control (MQC), and mitochondrial dysfunction, as Figure 12 shows (Guo et al., 2023). These aberrant changes ultimately lead to cellular senescence and age-related disorders. For example, in neurons high levels of mitochondrial ROS can induce neuroinflammation and neuronal death (Stefanatos and Sanz, 2018).

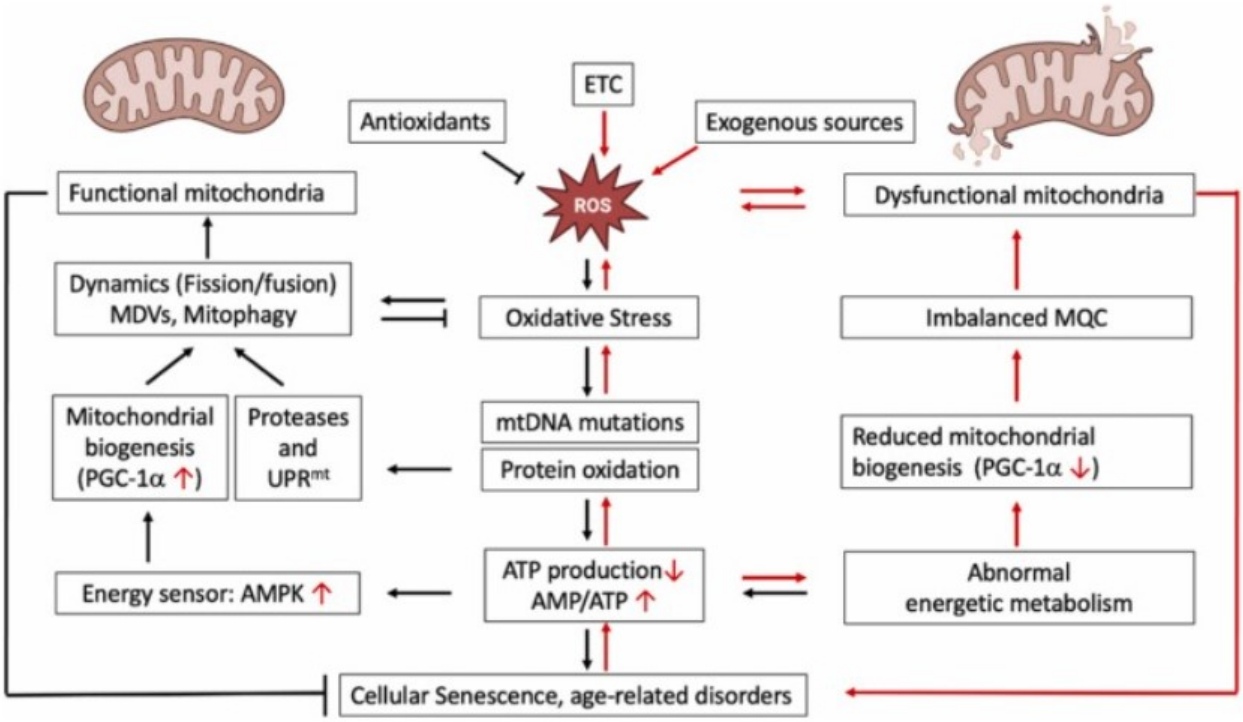


Figure 12: Mitochondria dysfunction in aging. ROS are generated by mitochondrial ETC and exogenous sources. Excessive ROS induces oxidative stress, which leads to mutations in mitochondrial DNA and protein oxidation. Abnormal products in the mitochondria reduce ATP production and increase the AMP/ATP ratio. However, persistently reduced ATP production induces abnormal energy metabolism, resulting in reduced mitochondrial biogenesis, imbalanced MQC, and mitochondrial dysfunction. These aberrant changes ultimately lead to cellular senescence and age-related disorders. ETC, electron transport chain; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; MQC, mitochondrial quality control, PGC-1α, peroxisome alpha-1 proliferator-activated receptor gamma coactivator; UPRmt, unfolded mitochondrial protein response. From Guo et al., 2023

Mitochondrial respiratory capacity and MQC decline with age (Porter et al., 2015). Moreover, the exposure to ROS can inactivate the tricarboxylic acid-cycle aconitase and the iron–sulfur centers of electron transport chain (ETC) at complexes I, II, and III, resulting in a shutdown of mitochondrial energy production (Reddy, 2008). Therefore, mitochondria undergo morphological and functional changes with age, including declines in ETC function, mitochondrial integrity, and mitochondrial quality, which results in impairments of cellular energy production and activity, Figure 13 (Webb and Sideris, 2020).

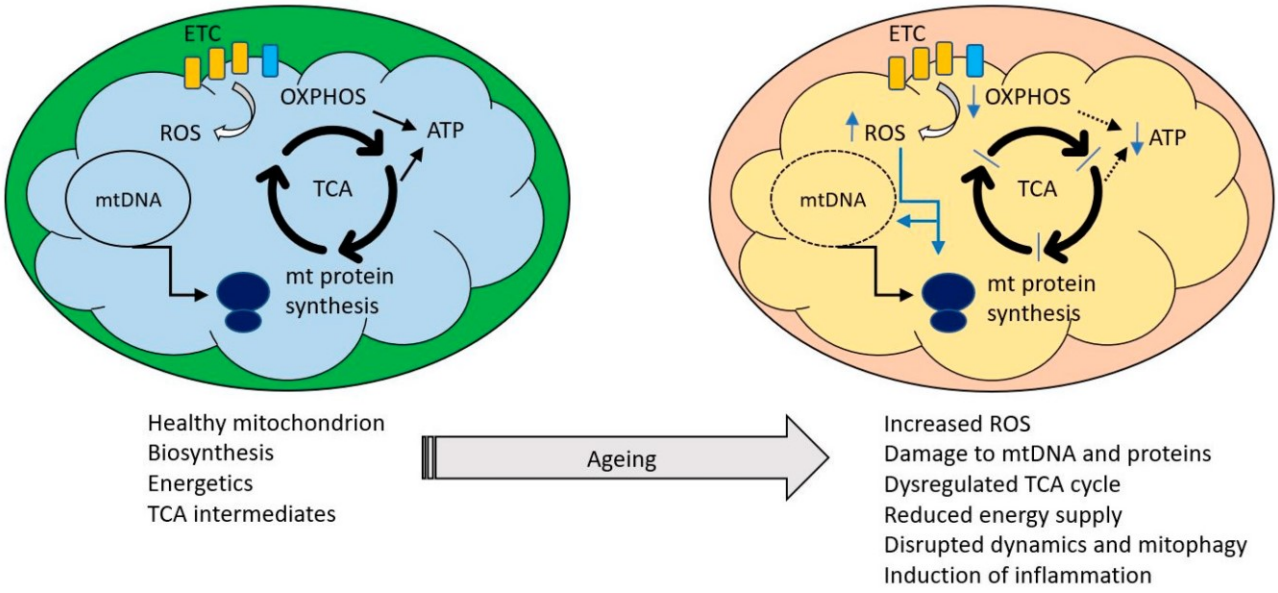


Figure 13: Defective function of old mitochondria in comparison with healthy mitochondria. Healthy mitochondria perform a variety of functions including the production and synthesis of ATP and metabolites necessary for cellular maintenance. Aging is associated with increased impairment of these functions, manifested as excessive production of reactive oxygen species (ROS), Oxidative damage to mitochondrial components, reduced ability to supply ATP, dysregulation of the tricarboxylic acid cycle, and increased mitochondria-driven inflammation. The main processes are indicated in black arrows, the blue arrows indicate the changes that occur during aging. From Webb and Sideris, 2020

The relationship between mitochondrial respiration and autophagy is bidirectional and is the basis of longevity (Wong et al., 2023). Cargo degradation products are released from autolysosomes to be reused by the cell to fuel various metabolic pathways (Kumar et al., 2021; Wilson et al., 2023). Glucose and free fatty acids released by autophagy fuel the TCA cycle and oxidative phosphorylation to support mitochondrial ATP generation (Lahiri et al., 2019), particularly during cellular energy crisis (King et al., 2021), as Figure 14 shows. Furthermore, a shift towards oxidative phosphorylation (OXPHOS) induces autophagy while the preference for aerobic glycolysis dampens autophagy (Thomas et al., 2018; Wong et al., 2020). In detail, NAD directly regulates autophagy and mitochondrial quality control. Likewise, autophagy has been shown to preserve NAD levels by modulating cellular stress (Wong et al., 2020). The interaction between mitochondrial respiration and autophagy is involved in the regulation of longevity.

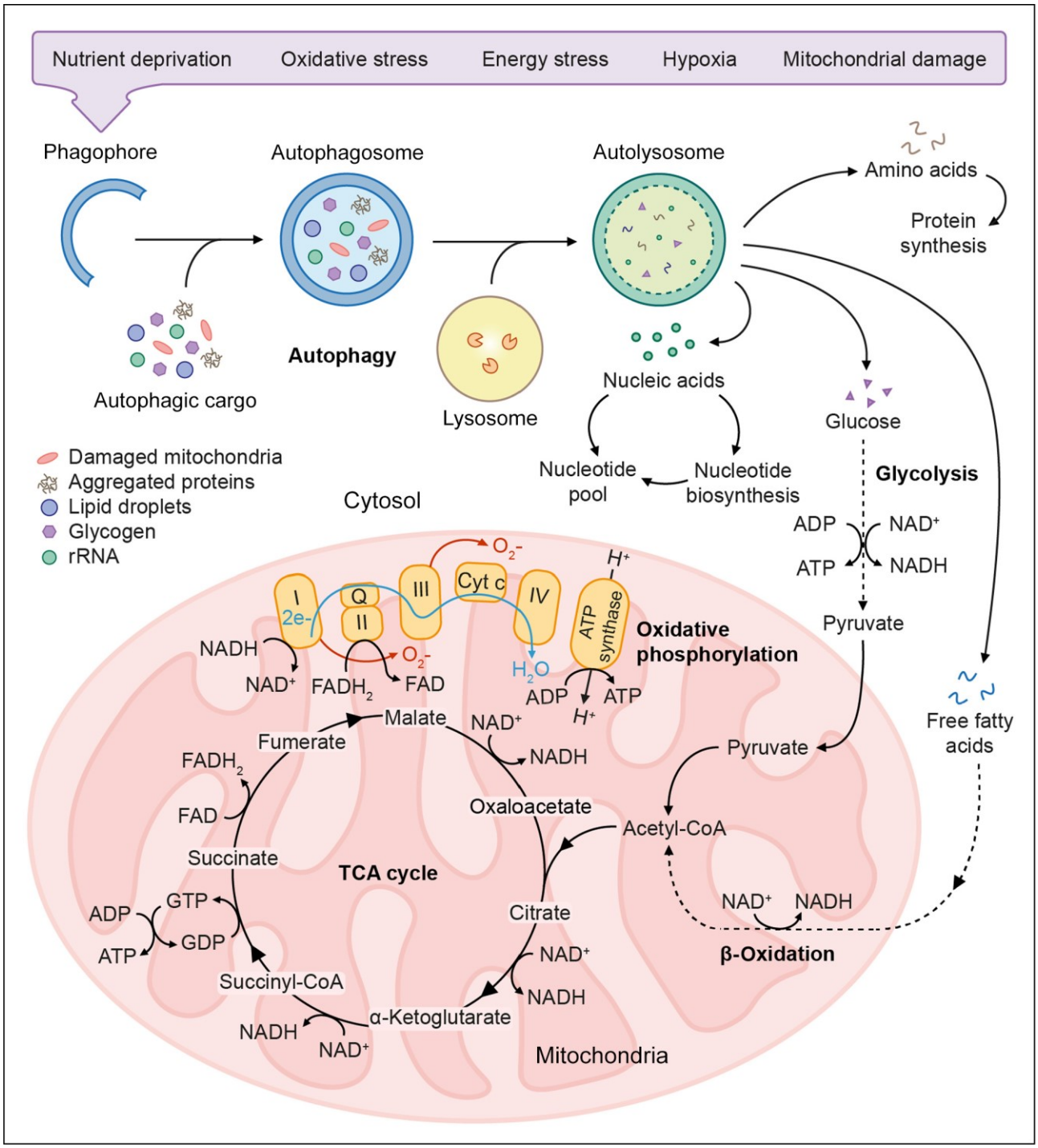


Figure 14: Autophagy and Mitochondrial respiration. Autophagy is activated in cells by numerous stimuli, from nutrient deprivation to mitochondrial damage. This triggers the formation of a phagophore that extends into a sealed double-membrane autophagosome. During this process macromolecules are selectively engulfed into autophagosomes and subsequently degraded following fusion with lysosomes. Cargo degradation products are released from autolysosomes to be reused by the cell to fuel various metabolic pathways. Glucose and free fatty acids released by autophagy fuel TCA cycle and oxidative phosphorylation to support mitochondrial ATP generation. I/II/III, respiratory complexes I/II/II; Cyt c, cytochrome c; FAD, flavin adenine dinucleotide; Q, coenzyme Q cytochrome c reductase; rRNA, ribosomal RNA; TCA cycle, tricarboxylic acid cycle. From Wilson et al., 2023

1.5.2 Autophagy in brain aging

The role of autophagy is particularly important for long-lived post-mitotic cells such as neurons. Without the aid of cell division, neurons require efficient autophagy to remove the waste material that would otherwise accumulate in the cytosol. Additionally, neuronal autophagy has been shown to be essential not only for proper neuronal function, but also for prevention of inflammation in glial cells (Lee, 2012). In fact, autophagic flux disruption in primary cortical neurons causes changes that closely resemble the accumulation of autophagic organelles and neurite dystrophy associated with AD (Boland et al., 2008). Studies of human brain gene expression have found age-associated reductions in markers of macroautophagy. Shibata et al. (2006) found downregulation of Beclin 1 and Lipinski et al. (2010) found decreases in genes regulating autophagy, including Atg5 and Atg7. Guebel and Torres (2016), while studying the effects of gender and aging on gene transcription in the hippocampus, reported decreased expression of LC3, HDAC6 (a deacetylaser required for autophagosomal maturation and fusion with lymphosomes; Lee J.H. et al., 2010), and PINK1 (a mitochondrial kinase whose activity is crucial for mitochondrial function; Qu et al., 2015) in elderly women. Conversely, in elderly men the expression of Bcl-2, which inhibits Beclin 1 (Liang et al., 1999), increased, suggesting a decrease in macroautophagic activity. The study of aging brain autophagy in mammalian and non-mammalian animal models provides similar results. For example, macroautophagy has been shown to be required for normal lifespan in the lysosomal block of *Caenorhabditis elegans* and a decline in autophagic flux, including mitophagy, has been observed during brain aging of mice and other animal model. Specifically, reductions in Beclin-1, VPS34, and Atg5 were observed in the hippocampi of 16-month-old mice compared to 3-month-old mice (Glatigny et al., 2019). In the brain of elderly mice (Ott et al., 2016) and in the hippocampus of elderly rats (Yu et al., 2017), a decrease in autophagic markers was observed, leading to an increase in mTOR. Furthermore, a decrease in Beclin-1 and an increase in the LC3-II/LC3-I ratio were found in the hippocampi of older cows, which may indicate excessive accumulation of autophagosomes and reduced autophagosomal degradation (De Biase et al., 2017). Furthermore, a decrease in mitophagic activity was observed in the brains of aged mice (Su et al., 2015).

In detail, the reduction of autophagy during aging promotes the aggregation and accumulation of misfolded proteins in the aged brain. Elimination of misfolded proteins is crucial for cell survival because protein misfolding impairs biological function and increases the form toxic aggregates (Hart, 2017), one of the hallmarks of age-related neurodegenerative disorders (Lou et al., 2020). Under physiological conditions, misfolded proteins are initially removed by the ubiquitin-proteasomal system (UPS). However, larger cargoes, such as protein oligomers, aggregates, and damaged mitochondria, are too large to

enter the proteasome of the UPS system and are degraded by autophagy. Furthermore, growing genetic and biochemical evidence implicates dysfunction of the autophagic mechanism during the pathogenesis of many neurodegenerative diseases, including AD, PD, Huntington disease (HD), amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD; (Niccoli and Partridge, 2012; Wolfe et al., 2013; Frakes et al., 2015). Figure 15 illustrates the defects of autophagy reported in different neurodegenerative diseases.

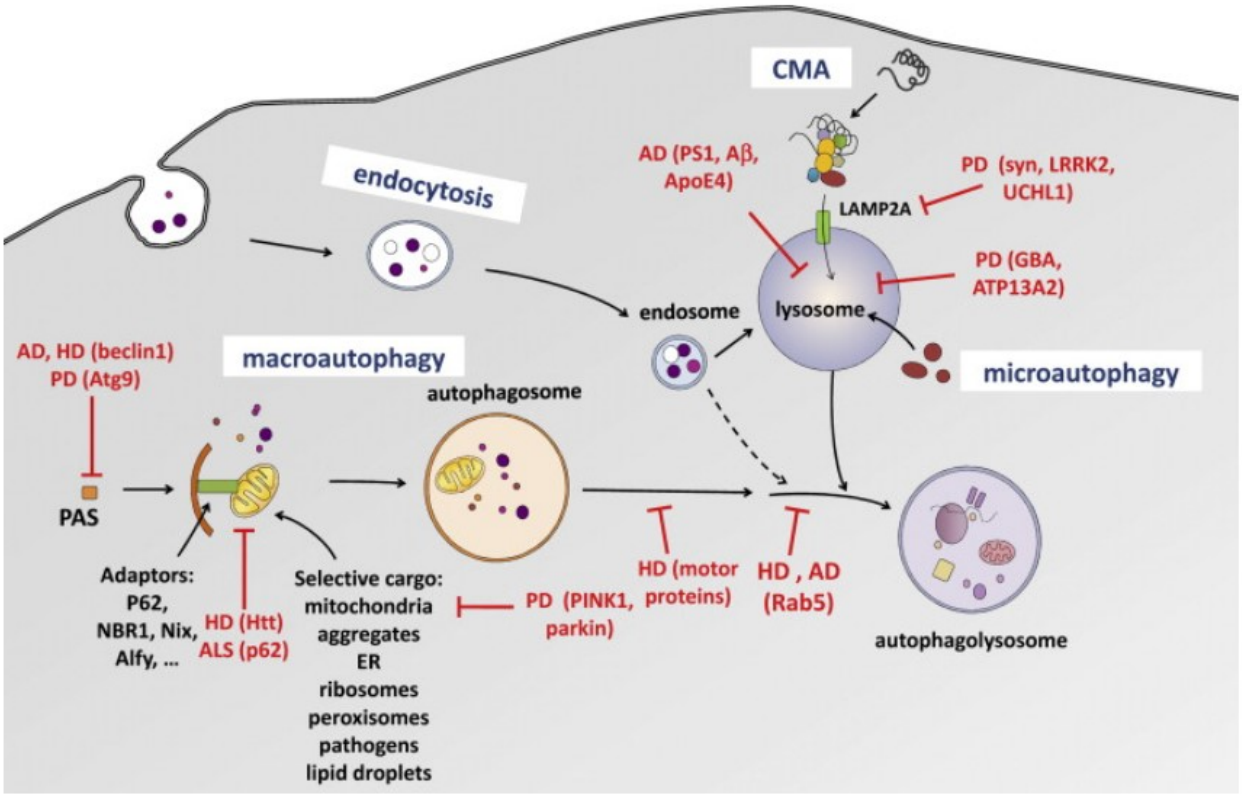


Figure 15: Alterations of autophagy pathways in neurodegenerative diseases. The figure describes the sequestration and degradation of proteins and organelles through direct invagination of the lysosomal membrane. Defects in the autophagy machinery for several neurodegenerative diseases can occur at different steps along the pathways (proteins involved are labelled in red). From Martinez-Vincente, 2015

For example, a familiar form of AD is caused by a mutation of presenilin 1 (PS1) that causes impaired glycosylation and impaired lysosomal acidification (Lee et al., 2010). The impairment of this mechanism involves reduced degradation of protein aggregates and autophagic vesicles accumulation. Moreover, during AD, Phosphatidylinositol binding clathrin-assembly protein (PICALM) can be truncated and downregulated, causing the impaired regulation of endocytosis (Moreau et al., 2014). The impairment of this mechanism also impairs phagophore elongation, autophagosome formation and autophagosome-lysosome fusion, resulting in reduced aggregates degradation. During AD, high levels of aβ and APP (Amyloid Precursor Protein) increase PI3/AKT signalling,

phosphorylation of the proline rich AKT substrate 40 (PRAS40) and increase mTOR signalling (Caccamo et al., 2011). This mechanism determines autophagy inhibition and accumulation of Aβ and tau. In addition, decreased autophagy results from abnormal level and abnormal phosphorylation of p62/SQSTM1 (Tanji et al., 2014). In PD, the over-expression of α-synuclein causes inhibition of autophagy by mislocated AT9 (Son et al., 2012). Moreover, α-synuclein mutations inhibit Chaperon mediated autophagy, trigging the macroautophagic compensation (Cuervo et al., 2004; Xilour et al., 2008; Xilour et al., 2009). Mutation of ATPase type 13A2 reduces the lysosomal acidification causing decrease of autophagy (Ramirez et al., 2006; Di Fanzo et al., 2007). In addition, PINK1 mutation reduces ubiquitin labelling of mitochondrial proteins, decreasing mitophagy (Gasser, 2009; Nixon and Yank, 2012; Son et al., 2012). In HD, htt (huntingtin) sequesters mTOR (Ravikumar et al., 2009) and Beclin-1 (Jeong et al., 2009; Ashkenazi et al., 2017). These impaired mechanisms implicate induction and inhibition of autophagy, respectively. The reduction of ALFY (Autophagy-linked FYVE protein) causes decreases of protein's clearance (Isakson et al., 2013). Moreover, the altered p62/SQSTM1 and phosphorylated at UBD increase protein aggregates clearance (Matsumoto et al., 2011; Lim et al., 2015).

1.5.2.1 Autophagy and microglia in brain aging

Microglial cells are central nervous system (CNS) resident macrophages that play an important role in development, homeostasis, response to damage, infection and microenvironmental perturbations. Microglia differ from other types of brain cells because they originate from the yolk sac rather than the neural tube (Priller et al., 2001; Ginhoux et al., 2013; Prinz and Priller, 2014). Once the nervous tissue is reached, microglial cells can replicate and reconstitute in the brain, forming the resident population (Priller et al., 2001). In concert with other glial cells, microglia perform important functions in brain homeostasis and regeneration (Kent and Miron, 2023). In addition to being critical in innate immunity and being the first line of defence of the central nervous system against pathogens and internal injury, microglia are involved in myelination, synapse remodelling and tissue repair (Kalafatakis and Karagogeos, 2021). Microglia and astrocytes are part, together with the blood-brain barrier, of the so-called "neurovascular unit" and are involved in the response to brain insults that orchestrate neuroinflammation processes (Liu et al., 2020). Due to their critical role in central nervous system defence and immunity, microglia promote inflammation and present antigens to lymphocytes (Gomez-Nicola and Perry, 2015), but may also have anti-inflammatory properties. Furthermore, microglia were classified as "resting" and "active" (Río-Hortega, 1919; Sierra et al., 2016; Sierra et al., 2019; Paolicinelli et al., 2022). More recently, microglia have also been classified as M1- and M2-phenotype to support the study of microglial activation, as Figure 16 shows (Varnum and Ikezu, 2012; Heneka et al., 2015; Ransohoff, 2016; García-Revilla et al., 2019; Song and Suk, 2019; Molina-Martínez et al., 2020; Paolicinelli et al., 2022; Wrang et al., 2023). The classic

proinflammatory pattern “M1” is considered neurotoxic and the anti-inflammatory “M2” pattern is considered neuroprotective (Michelucci et al., 2019). Moreover, the different phenotypes are associated with a specific mitochondrial respiration. M1-phenotype involves an increase of glycolysis. On the contrary, M2-phenotype is associated with OXPHOS (Hickman et al., 2023). M1-M2 polarization of microglia corresponds to different active phenotypes implicated in harmful inflammatory effects or repair tasks (Colton et al., 2006¸ Masuda et al., 2019). Furthermore, the term “M0′” probably represents a homeostatic physiological state of microglial cells involved in functions other than those typical of M1 and M2. In any case, M1-M2 phenotypes represent the two extremes of a wide range of possible intermediate cellular states, which *in vivo* perform numerous functions that cannot be reproduced *in vitro* (Ransohoff, 2016).

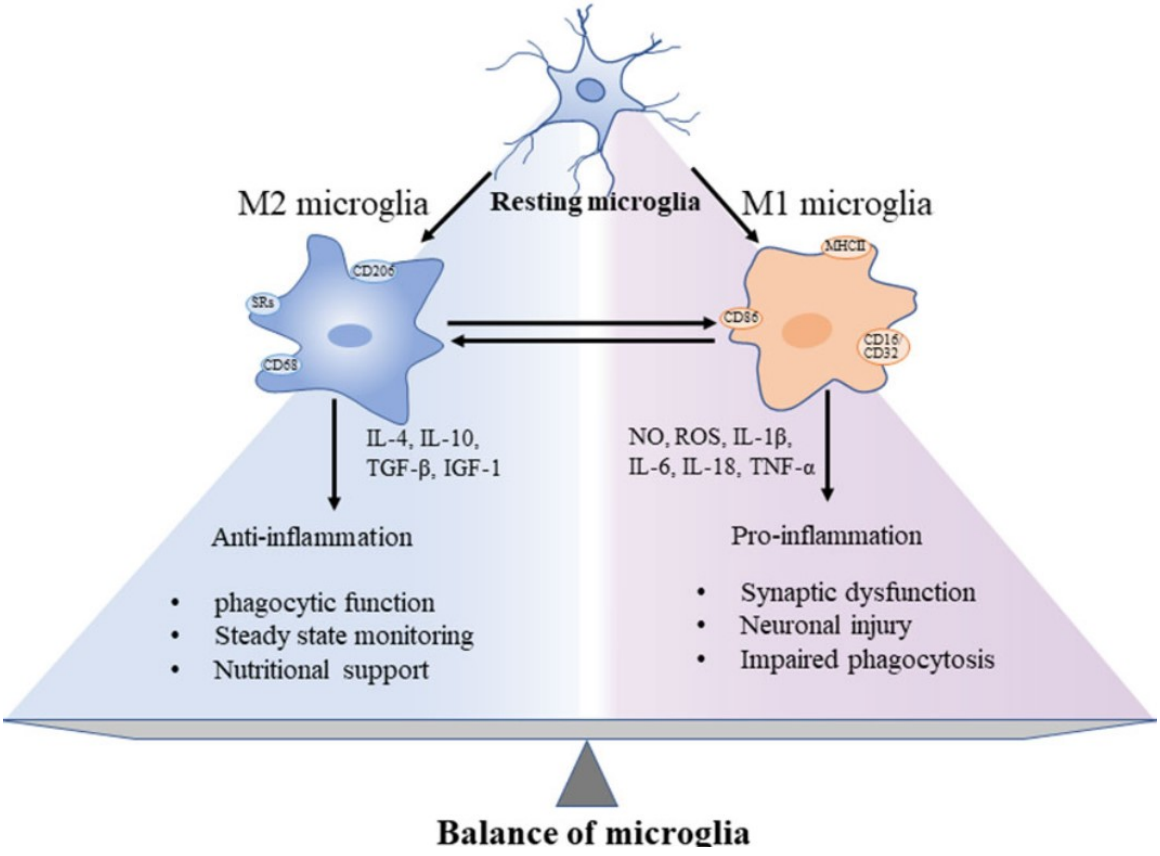


Figure 16: M1 and M2-phenotype of microglia. The figure describes the different functions of microglia and the balance between pro- and anti-inflammation in the progression of neuroinflammation of the central nervous system. These microglial phenotypes show different cell surface receptors and secreted factors, such as anti-inflammatory cytokines and trophic factors promote phagocytosis, maintain homeostasis and nourish nerves. From Wang et al., 2023

The microglial population is vulnerable to physiological disorders such as aging which can contribute to the development of psychiatric and neurodegenerative diseases [Spittau, 2017; (Fig. 17)]. Age-related microglial alterations with a moderately active phenotype are present during aging (Sheng et al., 1998; Streit et al., 2004; Streit et al., 2009). Furthermore,

microglia have been linked to the pathological processes that underlie age-related neurological dysfunction and disease. Aged microglia are classified as senescent or dystrophic (Streit and Xue, 2014). Senescent microglia may lose supportive activities that protect neurons and mimic reactive microglia. On the other hand, the definition of "dystrophic microglia" implies morphological change related to senescence (Streit et al., 2004). These morphological changes are debranching and retraction of processes, the formation of aberrant bulges in residual processes and cytoplasmic fragmentation or cytorhexis (Streit et al., 2009; Greenwood and Brown, 2021). Aged microglia have an altered surveillance phenotype with fewer dendritic branching and reduced process motility. When responding to injury, they show lower migration rates and have a more sustained inflammatory response in reaction to the damage (Damani et al. 2011). Furthermore, aged microglia not only change their cytokine signature to a proinflammatory one, but also show impaired phagocytosis and increased ROS production (Koellhoffer et al., 2017). These changes cause microglia not only to fail to maintain neuronal health, but to compromise it, thus contributing to the possible development of neurodegenerative diseases. Senescence Associated Secretory Phenotype (SASP) is a more recently described phenotype that characterizes aged microglia (Streit et al., 2014). The SASP is an integral part of the phenotype that defines a senescent cell. It includes a variety of molecules, such as proinflammatory cytokines, chemokines, ROS, growth factors, and proteases that can cause significant negative impact on surrounding cells and is linked to aging and neurodegenerative diseases (Chinta et al., 2015). In microglia, SASP entails elevated levels of (TNFα), IL1β, IL-6 and IL-8 (Sierra et al., 2007), increased DNA damage (Coppe et al., 2010), particularly of mitochondrial DNA (Hayashi et al., 2008; von Bernhardi et al., 2015) and telomere shortening (Flanary and Streit 2004). Aged microglia show an increased production of inflammatory cytokines and free radical species, and a poor induced phagocytosis, which together with a reduced lysosomal activity, decrease clearance, as figure 16 shows. Those changes shift the balance towards decreased neuroprotective functions and increased neurotoxicity (Tichauer et al., 2014; von Bernhardi et al., 2015). Hallmarks of aged microglia activation are increased expression of MHCII (Henry et al., 2009; VanGuilder et al., 2011), CD68 (Wong et al., 2005; Griffin et al., 2006) as well as increased TLR levels (Letiembre et al., 2007). Aged microglia were characterized by the presence of lipofuscin inclusions and reduced process complexity. In general, the responsiveness of microglia to stimulation appears increased during aging, which is further reflected in the increased activation of aged microglia after injection of activating cytokines IL1β and IL12 into the hippocampus (Lee et al., 2013). This phenomenon has been defined as "microglial priming", and TLR2, TLR3, and TLR4 appear to be essential for priming microglia, but not astrocytes, for ATP-dependent interleukin-1β release (Facci et al., 2014). Microglial priming induces a highly conserved transcriptional signature with specific aspects of aging (Holtman et al., 2015), which is dependent on high mobility group box 1 (HMGB1). HMGB1 mediates

neuroinflammatory priming in the aged central nervous system. Inhibition of HMGB1 functions appears to desensitize aged microglia to an immune challenge, thus preventing exaggerated behavioural and neuroinflammatory responses following microglial stimulation (Fonken et al., 2016).

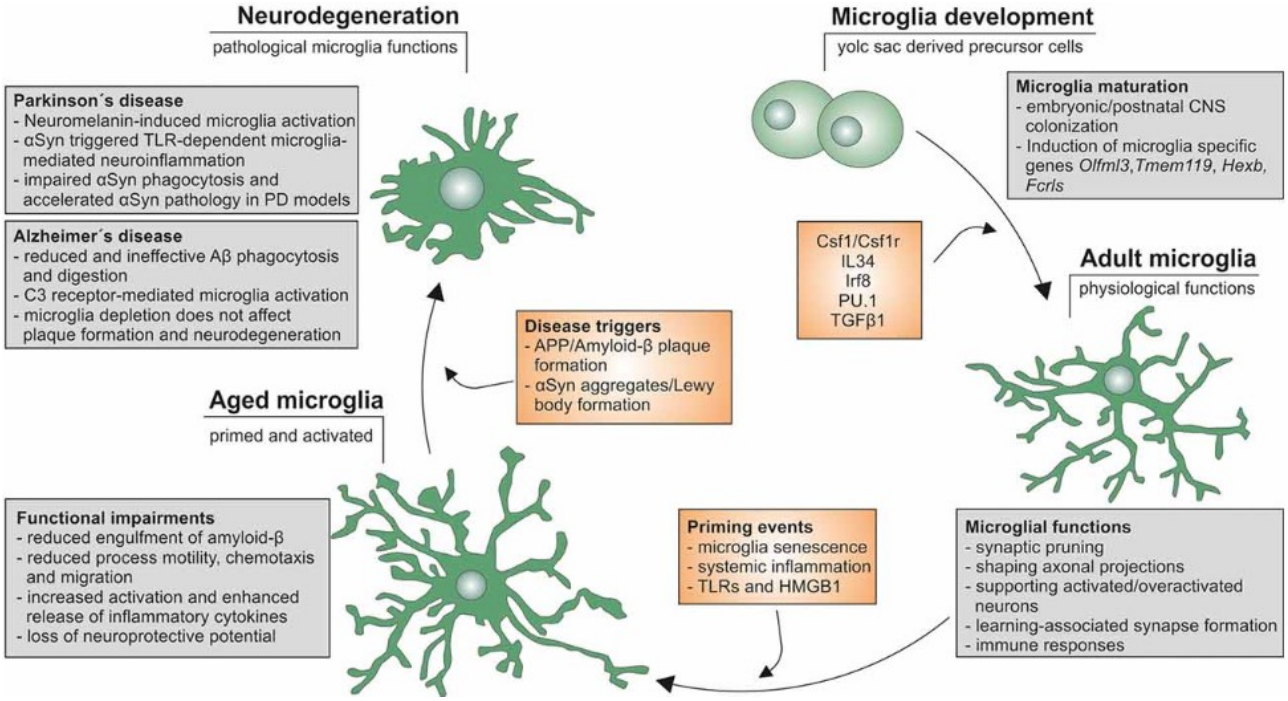


Figure 17: Microglia life cycle. Schematic summary of microglial development, maturation, and aging and functional changes that influence the onset and progression of neurodegeneration in Alzheimer's disease (AD) and Parkinson's disease (PD). From Spittau, 2017

The inflammation in aged microglia with increased a production of pro-inflammatory cytokines determines impaired autophagic flow and drives the shift of microglia towards the M1 phenotype through the activation of AKT/mTOR signalling (Li et al., 2018). Impaired of autophagy causes an increase of lipofuscin granules in aged microglia, although to a lesser extent than neurons (Sierra et al., 2007; Eichhoff et al., 2008; Xu et al., 2008). Lipofuscin accumulation is linked to increased oxidative stress, decreased antioxidant defence system, lysosomal iron overload and mitochondrial dysfunction (Brunk and Terman, 2002; Terman and Brunk, 2006). Furthermore, intracellular ROS can act as second messengers to activate the p38 mitogen-activated protein kinase cascade in various cell types including microglia (Hashimoto et al., 2001; Hsu et al., 2002; Pawate et al., 2004). Therefore, increased lipofuscin accumulation is a potential candidate for the executive factor for microglial priming in the aging brain. Furthermore, the turnover rate of mitochondria in microglia is extremely high. The other is that lysosomal dysfunction in microglia more readily leads to the observed autophagic dysfunction. The decline in lysosomal functions caused by ceroid-lipofuscin accumulation may further influence mitochondrial turnover. The accumulation

of ceroid-lipofuscin over time in lysosomes can cause an alteration of lysosomal enzymes in lysosomes with lipofuscin, to attempt to degrade this material. The resulting lack of lysosomal enzymes for autophagy then leads to a reduced ability to recycle other cellular organelles such as mitochondria. The lysosomes with poorly acidic lumen (impaired lysosomal acidification) have inefficient fusion with autophagosomes or phagosomes, or even no fusion, leading to reduced phagocytic and autophagic functions. Moreover, overloaded mitochondrial ROS impairs lysosomal function to block autophagic flux and drive microglia to M1-type polarization (Yuan et al., 2019). The theory of "mitochondrial-lysosomal axis theory of aging" (Brunk and Terman, 2002; Terman and Brunk, 2006) link the dysfunction of mitochondria and autophagic processes (Brunk and Terman, 2002; Terman and Brunk, 2006). Therefore, autophagic activity decreases and mitochondria in aging produce less ATP and more ROS in aged microglia.

An autophagy-dependent neuroprotective microglia population is a potential target for treating age-related neuroinflammatory conditions. In fact, autophagy stimulation can polarize microglia into the M2-like phenotype and inhibit subsequent inflammation (Jin et al., 2018).

1.6 Autophagy: potential therapeutic targeting

Increasing evidence supports the bidirectional interactions of autophagy and multiple aging factors. Indeed, aging hallmarks are influenced by autophagic activity modulations and, symmetrically, aging phenotypes influence the autophagic process. The figure 18 highlights aging processes that are sensitive to autophagy, for example the relationship between autophagy and metabolism, or proteostasis, or senescence etc (Kaushik, 2021). Impaired autophagy during aging is likely not a just a hallmark but a driver of brain deterioration and dysfunction, as maintenance/restoration of autophagy delays aging and prevents brain diseases.

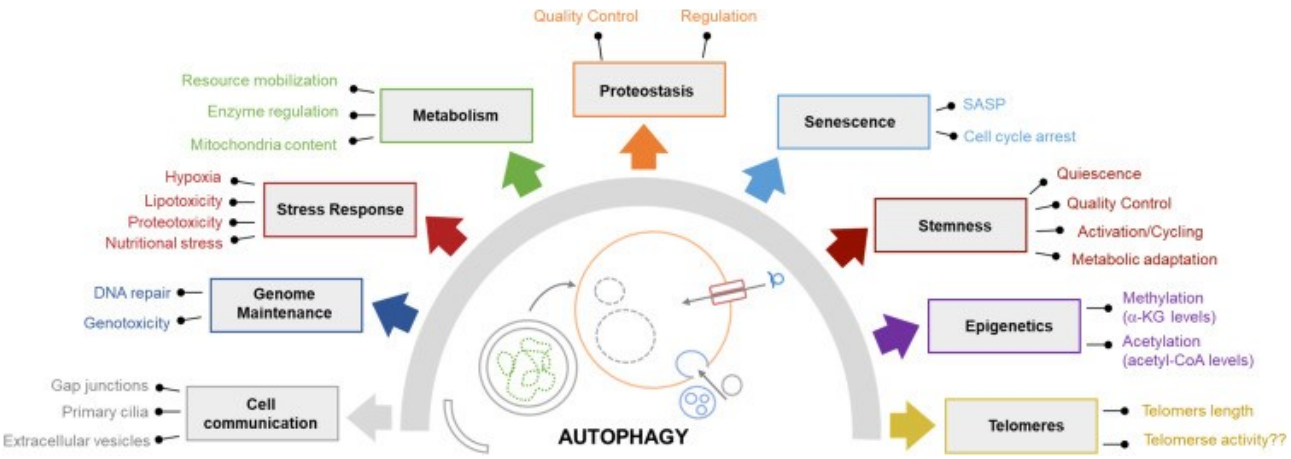


Figure 18: Modulation of autophagy to prevent process of aging. The scheme illustrates the characteristics of aging influenced by changes in autophagic activity (Cell Communication, Genome Maintenance, Stress Response, Metabolism, Proteostasis, Senescence, Stemness, Epigenetic, Telomeres), and we indicate the mechanisms by which autophagy modulates these processes (indicated with black dot). From Kaushik, 2021

Autophagy enhancement can be a therapeutic strategy to reduce aging phenotypes and neurodegenerative disease associated with aging. Drug and nutraceutical compound discovery aimed at reversal of age-associated effects via autophagy should therefore represent a priority. Clinical implementation of autophagy inducers may provide high therapeutic potential. Interventions, that improve lifespan and healthspan via autophagy, are caloric restriction and treatments with rapamycin or spermidine.

Spermidine is a naturally occurring polyamine abundant in soybeans, peas, corn, chicken liver, shellfish, and blue cheese (Atiya et al., 2011). Spermidine supplementation improves longevity in several species, such as yeast, flies, worms, and mice, through the induction of autophagy that occurs independently of mTOR (Eisenbertg et al., 2009; Morselli et al., 2011; Eisenbertg et al., 2016; Madeo et al., 2019). Spermidine triggers autophagy mainly by promoting AMPK phosphorylation and mitophagy via PINK1/Parkin pathway. Specifically, spermidine reduce brain aging phenotypes and ameliorates cognitive ability (Maglione et al., 2019; Wirth et al., 2019; Xu et al., 2020; Schroeder et al., 2021; Hofer et al., 2022). A reduction of aging phenotypes in the brain is determined by improving autophagy and mitochondrial function. Spermidine through induction of autophagy has benefits in neurodegenerative diseases, for example the compound reduces neuroinflammation and soluble Aβ in APP/PS1 mice and prevents Tau fibrillization in rTg4510 mice (Guebel and Torres, 2016; Fang et al., 2019). According to recent animal studies, spermidine also appears to improve the immune response to infections (Puleston et al., 2015), counteracting the formation of defective CD8+ T cells that increase with aging. Although mTOR increases both the quantity and quality of CD8+ T cells against viral infections (Araki et al., 2009), spermidine may not depend on the mTOR pathway (Puleston et al., 2015).

Rapamycin (also known by the trade names of sirolimus or rapamune) is a macrocyclic lactone produced by *Streptomyces hygroscopicus*. Rapamycin extends lifespan and improves health in evolutionarily divergent species, including some models of neurodegeneration (Kaeberlein et al., 2005b; Bjedov et al., 2010; Miller et al., 2011; Wilkinson et al., 2012), and has a positive effect on cognition in aging models and has shown promise as an intervention to improve health and cognition (Caccamo et al., 2010; Spilman et al., 2010; Halloran et al., 2012; Majumder et al., 2012; al., 2012). The benefits of rapamycin are potentially through immunomodulation and autophagy induction. Most of rapamycin's effects on the immune system are mediated by its ability to inhibit mTOR

(Saemann et al., 2009), although recent studies also show that rapamycin can also influence the immune system independently of mTOR (Janes and Fruman, 2009).

1.7 β-Glucans

β-glucans are a group of biologically active natural molecules and are gaining increasing attention both as an important dietary supplement and as an immunostimulant and potential drug.

1.7.1 History of β-glucans

Interest in β-glucans as so-called biological response modifiers (BRMs), with beneficial effects on human and animal health, has historical origins (Novak and Vetvicka, 2008). In the 1940s, Pillemer and Ecker prepared and studied zymosan, a crude mixture of polysaccharides, proteins and lipids, isolated from the cell wall of the yeast *Saccharomyces cerevisiae* (Pillemer and Ecker, 1941). Zymosan was found to improve immune reactions. Subsequent research identified β-glucan as the main component of zymosan responsible for the observed biological effects. Furthermore, the composition and biological effects of mushrooms used in Asian medicine were studied, and glucan was once again discovered to be the main immunomodulatory constituent. Starting from the pioneering work of Di Luzio and Chihara in the 1960s and 1970s on glucans derived from yeasts and fungi, respectively, the beneficial effects of various β-based preparations have been reported. (Chihara et al., 1969; DiLuzio et al., 1970).

1.7.2 Structure of β-Glucans and sources

β-Glucans are a group of naturally occurring polysaccharides produced by yeast, bacteria, fungi, algae, and cereals and are constituents of their cell walls, as Figure 19 shows (Kaur et al., 2020).

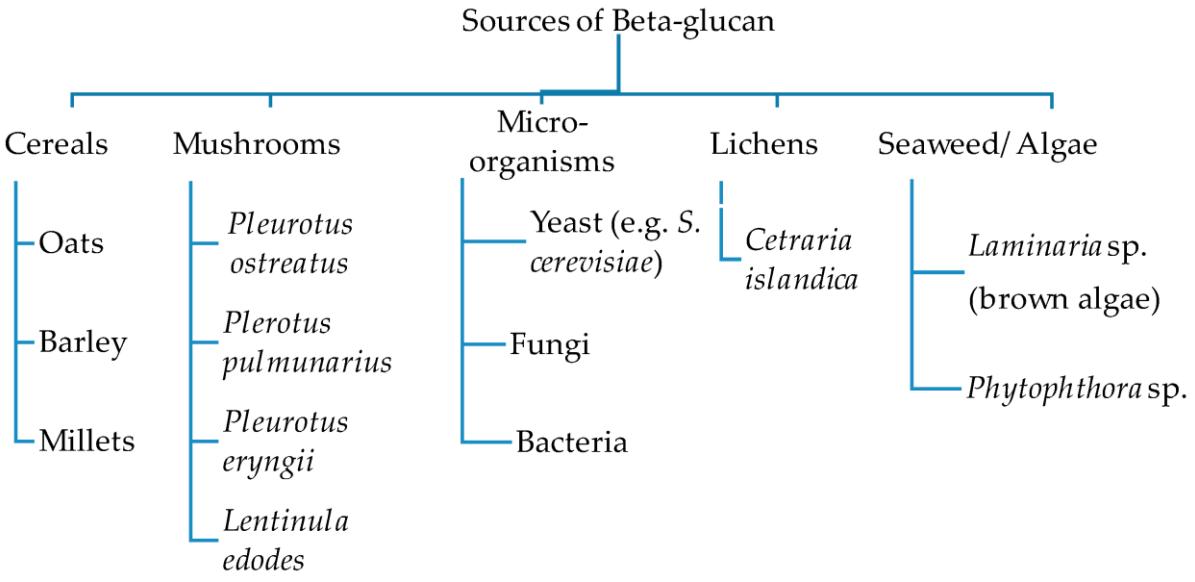


Figure 19: Sources of β-glucans, as such Cereals, Mushrooms, Micro-organism (Yeast, Fungi and Bacteria), Lichens and Seaweed/Algae. From Kaur et al., 2020

Structurally, β-glucans are long or short-chain polymers of β-(1,3) or β-(1,4) linked glucose subunits which may be branched, with the side chains branching from the six-position of the backbone (Barsanti et al., 2009), as Figure 20 shows (Du et al., 2019). Cereal-derived β-glucans are predominantly mixtures of β(1→3)(1→3) and β (1→4)(1→4) glycosidic linkages without any β (1→6)(1→6) bonds (Johansson et al., 2000; Nakashima et al., 2018). β-glucans from yeasts are mixtures of linear β (1→3)(1→3) backbones with 30-residue straight chains and connected to these are long branches attached via β (1→6)(1→6) linkages (Liu et al., 2021). Fungal β-glucans are made of straight β (1→3)(1→3) glucan with short-branched chains connected through β (1→6) (Synytsya and Novak, 2013). Bacterial β-glucans have straight and unbranched β (1→3)(1→3)-D-glucan backbones (McIntosh et al., 2005). Seaweed β-glucans are species-dependent and may contain straight chain β (1→3)(1→3) residues or the straight chain backbone together with high levels of β (1→6)(1→6) branches (Usoltseva et al., 2020).

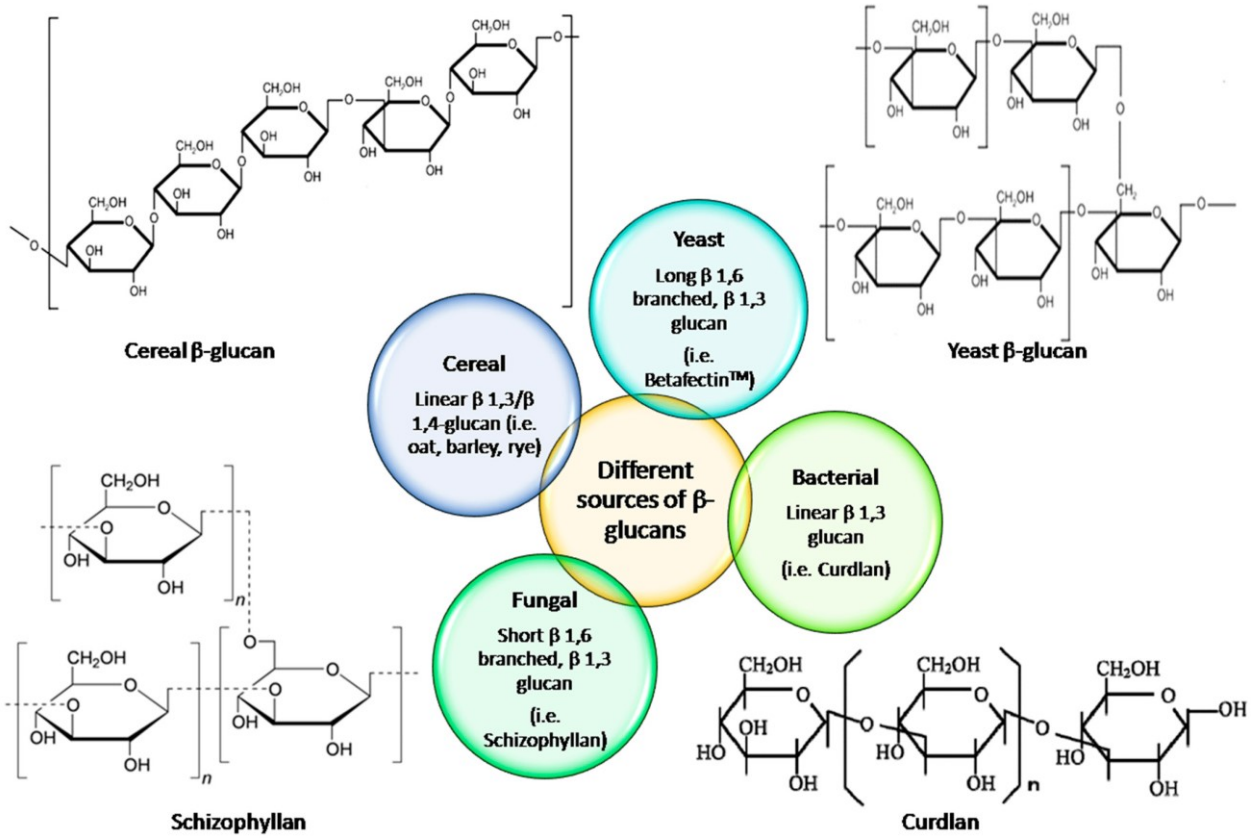


Figure 20: Structure of beta-glucans extracted from various sources. From Du et al., 2019

β-glucans are powerful immunomodulators that have multiple activities such as anti-neoplastic, anti-infective, anti-inflammatory and antioxidant (Novak and Vetvicka, 2008; Kim et al., 2011; Murphy et al., 2020; Steenwijk et al, 2021). However, knowledge of the mechanisms of action and pharmacokinetics of β-glucans remain insufficient. The binding of β-glucan to specific receptors in immune cells, such as macrophages and dendritic cells, modulates their immune response. In case of hyperstimulation of the immune system, such as acute inflammation, β-glucans reduce inflammation. At the same time, in case of immunodeficiency, β-glucans stimulates the immune system (Kim et al., 2011).

1.7.3 Mechanism of action

β-glucans cannot directly penetrate the cell membrane due to their large molecular size and bind receptors on the cell surface called pattern recognition receptors (PRRs). These receptors are mainly expressed in immune cells, such as macrophages and dendritic cells, but can also be found in other cell types such as neurons. The main PRRs that bind β-glucans and could be involved in their action are dectin-1, toll-like receptor (TLR), complement receptor 3 (CR3), scavenge receptors (SR) and lactosylceramide (LacCer), (Brown and Gordon, 2005). Specifically, orally-administered β-glucans are adsorbed in the intestine, either by via M cells, or by binding to the projecting tips of dendritic cells (DCs) in the follicle-associated epithelium (FAE) of Peyer's patches [Han et al., 2022; (Fig. 21)]. Once β-glucans have penetrated the Peyer's patches, they are fragmented and released in the form of fragmented β-glucans (FBGs). FBGs, like soluble β-glucans, trigger a series of biological responses, including immune system modulation (Hong et al., 2004; Sainkhuu et al., 2012) and are then transported to the lymph nodes, spleen and bone marrow (Hong et al., 2004).

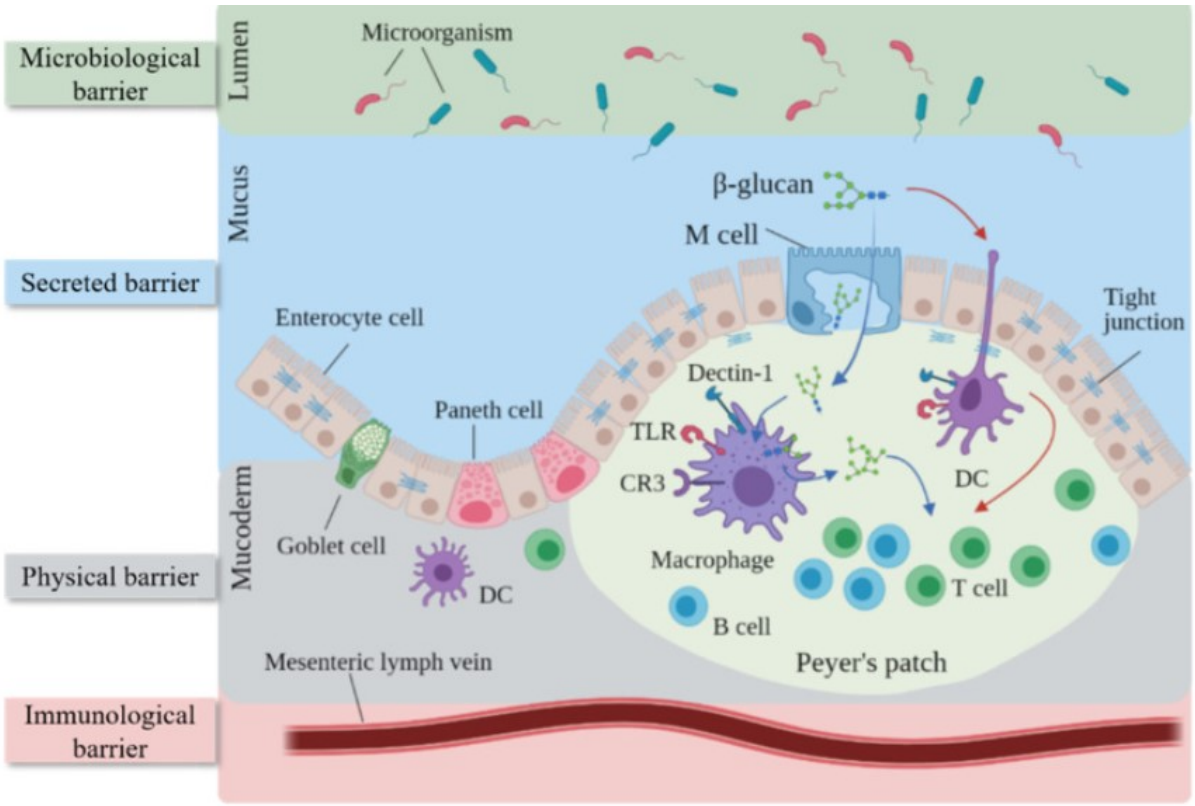


Figure 21: Absorption and transportation mechanism of β-glucan in the gastrointestinal tract. β-glucans have positive regulatory effect on the intestinal mucosal barrier and the immune barrier, to play an immunoregulatory role. β-glucan enters the intestine and intestinal immunity cells. The absorption of β-glucan in the intestinal tract has been divided into two levels: intestinal absorption and cellular absorption. Intestinal absorption occurs the segment was ileum > jejunum > duodenum > colon. Cellular level: β-glucan enter through latex-mediated giant endocytosis and pinocytosis protein structure. The microfold cell (M cell) is a specialized epithelial cell found primarily in the mucosa-associated lymphoid tissue of the gastrointestinal tract. Through uptake, pinocytosis, and phagocytosis, M cells take up bg and present it to Peyer's plaque without degrading it. β-glucan penetrated the Peyer's patches are fragmented and released in the form of fragmented β-glucans (FBG). FBGs, like soluble β-glucans, trigger a variety of biological responses, including modulation of the immune system. Peyer's patches are considered the primary site of the mucosa immune response because they are closely connected to the intestinal lumen and are rich in macrophages, DCs and lymphocytes. The receptor specifically recognizes β-glucan transport and internalizes it as an internal fragment. Furthermore, β-glucan can be taken up by extracellular matrix cells. From Han et al., 2022

1.7.4 Activities of β-glucans

β-glucans are biologically active biopolymers that can interact with different protein receptors, present in both microorganisms and multicellular organisms, eliciting a broad spectrum of biological responses. These responses often result in important bioactivities including immunomodulatory, anticancer, antioxidant, cholesterol-lowering and prebiotic effects (Wang et al., 2017; Ahnen et al., 2019; Frank et al., 2019; Murphy et al., 2020;

Duysburgh et al., 2021). Numerous studies have attempted to exploit the potential therapeutic properties of β-glucans, which can generally be divided into immunomodulatory or metabolic properties (Yamada et al., 2007; Fang et al., 2012; Fuller et al., 2012; Kofuji et al., 2012; Vetvicka et al., 2019; Chan et al., 2021; Han et al., 2020; Sivien et al., 2022). The health benefits of β-glucans have been extensively documented over the past two decades. β-glucans are used as a disease prevention agent, as well as part of anticancer or anti-inflammatory therapy. β-glucans are the most consumed immunomodulators with strong anti-tumor, insulin resistance, anti-hypertension, and anti-obesity effects. β-glucans are recognized and effective inducer for trained immunity to protect the organism from viral, bacterial, and fungal pathogens (Davis et al., 2004; Khan et al., 2016; Medina-Gali et al., 2018; Vetvicka et al., 2020; Konusova et al., 2021; Schwartz et al., 2021; Liang et al., 2022; Krishnan et al., 2022; Wang et al., 2023; Wzorek-Lyczko et al., 2023), and its immunomodulatory effect (Di Luzio et al., 1985; Vetvicka et al., 2008; Jang et al., 2009; Murphy et al., 2020; Lee et al., 2021; Thomas et al., 2022). Specifically, 1.3, 1.6 β-glucans have greater immunomodulatory activity than the other forms and can exert a bidirectional action on the immune system by either boosting or suppressing immune responses. It was demonstrated that the immunostimulatory activity of β-glucans is exerted by activating macrophages and increasing the number of immunoglobulins (Daou et al., 2012). The immunomodulatory effects of β-glucans and their subsequent benefits were investigated in the context of infectious diseases and cancer (Murphy et al., 2012). Furthermore, during hyperactivation of the immune system, for example microglial activation during infection, they re-establish the immune system and reduce inflammation (Chen et al., 2023). LPS-induced immune system and inflammatory activation is also restored by β-glucans (Novakovic et al., 2016). The modulation of the immune system by β-glucans also determines the modulation of inflammation. In fact, during the immune stimulation of β-glucans, i.e. during infection, it is possible to observe an increase in pro-inflammatory cytokines to fight infection. Instead, in conditions in which β-glucans reduce immune hyperactivation, it is possible to observe a reduction in pro-inflammatory- and activation of anti-inflammatory-cytokines, thus reducing inflammation (Camilli et al., 2022; Chen et al., 2023; Zhao et al., 2024). Moreover, the immunomodulation by β-glucans can induce axons regeneration in the central nervous system, i.e. the optic nerves, by binding to dectin 1 (Baldwin et al., 2015). In addition to their immunomodulatory action, β-glucans are metabolized by some gut bacteria and therefore modulate the composition of the gut microbiota with positive actions of the host (Xu et al., 2020; Shi et al., 2020; Golish et al., 2021; Zhen et al., 2021; Hu et al., 2022; Karimi et al., 2023; Singh et al., 2023; Sung et al., 2023; Zhang et al., 2023). The antioxidant effects of β-glucans have been extensively studied using *in vitro* and *in vivo* animal models (Song et al., 2006; Kogani et al., 2008; Jaehing et al., 2008; Blaszczyk et al., 2015; Giese et al., 2015; Ciecierska et al., 2019; Liu et al., 2021; Han et al., 2022). Some studies suggest anti-neoplastic effects of β-glucans (Chen et al.,

2007; Albeituni et al., 2016; Wang et al., 2020). They could be useful as adjuvants during chemotherapy or monoclonal antibodies to improve the quality of life of cancer patients (Hong et al., 2003; Tian et al., 2013; Jacobson et al., 2019; O'Day et al., 2019; Cheung et al., 2022). Moreover, it was examined the ability of β-glucans in the prevention and treatment of metabolic syndrome, their underlying mechanisms of action, and their potential in food applications (El Khoury et al., 2012; Mosikanon et al., 2017; Ferreira et al., 2022; Mitchelson et al., 2022). In fact, β-glucans can reduce risk factors associated with diabetes mellitus and benefit diabetes therapy (Chen et al., 2008). β-glucans have been shown to reduce total cholesterol and blood lipid profile, as well as maintain body weight (Liatis et al., 2009). Given the important activities of β-glucans, these compounds have benefits in many conditions and pathologies. β-glucans can promote wound healing, alleviate ischemic heart damage and cardiac disease (Qian et al., 2009; Esefoglu et al., 2016; Wang et al., 2016; Kaya et al., 2019). β-glucans are also able to speed up the healing of wounds in different contexts (Majtan and Jesenak, 2018; Fronte et al., 2019; Seo et al., 2019; Edirisinghe et al., 2023). Finally, a positive action of β-glucans on cognitive impairment in neurodegenerative diseases, such as Alzheimer disease (Xu et al., 2020; Hu et al., 2022; Shi et al., 2023; Zhang et al., 2023).

1.7.5 β-glucans and autophagy

The strong relationship between autophagy and immunity (Zhou and Zang, 2012; Oh and Lee, 2014; Jang et al., 2019) has increased the evidence for autophagy induction by β-glucans. Interestingly, experiments have also shown that the induction of acquired immunity by β-glucans depends on autophagy. Both pharmacological and genetic inhibition of autophagy leads to blockade of acquired responses (Buffen et al., 2014). Indeed, individuals with autophagy defects are unable to develop a complete and potent acquired immunity (Buffen et al., 2014). Furthermore, autophagy itself has been shown to be a crucial component of macrophage activation/polarization. β-glucans induces autophagy in dendritic cells involving the maturation of these cells and facilitating the immune response (Ding et al., 2019). Furthermore, β-glucans determine the appearance of acidic vesicular compartments (AVO) and the accumulation of protein light chain-3 (LC3) within macrophages. β-glucan exposure triggers autophagy through LC3-associated phagocytosis and directs LC3 recruitment to phagosomes containing material to be digested (Ma and Underhill, 2013). Therefore, β-glucans can be exploited to trigger autophagy (Fatima et al., 2017; Blaszczyk et al., 2022). In addition, β-glucan regulates metabolism by inducing autophagy. For example, in, β-glucans have role in lipid metabolism in zebrafish larvae decreasing adipose mass by autophagy induction (Li et al., 2019). Induction of autophagy by β-glucan has also been observed as a mechanism for viral defence (Liang et al., 2022). Furthermore, it has been observed that β-glucans induce autophagy by downregulating the expression of proinflammatory cytokines in diverse pathologies, such as inflammatory

bowel disease (IBD; Xu et al., 2023), colitis (Kopiasz et al., 2021) or in cancer (Kopiasz et al., 2024).

1.8 Nothobranchius furzeri

Model organisms are unavoidable for aging research. For years, yeast, fruit flies, roundworms, mice, rats, dogs and monkeys enabled scientists to make fundamental discoveries into the biology of aging. Fruit flies, and nematodes are tiny and inexpensive, have a short lifespan and their genetic manipulation is straightforward, that made them instrumental in the discovery of fundamental conserved mechanisms of aging, but they lack vertebrate specific genes (e.g. APOE, the main genetic determinant of human aging) and have body plans entirely different from those of vertebrates as they lack bones, adaptive immunity and myelinated axons, among the other things. On the other hand, common vertebrate models such as mice or zebrafish have lifespan of years, which represents a limit for aging studies. In 2003, *Nothobranchius furzeri (Nfu*) was presented as the vertebrates with the shortest lifespan and was proposed as an alternative animal model for studying aging (Valdesalici and Cellerino, 2003) as it became increasingly accepted and used to investigate the effects of interventions on aging and lifespan (Valenzano et al., 2006; Terzibasi et al., 2007; Kim et al., 2016; Poeschla and Valenzano, 2020).

1.8.1 Lifecycle of *Nfu*

Nfu lives East Africa in seasonal pools that fill during the short rainy season and dry up completely during the subsequent, longer dry season (Genade et al., 2005). To survive and reproduce in this environment, *Nfu* have evolved an annual life cycle, characterized by a prolonged period of embryonic stasis called diapause, followed by rapid growth and sexual maturation. Once the dry season ends and the natural pools are filled with rainwater, the embryos hatch and quickly reach sexual maturity. Once they reach maturity, killifish reproduce and lay eggs daily, as Figure 22 shows (Cellerino et al., 2016). Overall, annual *Nfu* have adapted to their unique environment by evolving a life cycle characterized by prolonged arrest of embryonic development during dry seasons and explosive growth and sexual maturation during wet seasons.

Several strains of *Nfu* have been bred in the laboratory and their lifespan has been studied. Specifically, two strains are more studied, the GRZ strain and the MZM0410 strain (Terzibasi et al., 2008). The GRZ strain derives from a population collected in 1968 in Zimbabwe. The recorded lifespan of the GRZ strain is the shortest among all *Nfu* species and ranges from 12 to 20 weeks. The MZM0410 strain was collected in 2004 in Mozambique and has a longer lifespan that can reach up to 30-40 weeks of average lifespan. A more recent strain, MZCS222, was collected in Mozambique in 2012 and has a similar lifespan as the

MZM04010 strain (Blazek et al. 2017). Lifespan is also influenced by husbandry conditions so that the same strain can have lifespan in different laboratories.

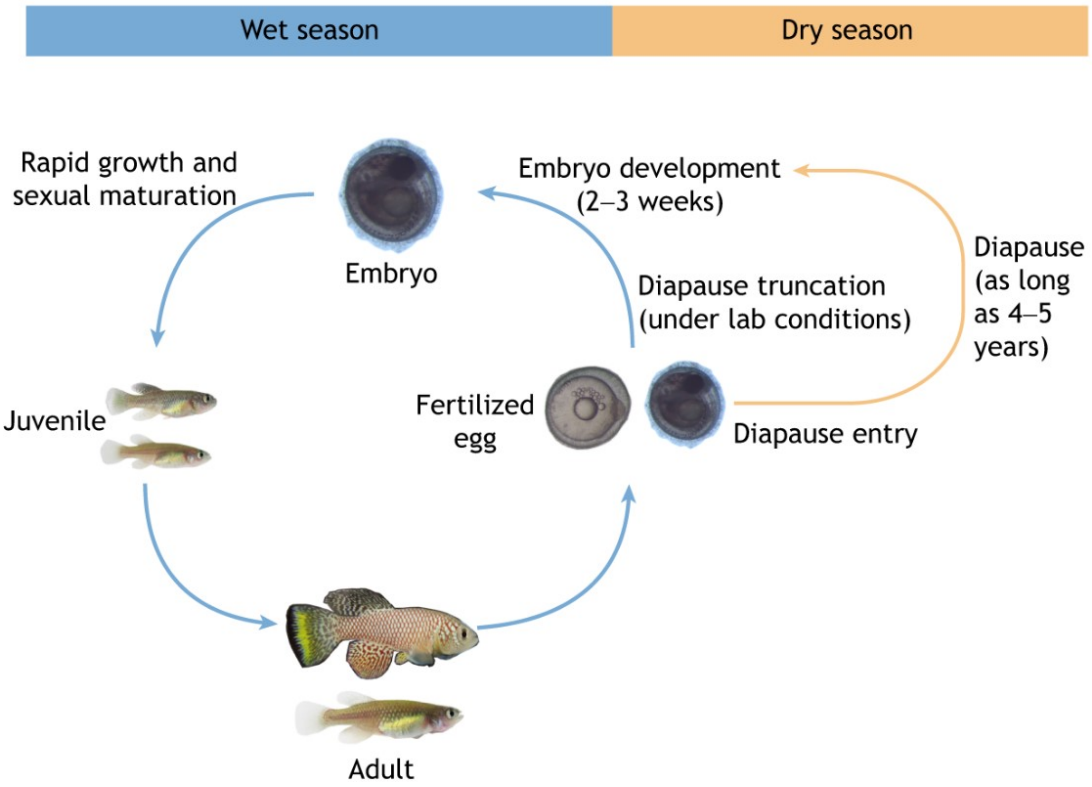


Figure 22: Lifecycle of *Nfu*. After hatching, *Nfu* mature rapidly and begin reproducing within a few weeks. After sexual maturation, *Nfu* continue to grow throughout their lives. Fertilized eggs may arrest in diapause development at various developmental stages and may persist in diapause for several years. After escaping from diapause, the embryos mature in 2-3 weeks before hatching and the *Nfu* life cycle begins again. From Poeschla and Valenzano, 2020

1.8.2 *Nfu* aging phenotypes

The rapid aging of *Nfu* entails hallmarks of aging at the molecular, cellular and physiological level shared with other vertebrates, including humans (Genade et al., 2005; Valenzano et al., 2006b; Terzibasi et al., 2007; Hartmann et al., 2009; Terzibasi et al., 2009; Hartmann et al., 2011). The table below (Tab. 1) summarizes the main aging phenotypes shared with other organisms.

Description	Other species
Integrative phenotypes	
Spinal curvature[a, b]	Zebrafish[c], medaka[d],
Emaciation[a, b]	Zebrafish[c], medaka[d]
Disrupted circadian rhythm[b]	*Drosophila*, zebrafish[e], mammals
Reduced spontaneous activity[a, b]	*Drosophila*, mammals
Cognitive impairments[f]	*Drosophila*, zebrafish, mammals
Cellular phenotypes	
Apoptosis (liver)[g]	Medaka[h]
Lipofuscin (liver, brain, gills)[a, i, j]	Nearly universal
Cardiac hypertrophy[g]	Guppy[k], mammals
Kidney tubule dilatation[g]	Guppy[l]
Liver neoplasias[g]	Medaka (low incidence)[m]
Senescence-associated β-Gal[a, i, j]	Zebrafish[n], human[o]
Gliosis[p]	Mammals
Quiescence of neuronal stem cells[p]	Zebrafish[q], mammals
Molecular phenotypes	
Telomere shortening[r]	Medaka[d], human
Lipid peroxidation[s]	Nearly universal
Mitochondrial impairments[t]	Mammals

β-Gal, β-galactosidase.
[a]Genade *et al.* (2005).
[b]Lucas-Sánchez *et al.* (2011).
[c]Gerhard *et al.* (2002).
[d]Hatakeyama *et al.* (2008).
[e]Zhdanova *et al.* (2008).
[f]Valenzano *et al.* (2006*a*,*b*).
[g]Di Cicco *et al.* (2011).
[h]Ding *et al.* (2010).
[i]Hsu *et al.* (2008).
[j]Liu *et al.* (2012).
[k]Woodhead (1984).
[l]Woodhead *et al.* (1983).
[m]Masahito *et al.* (1989).
[n]Kishi *et al.* (2003).
[o]Dimri *et al.* (1995).
[p]Tozzini *et al.* (2012).
[q]Edelmann *et al.* (2013).
[r]Hartmann *et al.* (2009).
[s]Lucas-Sánchez *et al.* (2014).
[t]Hartmann *et al.* (2011).

Table 1: Aging phenotypes conserved in Nothobranchius spp. and their occurrence in other model taxa. From Cellerino and Valenzano, 2015

The most relevant aging phenotypes for this thesis will be described in detail below.

Nfu males progressively lose body and tail colour as well as their distinct aging pattern, like blind mammals that progressively lose fur and skin pigment with age (Geyfman and

Andersen, 2010). Aging phenotypes in *Nfu* are also associated with abnormal, defective vision (Vanhunsel et al., 2021), structural deterioration of the fins, reduced spontaneous locomotion activity (Genade et al., 2005; Evsiukova et al., 2021) and impaired learning (Valenzano et al., 2006b; Mariën et al., 2024). Additionally, *Nfu* regeneration ability are reduced with age both in fins (Wendler et al., 2015) and central nervous system (Van Houcke et al., 2021; Vanhunsel et al., 2021). In addition, an increased concentration of lipofuscin, a canonical aging marker (Goyal, 1982), was observed in the brain, liver, heart, and skeletal muscle of old *Nfu* (Terzibasi et al., 2008; Ahuja et al., 2019; Ruparelia et al., 2023). Staining of senescence-associated β-galactosidase (SA-β-gal), a marker of cellular senescence and stress response in human cells (Cristofalo, 2005; Dimri et al., 1995; Kurz et al., 2000; Untergasser et al., 2003; Yegorov et al., 1998), increases significantly in the skin and brain of aged fish (Genade et al., 2005; Graf et al., 2013; Van Houcke et al., 2023). Neoplastic lesions have been measured in *Nfu* using several neoplasms associated proteins, including Bcl-2, cytokeratin-8, carcinoembryonic antigen, and mutated p53 (Di Cicco et al., 2011). In this study, the liver showed the highest incidence rate of neoplasms, specifically in the short-lived GRZ strain. Furthermore, the frequency of these lesions increases with the aging of the fish (Di Cicco et al., 2011). In addition, *Nfu* shows shortening of telomere during aging (Hartmann et al., 2009). Interestingly, *Nfu* telomeres, which are over four times shorter than those of mice (Zhu et al., 1998), are comparable in length to human telomeres and shorten with age (Hartmann et al., 2009). In addition, a telomerase mutant line of *Nfu* showed premature infertility, a dramatic decrease in the number of red and white blood cells, abnormalities in the epithelial cells of the intestine, including a decrease in polarity, and an increase in the nuclear/cytoplasmic ratio (Harel et al., 2015). These findings suggest that telomerase plays a key role in maintaining organismal homeostasis in the turquoise killifish, as in other organisms. Moreover, Mitochondrial DNA (mtDNA) instability was observed in *Nfu*. mtDNA copy number was found to be significantly reduced in different organs including brain, liver, and muscle, and mitohcondrial biogenesis was also impaired in muscles of elderly individuals (Hartmann et al., 2011).

1.8.2.1 Nfu brain aging phenotypes

Nfu brain aging has seen an increase in interest in recent years (Matsui et al., 2019; Kelmer Sacramento et al., 2020; Van Houcke et al., 2021; Vanhunsel et al., 2021; Bagnoli et al., 2022; Louka et al., 2022; Vanhunsel et al., 2022; de Bakker and Valenzano, 2023; Mariën et al., 2024). *Nfu* aging reveal a multifaceted degenerative process sharing key aspects with human neurodegenerative diseases associated with aging (Fig. 23). The main brain aging-related phenotypes of *Nfu* are discussed below.

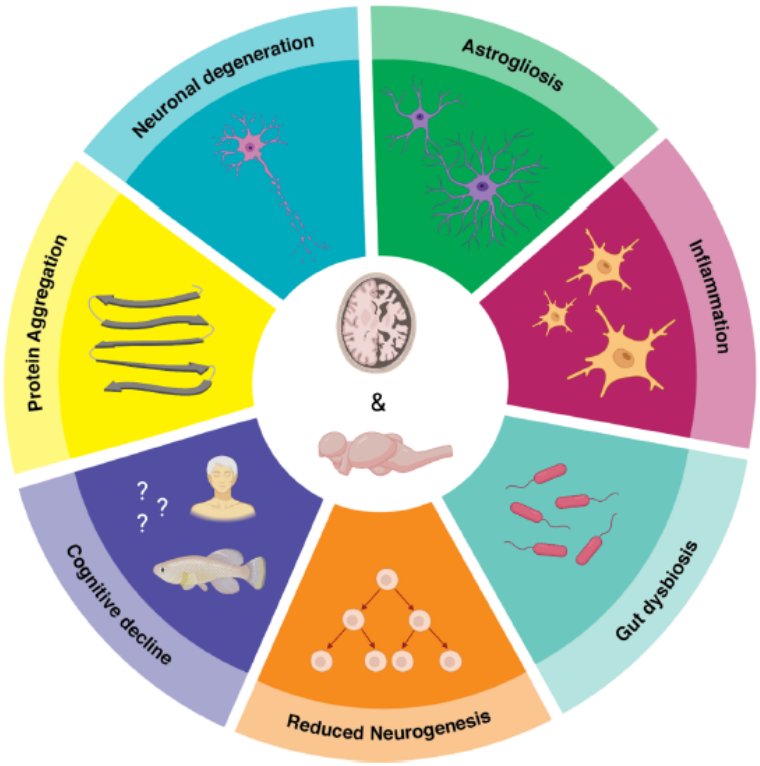


Figure 23: *Nfu* brain aging phenotypes in common with human brain aging. From Valenzano and de Bakker, 2023

- Reduced neurogenesis:

The reduced ability to produce new neurons during aging is associated with neurodegenerative diseases in mammals (Rodríguez and Verkhratsky, 2011; Winner et al., 2011). *Nfu* brain shows a drastic decrease in neurogenic potential with age (Tozzini et al., 2012). In detail, a decrease in the homeostatic neurogenerative potential primarily of dividing nonglial progenitors, as Figure 24 shows (Van Houcke et al., 2021). In addition, to the decline in homeostatic neurogenesis, the *Nfu* telencephalon also shows a strong age-related reduction in regenerative capacity after a stab wound, resulting in permanent glial scar formation. A decrease in the division of non-glial progenitor cells and induction of cellular senescence is thought to be primarily responsible for the loss of regenerative capacity (Van Houcke et al., 2021). Indeed, the use of senolytics improves brain regeneration in *Nfu* (Van Houcke et al., 2023). It is important to underline that the loss of regenerative capacity could be the basis of the accumulation of tissue damage in *Nfu* aging. These results may indicate that the decrease in neurogenic potential may contribute to the overall physiological decline of old *Nfu*.

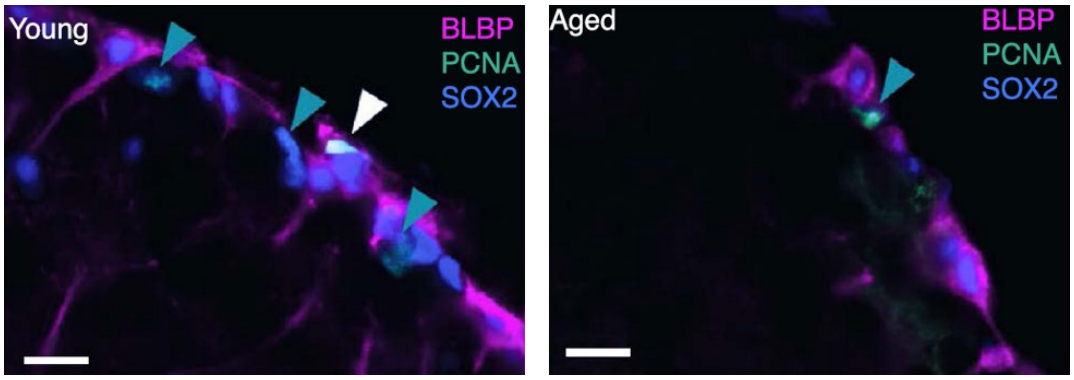


Figure 24: Reduction of neurogenesis in old *Nfu* compared to young (scare bar 100 μm). The images show a reduction of marker related to neurogenesis, such as BLBP (Fuchsia), PCNA (Green) and SOX2 (Blue). From Van Houcke et al., 2021

- Neuronal degeneration:

Nfu shows neuronal degeneration in the aging brain, as observed using Fluoro-Jade B staining, as Figure 25 A shows (Valenzano et al., 2006b; Terzibasi et al., 2008). Specifically, neurodegeneration also increases in the brain of GRZ strain as early as 2 months of age, strongly suggesting a spontaneous age-dependent increase in neurodegeneration (Valenzano et al., 2006b; Terzibasi et al., 2007). Furthermore, it has been identified that *Nfu* shows an age-dependent decrease in the number of dopaminergic in the locus coeruleus [Matsui et al., 2019; Bagnoli et al., 2022; (Fig. 25)].

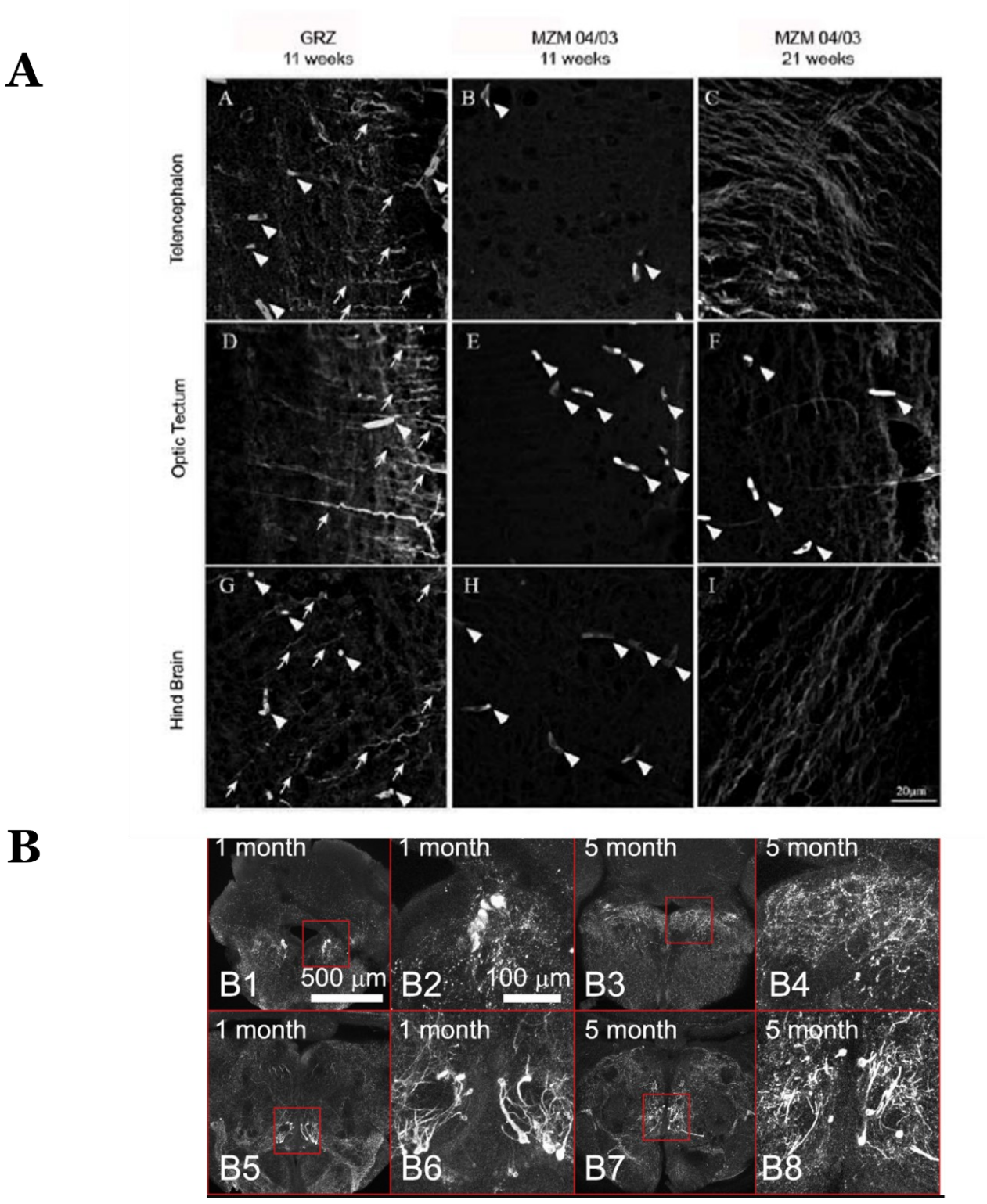


Figure 25: Neurodegeration of old *Nfu*. A, Neurodegeration of *Nfu* using Fluoro-Jade B (white) in Telencephalon, Optic tectum and Hind brain. The white arrows indicate Fluore-Jabe B staining. From Terzibasi et al., 2008. B, Degeneration of dopaminergic neurons (TH staining, white) in locus coerelus comping young (1 month) and aged (5 months) brains. From Matsui et al., 2019

- Protein aggregation:

A typical neurodegeneration phenotype, protein aggregation, was observed in the aging *Nfu* brain (Kelmer Sacramento et al., 2020; Matsui et al., 2019). Moreover, *Nfu* shows accumulation of lipofuscin (residues of lipid and protein oxidation) in aging tissues (Fig. 26A), including the brain (Terzibasi et al., 2008). Age-related increases in protein aggregation (an example in Fig. 26B), including ubiquitinated proteins, have been associated with age-related declines in proteasome activity (Kelmer Sacramento et al., 2020; Matsui et al., 2019). For example, an accumulation of human disease-specific proteins such as TDP-43 has been observed, indicative of FTD and ALS (Jo et al., 2020), or α-synuclein, indicative of AD (Louka et al., 2022; Matsui et al., 2019; Stefanis, 2012). Aggregated intracellular TDP-43 accumulates with age and colocalizes with stress granule markers in the telencephalon and optic tectum (Louka et al., 2022). Accumulation of α-synuclein was detected in the medulla oblongata before expanding towards the rostral side of the brain, showing a similar spatiotemporal sequence of events in the brains of PD's patients (Matsui et al., 2019).

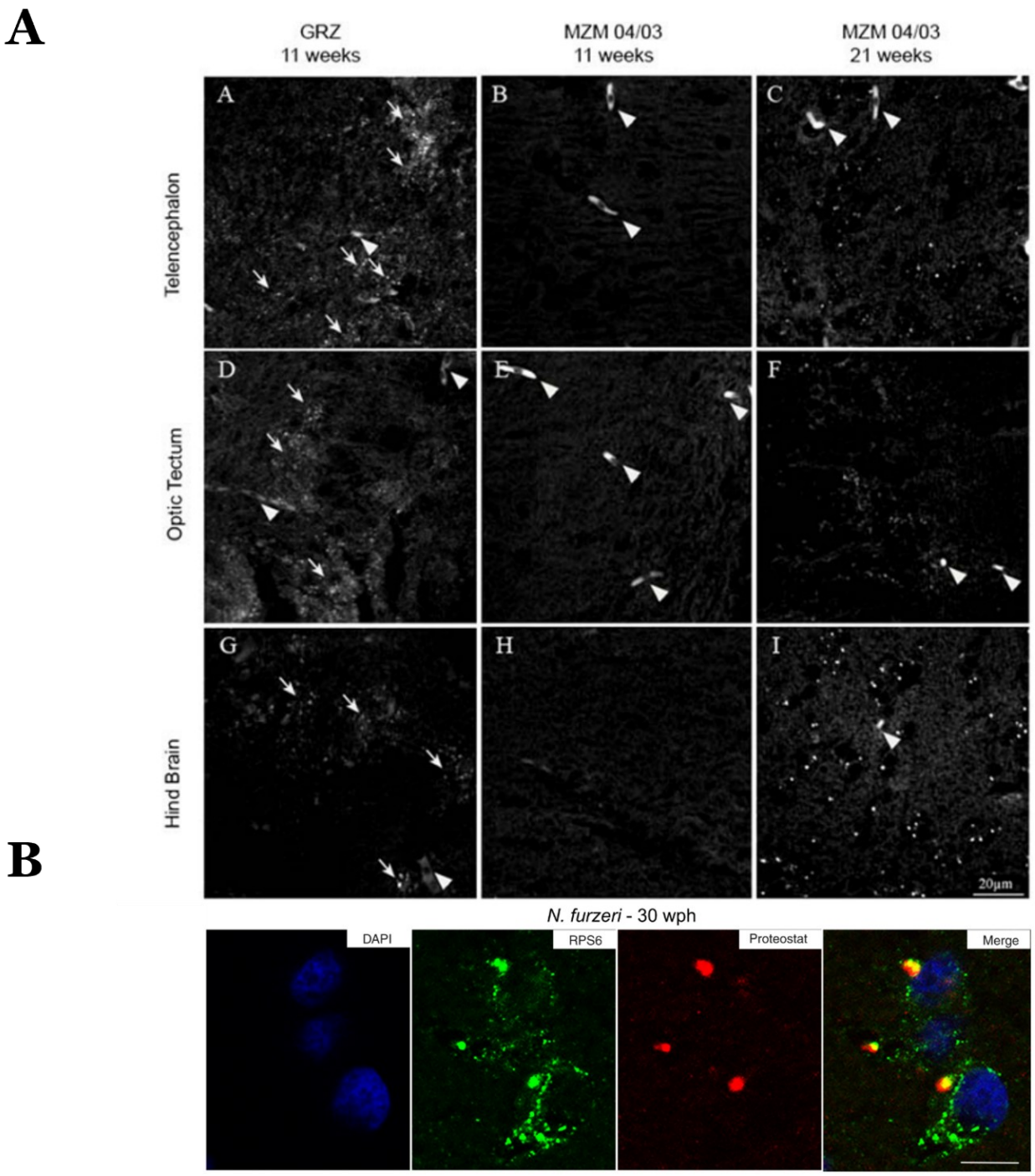


Figure 26: Aggregation proteins in old brain of *Nfu*. A) Lipofuscin aggregation during aging in *Nfu*, the white arrows indicate the granules of lipofuscin in Telencephalon, Optic Tectum and Hind Brain in different strains (GRZ and MZM03/04) and ages (11 and 21 weeks). From Terzibasi et al., 2008. B) Ribosomal protein aggregates in old *Nfu*. Protesostat (red) indicates the aggregates of proteins (scare bar 10 µm). From Sacramento et al., 2020

- Inflammation:

Increased protein aggregation and neuron degeneration it is often accompanied by an increase in activated microglia (Hickman et al., 2018). In the telencephalon and optic tectum of *Nfu*, the quantity of microglia increases in the aging *Nfu* brain (Fig. 27). In specific, an increased number of neonatal microglia was observed in aged *Nfu* (Vanhunsel et al., 2021). The age-dependent increase in microglia suggests a possible increase in the inflammatory state. Similarly, increased expression of complement, a generally of inflammatory response is observed both at the level of transcripts (Baumgart et al., 2014) and proteins (Kelmer et al., 2020)

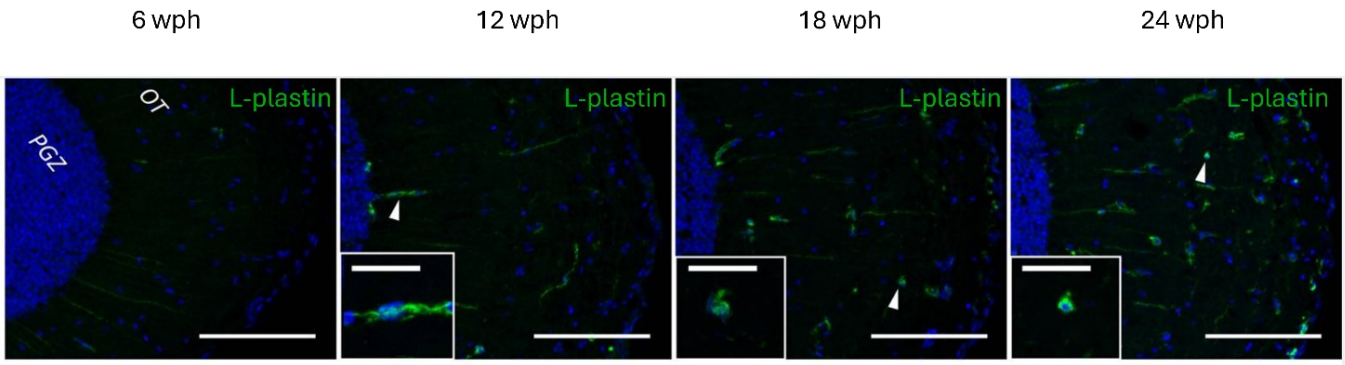


Figure 27: Inflammation (L-plastin) in Optic tectum of *Nfu* during aging. Comparison of l-plastin, marker of microglia/macrophages in different ages, or 6 wph, 12 wph, 18 wph and 24 wph (scare bar 20 μm). From Vanhunsel et al., 2021

- Astrogliosis:

As the process of astrogliosis is observed in neurodegenerative diseases, and it is a consequence of the induction of M1 inflammatory phenotype in microglia cells that –via inflammatory cytokines- induces the so-called A1 phenotype of astrocytes that is characterized by increased expression of GFAP (Liddelow et al., 2017; Clarke et al., 2018; Lawrence et al., 2023). Astrogliosis is also observed in the aging brain of *Nfu* as demonstrated by the dramatic increase of throughout the optic tectum and telencephalon, as Figure 28 shows (Tozzini et al., 2012; Vanhunsel et al., 2021) and was confirmed by proteomics (Kelmer et al., 2020). Moreover, it is interesting to specify that the increase in GFAP signalling in *Nfu* may not be indicative of the same processes as those underlying the increase in GFAP signalling in humans, including neuronal degeneration.

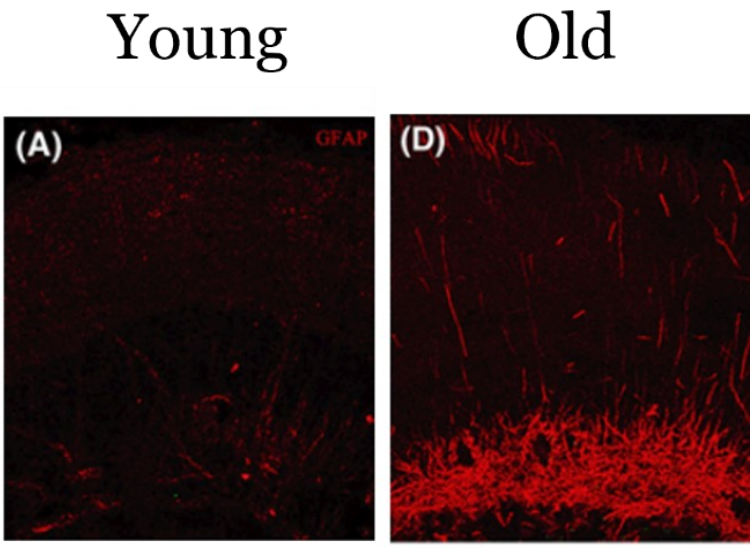


Figure 28: Glial Fibrillar Acid Protein staining in optic tectum of young and old *Nfu*. GFAP is labelled in red. From Tozzini et al., 2012

- Gut dysbiosis:

In recent years it has become increasingly clear that intestinal dysbiosis is correlated with aging and age-associated neurodegenerative diseases (Biagi et al., 2016; Qian et al., 2018; Barichella et al., 2019; Li et al., 2019; Liu et al., 2020; Kaiyrlykyzy et al., 2022). Gut dysbiosis also occurs naturally in *Nfu* (Smith et al., 2017). Gut bacterial taxonomic diversity within an individual decreases in *Nfu* during aging (Smith et al., 2017). In *Nfu*, a decrease in *Bacteroidetes*, *Firmicutes* and *Actinobacteria* and an increase of *Proteobacteria* was observed (Smith et al., 2017), as also observed in AD patients (Vogt et al., 2017). Age-related changes in intestinal microbiology share similarities with those observed in patients suffering neurodegenerative diseases and could impact systemic health.

- Cognitive decline:

The decrease in cognitive abilities decreases dramatically with aging and in neurodegenerative disorders (Selkoe, 2021; van Dyck et al., 2022). *Nfu* show a significant age-dependent decrease in learning ability (Valenzano et al., 2006; McKay et al., 2022). Active avoidance test was used, in which fish must learn to avoid red light that is associated with a negative stimulus such as air bubbles. Unlike old *Nfu*, the young fish have learned to avoid red light. Besides this test, a positive associative learning test (McKay et al., 2022) was performed which showed reduced learning ability in old *Nfu*. Furthermore, life-extending interventions, such as dietary restrictions or resveratrol treatment, improved learning abilities in aged *Nfu* (Valenzano et al., 2006; Terzibasi et al., 2009; Yu and Li, 2012).

Age-dependent spontaneous phenotypes in *Nfu* are consistent with an overt degenerative process in the brain. Together these phenotypes are consistent with a degenerative process in the brain. *Nfu* brain aging trajectory resembles that of humans suffering from neurodegenerative diseases compared to that of healthy humans displaying a normal, non-disease brain (Valenzano and de Bakker, 2023). *Nfu* is used as a natural model to study the underlying mechanisms of aging and the spontaneous onset of neurodegenerative diseases. The advantages of *Nfu* involve this animal useful for screening aimed at identifying diets, nutraceutical compounds and drugs potentially capable of reducing age-related neurodegenerative symptoms.

1.8.2.2 Nfu a powerful model for invention in aging studies

Nfu has emerged as a powerful model to investigate interventions in aging research for pharmacological and dietary interventions aimed at prolonging longevity. This short-lived vertebrate offers a unique combination of rapid aging and pronounced age-related phenotypes, making it ideal for lifespan studies. *Nfu* is utilized to explore the effects of various drugs and dietary supplement on lifespan extension, uncovering insights into the mechanisms of aging and potential therapeutic targets.

The use of the *Nfu* animal model has allowed us to observe the significant effects of resveratrol on aging. For example, Valenzano et al. (2006) reported that resveratrol treatment led to a dose-dependent increase in the median and maximum lifespan of *Nfu*. Additionally, resveratrol was found to improve markers of healthspan, including enhanced locomotor activity and reduced cognitive decline (Valenzano et al., 2006a). *Nfu* was a good model to demonstrate the effect of resveratrol on prolonging lifespan.

Furthermore, *Nfu* has been used in studies on the effect of Dietary Restriction (DR) on aging. DR significantly prolonged the lifespan of *Nfu* (Terzibasi et al., 2009a). Furthermore, DR was associated delayed onset of age-related phenotypes, and preservation of cognitive function (Terzibasi et al., 2009a). *Nfu* has demonstrated the effect of DR on increasing lifespan and slowing down aging phenotypes.

In recent years, the different activities of BGs in different conditions, such as cancer, diabetes, cardiovascular diseases, etc. have been investigated in detail (Wang et al., 2017; Ahnen et al., 2019; Frank et al., 2019; Murphy et al., 2020; Duysburgh et al., 2021). Although, BGs have various activities that can act in the mechanisms of aging, there are few studies regarding their anti-aging function. Specifically, there are no studies on the effect of BGs in aging using *Nfu*. For contrary, Song et al. (2020) studied the effect of 1.3 BGs (from alga *Euglena gracilis*) in aging using *Nothobranchius guentheri* as a model animal. *Nothobranchius guentheri* were fed 1.3 BGs (1.25 g/kg body weight) were included in the worm from 36 wph. 1.3 BGs prolonged the lifespan of the *Nothobranchius guentheri*, in fact

treated live up to 54 wph, for contrary the controls 52 wph. Furthermore, it was shown that 1.3 BGs reduced lipofuscin accumulation and β-galactosidase. Finally, 1.3 BGs reduced lipid and protein peroxidation by providing antioxidant activity in old *Nothobranchius guentheri*. This study demonstrated for the first time the potential utility in prolonging lifespan and the strong antioxidant activity of 1.3 BGs in aging using *Nothobranchius guentheri* (Song et al., 2020). This study laid the foundation for idealizing my thesis project, and for investigating widely the effect of 1.3, 1.6 BGs in other aging phenotypes, specifically brain aging phenotypes, used *Nfu* as animal model.

2 THESIS AIMS

The above scenario is suggestive of 1.3, 1.6 β-glucans (BGs) implication in processes reduced during aging, i.e. in autophagy, immunomodulation, antioxidant, and anti-inflammatory processes. Therefore, the aim of this project is to investigate the effect of BGs in a naturally aging animal using *Nothobranchius furzeri (Nfu)* as a model organism.

The first objective was to investigate if chronic low-concentration supplement on of BGs in the diet could retard the progression of aging-related phenotypes.

The second object was to concentrate on brain aging and investigate whether acute administration of BGs in older subjects was also capable of inducing autophagy in neurons. The third objective was to investigate if BGs can act directly on the brain. To this end, I used cultured brain sliced from *Nfu*.

The fourth aim was to understand we BGs where entirely mediated by microglia or there was also a direct action on neurons. To this end, I cultured iPSC-derived human neurons and investigated whether BGs induced autophagy in these cells.

This last aim was fundamental to confirm the effect of BGs in neurons and to suggest a possible translation into humans in the future.

3 MATERIALS AND METHODS

3.1 Ethics Statement

The study has been performed in accordance with the European Union (EU) Directive 2010/63/EU for animal experiments and according to the procedures established by the Institutional Animal Care and Use Committee (IACUC) of the University of Pisa. Ministry authorization B290E.N.VIC and B290E.N.TUN approves research on the effect of 1.3, 1.6 β -glucans (BGs) on aging using the *Nothobranchius furzeri (Nfu)* and zebrafish (respectively) as the experimental animal.

3.2 Setting and Study Design

The present study concerned the effect of 1,3-1,6 β-glucans (BGs) on aging in vivo and in ex-vivo using *Nothobranchius furzeri* (*Nfu*) and zebrafish as animal models. It was carried-out at the Bio@SNS of Scuola Normale Superiore, Pisa (Italy), in collaboration with "AquaLab" of the Department of Veterinary Sciences of the University of Pisa, "Zebralab"of IRCCS Stella Maris and Biogem.

3.3 Killifish Care and Maintenance

All animal experiments were performed on *Nothobranchius furzeri (Nfu)* strain MZCS-NF222 (Blažek et al., 2017). Embryos were hatched and housed locally in a recirculating Tecniplast Zebtech system with automatized water flow, pH and salinity control. Water parameters were set temperature of 28 °C, pH 7 and conductivity 800 μs/cm. The photoperiod was set to 12 h light 12 h dark in phase with external day/night cycle (Nath et al., 2023; Polacik et al., 2016; Dodzian et al., 2018). Fertilized eggs were maintained on wet peat moss at a temperature of 28 °C in sealed Petri dishes. When embryos reached the final stage of development, eggs were hatched by transferring them in 1: 1 humic acid (1g /L) at temperatures of 4 °C. Hatched embryos were transferred to a clean vessel with static water with daily water changes where they were kept until 2 weeks of age. For the first two weeks, fry were fed with newly hatched *Artemia nauplii* and were maintained in the incubator with the same parameters mentioned above. After age 2 weeks the diet was gradually shifted to frozen *Chironomus* larvae.

3.4 Zebrafish Care and Maintenance

The study was carried out using Tg(mpeg1.1: EGFP) zebrafish (Ellett et al., 2011; Renawat and Masai, 2021; Ge et al., 2023). Zebrafish were maintained at 28 °C in a water recirculating system under a 12 h light: dark photoperiod. The pH level of the water is

maintained at 7.0 – 8.0 and conductivity at 800 μs/cm (Lawrence, 2007). Adult were fed 4 times a day alternating Zebrafeed (400-600 μm, Sparos, Portugal) and *Artemia nauplii*. Mating was performed using a single couple tank 0.8 L capacity and eggs were collected, washed with egg water, and incubated at 28 °C until hatching. At 10 dpf (days post fertilization) zebrafish were introduced into the recirculating system. Larvae were fed from 5 dpf with Zebrafeed (<100 μm). During growth, the size of the feed was increased and *Artemia nauplii* was introduced (from 10 dpf).

3.5 Inclusion of 1.3, 1.6 β-glucans in the food

The 1.3, 1.6 β-glucans (from the cell wall of *Saccharomyces cerevisiae*, MacroGard®) utilized in our experiments were bought from Biorigin© (São Paulo, Brazil). To include it in the food, we ground up the Zebrafeed (Sparos, Portugal), added the concentration of 1.3, 1.6 β-glucans and water (125 mL/ 100g Zebrafeed) to create a pliable paste. The paste was injected into a syringe to form threads that were left in an oven overnight at 68 °C. The following day, we crushed the feed strands to re-create the feed's original size (400–600 μm).

3.6 Experimental design

The project comprises three aims:

3.6.1 Chronic effects of 1.3, 1.6 β-glucans on aging in vivo

Starting at the second week of life, fish were fed until 27 weeks post hatching (wph) with dry belonging to either of these tree groups (Fig. 29A): Zebrafeed (400-600 μm), Zebrafeed fortified with 0,125 μg/gr of 1.3, 1.6 β-glucans (corresponding to a doses of 12.5 mg/Kg BW assuming the consumption of 100mg per day) and Zebrafeed fortified with 1,25 μg/gr of 1.3, 1.6 β-glucans (corresponding to a doses of 125 mg/Kg BW assuming the consumption of 100 mg per day).

3.6.2 Acute effects of 1.3, 1.6 β-glucans on aging in vivo

Nothobranchius furzeri were fed with Zebrafeed fortified with 1,25 μg/gr of 1.3, 1.6 β-glucans (corresponding to a doses of 125 mg/Kg BW assuming the consumption of 100 mg per day) for a week starting at age 26 weeks (Fig. 29B).

At age 27 weeks, fish were euthanized with MS-222 and organs were extracted for analysis.

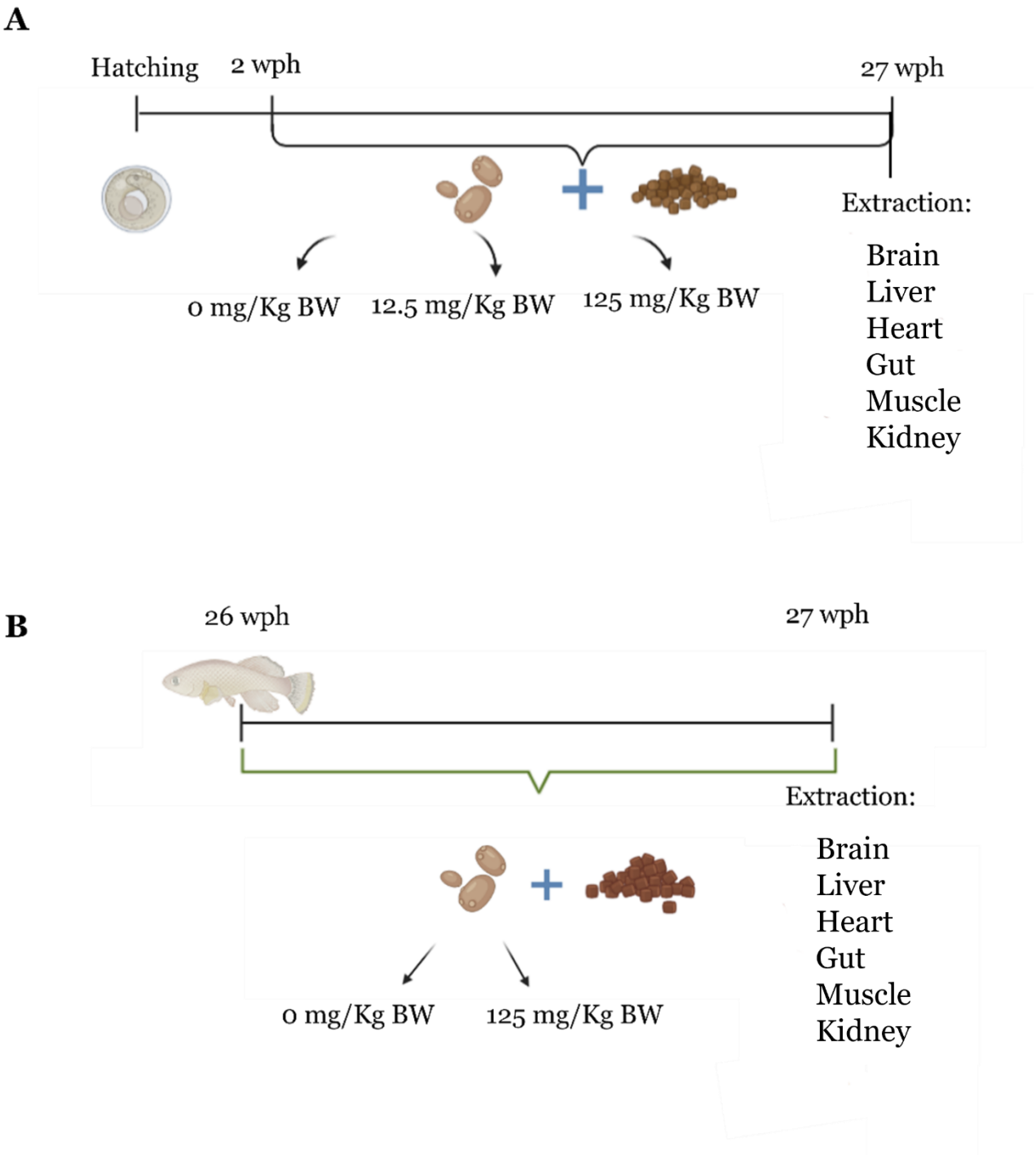


Figure 29: Experimental design of diet fortified with 1.3, 1.6 β-glucans. A) Chronic diet fortified with 1.3, 1.6 β-glucans, B) Acute diet fortified with 1.3, 1.6 β-glucans

3.7 Tissue fixation and processing

Fish were euthanized with MS-222 and cooled on crushed ice for 5 minutes before dissection. Target tissues were dissected and fixed by immersion in 4% paraformaldehyde in 100mM PBS for a day. Some organs were then included in paraffin and cut at a thickness of 5 µm on a microtome. Other organs were immersed in 30% sucrose for two days and then

frozen in OTC embedding matrix (Carl Roth, Germany). Frozen blocks were cut with a cryostat (CM186 UV, Leica) at a thickness of 25 µm. The paraffin slides were deparaffinized in accordance with defined procedures before beginning histology or immunolabelling (Krogerus and Andersson, 1988). Furthermore, 1% PBS was used to wash the cryosections to eliminate OTC.

3.7.1 Lipofuscin detection

Unstained slices were mounted using a mounting solution for lipofuscin detection. As lipofuscin is autofluorescent, no staining is required to produce its emission when it is stimulated at 488 nm. Via the use of the apotome microscope, lipofuscin was obtained.

3.7.2 Histology

According to the established protocols, slides were stained with haematoxylin and eosin (H&E) and Sirus Red.

3.7.2.1 Protocol for H&E staining:

Immerse the sections in Hematoxalin for 3 minutes. The sections are rinsed with water for 5 minutes. Then, the sections are stained with Eosin for 45 seconds. The sections are rinsed with water for 5 minutes. Subsequently, the sections were dehydrated at different concentrations of ethanol (3 x 5´ 95% ethanol, 3 x 5´ 100% ethanol, 2 x 10´ xylene. The sections were mounted using Permount (xylene based).

3.7.2.2 Sirus Red Fast Green protocol:

Sirius red and fast green staining (9046, Chondrex, Inc.) was used to detect muscle collagen deposits. Collagen fibers appeared magenta, while the non-collagen proteins were green. The slices are load with 0.3 ml Dye Solution for 30 minutes. After, the Dye Solution was aspirated, and the slices were rinse until the water runs clean. The slices were dehydrated and covered with coverslip.

3.7.2.3 Immunofluorescence and and Proteostat™ Aggresome staining:

Slides were subject to antigen unmasking using “Antigen Retrieval” acid (2,94g Trisodium citrate dihydrate, Tween 20 0,05%, 1L H_2O_{mq}, pH= 6)”. Antigen Retrival solution was put into a receptor, which was then heated in a microwave to 95 ° C. The slides were submerged in warm antigen retrival solution for two minutes. Three times this unmasking cycle was carried out. Only for slides stained with a with Aggresome (ProteoStat ™ Aggresome Detection Kit, Enzo Life Sciences Inc.) we applied a 1: 2000 solution of Aggresome stain in PBS for 3 minutes, rinsed in 1% PBS and left the sections immersed in 1% acetic acid 40 minutes for de-staining. To prevent non-specific staining, slides were treated with normal serum blocking solution (BSA 5%, Triton 0.3% diluted in 1% PBS) for two hours at RT (Room Temperature). Sections were then incubated with primary antibody (Tab. 2) at appropriate dilution in primary antibody dilution buffer (BSA 1%, Triton 0.1% in 1% PBS) overnight at

4 °C. Sections were rinsed three times for 5 minutes with 1% PBS. Secondary antibodies (diluted 1:500) were stained at RT in in the same dilution buffer used for the primary antibodies for 2 hours. The slides were rinsed again with 1% PBS (3 times, 5 minutes) and DAPI (10 µg/ml; 10236276001, Sigma Aldrich) was added for 10 minutes. The slices were rinsed with 1% PBS (3 times, 5 minutes) and mounted with a specific mounting medium (Fluoroshield mounting, Sigma-Aldrich).

Antibody Primary	Marker of	Working Solution	Product type	Code
GFAP	Gliosis	1:500	Rabbit Polyclonal	GTX128741, Genetex
LAMP1	Lysosomes	1:500	Rabbit Polyclonal	ab24170, Abcam
LC3B	Autophagy	1:500	Rabbit Polyclonal	ab48394, Abcam
LCP1	Inflammation	1:200	Rabbit Polyclonal	GTX124420, Genetex
Nitrotyrosine	Peroxidation protein	1:500	Rabbit Polyclonal	A-21285, Thermo Fisher

Table 2: Antibodies

3.8 Direct effects of 1.3, 1.6 β-glucans on the brain aging

3.8.1 Cultured brain slides

We created an ex-vivo model using the brains of *Nfu* aged 5, 10, and 27 weeks.

Experiments are performed under sterile conditions with tools sterilised by autoclaving prior to use. 70% EtOH is used to sterilize equipment and surfaces throughout the experiment.

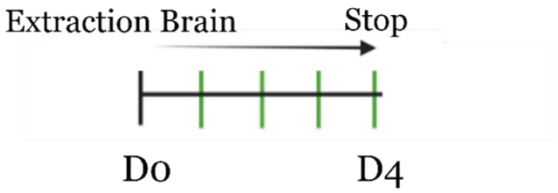
1. The fish were euthanized in MS-222 (200 mg/L), and placed in 0.05% NaOH for 30 seconds, followed by 70% EtOH for 30 seconds. This is done to sterilize the body and prevent the brain from becoming contaminated with bacteria during dissection.
2. The fish were decapitated using scissors.
3. The heads were transferred in the dissecting medium and brains were dissected using a stereomicroscope. The longer it takes to extract the brain, the less likely it is that the slices will survive.
4. The brains extracted were placed it on the micrometric slide and, using a precision micro-knife, cut it in half sagittally.
5. The cultured brain slices were extracted and appropriately positioned on the semipermeable membranes (PICM0RG50, Merk Life Science, Germany) containing

culture medium, place the six well plate into the incubator set at a temperature of 28°C and 5% CO_2.

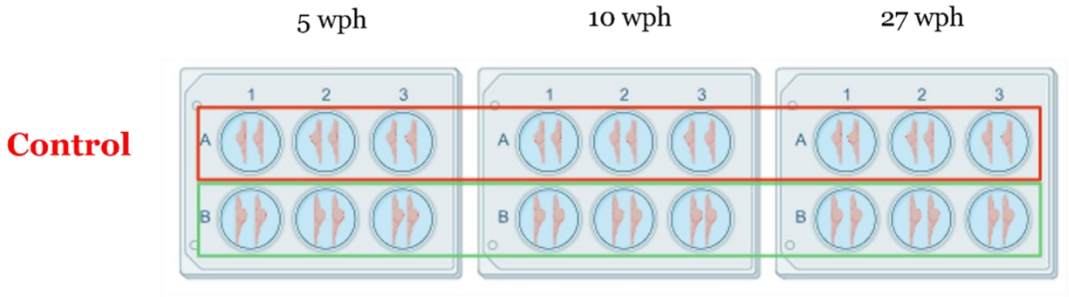
The culture medium used for cultured brain slices is composed of: DMEM/F12 (21331020, Thermofisher, USA), FCS 10% (ECS5000L, Euroclone, Italy), Sterile milliQ water 10%, Insulin 0.033% (L9278, Merk Life Science, Germany), Ascorbic acid 1% 511 μM, PenStrep 1% (ECB3001D, Euroclone, Italy), Glucose to adjust the solution at 0,4% (A2494001, Thermofisher, Italy). This medium was changed every day, and I put in the medium some treatment, like 1.3, 1.6 β-glucans, Bafilomycin A1, plx5622 and DMSO (control), to observe their effect.

The slices were treated with 1.3, 1.6 β-glucans (BGs) at 8 mg / L concentration (Brogi et al., 2021) in culture medium for three days to observe the effects of 1.3, 1.6 β-glucans in autophagy at different aging (Fig. 30A). Moreover, the cultured brain sliced extracted of Nothobranchius of 5-week and Tg(mpeg1.1.: EGFP+) zebrafish were also treated with 100 nm of Bafilomycin A1 for 3 days (Fig. 30B) to observe if 1.3, 1.6 β-glucans is inductor of autophagy and restore autophagy in condition of impairment of this mechanism. Finally, the cultured brain slices of Nothobranchius of 10-week were treated for 3 days with 20 μm of plx5622 to deplete microglia (Fig. 30C). This experiment was useful to understand if BGs induce autophagy activating cells other than microglia.

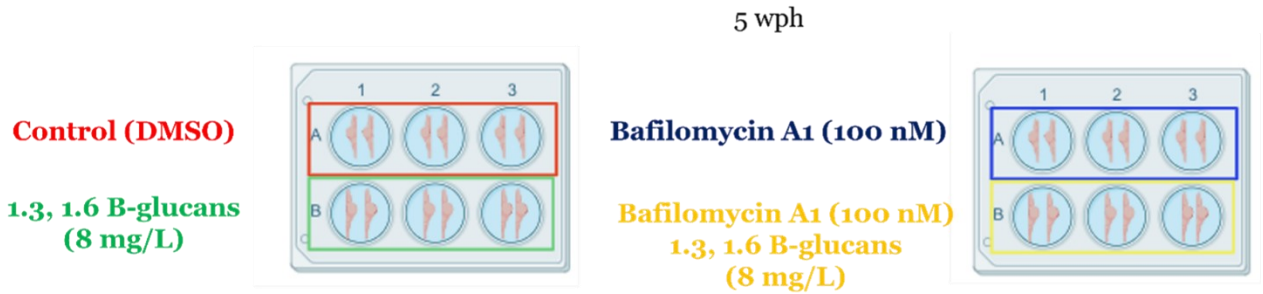
A


Extraction Brain
Stop
Do
D4

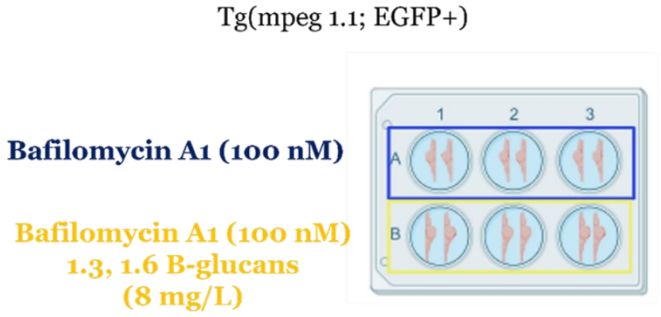
B


5 wph
10 wph
27 wph
Control
1
2
3
A
B

C


5 wph
Control (DMSO)
1.3, 1.6 B-glucans
(8 mg/L)
Bafilomycin A1 (100 nM)
Bafilomycin A1 (100 nM)
1.3, 1.6 B-glucans
(8 mg/L)
1
2
3
A
B

D


Tg(mpeg 1.1; EGFP+)
Bafilomycin A1 (100 nM)
Bafilomycin A1 (100 nM)
1.3, 1.6 B-glucans
(8 mg/L)
1
2
3
A
B

E

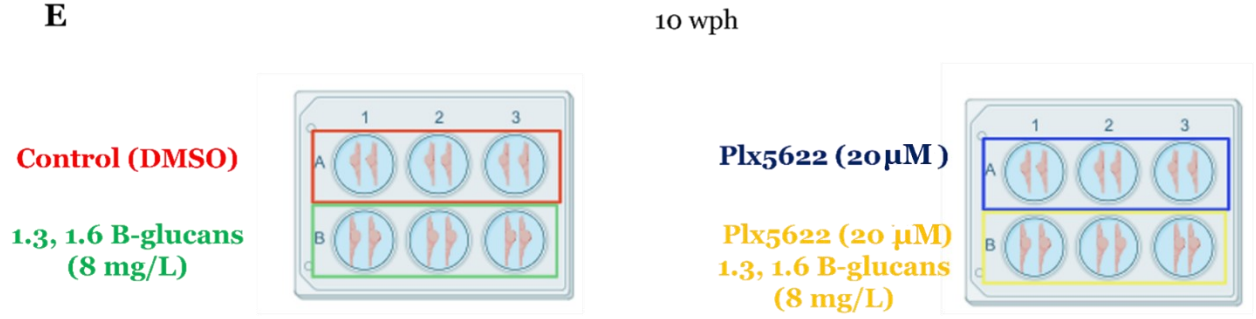

10 wph
Control (DMSO)
1.3, 1.6 B-glucans
(8 mg/L)
Plx5622 (20µM)
Plx5622 (20 µM)
1.3, 1.6 B-glucans
(8 mg/L)
1
2
3
A
B

Figure 30: Experimental design of treatment of cultured brain slices. A) Timeline of experimental design, B) Experimental design of cultured brain slices treated with BGs in different ages (5, 10, 27 wph), C) Experimental design of cultured brain slices at 5 wph treated with BGs, Bafilomycin A1, Bafilomycin A1 and BGs, D) Experimental design of cultured brain slices of Tg(mpeg1.1.: EGFP+) treated with Bafilomycin A1, Bafilomycin A1 and BGs, E) Experimental design of cultured brain slices at 5 wph treated with BGs, plx5622, plx5622 and BGs

3.8.1 Preparation of treatment for Culture brain slices

3.8.1.1 1.3, 1.6 β-glucans solution and sonication

1.3, 1.6 β-glucans (from the cell wall of *Saccharomyces cerevisiae*, MacroGard®) in water were sonicated for 2 minutes in 30 second on/off cycles, to have microparticle of 1.3, 1.6 β-glucans in solution stock. 1.3, 1.6 β-glucans solution stock was diluted in medium to have working solution of 8 mg/L.

3.8.1.2 Bafilomycin A1 solution

The lyophilized powder of Bafilomycin A1 (19-148; Merck; 50ug) was dissolved in 80.28 µl of DMSO to have a stock of 1mM Bafilomycin A1 solution stock. Bafilomycin solution stock was stored at -20°C. Bafilomycin A1 working concentration was diluted in medium of cultured brain slices or neuronal cultures to 10 or 100 nM.

3.8.1.3 Plx5622 solution

The crystals of plx5622 (1303420-67-8; Cayman Chemical; 5mg) was dissolved in 1.26 mL of DMSO to have a stock of 10mM plx5622 solution stock. Plx5622 solution stock was stored at -20°C. plx5622 working concentration was diluted in medium of cultured brain slices or neuronal cultures to 20 µM.

I performed immunofluorescence including some kits “Lysotracker” and “Mitotracker” and “CYTO-ID Autophagic detection”, proteomic analyses.

3.8.1.4 Immunofluorescence staining

I fixed the slices for 15 minutes by placing 1 ml of PFA 4% above and 1 ml below the membrane. After several washes we added the blocking solution (BSA 5%, Triton 0.1% dissolved in 1% PBS) for 4 hours to RT on a rocker in gentle agitation. We added the primary antibody (LC3B, ab38354, Abcam; LCP1, GTX124420, Genetex) into the antibody solution (BSA 1%, Triton 0.1% dissolved in 1% PBS) for 3 days at 4 ° C on a rocker agitation. After 3 days, the slides were washed in 1 %PBS and added the secondary antibody in the antibody solution. After washing the slides in 1% PBS, DAPI (10 µg/ml; 10236276001, Sigma Aldrich) was put on a rocker agitation at 4 ° C. After 3 washes in 1% PBS for 10 minutes, e A2 Sca/e clarity solution (Glycerol 10%, Urea 4M, Triton 0.1%) was added until the brain slices were transparent (util a week). For the maintenance of fluorescence, the S4D25(0) Sca/e solution [D(–)-Sorbitol 40% (wt/vol); Glycerol 10% (wt/vol); Urea 4 M; DMSO 25% (vol/vol)] was substituted with an A2 solution.

3.8.1.5 Lysotracker and Mitotracker staning

First of all, Lysotracker (LysoTracker™ Green DND-26, special packaging; L7526; Thermo Fischer), and Mitotracker was validated the use of the in the ex-vivo model and discovered the best parameters of use by observing the penetration of the kits every 5 minutes (Lysostracker) or every 30 minutes (Mitotracker). Not fixed cultured brain slices (at 27 wph) were stained with Lysotracker, marker of lysosomes, or Mitotracker, marker of Mitochondria. Lysotracker was introduced to the medium at the concentration of 75 µM for 45 minutes at 28 ° C in an incubator. Then, 3 washes of 10 min with medium were performed. The lyophilised dried powder of Mitotracker reagent was reconstituted in DMSO, as described in the Product Datasheet. The Mitotracker was added at the concentration of 500 nM in medium for 90 minutes at 28 ° C and 5% CO_2 in an incubator, followed by three 10-minute washes. The cultured brain slices were fixed in PFA 4% as for immunofluorescence analyses and they are viewed using confocal Zeiss LSM 900 with Airyscan.

3.8.1.6 CYTO-ID Autophagic detection

First of all, the use of the CYTO-ID® Autophagy detection kit 2.0 (ENZ-KIT175, Enzo) in the ex-vivo model was validated, given that this kit is designed for cell cultures. Then, in the medium of the cultured brain slices every 1add 2 µL of CYTO-ID® Green Detection Reagent was added and 1 µL of Hoechst 33342 Nuclear Stain. Initially, every 30 minutes it was observed whether the Reagent had penetrated the cultured brain slices in confocal instrument. I have observed that 2 µL of CYTO-ID® Green Detection Reagent 2 for 90 minutes at 28 ° C and 5% CO_2 in the incubator are the best parameters for the action of this kit. The cultured brain slices were rinse three times for 10 minutes and were fixed in PFA 4% as for immunofluorescence analyses and they are viewed using Confocal (Stellaris 5, Leica).

3.9 Direct effects of BGs on iPSC-derived human neurons

Human cortical neurons were cultured from 10 DIV 10 to 90 DIV, as described below.

3.9.1 Cultivation of IPSC-derived human cortical neurons

Day 1

Dilute the Poly-D-Lysine (A-003-E, Sigma-Adrich) solution with sterile dH_2O to a final concentration of 50 µg/mL was incubated plates ON in a 37 °C, 5% CO_2 humidified incubator.

Day 2

Dilute the Mouse Laminin I solution (C095-M, Merck Millipore) with sterile PBS to a final concentration of 10 µg/mL was prepared. Dilute the Poly-D-Lysine solution was aspirated

and Mouse Laminin I solution was incubate the plates ON in a 37 ˚C, 5% CO_2 humidified incubator.

Day 3

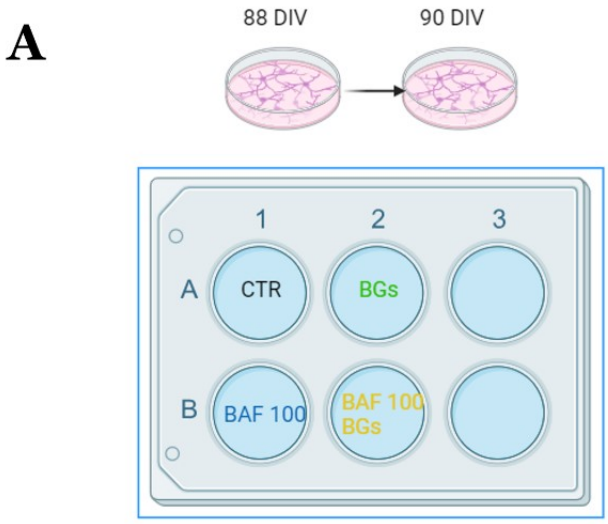
Neurons were thawed in the 37°C, then cells were diluted (1:5) in Hanks' Balanced Salt Solution (HBBS; 14025092, ThermoFisher Scientific) and centrifuged at 200g for 4 minutes at RT. The solution was aspirated, and the cell pellet was resuspended in maintenance medium (WiBiTi) with 2 µM Y-27632 (or rock-inhibitor, SM02-5, Cell Guidance Systems), and 20 µM DAPT (Notch inhibitor; D5942, Sigma Aldrich). Neurons were plated at 200000 cells/cm^2 in plates aspirated from the lamina I solution.

iPSC-derived human cortical neurons were maintained in WiBiTi medium (Table xxx) from 10 to 15 DIV. WiBiTi medium based in DMEM/F-12 (21331-046, ThermoFisher Scientific) containing 2mM Glutamine (25030, ThermoFisher Scientific), 1 mM Sodium Pyruvate (11360070, ThermoFisher Scientific), 1mM Non-essential amino acids (11140, Sigma Aldrich), 0.05mM β-mercaptoethanol (M3148, Sigma Aldrich), 10 µM 53AH (C5324-10, Cellagen Technology), 10 µM LDN193189 hydrochloride (SMLO559, Sigma-Adrich), 1 µM RepSox (Sigma-Aldrich R0158-5MG), N-2 Supplement 100X (175020, ThermoFisher Scientific), and B-27 Supplement minus Vitamin A 50X (125870, ThermoFisher Scientific). N2B27 medium was used from 16 to 32 DIV. N2B27 medium based DMEM/F-12 containing 2mM Glutamine, 1 mM Sodium Pyruvate, 1mM Non-essential amino acids, 0.05 mM β-mercaptoethanol, N-2 Supplement 100X, and B-27 Supplement minus Vitamin A 50X. iPSC-derived human neurons (32-70 DIV) were maintained with Neurobasal Young medium, changing it half the volume of the soil every 2 or 3 days as needed. Neurobasal medium based Neurobasal (21103049, ThermoFisher Scientific) containing 2mM Glutamine, 1 mM Sodium Pyruvate, 0.05mM β-mercaptoethanol, N-2 Supplement 100X, B-27 Supplement minus Vitamin A 50X (for "Young Medium") or B-27 Supplement 50X (17504044, ThermoFisher Scientific; for Old medium), 0.5 mM Ascorbic acid (A92902, Sigma Aldrich), and Recombinant human BDNF (20 ng/ml, NBP2-52006, Novus Biologicals). From 70 DIV to 90 DIV iPSC-derived human cortical neurons were maintained with Neurobasal Old medium. Every 2 weeks the neurons were split. After washing the cells with Versene for minutes and detaching with Acutase for 10 minutes at 37°C (incubator), the cells were aspirated and diluted in 1% PBS (1:5). Cell suspensions were centrifuged at 200 g for 4 minutes at RT, solution was aspirated, and the cell pellet was resuspended in maintenance medium (depending on the DIV) supplemented with 2 µM Y-27632, 20 µM DAPT and 10 µM LDN193189 hydrochloride. Less than 24 hours later, maintenance medium containing 2 µM Y-27632, 20 µM DAPT and 10 µM LDN193189 hydrochloride was aspirated and fresh, unsupplemented medium was added.

The last split was performed on 24-glass multiwell to be acquired confocally after immunofluorescence.

3.9.2 Experimental design of iPSC-derived human cortical neurons

After maintenance of iPSC-derived human cortical neurons up to 87 DIV, treatment with BGs and Bafilomycin A1 (10 and 100 nm) was carried out. The experimental design is described below in Figure 31. The acute experiment (Fig. 31A) is divided into 4 groups, i.e. control, 8 mg/L BGs, 100 nm Bafilomycin and 100 nm Bafilomycin + 8 mg/L BGs. In the prolonged treatment experiment (Fig. 31B) there are 6 groups, namely control, 8 mg/L BGs, 10 nm Bafilomycin A1, 10 nm Bafilomycin A1 + 8 mg/L BGs, 100 nm Bafilomycin A1 and 100 nm Bafilomycin + 8 mg/L BGs.



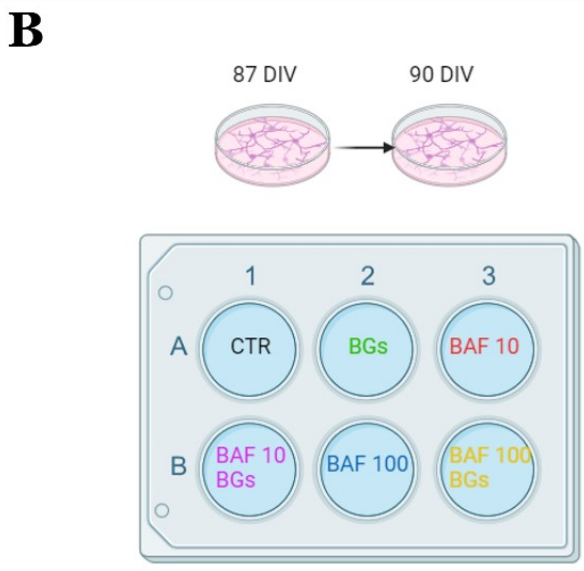


Figure 31: Experimental design about treatments of IPSC-derivate human cortical neurons. A) Acute treatment (24 hours) of BGs, Bafilomycin A1 100 nm and the combination of both, B) 3-day treatment of BGs, Bafilomycin A1 10 nm Bafilomycin A1 100 nm and the combination between BGs and each concentration of Bafilomycin A1. CTR (in black) = Control, i.e. no treatment. BGs (in green) = 8 mg/L of BGs. BAF 10 (red) = 10 nm of Bafilomycin A1. BAF 100 (blue) = 100 nm of Bafilomycin A1. BAF 10 + BGs (pink) = 10 nm of Bafilomycin A1 and 8 mg/L of BGs. BAF 100 + BGs (yellow) = 100 nm of Bafilomycin A1 and 8 mg/L of BGs

3.9.3 Immunofluorescence iPSC-derived human neurons

iPSC-derived human neurons DIV 90 were fixed with 2% PFA/1% PBS for 15 minutes at RT, after which the PFA was aspirated and replaced with fresh 1% PBS. Then, neurons were washed 3 times in 1X PBS after fixation and permeabilized and blocked in 10% Goat Serum/0.5% Triton-X in PBS and incubated at room temperature for 1 hour. The blocking buffer was aspirated and replaced with a primary antibody solution (Tab. 3) containing 10% Goat Serum in PBS and allowed to incubate ON at 4°C. The primary antibody solution was aspirated, the samples were washed 3 times in 1X PBS, 10 minutes per wash, and then allowed to incubate for 1 hour at room temperature in a secondary antibody solution (diliuted 1:1000) containing 0% Goat Serum in 1% PBS. Secondary antibody solution was aspirated, samples were washed 3 times in 1% PBS, 10 minutes per wash, and during the last wash DAPI (10 µg/ml; 10236276001, Sigma Aldrich) was included for 10 minutes after being washed in 1% PBS for 10 minutes. Samples were mounted with 3-4 drops of AquaPolymount (18606, Polysciences Inc.) and allowed to cure 72 hours before confocal acquisition.

Antibody Primary	Marker of	Working Solution	Product type	Code
Tubulin βIII	Neurons	1:10000	Mouse Monoclonal	801201, Biolegend
LC3B	Autophagy	1:500	Rabbit Polyclonal	ab48394, Abcam

Table 3: Antibodies used in iPSC-derived neurons

3.10 Microscopy

Histological images were acquired in bright field by Nixon optical microscope (Eclipse E400). Slides were acquired using a Zeiss Apotome.2 with Axiovision microscope equipped with Axiocam 807 mono. camera (fluorescence), a camera (Bright field) and Apotome slide. Subsequently, the images were processed using the software suite Zen Blue. Two distinct magnifications (objectives 40x and 63x, both oil immersion) were used to acquire z-stacks of multiple focal planes at 0.5 um each. Three areas were acquired for three sections for each

sample. Cultured brain slides were acquired using a confocal Zeiss LSM 900 with Airyscan 2 at 63x oil immersion using modality Airyscan. Z-stacks consisting of 10 focal planes of 2.5 µm each. Z-stack images of cultured brain slices Tg(*mpeg1.1.1:*EGFP +) were acquired with a step size of 2 µm using a 63× objective (NA 1.28) on an Leica Stellaris 5 spanning a thickness of 20 µm. Z-stack images of cultured brain slices treated with plx5622 were acquired with a step size of 2.5 µm using a 63× objective (NA 1.28) on a Leica Stellaris 5 spanning a thickness of 25 µm. Six areas were acquired for three/five (depending on the experiment) cultured brain slices. IPSC-derived human neurons were acquired with 10 stacks a 0.5 µm pitch using a 63 objective (NA 1.28) on a Leica Stellaris 5. 3 wells (multi-well 24) were quantified for each group, 10 images were acquired for each well.

3.11 Quantification of each phenotype

Images were quantified using Fiji software. The fluorescence area percent was the parameter used to compare the 3 groups (Control, 12.5 mg/Kg BW and 125 mg/Kg BW) for chronic diet and 2 groups (Control and 125 mg/Kg BW) for acute diet. For the quantification of each individual phenotype, 3 images per section were used for 3 sections (9 images total) for each sample.

3.11.1 Histology quantification

3.11.1.1 Hepatocyte vacuolization

Hepatocellular vacuolation would be considered as lipidosis or steatosis. Cytoplasmic inclusions of lipid are found in hepatocytes. Fish tend to accumulate more glycogen/lipids, in fact have larger vacuoles than mammals. The high hepatocellular vacuolation in *Nfu* is a natural phenomenon (De Cicco et al., 2009; Godoy, R. S. *et al.* 2019). Histologically we observe cytoplasmic inclusions of lipid in hepatocytes, as shown in Figure 32. In agreement with De Cicco, I semi-quantitatively measured hepatocytes vacuolization by defining 4 different degrees, as described in the Figure 31 (De Cicco et al., 2009).

Degree	Definition
0- Normal	Normal apparence of Liver
1- Mild	Cellular swelling and accumulation of lipids, resulting in localized or microvesicular fat accumulation
2- Moderate	Localized areas of cellular swelling and medium-sized intracytoplasmic vacuoles;

3- Severe	Widespread and severe swelling of the macrovacuolated or swollen hepatocytes;

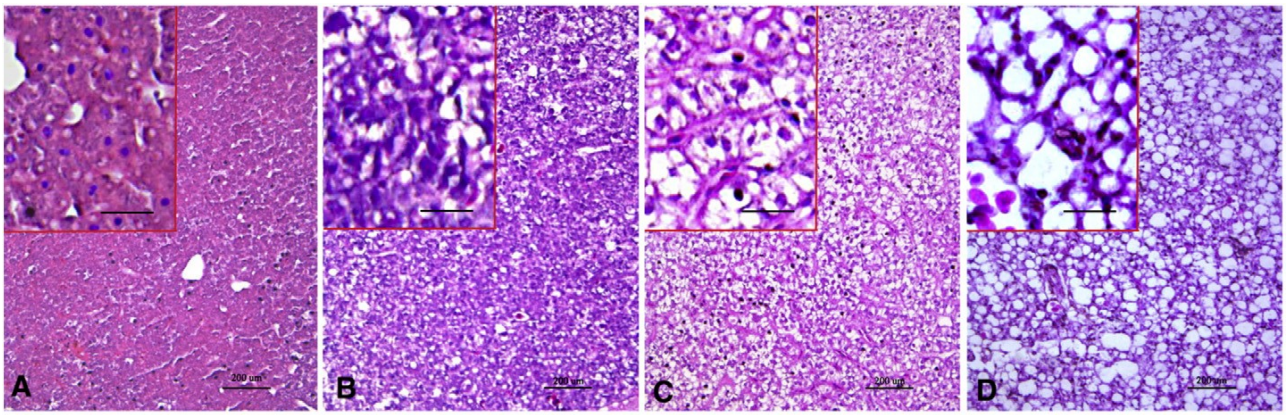


Figure 32: Representative images of different degrees of hepatocytes vacuolization, from De Cicco et al., 2009. A. Normal appearance of Liver, 0 degree. B. Mild, or 1, degree. C. Moderate, or 2, degree. D. Severe, or 3, degree

In addition, I classified the vacuoles, into glycogen-like or lipid vacuoles, as Figure 33 shows. The lipid type manifested itself as unstained cytoplasmic vesicles with sharp edges. The glycogen-like type was characterized by irregular vacuoles containing slightly flocculent material (Zac, et al., 2020).

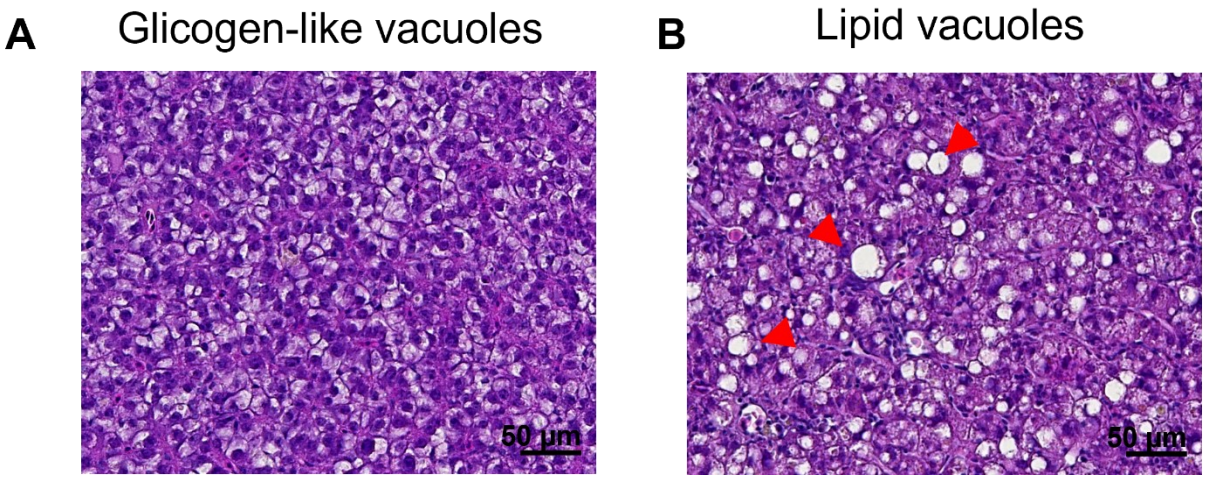


Figure 33: Representative image of the types of vacuoles in the liver. A. Glycogen-like type. B. Lipid type, the red arrows indicate some lipid vacuoles

3.11.1.2 Histology of Kidney

Dilation of the renal tubules is a typical condition of aging. I measured the diameter of each individual renal tubule (Fig. 33) using 3 images/section by 3 sections. Moreover, I calculated the presence of precipitate in renal tubules and necrotic tubules. As the figure shows, the

renal tubules show internal material (red arrow). Necrotic renal tubules show sloughing of tubular cells into the lumen and severe tubular leakage. I measured the increase in thickness of Bowman's capsule relative to the area of the glomerulus. The renal corpuscle is formed by a centrally located glomerulus surrounded peripherally by a Bowman's capsule. To quantify the increasement of in thickness of Bowman's capsule I calculated the ratio between the thickness of Bowman's capsule (pixels²) compared to the total area (pixels²) of the renal corpuscle (Bowman's capsule and Glomerulus), in Figure 34.

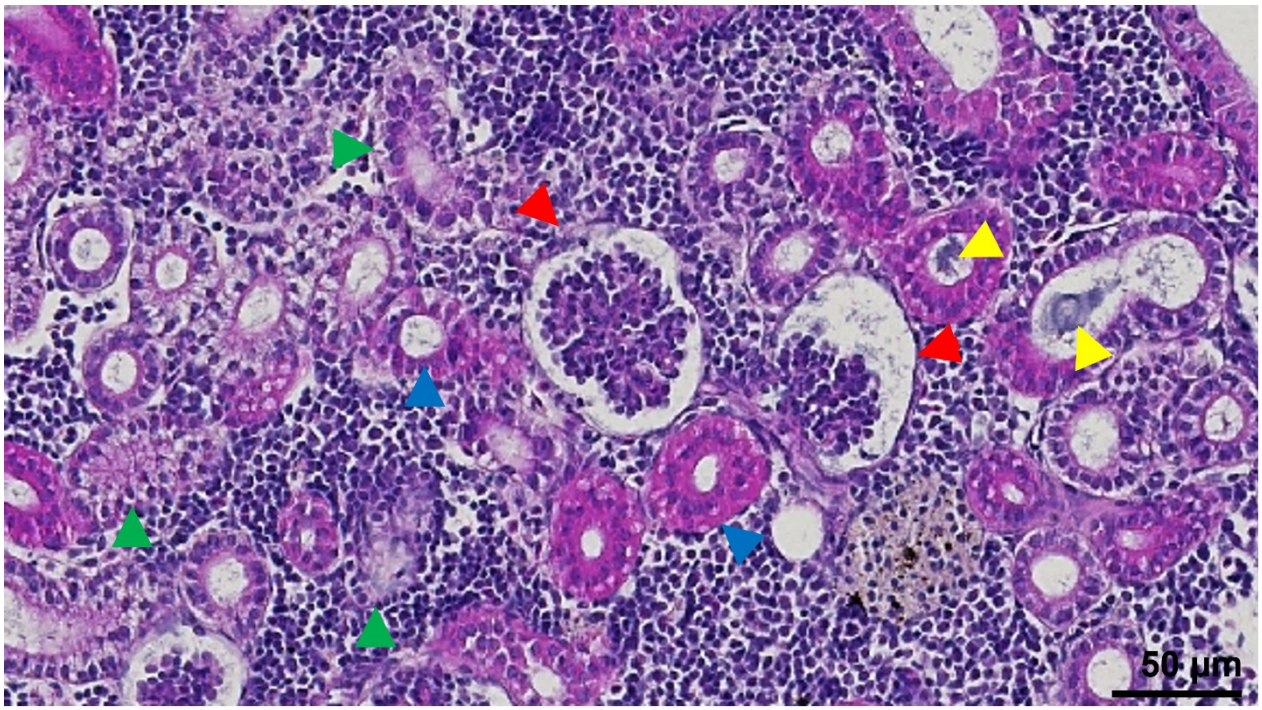


Figure 34: Representative image of kidney histology. Red arrows show glomeruli; Bowman's capsule appears as a white cup-like sack surrounding the glomerulus. The blue arrows indicate the renal tubules. The green arrows are degenerated renal tubules with tubular loss. Yellow arrows indicate renal tubules with precipitates

3.11.1.3 Feret's size

HE-stained muscle fibers were individually measured via Feret'size from imageJ. Feret'size measure diameters derived from the distance of two tangents to the fiber boundary in a well-defined orientation. All fibers from 3 images for 3 sections for each animal were analyzed.

3.11.1.4 Fibrosis

Collagen deposition was used to measure the fibrosis in the muscle. Collagen deposition was quantified analysing the percentage area of the magenta fibers (collagen). The percentage area of the image was measured by setting the same threshold for all images.

3.11.2 Immunofluorescence quantification

The fluorescence area of each phenotype was quantified measuring the area of fluorescence respect to the total area (pixels²) for each image (percentage area). The fluorescence area of

the image was measured by setting the same threshold for all images. We used threshold for each phenotype, specially:

- Nitrotyrosine: 150-255 for cardiac sections and 100-255 for hepatic sections;

-GFAP (Gliosis): 75-255 in the brain;

- LC3: 110-255 for liver, 120-255 for brain and 100-255 for muscle;

-Lipofuscin: 90-255 for the hepatic sections, 85-255 for the brain, 100-255 for cardiac sections.

-Lysosomal activity was analysed calculating the ratio of Area of aggregates respect to Area of lysosomes. To quantify the area of aggregates and lysosomes, or LAMP1, I set threshold, specific:

LAMP1: 100-255, Aggressom: 90-255

-The inflammation was quantified counting manually the $LCP1^+$ cells respect the total cells. Also in cultured brain slides the depletion of microglia by PLX5622 was quantified counting manually the $LCP1^+$ cells respect the total cells using 3D images.

-Measuring LC3, Lysotracker, Mitotracker and CYTO-ID in 3D images of cultured brain slices:

To quantify the LC3, Lysotracker and Mitotracker pucta in 3D images system, Fiji's 3D Object Counter was used, as described by Elbaly, 2021. In detail, the number of Objects (LC3, lysosomes and mitochondria), Volume of Objects, Percentage area of Objects (normalizing with cells), Integrity Density of Objects. The z-stacks of acquisitions (JPG) are open in Fiji, convert them to 8 bit. After selecting the 3D object counter, the threshold was adjusted and the size filter to 1 pixel. Threshold applied, Lc3: 25-255. Lysotracker: 50-255, Mitotracker: 30-255, CYTO-ID: 20-255. In all samples, the threshold and size filter values were the same.

-Measuring of sphericity of mpeg+ cells in 3D images of cultured brain slices:

Microglia sphericity were quantified using Imaris software (Bitplane) by creating 3D surface reconstructions of *mpeg1.1:*$EGFP^+$. All images were set to a standard threshold to accurately maintain morphology for quantifications.

-Measuring of survival and LC3 in iPSC-derived human neurons:

3 wells (multi-well 24) were quantified for each group, 10 images were acquired for each well. For each image, 6 stacks spaced by 0.5 µm were considered to quantify neuronal survival and LC3. For each image the number of neuronal nuclei and the total number of nuclei (including progenitor cells) were counted. Neuronal survival was determined by calculating the percentage of the ratio between the number of nuclei of neurons compared to the number of total nuclei. The quantification of LC3 was performed via Imaris software (Bitplane), calculating the number of objects of LC3 on the surface (µm2) of neurons, i.e. β-III Tubulin surface. β-III Tubulin surface (threshold, absolute intensity: 11-60) was created and measured by "Surface" tool, then mask was applied to the LC3 canal. At this point, the objects of LC3 within the surface of β-III Tubulin using "Spot" tool were selected by determining the threshold (2.5-60).

3.12 Proteomics analysis of cultured brain slices

3.12.1 Preparation of cultured brain slices for proteomics analyses

After 3 days of cultivation the cultured brain slices were collected and twice for one minute in fresh 1% PBS with 1% phosphatase and protease inhibitors. Subsequently, they were weighed in the precision balance. The cultured brain slices were collected and snap-frozen in liquid nitrogen and stored at -80 ° C.

3.12.1.1 Sample preparation for proteomics analysis

Tissues (cultured brain sliced) were resuspended in PBS to a concentration of 200 µg/µl cells before adding 2x lysis buffer (final concentration of 5% SDS, 100 mM HEPES and 50 mM DTT). The samples were sonicated (Bioruptor Plus, Diagenode, Belgium) for 10 cycles (30 sec ON/60 sec OFF) at a high setting at 20°C, followed by boiling at 95°C for 5 min. Reduction was followed by alkylation with iodoacetamide (final concentration 15 mM) for 30 min at room temperature in the dark. Samples were acidified with phosphoric acid (final concentration 2.5%), and seven times the sample volume of S-trap binding buffer was added (100 mM TEAB, 90% methanol). Samples were bound on S-trap micro spin columns (Protifi) and washed three times with binding buffer. Trypsin in 50 mM TEAB pH 7.55 was added to the samples (1 µg per sample) and incubated for 1 h at 47°C. The samples were eluted in three steps with 50 mM TEAB pH 7.55, elution buffer 1 (0.2% formic acid in water) and elution buffer 2 (50% acetonitrile and 0.2% formic acid). The eluates were dried using a speed vacuum centrifuge (Eppendorf Concentrator Plus, Eppendorf AG, Germany). The samples were resuspended in Evosep buffer A (0.1% formic acid in water) and sonicated (Bioruptor Plus, Diagenode, Belgium) for 3 cycles (60 sec ON/30 sec OFF) at a high setting at 20°C. The samples were loaded on Evotips (Evosep) according to the manufacturer's instructions. In short, Evotips were first washed with Evosep buffer B (acetonitrile, 0.1% formic acid), conditioned with 100% isopropanol and equilibrated with Evosep buffer A.

Afterwards, the samples were loaded on the Evotips and washed with Evosep buffer A. The loaded Evotips were topped up with buffer A and stored until the measurement.

3.12.1.2 LC-MS Data independent analysis (DIA)

Peptides were separated using the Evosep One system (Evosep, Odense, Denmark) equipped with a 15 cm x 150 µm i.d. packed with a 1.9 µm Reprosil-Pur C18 bead column (Evosep Endurance, EV-1106, PepSep, Marslev, Denmark). The samples were run with a pre-programmed proprietary Evosep gradient of 44 min (30 samples per day) using water and 0.1% formic acid and solvent B acetonitrile and 0.1% formic acid as solvents. The LC was coupled to an Orbitrap Exploris 480 (Thermo Fisher Scientific, Bremen, Germany) using PepSep Sprayers and a Proxeon nanospray source. The peptides were introduced into the mass spectrometer via a PepSep Emitter 360-µm outer diameter × 20-µm inner diameter, heated at 300°C, and a spray voltage of 2.2 kV was applied. The injection capillary temperature was set at 300°C. The radio frequency ion funnel was set to 30%. For DIA data acquisition, full scan mass spectrometry (MS) spectra with a mass range of 350–1650 m/z were acquired in profile mode in the Orbitrap with a resolution of 120,000 FWHM. The default charge state was set to 2+, and the filling time was set at a maximum of 60 ms with a limitation of 3×10^6 ions. DIA scans were acquired with 40 mass window segments of differing widths across the MS1 mass range. Higher collisional dissociation fragmentation (stepped normalized collision energy; 25, 27.5, and 30%) was applied, and MS/MS spectra were acquired with a resolution of 30,000 FWHM with a fixed first mass of 200 m/z after accumulation of 1×10^6 ions or after filling time of 45 ms (whichever occurred first). Data were acquired in profile mode. For data acquisition and processing of the raw data, Xcalibur 4.4 (Thermo) and Tune version 3.1 were used.

3.12.1.3 Proteomic data processing

DIA raw data were analysed using the directDIA pipeline in Spectronaut (v.16.2, Biognosysis, Switzerland). The data were searched against an in-house species-specific database (*Nothobranchius furzeri* 59.154 entries) and contaminant (247 entries) SwissProt database. The data were searched with the following variable modifications: oxidation (M) and acetyl (protein N-term). A maximum of 2 missed cleavages for trypsin and 5 variable modifications were allowed. The identifications were filtered to satisfy an FDR of 1% at the peptide and protein levels. Relative quantification was performed in Spectronaut for each paired comparison using the replicate samples from each condition. The data were exported, and further data analyses and visualization were performed with R studio using in-house pipelines and scripts.

3.12.2 Data Analysis of proteomics data

3.12.2.1 Identification of DEPs

DEPs were identified with |logFC| >0 and P value <0.05. The DEPs with logFC <0 or logFC >0 were considered downregulated or upregulated genes, respectively. DEPs common among datasets were screened using the Venn diagram software (Venny 2.0; https://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html).

3.12.2.2 Protein-protein interaction network construction and module analysis

Protein-protein interaction (PPI) information was obtained through an online STRING analysis (http://string-db.org). Proteins were clustered using "KMEANS" clustering algorithm. the KMEANS algorithm finds a defined number of clusters based on their centroids. The clusters were identified based on the Cellular Components.

3.12.2.3 Over Representation Analysis (ORA)

The Webgestalt (http://webgestalt.org/) was used to perform ORA of the identified DEPs, associated with biological processes. This website uses affinity propagation to group together similar gene sets; identifies the most enriched GO categories in each dataset and provides the p-value of the FDR-corrected enrichment. Categories from the GO database (Ashburner et al., 2000; Carbon et al., 2017) containing at least five genes were considered, and p values were corrected for multiple comparisons via the FDR method described in (Benjamini and Hochberg, 1995). Biological processes with FDR <0.10 were selected as significant.

3.12.2.4 Principal component analysis (PCA) and heatmap analysis

PCA and heatmap representation of their hierarchical clustering were performed using the ComplexHeatmap 2.0.0, pcaMethods 1.76.0, and ggpubr 0.2 packages of R.

3.13 Statistics Analysis

GraphPad Prism 8 program (GraphPad, San Diego, CA, USA) and ImageJ software were used to perform statistical analysis. All the data were expressed as Mean ± standard error of the mean (S.E.M). Data regarding survival curves were subjected to the log-rank test. Data regarding percentage of vacuole's type were calculated with Chi^2 test. Some data were analysed by One-way ANOVA trend analysis test. Other data were analysed by Two-way ANOVA test with Tukey's multiple comparison test to compare the different groups. Other data were analysed by Student's t-test calculated adjusted p-value with FDR. Differences between treatments were considered significant for $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.00001$ (****).

4 RESULTS

4.1 Chapter 1 Chronic effects of diet fortified with BGs in aging

4.1.1 Effects of BGs on survival

Nothobranchius *furzeri (Nfu)* were fed with dry food fortified with 1.3, 1.6 B-glucans (BGs) at two different concentrations: 12,5 mg/Kg body weight (n=26), 125 mg/Kg body weight (n=28) from 2 wph (weeks post hatching) until 27 wph. Fish fed with dry food (n=25) served as control. All surviving fish were sacrificed at week 27 (n =10, n= 9, and n =10 in the three groups). Survival analysis did not detect an effect of BGs on mortality, as Figure 35 shows (Log-rank, $\chi 2$=0,2286).

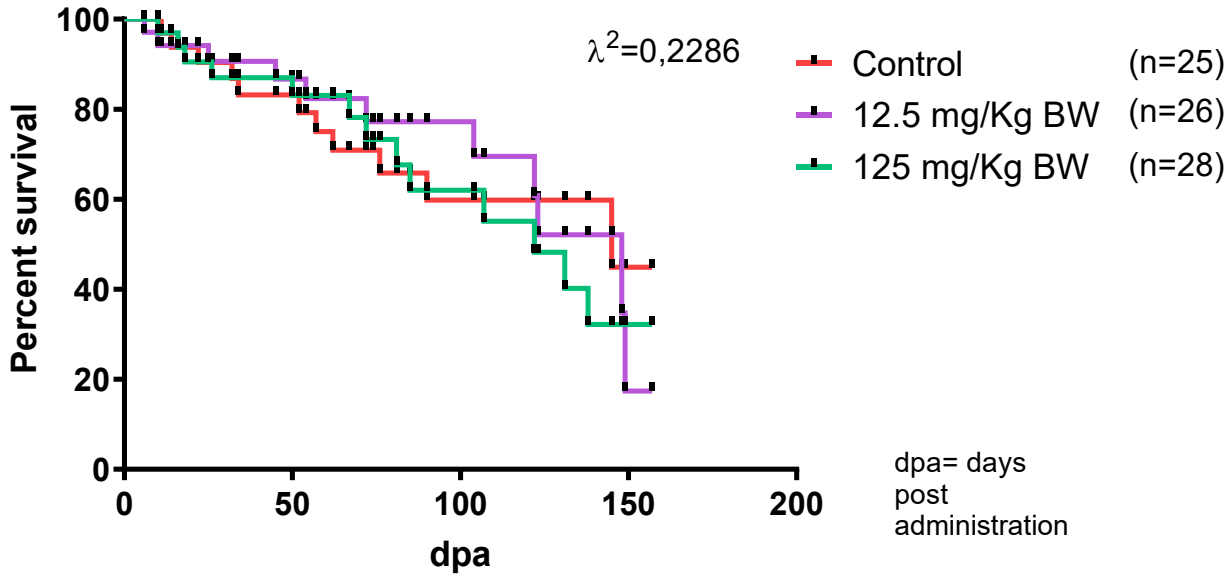


Figure 35. Survival curve of control and treated *Nfu*

Final body weight was influenced by sex, but not treatment, as Figure 36 shows (p<0.0001, Two-way ANOVA, p=0.4522, One-Way ANOVA).

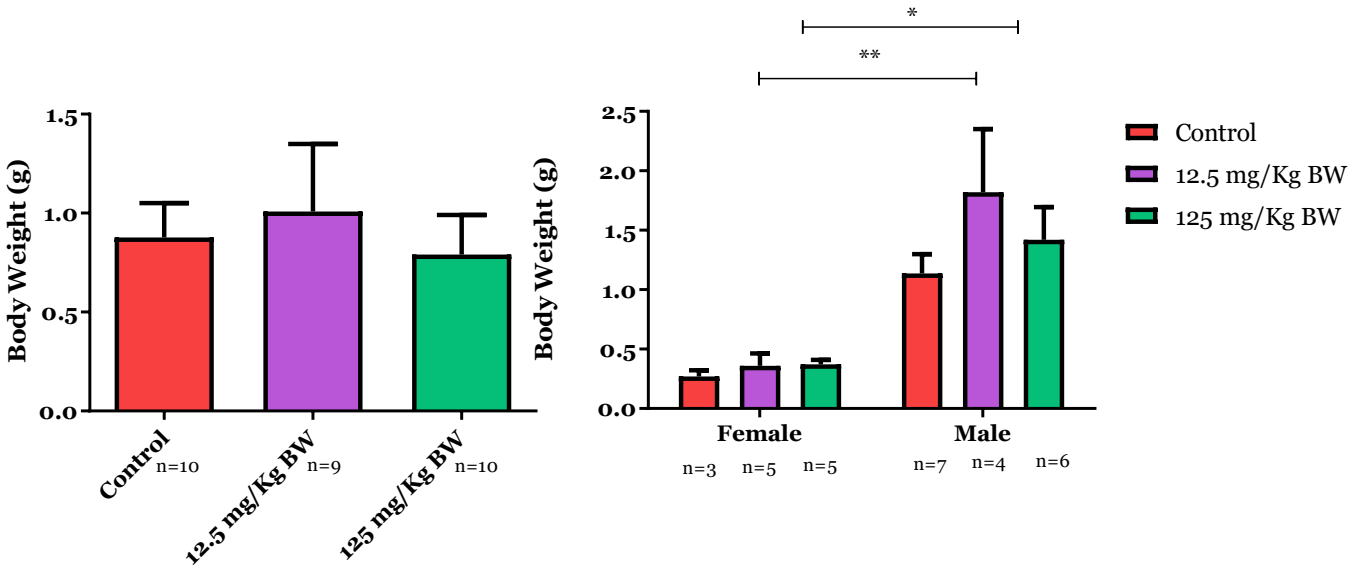


Figure 36. Effects of BGs in body weight. a. Body weight final; b. Body weight final of female and male

4.1.2 Effects of BGs on aging phenotypes

I analysed histological and immunohistochemical aging markers in liver, brain, muscle, and heart for a total of 19 different traits. These markers relate to the mechanism of autophagy, inflammation, and peroxidation of proteins. I addition, I analysed age-related histopathological lesions of kidney and liver (Tab. 4).

Aging Phenotypes	Organ	pvalue	FDR
Inflammation	Brain	0.0001	0.00027
Peroxidation protein	Liver	0.0001	0.00032
Inflammation	Gut	0.0001	0.00038
Lipofuscin	Brain	0.0001	0.00048
Autophagy	Liver	0.0001	0.00063
Lipofuscin	Liver	0.0003	0.00071
Autophagy	Brain	0.0004	0.00084
Peroxidation protein	Heart	0.0001	0.00095
Gliosis	Brain	0.0008	0.00152
Lysosomal activity	Liver	0.0001	0.0019
Fibrosis	Muscle	0.0089	0.01537
Feret's size	Muscle	0.0141	0.02233
Lipofuscin	Heart	0.0224	0.03274
Precipitation renal tubules	Kidney	0.0519	0.06574
Area renal tubules	Kidney	0.0518	0.07030
Necrotic renal tubules	Kidney	0.4116	0.48878
Hepatocytes vacuolization	Liiver	0.5544	0.61962
Bowman's capsule enlarged	Kidney	0.7818	0.82523
Autophagy	Muscle	0.9395	0.93950

Table 4. Aging phenotypes

In order to evaluate the global effect of BGs on aging phenotypes, the dimensionality of the data was reduced by principal component analysis (PCA), as Figure 37 shows. The PC1 axis (32.5% of variance explained) perfectly separates control-samples and samples treated with the higher concentration of BGs, with samples treated with lower concentration of BGs occupying an intermediate position. This result proves a positive dose-dependent preventive effect of BGs on the global aging process of *Nfu*.

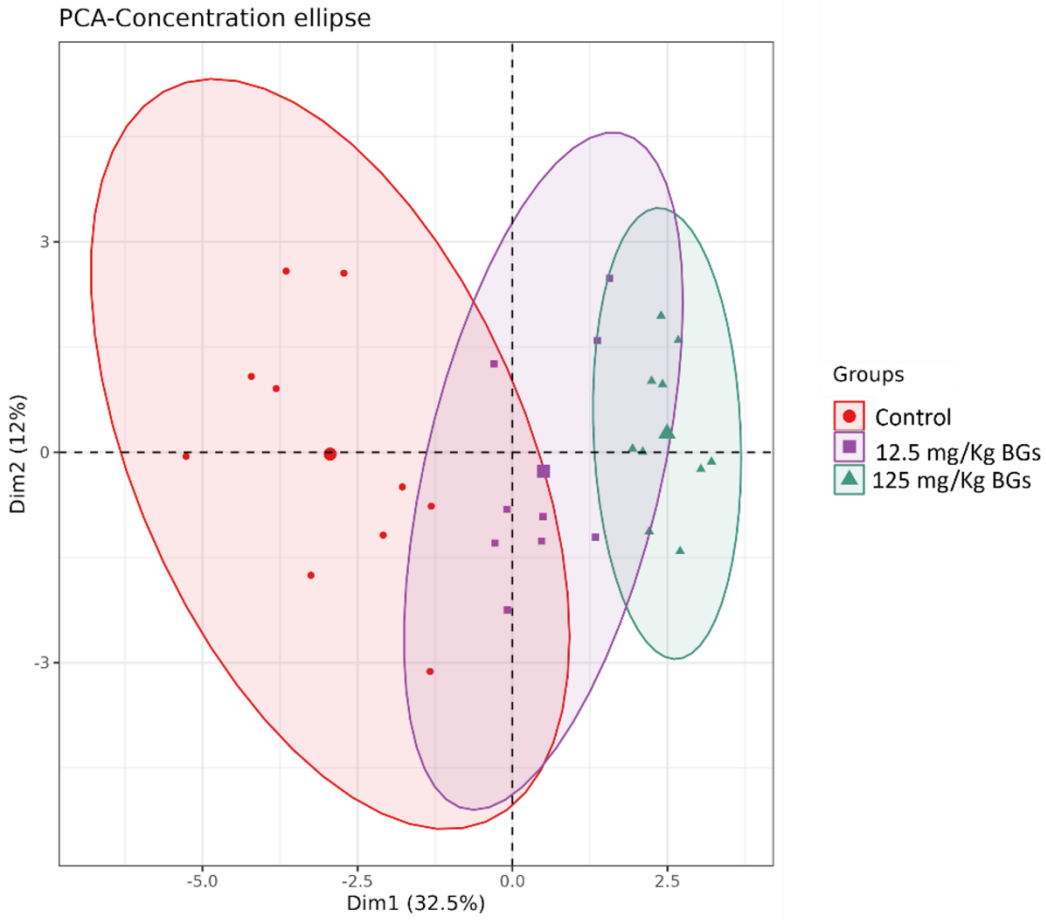


Figure 37. Principal components analysis (PCA) of effect of BGs phenotypes

A significant effect of treatment was detected for twelve the traits (FDR<0.05 two-way ANOVA). Table 4 and these traits are discussed singularly below. To visualize the traits that mostly contributed to the sample separation, I used a Biplot visualization (Fig. 38) where the arrows indicate the direction a datapoint would move by increasing only the variable of interest. Longer arrows aligned with PC1 correspond to the traits that are mostly responsible for the effects of BGs on aging. BGs act specifically on mechanism related to autophagy (i.e. LC3 Brain and Liver), peroxidation of proteins (i.e. Nitrotyrosine Heart and Liver, Lipofuscin in Brain and Liver) and inflammation (i.e. LCP1 Brain).

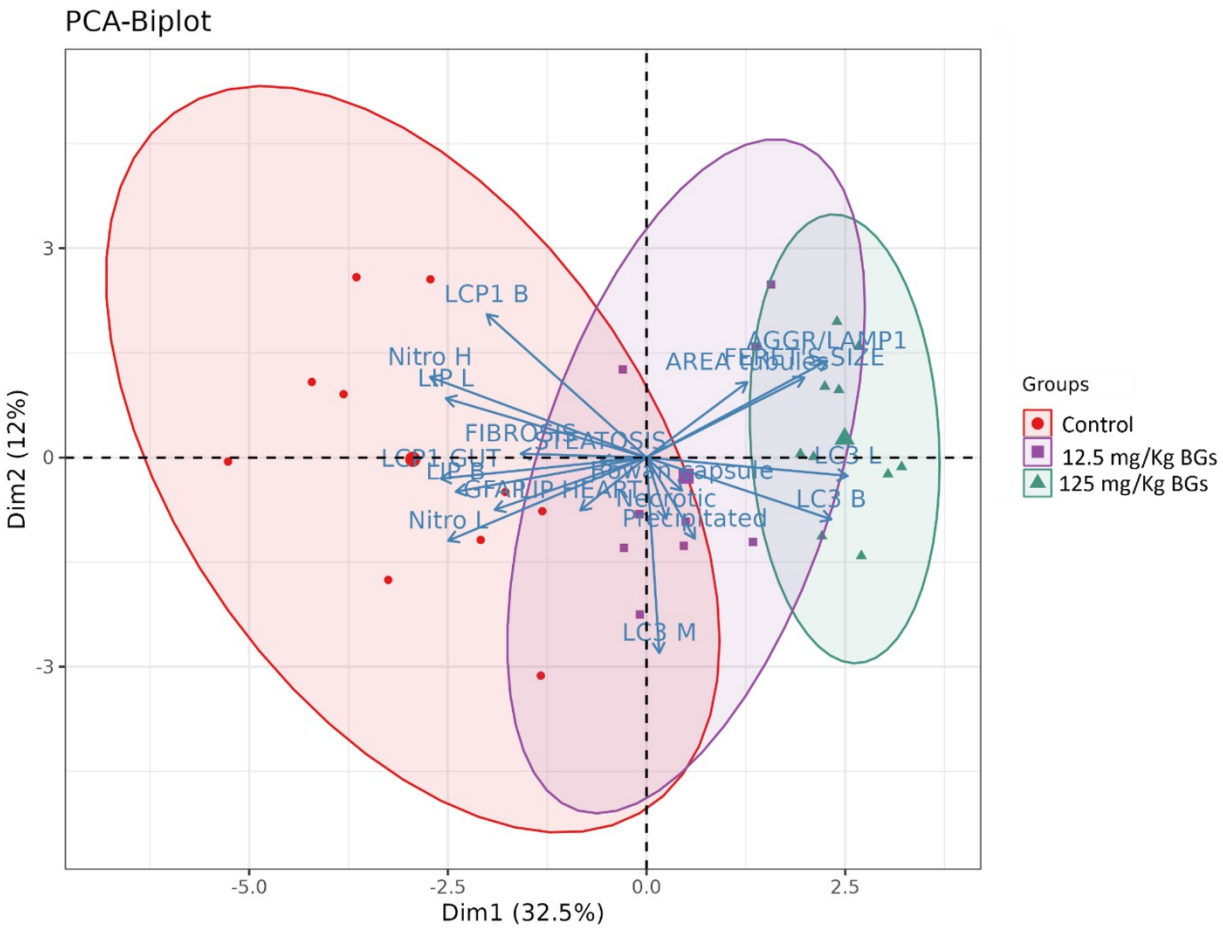


Figure 38: PCA biplot of singular phenotypes. LIP L=Lipofuscin Liver, LIP B=Lipofuscin Brain, LC3 L= Autophagy (LC3) Liver, LC3 B= Autophagy (LC3) Brain, LC3 M= Autophagy (LC3) Muscle, NITRO L=Peroxidation of proteins (Nitrotyrosine) Liver, NITRO H=Peroxidation of proteins (Nitrotyrosine) Heart, GFAP=Gliosis, AGGR/LAMP1=Lysosomal activity (Aggressom/Lysosomes) Liver, LCP1 B=Inflammation (LCP1) Brain, LCP1 G=Inflammation (LCP1) Gut, FIBROSIS M=Fibrosis Muscle, FERET'S SIZE M=Feret's size Muscle, AREA TUBULES K=Area tubules of Kidney, BOWAN CAPSULE K=Bowan's capsule engagement, PRECIPITATED K=Percentage of renal tubules with precipitated, NECROTIC K= Percentage of necrotic renal tubules

To unravel covariation between traits, I analysed the Sperman's correlation between phenotypes visualized as Heatmap (Fig. 39). Below I mention significant correlations that make biological sense. Lipofuscin and autophagy are negatively correlated in the brain, suggesting that induction of autophagy mitigates lipofuscin accumulation in the brain. Autophagy is positively correlated in brain and liver, implying that BGs increase autophagy in a coordinated fashion in multiple organs. In the brain, lipofuscin is correlated positively with gliosis and inflammation, [so an increase of lipofuscin during aging can induce reactivity of glia cells, as reported in aged rat (Hilbig et al., 2022)] and negatively with inflammation. In fact, the autophagic pathway can prevent tissue inflammation (Levine et al., 2011). Also, I observed that lysosomal activity (visualized as colocalization of Aggresome

and LAMP1) is negatively correlated with accumulation of lipofuscin and peroxidation of proteins that are in turn positively correlated, so as one increases/decreases the other phenotype increases/decreases. In the liver, autophagy and lysosomal activity are positively correlated as expected by the key involvement of lysosomal degradation in the autophagic mechanism (Yim and Mizushima, 2020). The decrease in lysosomal or autophagy activity leads to an increase in aggregates, such as lipofuscin. The Figure 39 shows in detail all correlation between phenotypes.

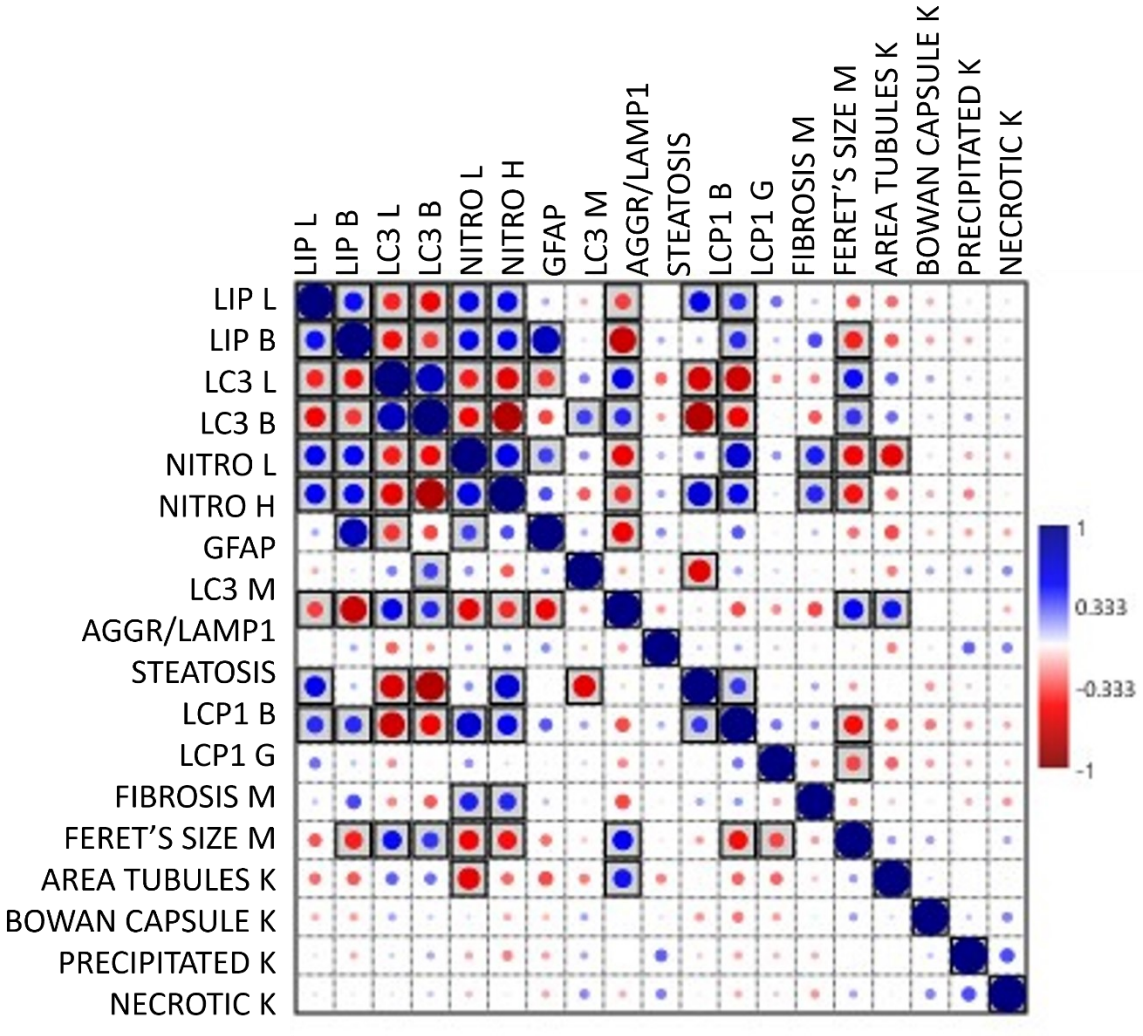


Figure 39: Heatmap between phenotypes. The positive correlation is marked with blue colour, for contrary the negative correlation is in red. And correlations outlined in black are statistically significant ($p<0.05$). LIP L=Lipofuscin Liver, LIP B=Lipofuscin Brain, LC3 L= Autophagy (LC3) Liver, LC3 B= Autophagy (LC3) Brain, LC3 M= Autophagy (LC3) Muscle, NITRO L=Peroxidation of proteins (Nitrotyrosine) Liver, NITRO H=Peroxidation of proteins (Nitrotyrosine) Heart, GFAP=Gliosis, AGGR/LAMP1=Lysosomal activity

(Aggressom/Lysosomes) Liver, LCP1 B=Inflammation (LCP1) Brain, LCP1 G=Inflammation (LCP1) Gut, FIBROSIS M=Fibrosis Muscle, FERET'S SIZE M=Feret's size Muscle, AREA TUBULES K=Area tubules of Kidney, BOWAN CAPSULE K=Bowan's capsule engagement, PRECIPITATED K=Percentage of renal tubules with precipitated, NECROTIC K= Percentage of necrotic renal tubules

4.1.3 BGs slow down the accumulation of lipofuscin during aging

Lipofuscin is an autofluorescent pigment resulting from lipid peroxidation that accumulates with age within the lysosomes (Gray and Woulfe, 2005; Ilie et al., 2020). Age-dependent accumulation of lipofuscin is one of the most conserved markers of aging (Goyal, 1982; Brunk and Terman, 2002; Jung et al., 2007; Ng'oma et al., 2014; Pincus et al., 2016; Kushwana et al., 2018; Moreno-Garcìa et al., 2018; Imler et al., 2019; Sujana et al., 2020; Prasanna et al., 2022). In *Nfu*, Lipofuscin content is much higher in the liver, where it accumulates as large puncta in the shape of "rosettes", than in the brain, where it appears as smaller dots (Fig. 40A). Treatment with BGs slows down the accumulation of lipofuscin in the liver ($p=0.0003$, One-Way ANOVA), the brain ($p<0.0001$, One-Way ANOVA) and the heart ($p=0.0224$, One-Way ANOVA) in dose-dependent manner (Figs. 40B-C).

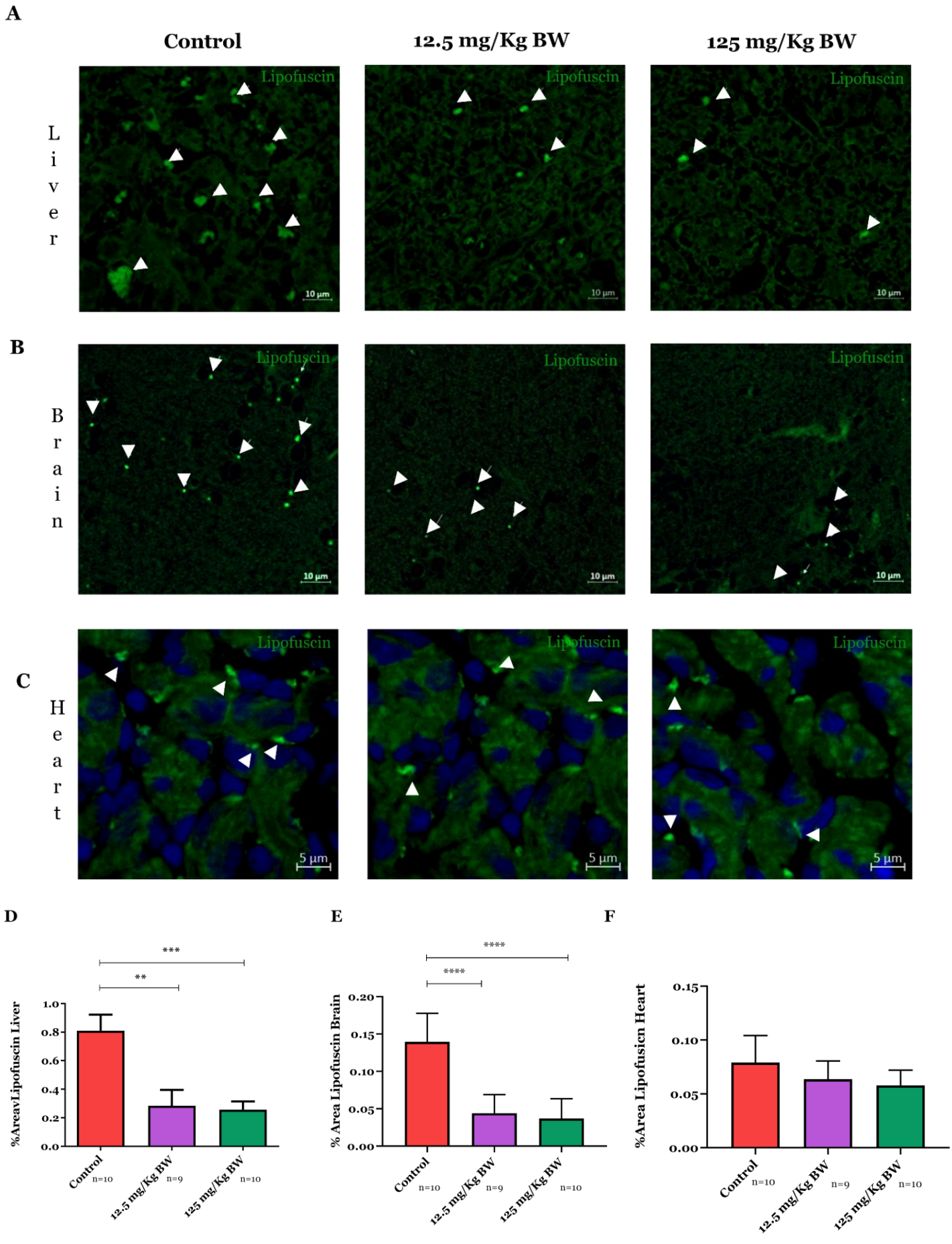
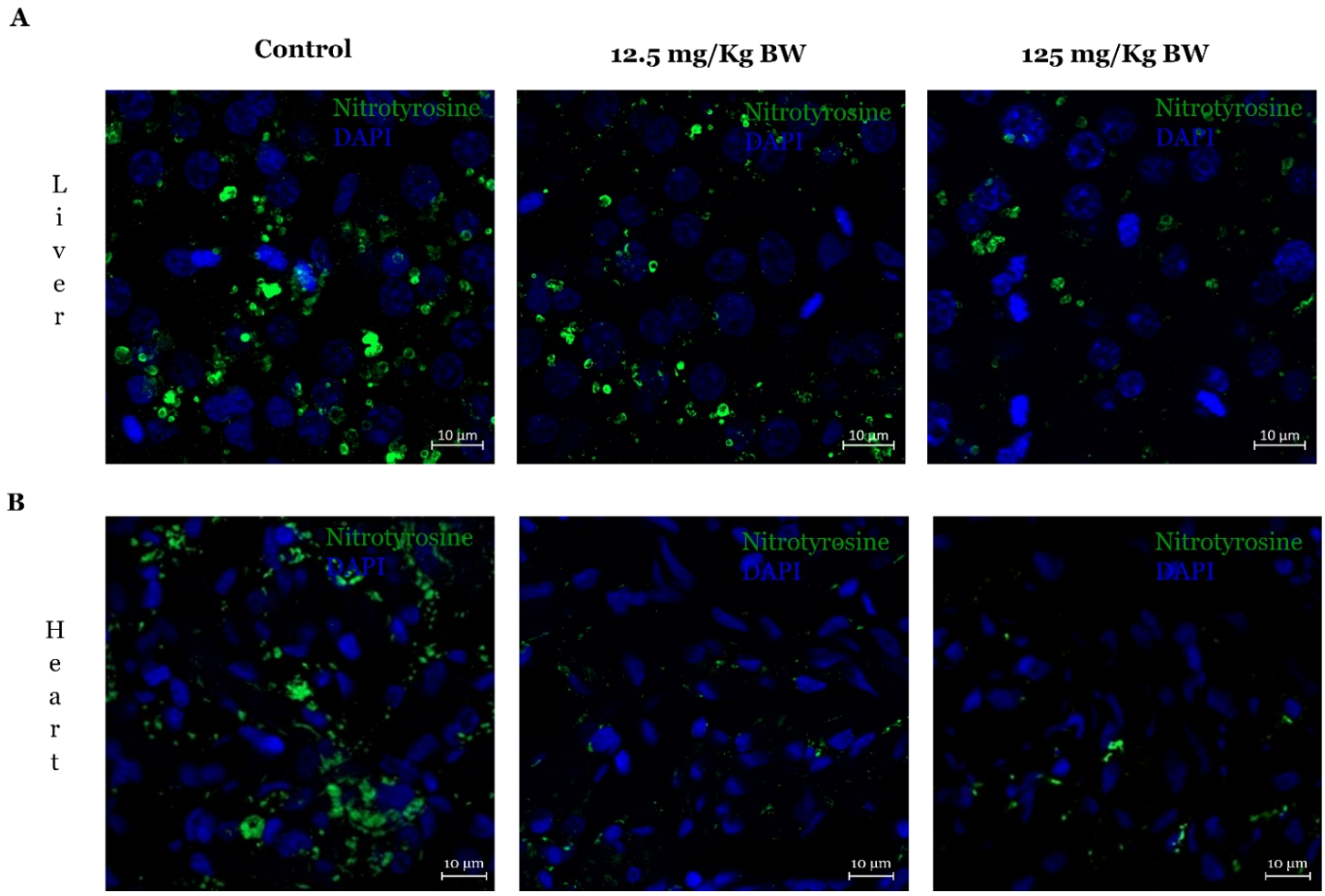

A
Control
12.5 mg/Kg BW
125 mg/Kg BW
Liver
Lipofuscin
10 µm
B
Brain
Lipofuscin
10 µm
C
Heart
Lipofuscin
5 µm
D
%AreavLipofuscin Liver
***
**
1.0
0.8
0.6
0.4
0.2
0.0
Control n=10
12.5 mg/Kg BW n=9
125 mg/Kg BW n=10
E
% Area Lipofuscin Brain
****
****
0.20
0.15
0.10
0.05
0.00
Control n=10
12.5 mg/Kg BW n=9
125 mg/Kg BW n=10
F
%Area Lipofusicn Heart
0.15
0.10
0.05
0.00
Control n=10
12.5 mg/Kg BW n=9
125 mg/Kg BW n=10

Figure 40: Effects of BGs on lipofuscin in A) hepatic, B) brain and C) cardiac sections derived from old *Nfu*. White arrows indicate lipofuscin aggregates. D)Quantification of lipofuscin in liver, E) Quantification of lipofuscin in brain, F) Quantification of lipofuscin in heart

4.1.4 BGs reduce protein oxidation during aging

Protein oxidative damage was analysed by means of immunofluorescence staining for Nitrotyrosine. Nitrotyrosine staining is apparent as green fluorescence puncta surrounding the nucleus in Figure 40. Nitrotyrosine straining was quantified in the liver (Figs. 41A-C) and heart (Figs. 41B-D). Both concentrations of BGs reduced Nitrotyrosine staining significantly in both organs ($p<0.0001$ and $p<0.0001$, respectively, One-Way ANOVA) in a dose-dependent manner.



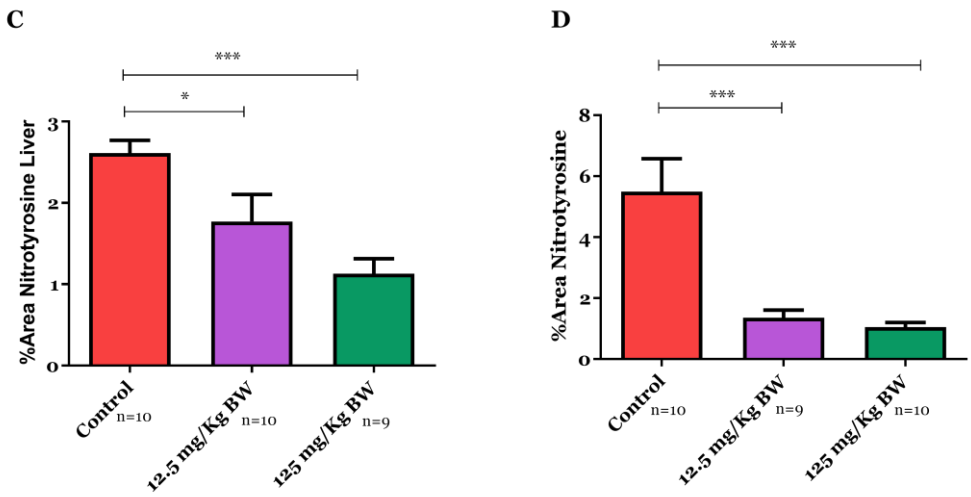
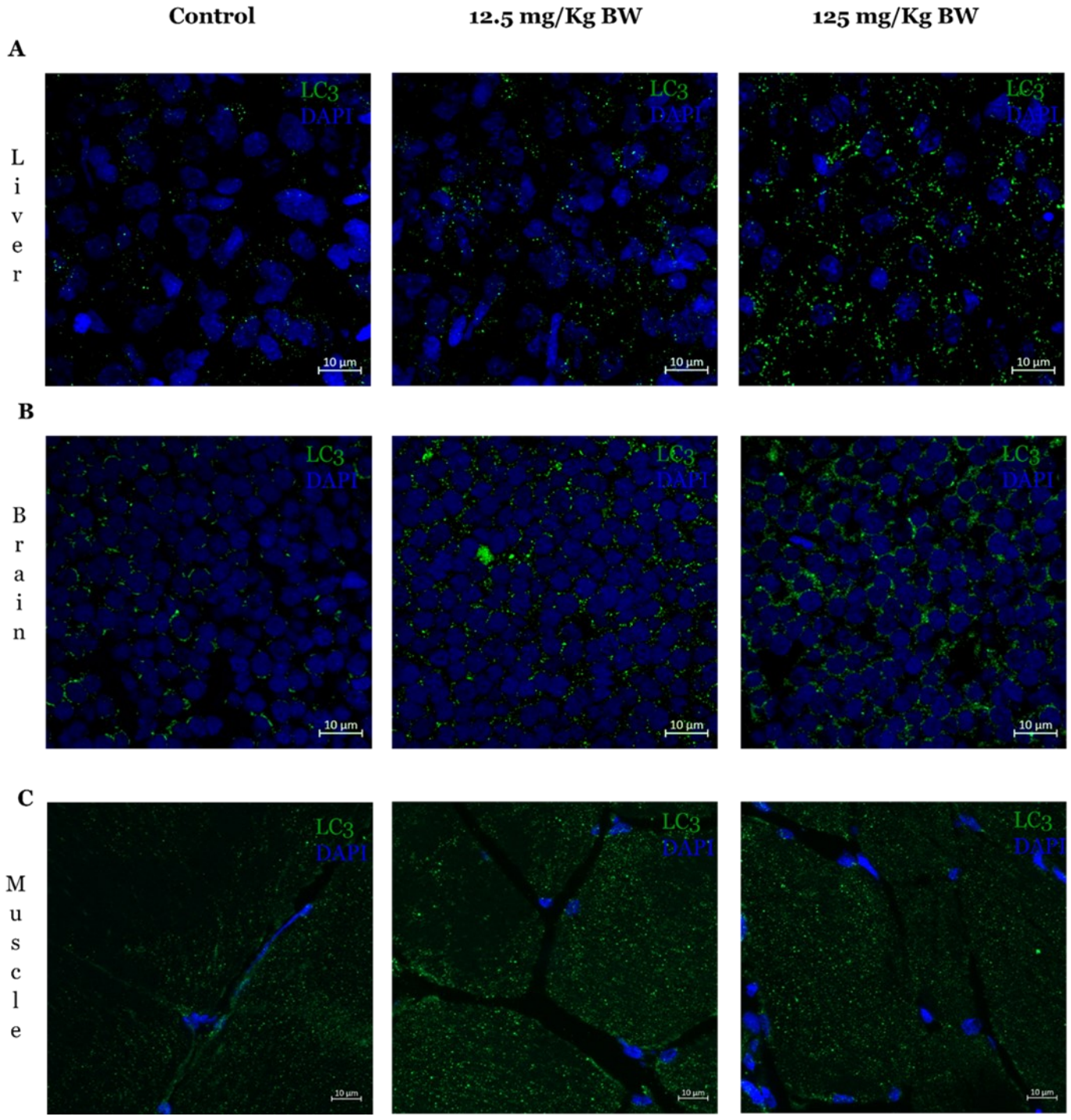


Figure 41: Effects of BGs on Peroxidation of proteins levels in A) hepatic and B) cardiac sections derived from old *Nfu,* C) Quantification of Peroxidation of proteins in liver, D) in heart

4.1.5 BGs induce autophagy during aging

Induction of autophagy by BGs was investigated by immunostaining of LC3, a protein localized to the inner and outer membranes of autophagosomes (Runwal et al., 2019). LC3 immunoreactivity is apparent as green fluorescent puncta surrounding the nucleus in Figure 42. Treatment with BGs at 125 mg/Kg BW induces a statistically significant increase of LC3 immunoreactivity in the liver ($p<0.0001$, One-Way ANOVA) and in the brain ($p=0.0004$, One-Way ANOVA) in dose-dependent manner, suggesting autophagy induction. For contrary, I didn't observe induction of autophagy by BGs in dose-dependent manner in muscle ($p=0.9395$, One-Way ANOVA).


Control
12.5 mg/Kg BW
125 mg/Kg BW
A
Liver
B
Brain
C
Muscle
LC3
DAPI
10 µm

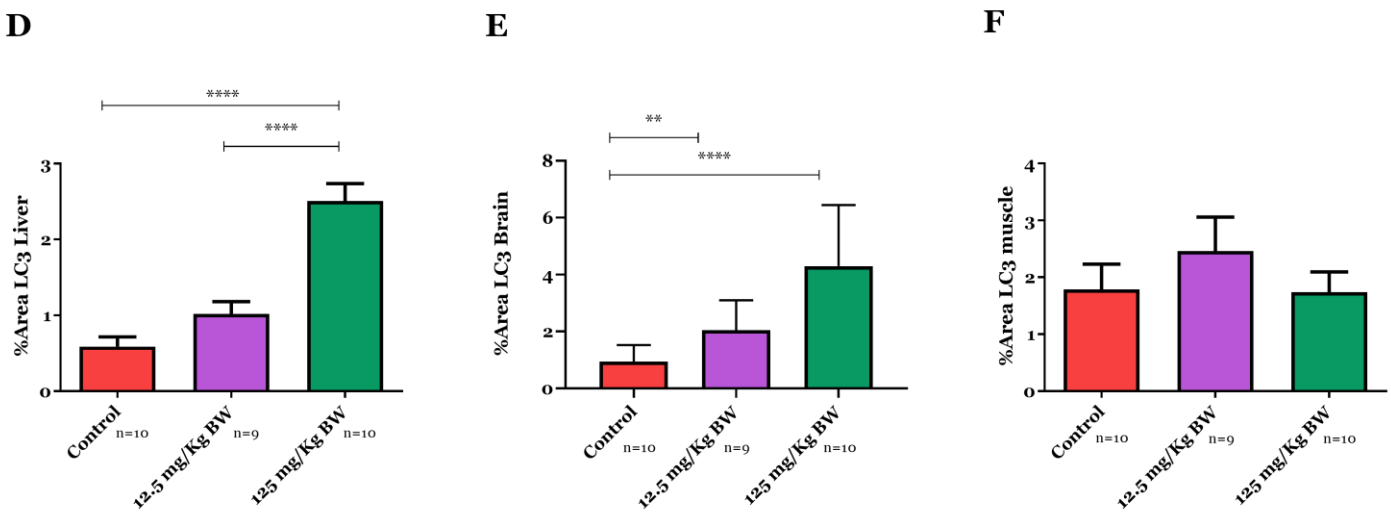
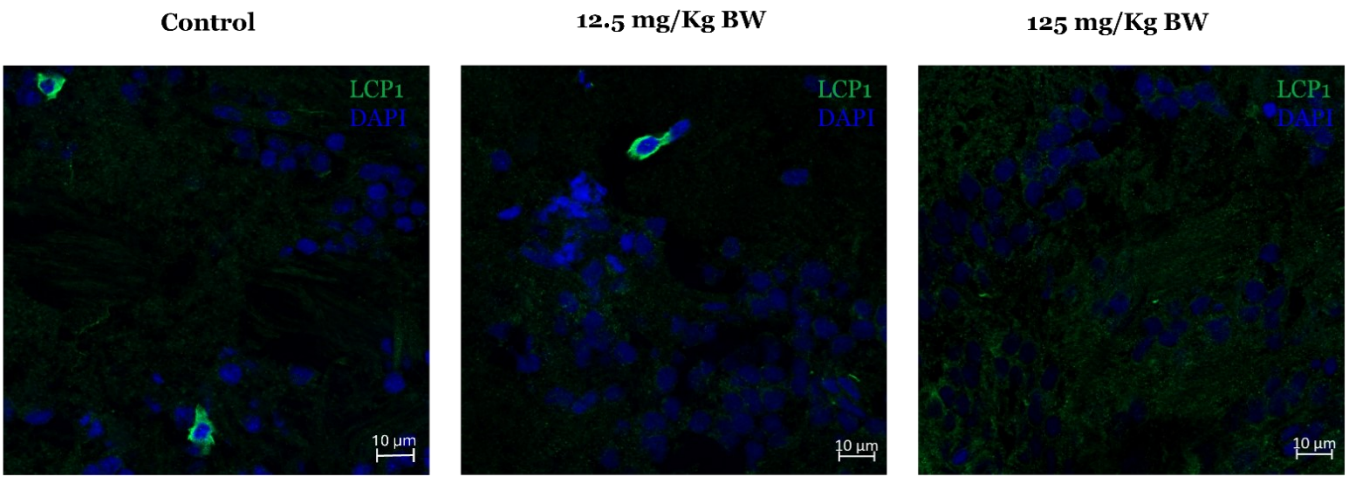


Figure 42: Effects of BGs on Autophagy in A) liver, B) optic tectum, C) muscle derived from old *Nfu,* D) Quantification of LC3 in liver, E) brain, F) muscle

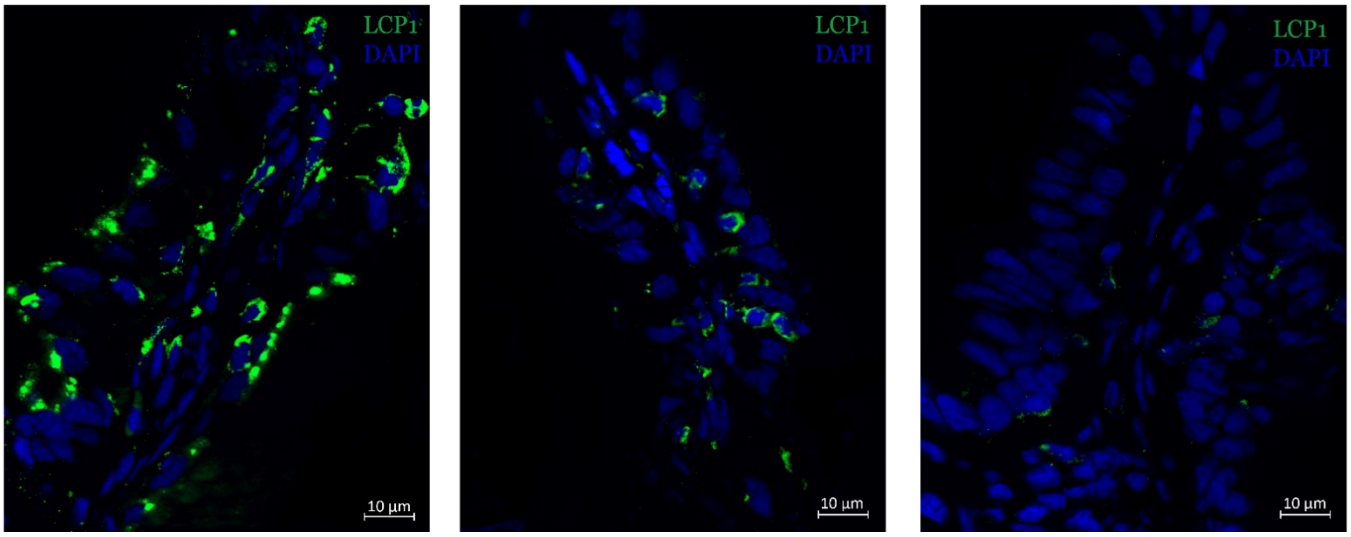
4.1.6 BGs reduce brain and gut inflammation during aging

Activation of a chronic low-intensity inflammatory response is a highly conserved phenotype of aging in vertebrates (Amarillo Irizar et al., 2018). I specifically focused on the telencephalon (brain) and gut to quantify the number of myeloid cells using L-plastin (or LCP1) as a marker (Van houcke et al., 2023; Van houcke et al., 2021). Figure 43 shows the appearance of $LCP1^+$ cells in the brain and gut in the three different experimental groups. The diet fortified with BGs (12.5 mg/Kg BW and 125 mg/Kg BW) reduced the number of $LCP1^+$ cells in brain and in the gut in a dose-dependent manner ($p<0.0001$ for both tissue, One-Way ANOVA). These results support the notion of an anti-inflammation activity of BGs in the brain and gut.

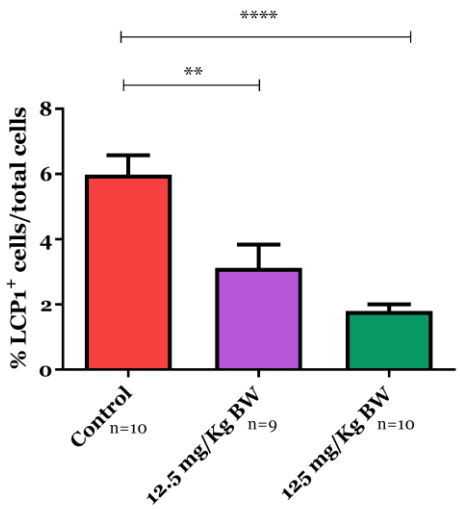
A


Control
12.5 mg/Kg BW
125 mg/Kg BW
LCP1
DAPI
10 µm

B


LCP1
DAPI
10 µm

C


****
**
% LCP1+ cells/total cells
0
2
4
6
8
Control n=10
12.5 mg/Kg BW n=9
125 mg/Kg BW n=10

D

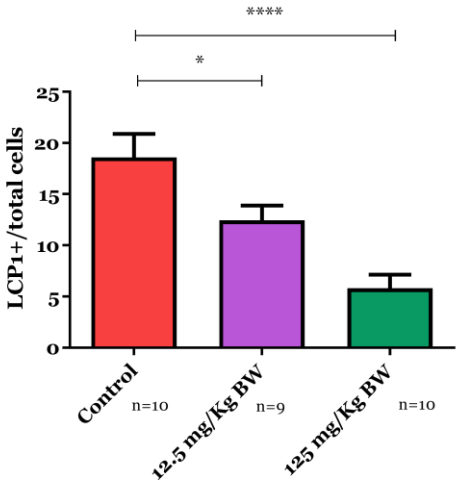

****
*
LCP1+/total cells
0
5
10
15
20
25
Control n=10
12.5 mg/Kg BW n=9
125 mg/Kg BW n=10

Figure 43: Effects of BGs on Inflammation in A) Telencephalon, B) Gut derived from old *Nfu, C*) Quantification of LCP1 in telencephalon, D) Quantification of LCP1 in gut

4.1.7 Gliosis is reduced in diets fortified with BGs

Gliosis is a reactive change of astroglia induced by neuronal damage and can be quantified using GFAP (Glial fibrillary acid protein) as a marker. Up-regulation of GFAP is observed in the aging brain of humans (David et al., 1997), rodents (Kohama et al., 1995) and killfish (Tozzini et al., 2012; Van houcke et al., 2021) and is thought to be induced by the production of pro-inflamatory citokines by activated microglia (Liddelow et al., 2017; Clarke et al., 2018). Typical appearance of GFAP immunostaining is illustrated in Figure 44 where staining is apparent as green filamentous structures in the radial processed of optic tectum glia. GFAP immunoreactivity is significantly reduced in brain tissues of *Nfu* that were fed with 125 mg/Kg BW of BG-fortified feed and 12.5 mg/Kg BW of BGs in a dose-dependent manner (p=0.0008, One-Way ANOVA).

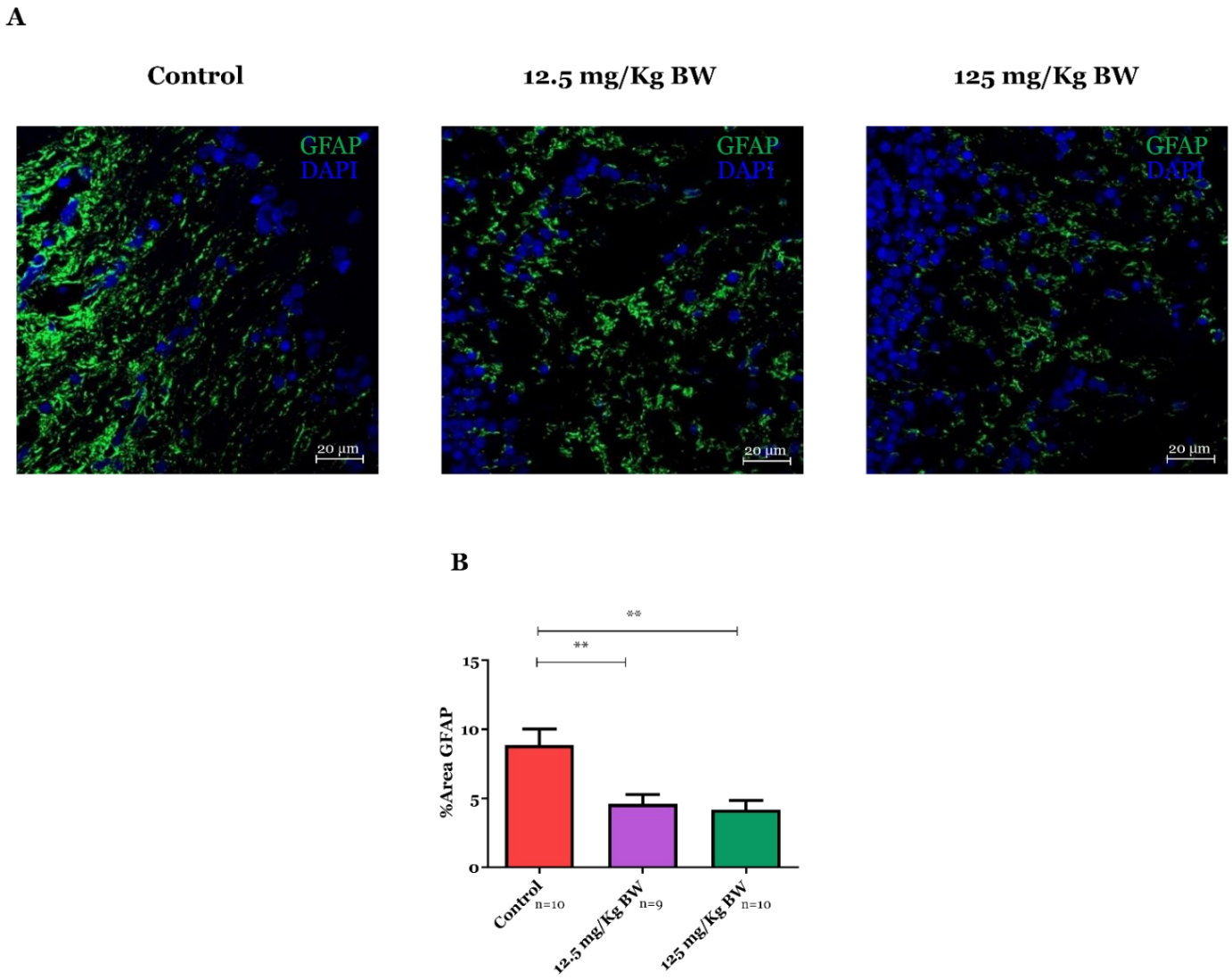


Figure 44: Effects of BGs on Gliosis in old *Nfu*. A) Gliosis in optic tectum, B) Quantification of gliosis

4.1.8 BGs increase lysosomal activity in the aged liver

Dysfunction of lysosomes is a highly conserved phenotype associated with aging and age-related diseases (Amarillo Irizar et al., 2018) associated with changes in the organelles' physical and chemical characteristics (Carmora-Gutierrez et al., 2016; Gomez-Sintes et al., 2016; Nikon, 2020; Guerrero-Navarro et al., 2022; Tan and Finkle, 2023). Immunoreactivity for LAMP1, a protein of the lysosomal membrane, was used to visualize lysosomes and is apparent as green fluorescence puncta in Figure 45. To visualize the lysosomal activity *in situ*, I double labelled liver slices with LAMP1 and aggresome and quantified the LAMP1+ area (green; Fig. 44) surrounding protein aggregates, (red dots in Fig. 45). BGs significantly increase lysosomal activity in dose-dependent manner ($p<0.0001$, One-Way ANOVA).

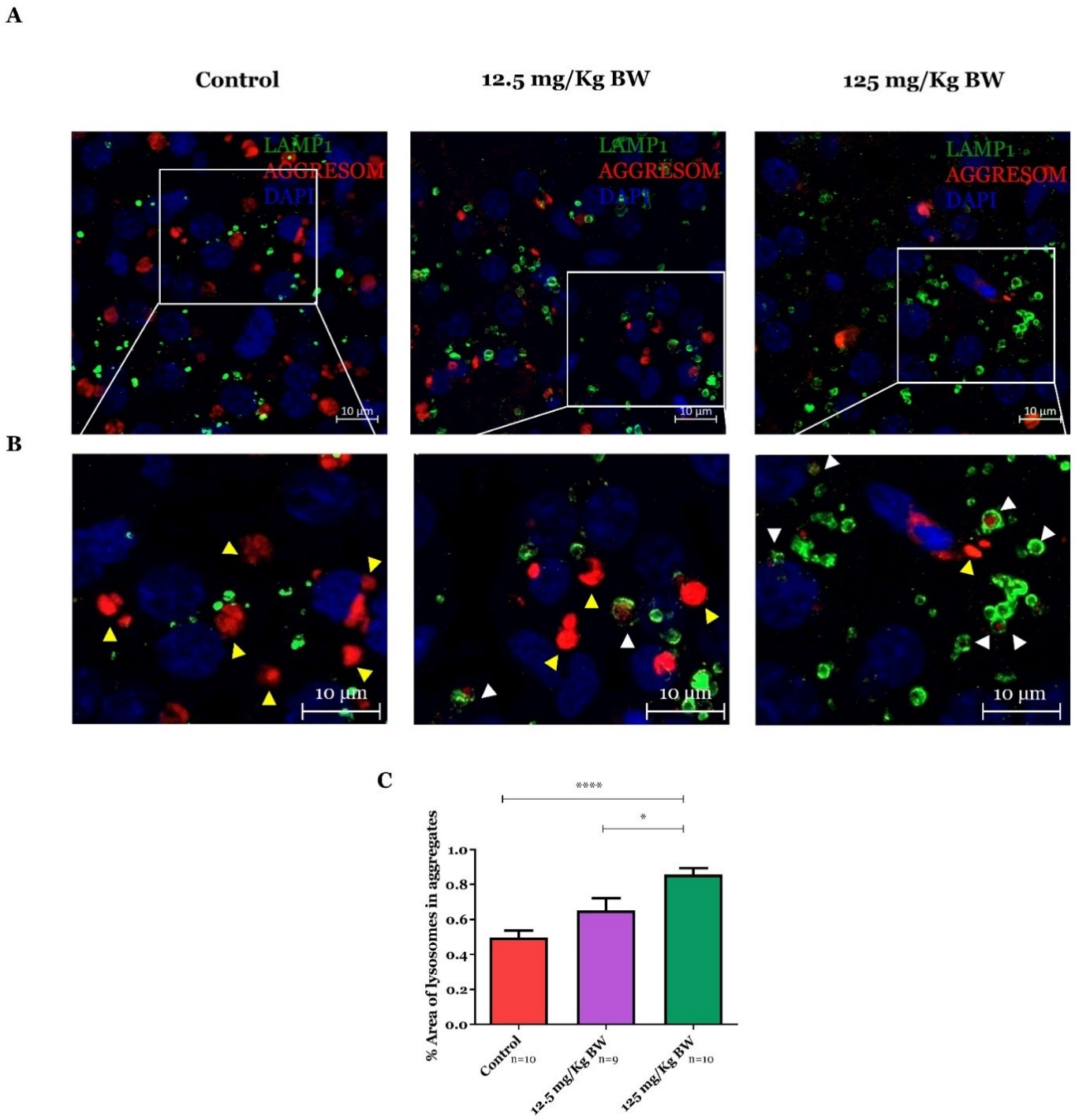


Figure 45: Effects of BGs on Lysosomal activity. A) Lysosomal activity in liver lysosomes (green) and aggregates (red), B) Enlargement of figure A. White arrows indicate lysosomes with inside aggregates, yellow arrows indicate only aggregates. C)Graph of Lysosomal activity

4.1.9 BGs do not affect hepatocytes vacuolization

Hepatocytes vacuolization, or steatosis, is a disorder of the aging liver that can lead to a decline in liver function (Ogrodinik et al., 2017; Nguyen et al., 2021). Hepatocytes vacuolization is prominent in *Nfu (*Di Cicco et al., 2009) and two types of vacuoles can be

distinguished based on their morphology: glycogen- and lipid-vacuoles (Zac et al., 2020). We failed to detect significant effects of BG-fortified feed on either hepatocyte's vacuolization ($p=0.5544$, One-Way ANOVA) or the type of vacuoles ($Chi^2=0.4475$, Chi^2-test, $p=0.7995$, One-Way ANOVA), as Figure 46 shows.

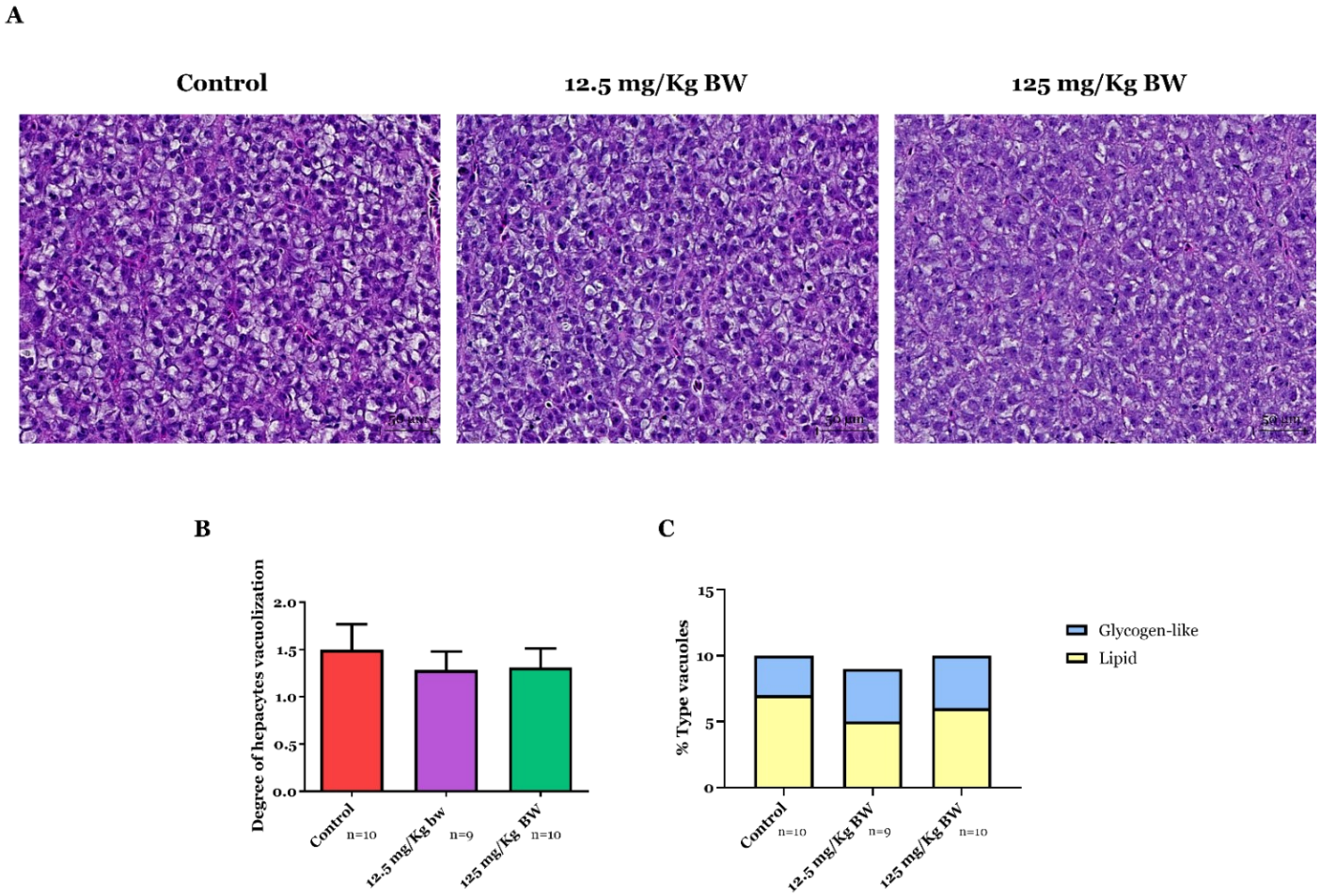


Figure 46: Effects of BGs on hepatocytes vacuolization. A) Representative figures of hepatocytes vacuolization. B) Degree of hepatocytes vacuolization in control and treated *Nfu,* C) Percentage of different type of hepatic vacuoles

4.1.10 BGs preserve muscle histology during aging

Skeletal muscle aging is characterized mainly by muscle atrophy and collagen deposition, or fibrosis (Gustafsson and Ulfhake, 2021; Selman and Pardo, 2021). Previous studies reported that aged killifish suffer from muscle fiber atrophy (Ruparelia et al., 2023). To investigate the structure of aged muscle, I measured muscle fiber size (Feret's size) as a marker of atrophy and collagen deposition as a marker of fibrosis. The Feret's size of muscle fibers is larger in fish fed with BG-fortified feed in dose-dependent manner ($p=0.0141$, One-Way ANOVA), as Figures 47A-C shows. Additionally, BGs significantly reduced collagen deposition in dose-dependent manner [area stained by Sirius red; ($p=0.0089$, One-Way ANOVA)], Figures 47B-D.

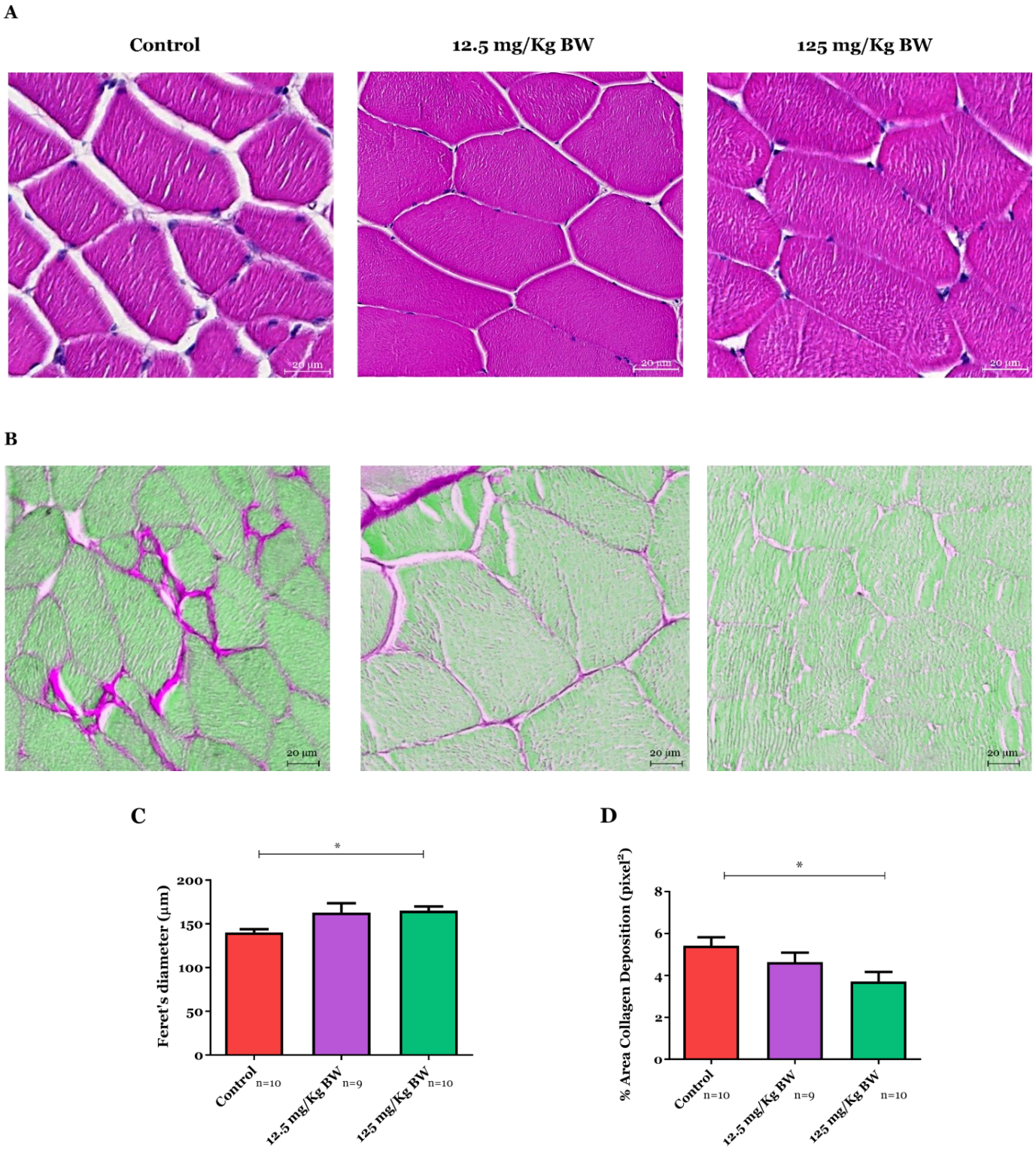


Figure 47: Effects of BGs on muscle histology. A) Feret's diameter, B) Collagen deposition in control and treated *Nfu*, C) Quantification of Feret's diameter, D) Quantification of Collagen deposition

4.1.11 BGs do not modify renal histopathology

Aging is associated with different molecular, morphologic, and functional changes in kidney tissue (Kotob et al., 2021). Markorfsky and Milstoc, and De Cicco et al. described the typical age-related renal histopathological changes in Nothobranchius, specifically: increment of Bowman's capsule thickness, dilatation of tubules, presence of precipitates in renal tubules and tubule necrosis (Markorfsky Milstoc, 1979; De Cicco et al., 2009). The histopathological lesions are not significantly influenced by BG-fortified diet: Bowman's capsule thickness (p=0.7818, One-Way ANOVA), incidence of precipitated in renal tubules (p=0.0519, One-Way ANOVA), incidence of necrotic renal tubules (p=0.4116, One-Way ANOVA) and area of tubules (p=0.0518, One-Way ANOVA), as Figure 48 shows.

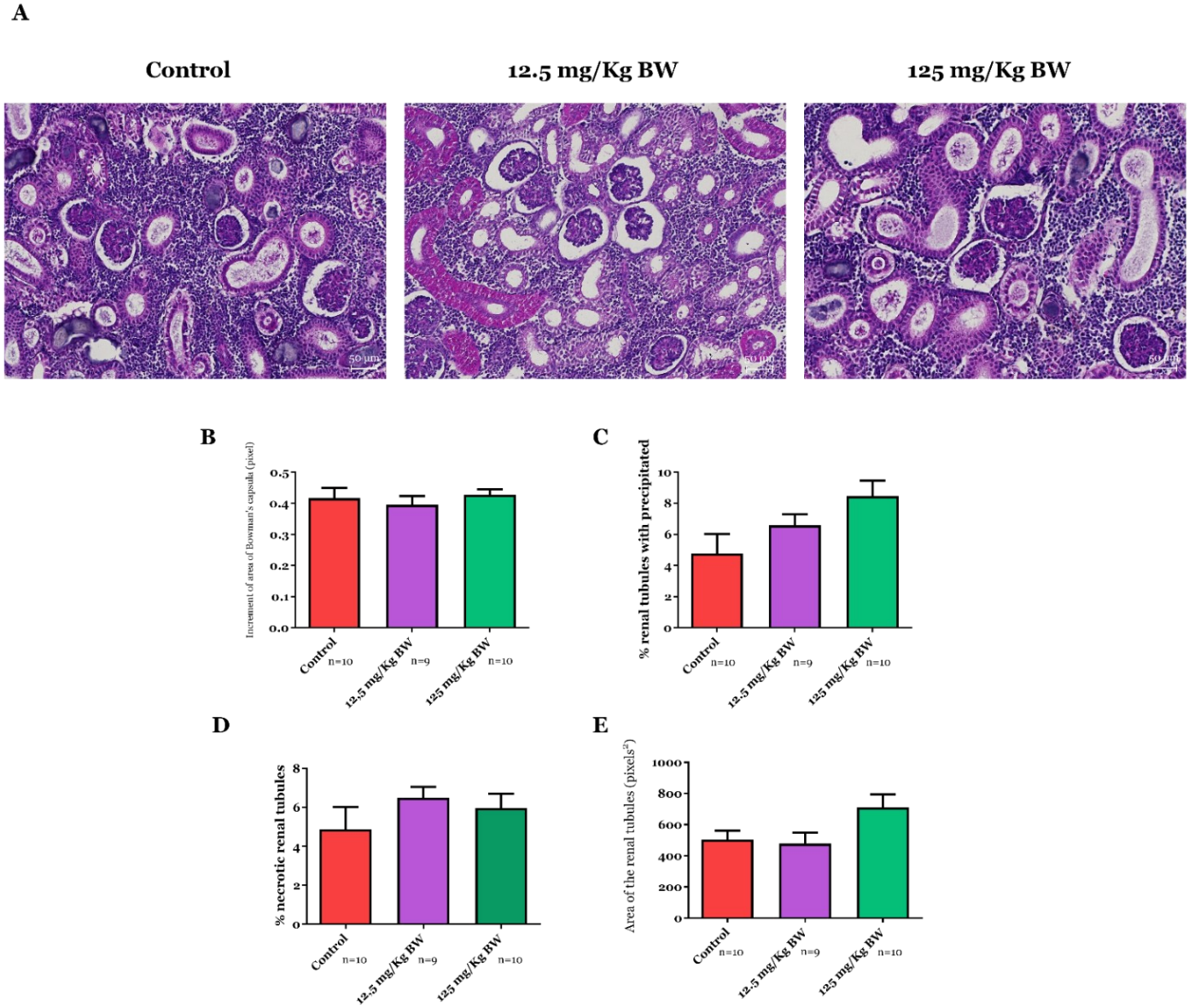


Figure 48: Effects of BGs on renal histology. A) Representative figure of renal histology, B) Increment of area Bowan's capsule, C) Percentage of renal tubules with precipitated, D) Percentage of necrotic renal tubules, E) Area of renal tubules in control and treated *Nfu*

4.2 Chapter 2 Acute effects of BGs on autophagy and organelles

After observing that chronic supplementation of the diet with BGs prevents some phenotypes associated with aging, I decided to investigate whether BGs could acutely reduce phenotypes already established in elderly subjects. Old *Nfu* (n=5) were fed with Zebrafeed fortified with 1.25 μg/gr of BGs for a week starting at age 26 weeks. I focussed the analysis on lipofuscin and autophagy in the optic tectum, as these phenotypes were regulated prominently by chronic BGs supplementation.

4.2.1 Acute diet switch with BGs fortified feeds does not reduce lipofuscin during aging

I measured lipofuscin content in brain sections of animals treated acutely with BGs and detected no significant difference (padj= 0.24216, t-test adjusted with False Discovery Rate, FDR). This is in contrast with the reduction of lipofuscin accumulation detected in aged brains of fish treated chronically with BGs (padj=0.0012, t-test adjusted with False Discovery Rate, FDR), indicating that BGs mitigate the accumulation of lipofuscin but fail to remove it once it is already accumulated (Fig. 49).

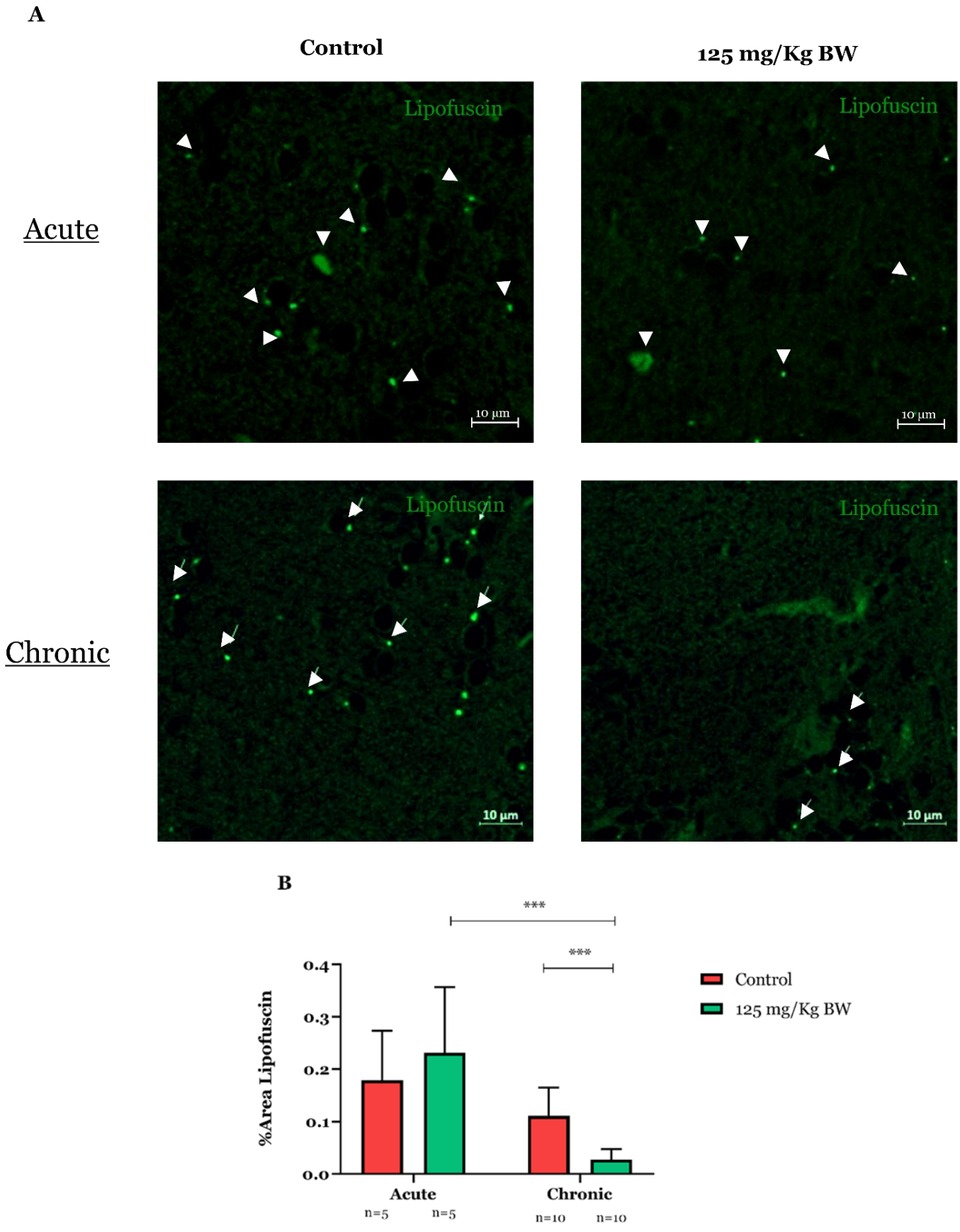
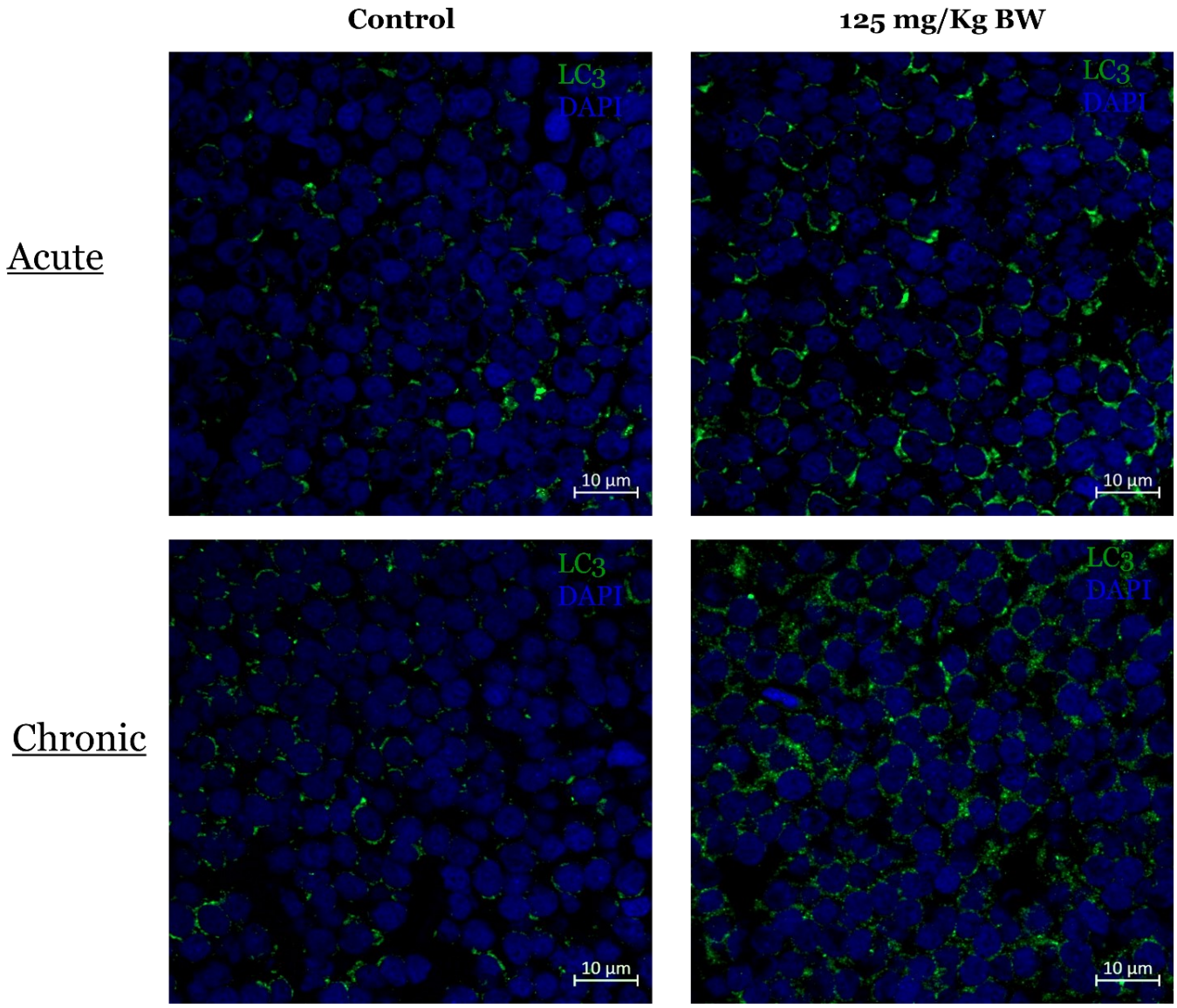


Figure 49: Acute effects of BGs on lipofuscin in optic tectum in old *Nfu*. A) Representative figures of lipofuscin, B) Quantification of lipofuscin in acute and chronic treatment

4.2.2 Acute treatment with BGs induce autophagy during aging

Acute treatment with BGs induces a statistically significant increase of LC3 immunoreactivity in the aged brain (padj=0.02475, t-test adjusted with False Discovery Rate, FDR), as Figure 50 shows. The magnitude of this induction is not significantly different between chronic- and acute-treatment (padj=0.09924, t-test adjusted with False Discovery Rate, FDR).

A



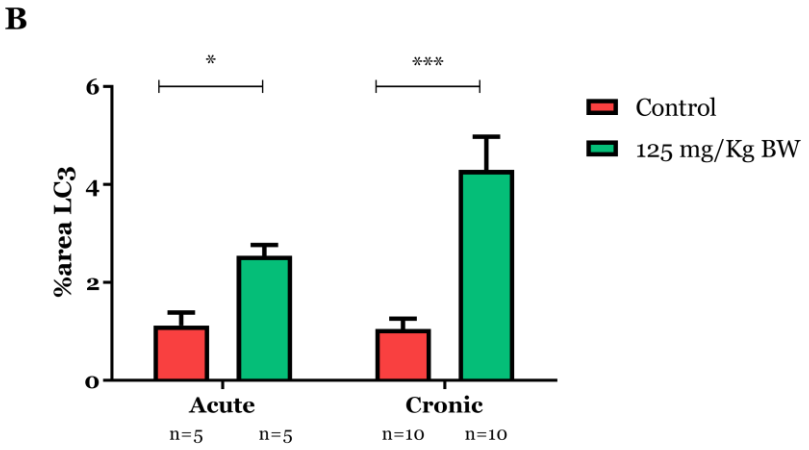


Figure 50: Acute effects BGs on autophagy in optic tectum in old *Nfu*. A) Representative figures of autophagy, B) Quantification of autophagy in acute and chronic treatment

The observation that an acute treatment with BGs is sufficient to induce autophagy enables to study the effects of BGs ex-vivo. and test whether BGs ex-vivo have a direct impact on the brain. To this end, I exploited an ex-vivo brain model previously developed in my laboratory (Bagnoli et al., 2023). I therefore exposed brain of the different ages (5 weeks, young; 12 weeks, adult; 27 weeks, old) to 8 mg/L of BGs for three days and then studied their impact on multiple parameters.

4.3 Chapter 3 role of BGs directly on the brain

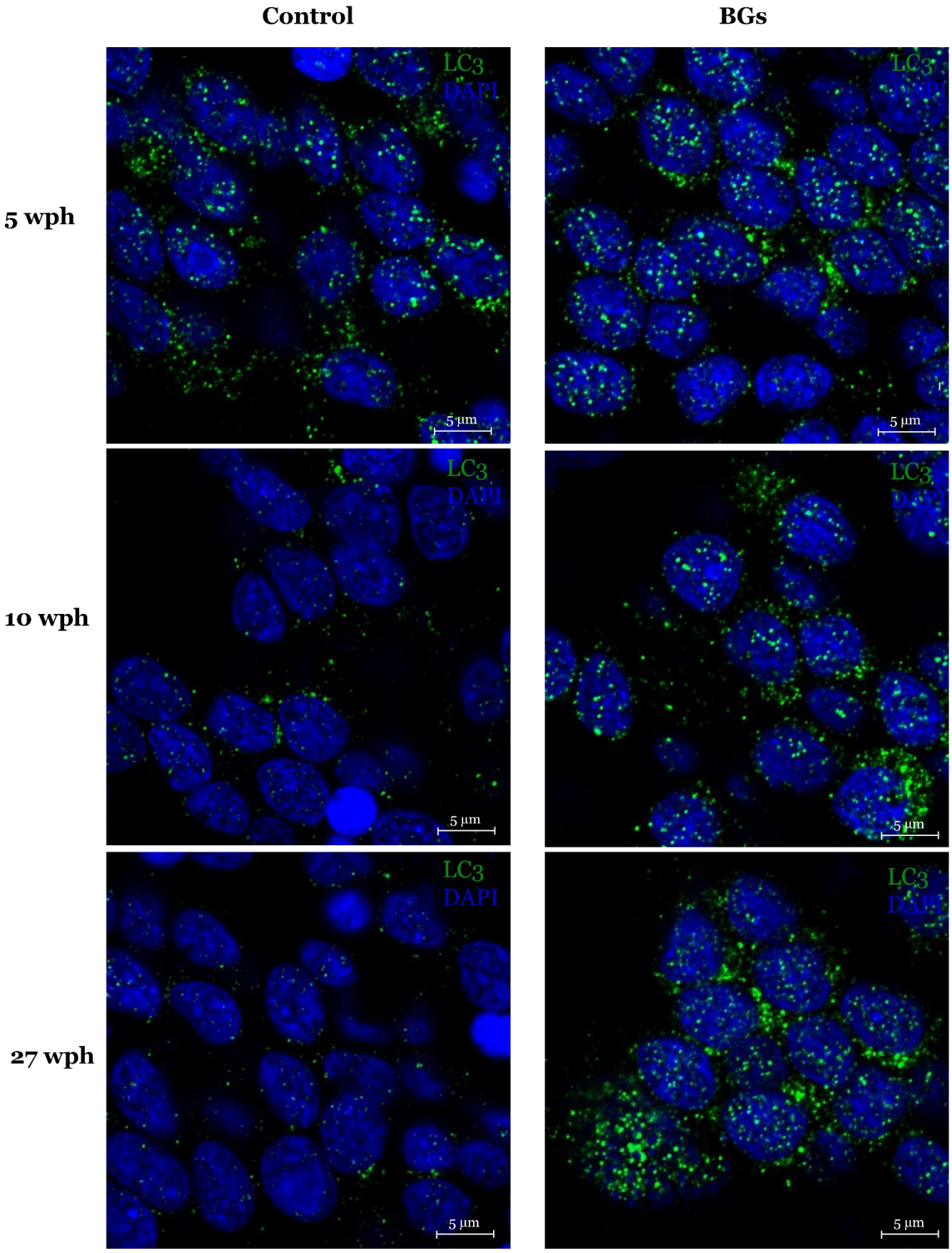
4.3.1 BGs act directly on the brain and restore defective autophagy during aging

Firstly, I investigated autophagy in *ex vivo* brain cultures of different ages (5, 10, 27 WPH), in the optic tectum since neuronal cells reside in the grey layer and no other cell types. I quantified quantifying Number and Volume, of LC3+ autophagosomes, Percentage of LC3+ area and LC3 Intensity density (Fig. 51). Aging resulted in a significant reduction of the number (young vs. adult p= 0.0003; young vs. old p= 0.0002; Two-way ANOVA), volume (young vs. adult p= 0.0025; young vs. old p=0.0010; Two-way ANOVA), percentage of area (young vs. adult p=0.0318; young vs. old p=0.0027; Two-way ANOVA) but not of the integrated density (young vs. adult p=0.0791; young vs. old p= 0.1647; Two-way ANOVA) of autophagosomes, indicating that autophagy is defective in cultured brain slices from adult and old fish.

Then, I investigated whether BGs induce autophagy in *ex vivo* brain slices. No significant induction of LC3+ autophagosomes was observed in young slices treated with BGs for 3-day (p=0.9518; Two-way ANOVA). On the other hand, BGs induced autophagy in adult- and old-brains. In fact, BGs increase the number (young vs. adult p<0.0001; young vs. old p<0.0001;

Two-way ANOVA), volume (young vs. adult p=0.0092; young vs. old p=0.0040; Two-way ANOVA), and percentage area of autophagosomes (young vs. adult p=0.0139, young vs. old p=0.0018; Two-way ANOVA). Therefore, BGs have a direct effect on the brain and normalize impaired autophagy in the old brain.

A



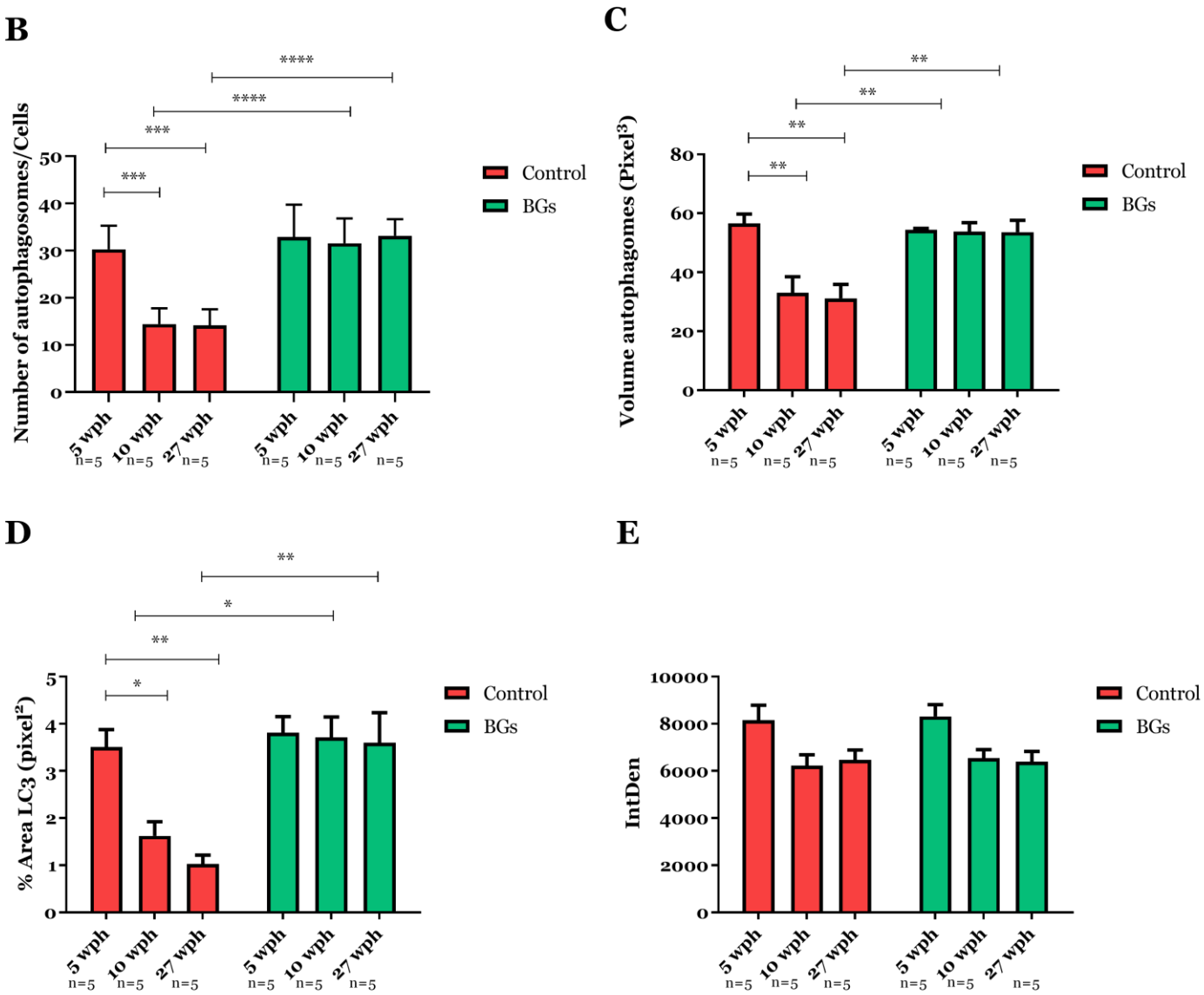


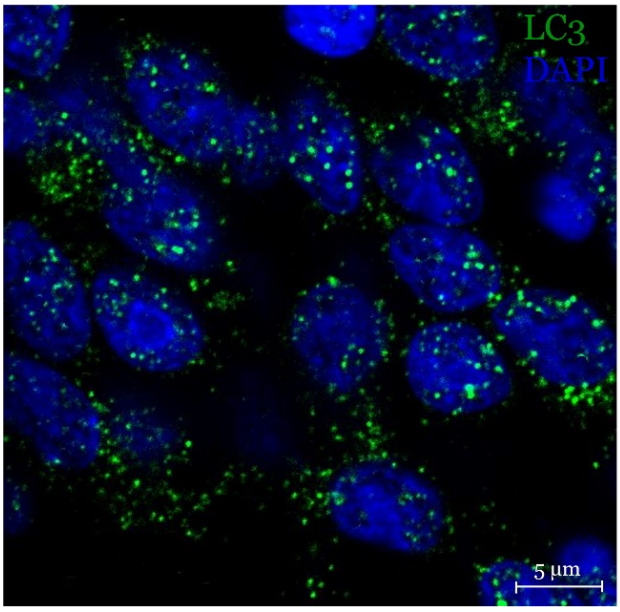
Figure 51: Direct effects of BGs on Autophagy in ex-vivo model. A) Representative figures of autophagy in different ages and treatment, B) Quantification of number of autophagosomes per cells, C) Quantification of volume of autophagosomes, D) Quantification of percentage area of LC3, E) Quantification of integrity Density in the different ages and treatment

Increased immunoreactivity for LC3+ could be the result of either higher number of phagosomes with normal autophagic flux (i.e. enhanced autophagy) or a block of autophagic flux that causes accumulation of autophagosomes despite normal autophagosome formation (i.e. blocked autophagy). To confirm that BGs increase autophagy, I blocked autophagy with Bafilomycin A1 in cultured brain slices from young brains and quantified the effects of BGs on LC3+ autophagosomes. Bafilomycin A1 is an inhibitor of lysosome H+ pumps and prevents the processing of LC3+ autophagosomes. I observed that Bafilomycin A1 induced accumulation of autophagosomes (Number of LC3/Cell) in comparison to control

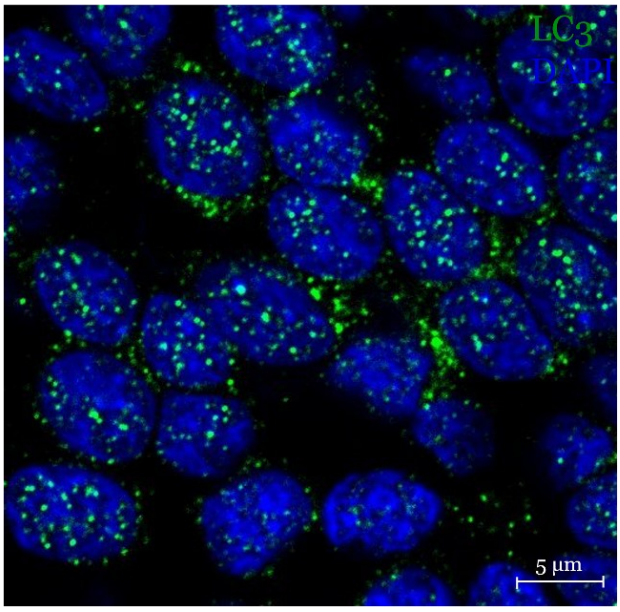
(p<0.0001, One-Way ANOVA) and BGs (p=0.001, One-Way ANOVA), an increase in the percentage of area of autophagosomes as compared to control (p=0.0159, One-Way ANOVA) and BGs (p=0.0253, One-Way ANOVA) and an increment in the volume of autophagosomes relative to control (p=0.0029, One-Way ANOVA) and to BGs (p=0.0023, One-Way ANOVA). Combined treatment with Bafilomycin A1 and BGs increased the number (p=0.0110, One-Way ANOVA), volume compared with Bafilomycin A1 alone. These results indicate that BGs increase the formation of autophagosomes. (p<0.0001, One-Way ANOVA) and percentage area (p=0.0162, One-Way ANOVA) of autophagosomes (Fig. 52) further as compared with Bafilomycin A1 alone. These results indicate that BGs increase the formation of autophagosomes.

A

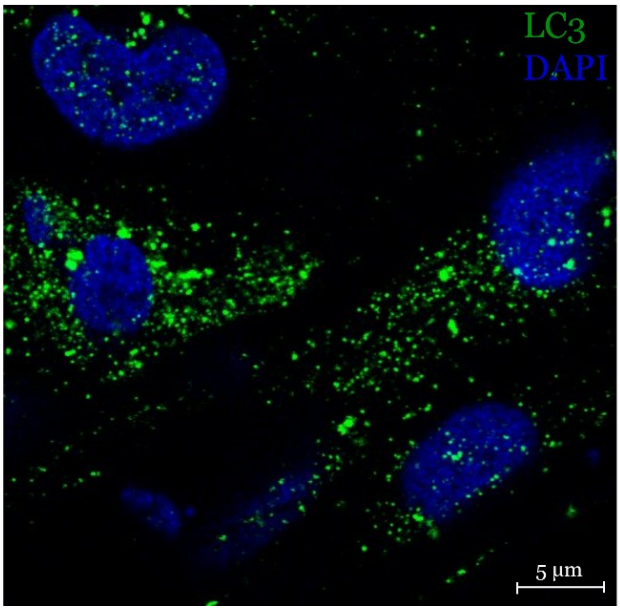
Control



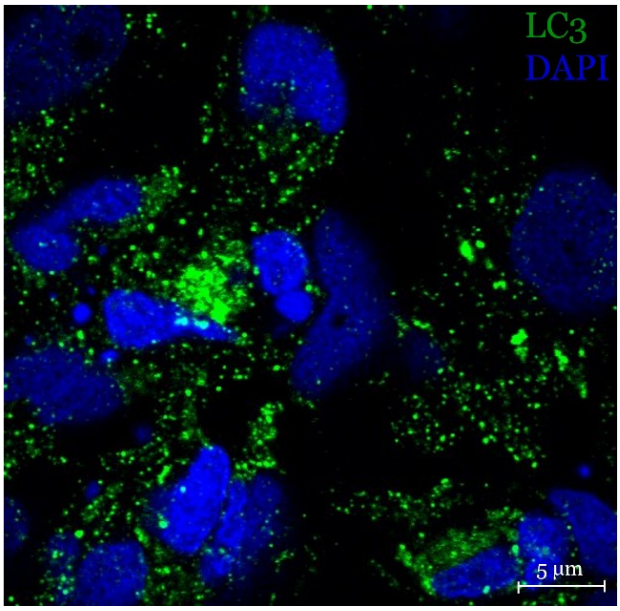
BGs



Bafilomycin A1



Bafilomycin A1 & BGs



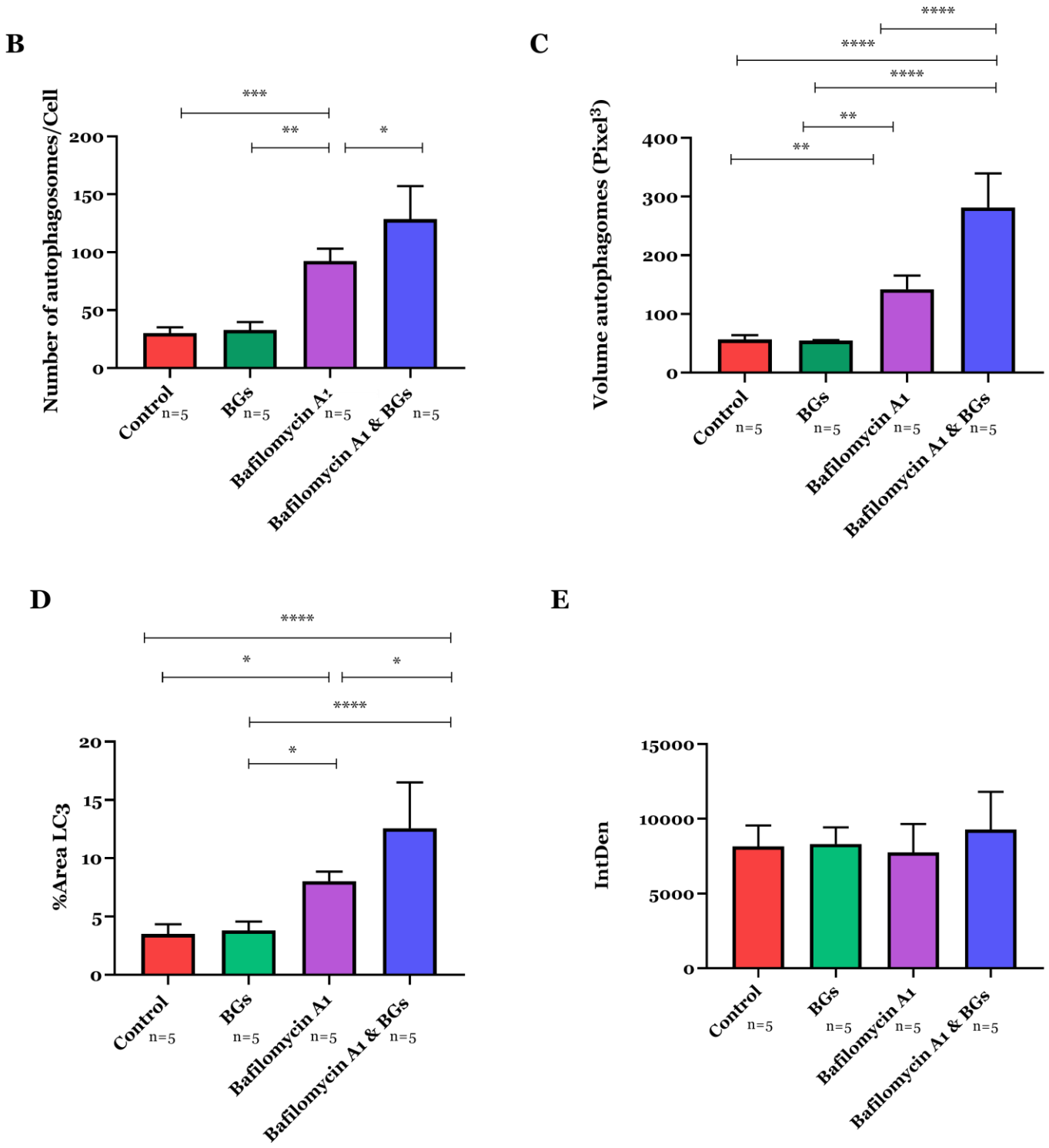


Figure 52: Direct effects of BGs on Autophagy in ex-vivo model after the block of autophagy by Bafilomycin A1. A) Representative figure of autophagy blocked by Bafilomycin A1, B) Graph of number of autophagosomes per cells, C) Graph of volume of autophagosomes, D) Graph of percentage area of LC3, E) Graph of integrity Density in young ex-vivo brain

To confirm this result, I used CYTO-ID Autophagy Detection kit as an independent method to label autophagosomes. Also in this case, BGs increased the number ($p=0.0344$, t-test), the percentage of area ($p=0.0307$, t-test) and volume ($p=0.0167$, t-test) of autophagosomes (Fig. 53). These results demonstrate that BGs induce autophagy in cultured brain slices.

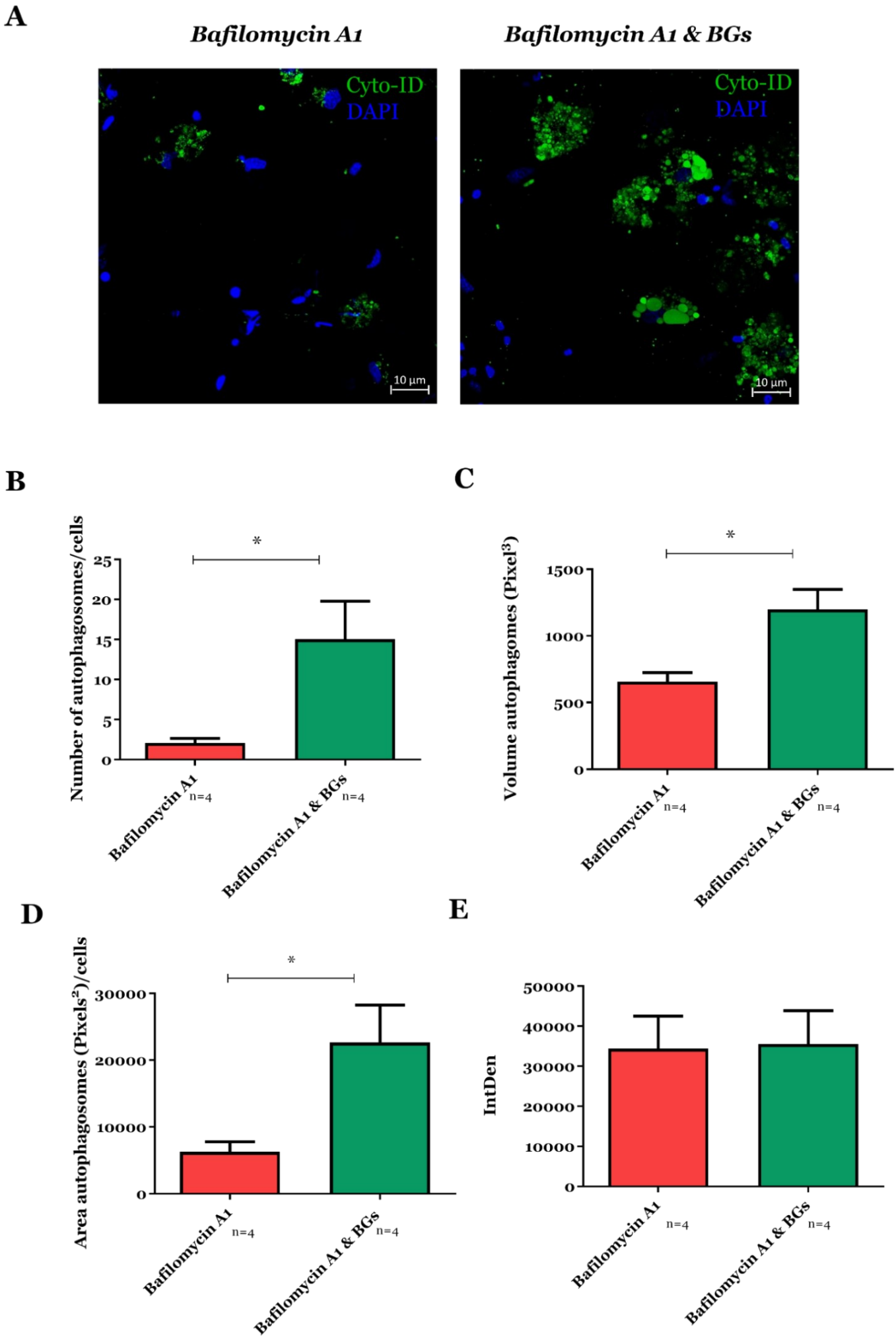

A
Bafilomycin A1
Bafilomycin A1 & BGs
Cyto-ID
DAPI
10 µm
B
Number of autophagosomes/cells
Bafilomycin A1 n=4
Bafilomycin A1 & BGs n=4
C
Volume autophagomes (Pixel³)
D
Area autophagosomes (Pixels²)/cells
E
IntDen

Figure 53: Direct effects of BGs on Autophagy in ex-vivo model after the treatment of Bafilomycin A1. A) Representative figures of Cyto-ID, B) Number of autophagosomes per cells, C) Volume of autophagosomes, D) Percentage area of LC3, E) Integrity Density in old ex-vivo brain

4.3.2 BGs improve the morphology of lysosomes and mitochondria in aged brains

To further characterized the effects of BGs in the aged brain, I investigated the morphology and number of acidic lysosomes using Lysotracker (Fig. 54). In cultured brain slices from old (27 wph) *Nfu,* treatment for 3 days with BGs increases the number (p=0.0014, t-test) and the area (p=0.0113, t-test) of acidic lysosomes, consistently with the in vivo results obtained by double labelling of LAMP1 and aggresome.

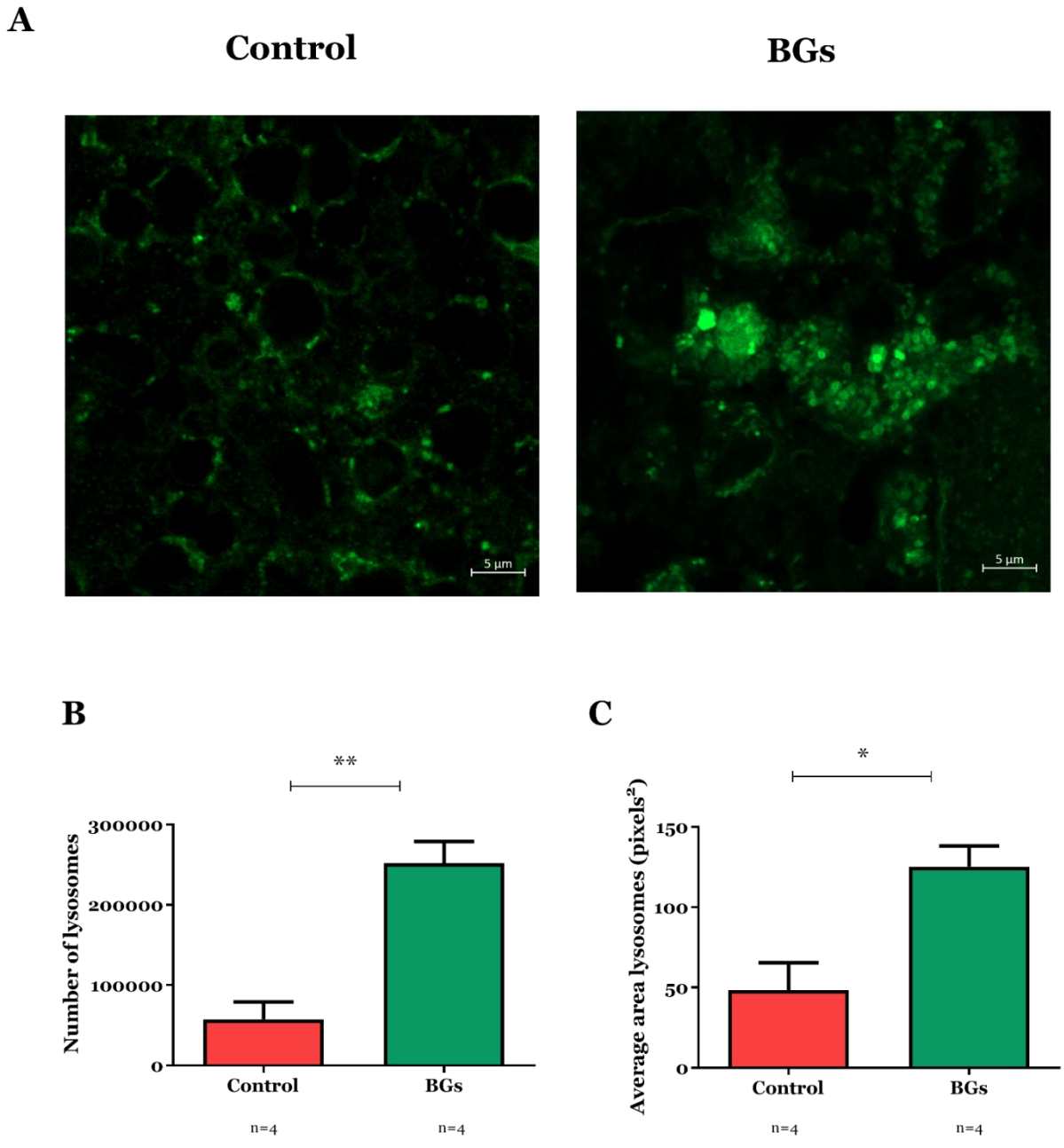


Figure 54: Direct effects of BGs on Lysosomes in ex-vivo model. A) Number of lysosomes, B) Area of Lysosomes in old ex-vivo brain

Autophagy is an important mechanism of mitochondria quality control that eliminates damaged mitochondria and recycles biomolecules of the cargo (mitophagy). Induction of autophagy induces an increment of functionality and energy production of mitochondria (Wilson et al., 2023) and treatment with BGs improved mitochondrial respiration in zebrafish larvae (Brogi et al., 2021). I therefore analysed the morphology and number of mitochondria using Mitotracker. Figure 55 shows the appearance of labelled mitochondria in cultured brain slices. The number (p=0.0071, t-test) and area of mitochondria (p=0.0366, t-test) are increased in old brains after 3-day of treatment of 8 mg/L BGs.

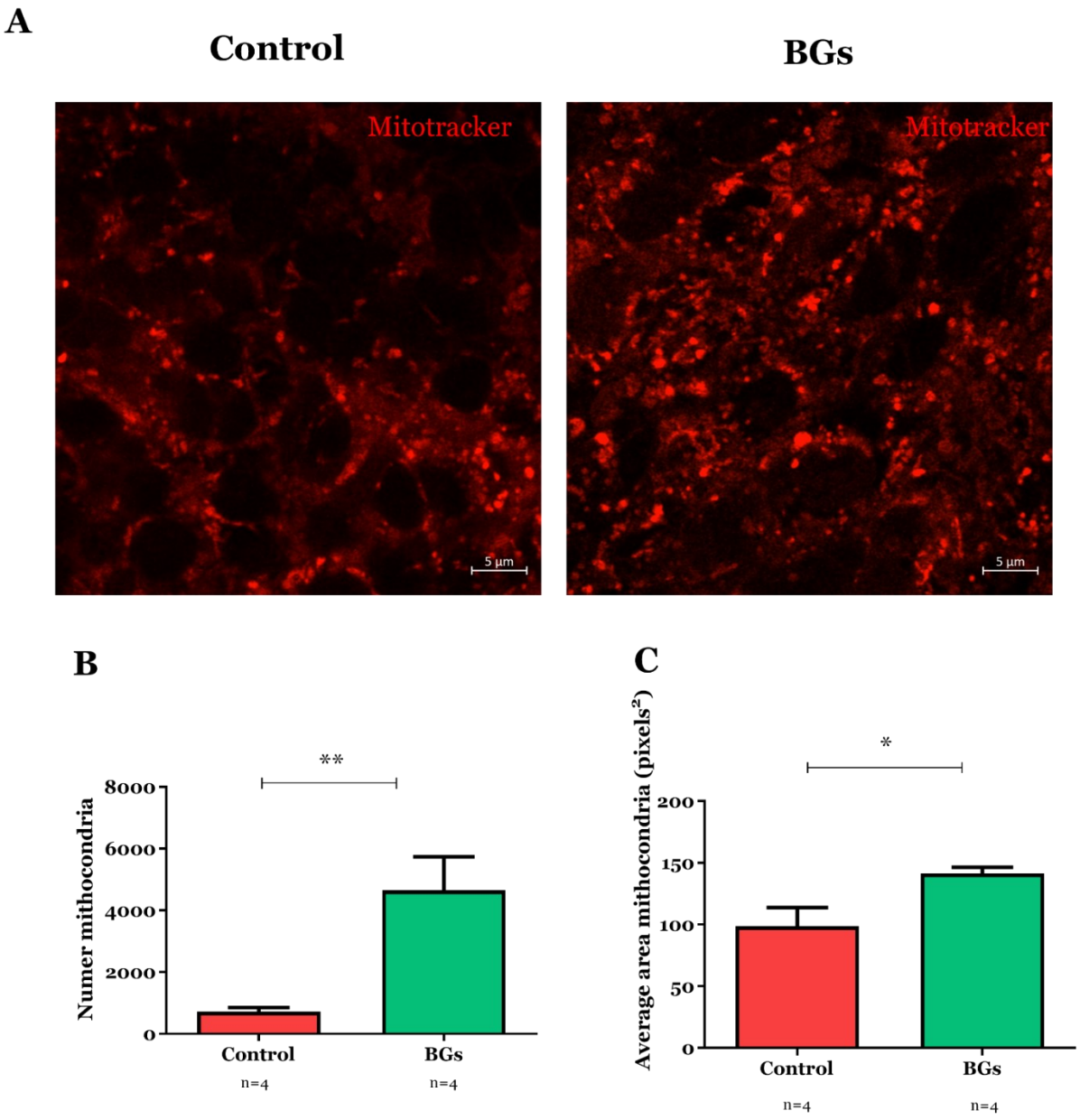


Figure 55: Direct effects of BGs on Mitochondria in ex-vivo model. A) Representative figures of Mitochondria, B) Number of Mitochondria, C) Area of Mitochondria in old ex-vivo brain

4.4 Chapter 4 role of microglia

4.4.1 BGs reduces microglia activation

Brain aging is associate with an increased production of pro-inflammatory cytokines and increased expression of inflammatory receptors by microglia (Norden and Godbout et al., 2014). The aged brain of *Nfu* is characterized by an increase expression of inflammation-related genes, especially complement (Baumgart et al., 2014; Amarillo Irizar et al., 2018; Kelmer et al., 2020) and increase in the number of microglia (Vanhunsel et al., 2021). Moreover, BGs significantly prevented the hyperactivation of pro-inflammatory microglia on the mice's brain infected (Cui et al., 2023). To analyse whether BGs reduce activation of microglia, I prepared organotypic cultures from adult Tg(mpeg1.1: EGFP) zebrafish that enable a fine analysis of microglia morphology and treated them with Bafilomycin A1 for 3-day. BGs reduce the sphericity ($p<0.0001$, t-test) of microglia cells in Tg(mpeg1.1: EGFP) zebrafish after treatment with Bafilomycin A1 (Fig. 56). This result demonstrates that BGs can directly modulate the phagocytic phenotype of microglia cells of teleost.

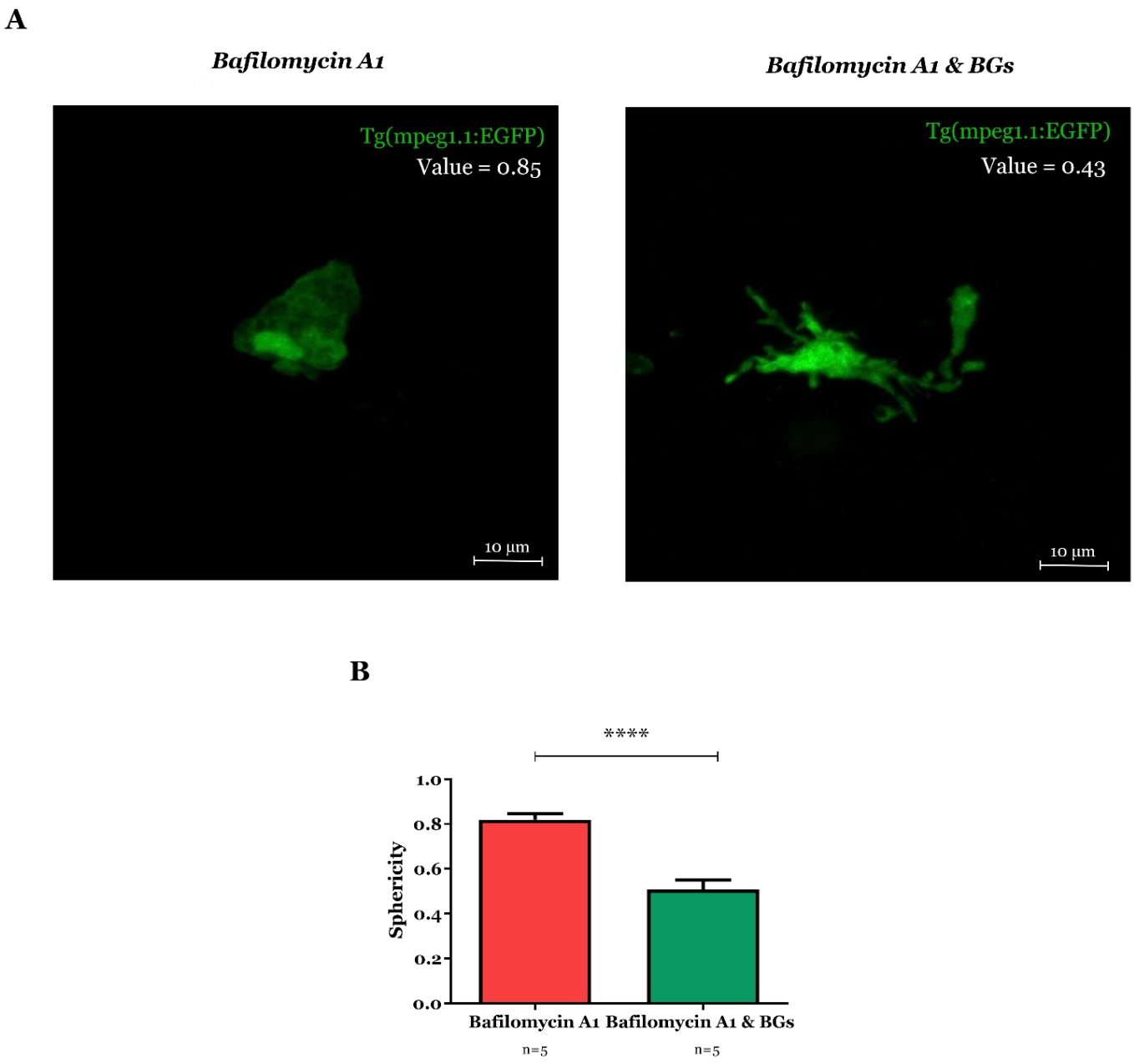


Figure 56: Direct effects of BGs on Microglia in ex-vivo model from Tg(mpeg 1.1: EGFP). A) Representative figure of microglia, B) Quantification Sphericity

4.4.2 Induction on neuronal autophagy by BGs is partially mediated by microglia

The previous result is in line with a large body of evidence (Shah et al., 2008; Goodridge et al., 2009; De Marco Castro et., 2020; Heng et al., 2021) indicating that BGs act on immune cells, particularly on microglia and macrophages, mostly by binding Toll Like Receptors (Sahasrabudhe et al., 2016; Zhang et al., 2022). Some of these receptors are expressed to a lesser extent in neurons as well. I therefore wondered whether BGs could act directly on

neurons. To answer this question, I treated cultured brain slices with PLX5622, a drug that eliminates microglia. Firstly, I quantify the effect of PLX5622 in culture brain slices of *Nfu*. The percentage of LCP1+ cells/Total cells is significantly reduced (p=0.007, t-test) in cultured brain slices treated with 20µM of PLX5622 compared to control (Fig. 57). This result confirms the depletion on microglia by PLX5622 in in culture brain slices of *Nfu*.

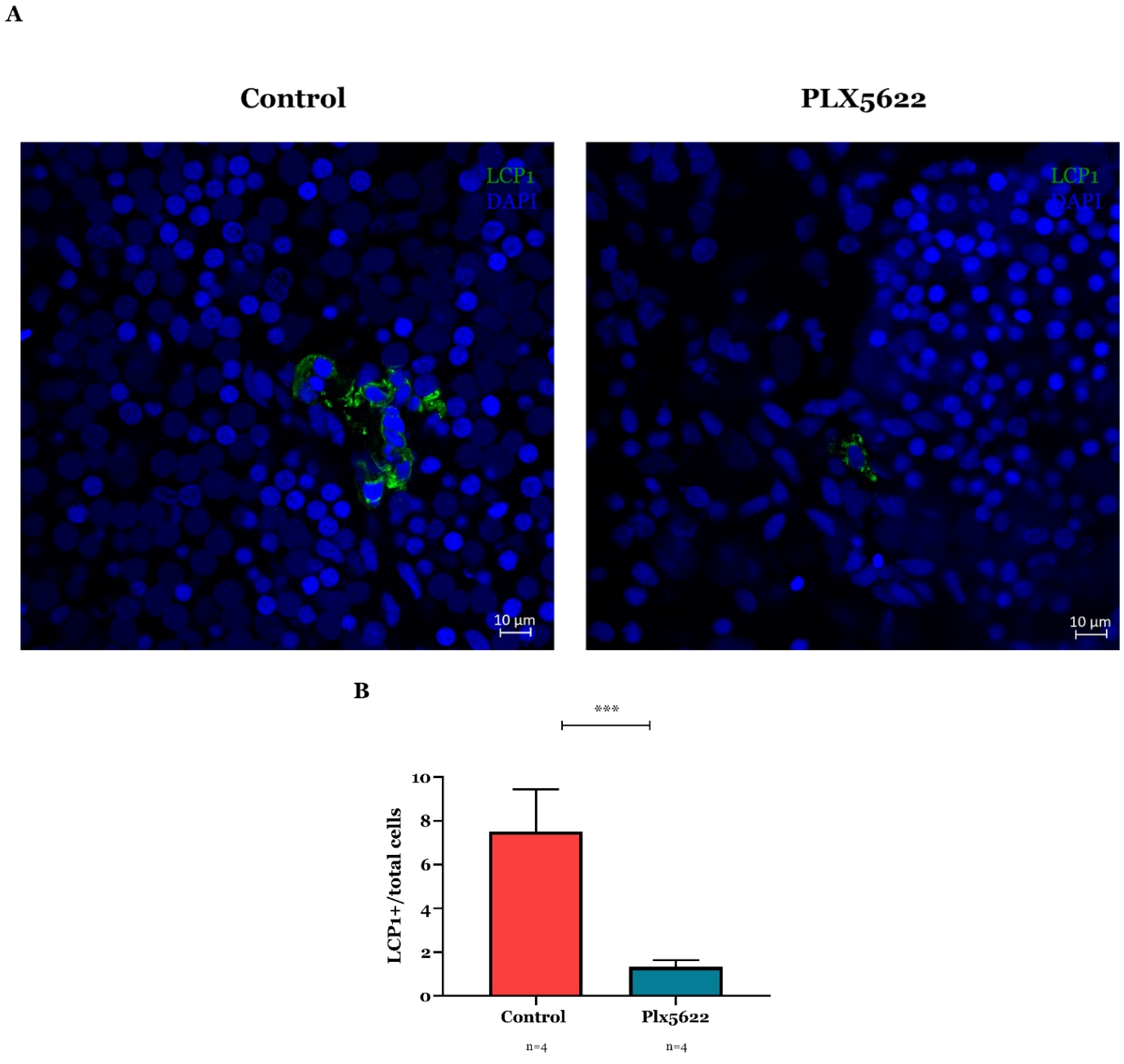
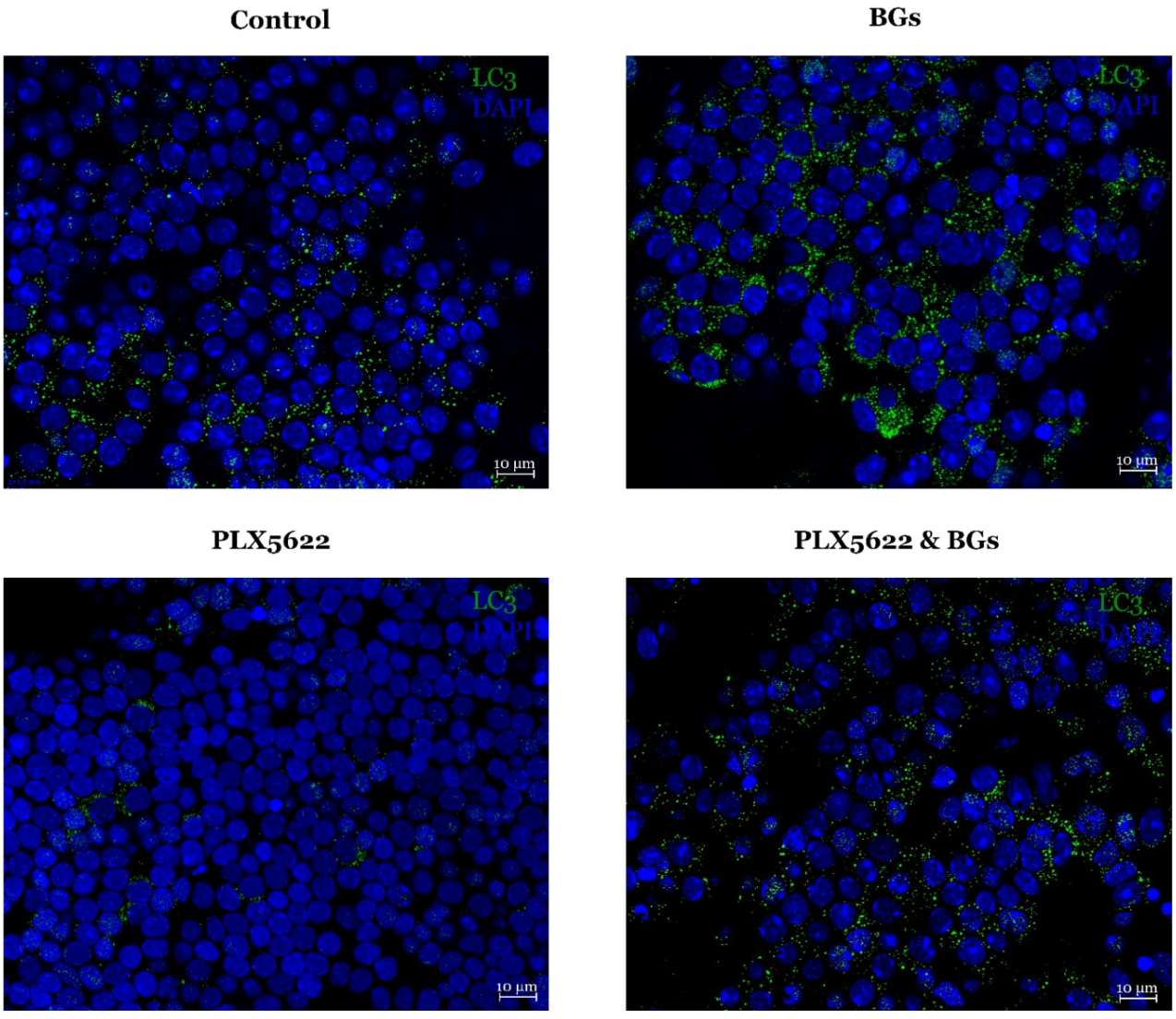


Figure 57: Depletion on microglia in cultured brain slices treated with 20µM PLX5622. A) Representative figure of LCP1 in culture brain slices, B) Quantification of LCP1 in culture brain slices treated with PLX5622 and Control

I then analysed the effect BGs on dysregulated autophagy in cultured brain slices devoid of microglia. Consistently with previous experiments, treatment of adult brain slices with 8

mg/L of BGs for 3 days increased the number of autophagosomes per cell ($p<0.0001$, Two-Way ANOVA). Depletion of microglia reduced the number of autophagosomes per cells in control slices ($p=0.0385$, Two-Way ANOVA), but do not change volume of autophagosome ($p=0.2615$, Two-Way ANOVA). BGs increase the number of autophagosomes in slices depleted of microglia ($p=0.0022$, Two-Way ANOVA), but to a lesser extent than in control slices treated with BGs ($p=0.0001$, Two-Way ANOVA). And BGs do not cause an increase in autophagosome volume ($p=0.1774$, Two-Way ANOVA). BGs increased the number of autophagosomes per cell in the same way in control and depleted of microglia slices ($p=0.89842$, Two-Way ANOVA). For contrary, BGs increased the volume of autophagosomes more strongly in controls than in depleted of microglia slices ($p=0.0215$, t-test). The findings suggest that BGs effects on neurons are mediated only partially by microglia (Fig. 58).

A



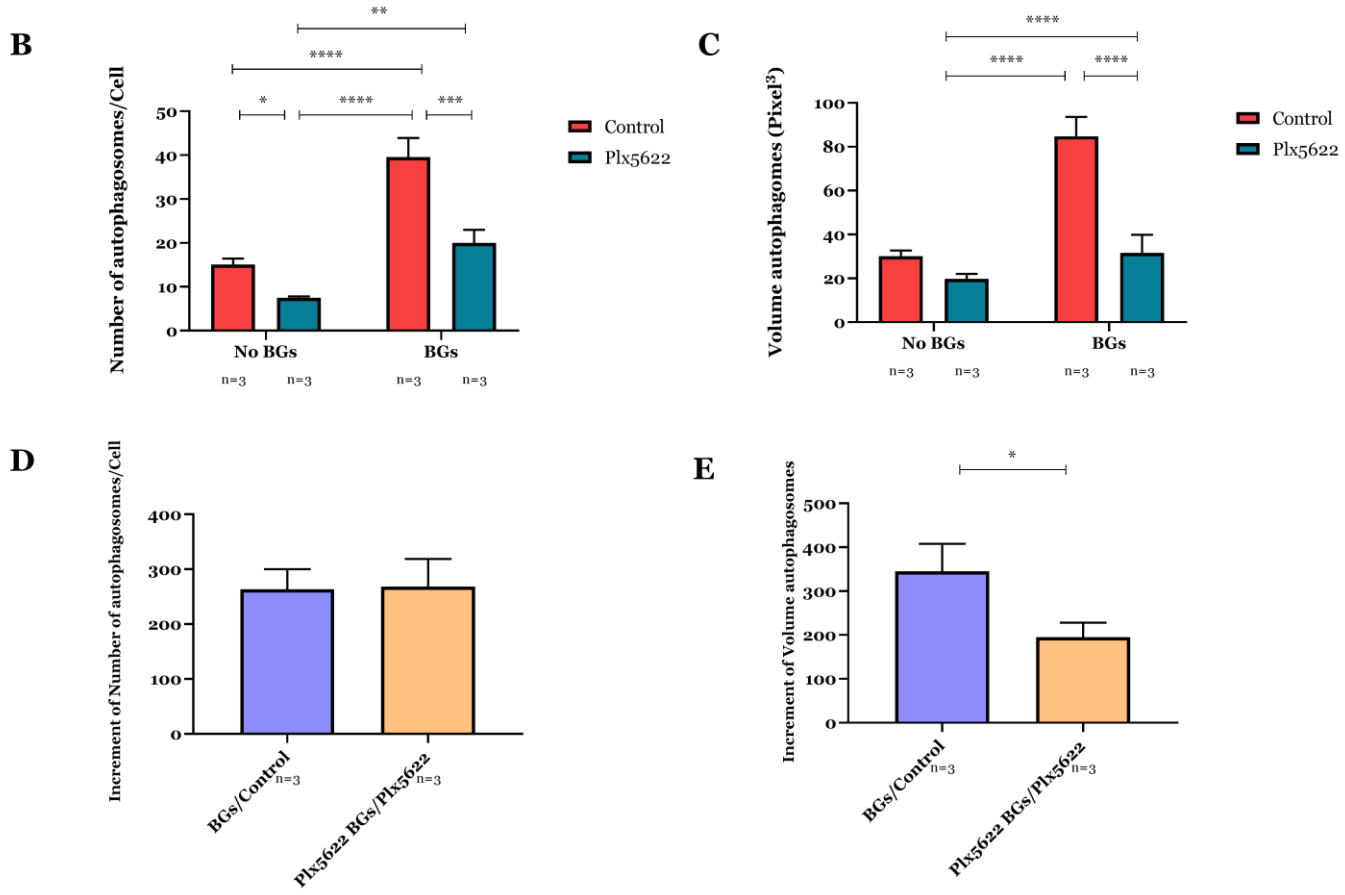


Figure 58: Direct effects of BGs in different cells than Microglia in ex-vivo model. Microglia was blocked by pl5622. A) Representative figure of autophagy in ex-vivo model treated with pl5622, B) Quantification of number of autophagosomes, C) Quantification of volume of autophagosomes, D) Quantification of ratio of BGs/Control and PLX5622 & BGs/PLX5622 about number of autophagosomes, E) Quantification of ratio of BGs/Control and PLX5622 & BGs/PLX5622 about volume of autophagosomes

4.5 Chapter 5 role of human BGs on iPSC-derived neurons

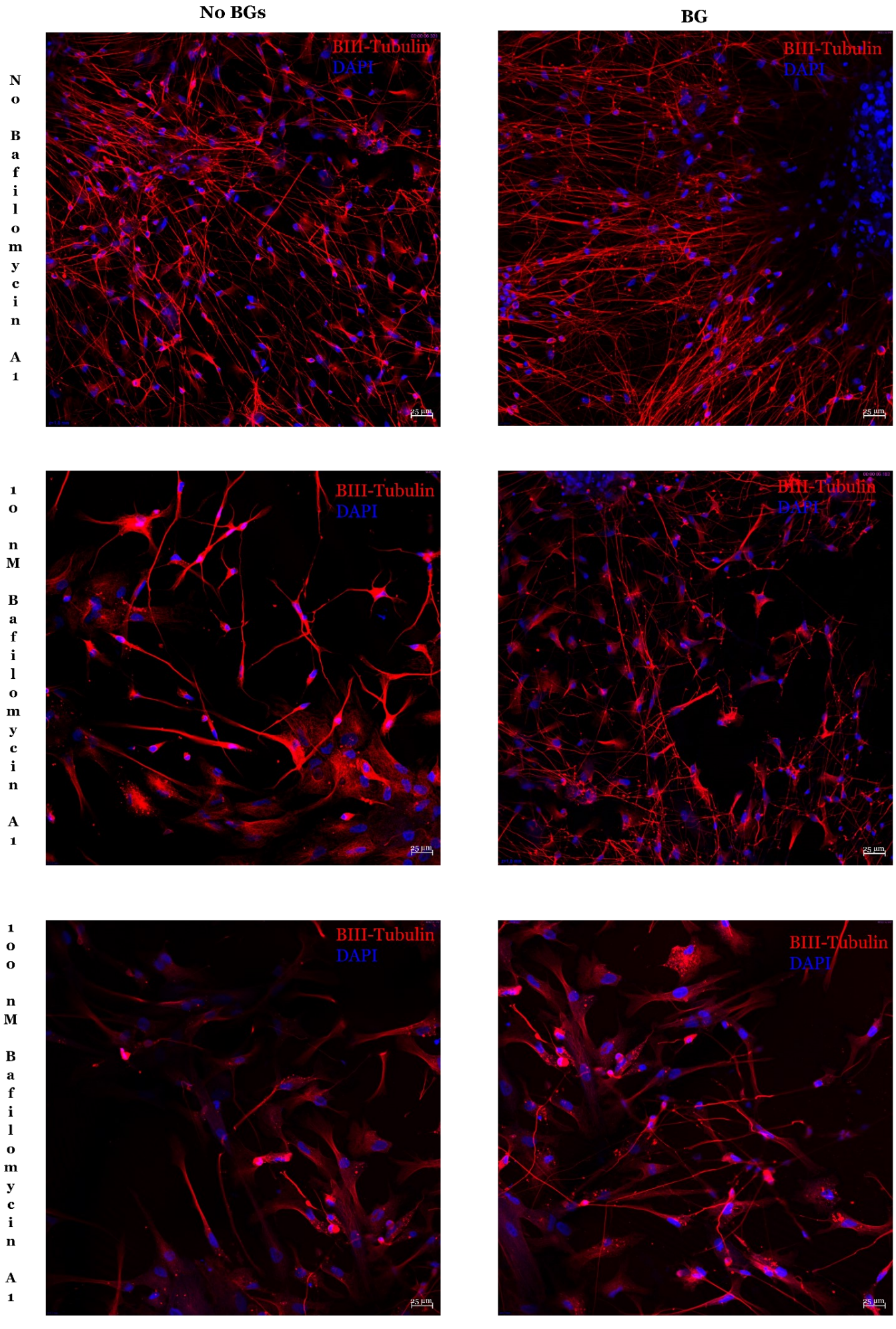
As the previous results demonstrated that BGs effects in killifish are not entirely mediated by microglia, I investigated the effect of BGs on human iPSC-derived cortical neurons. The cells were cultured for 90 days with a differentiation protocol, and they are similar to human foetal neurons. Considering that these neurons do not suffer from impaired of autophagy, I treated human neurons with 10 and 100 nM Bafilomycin A1 for three days to block autophagy.

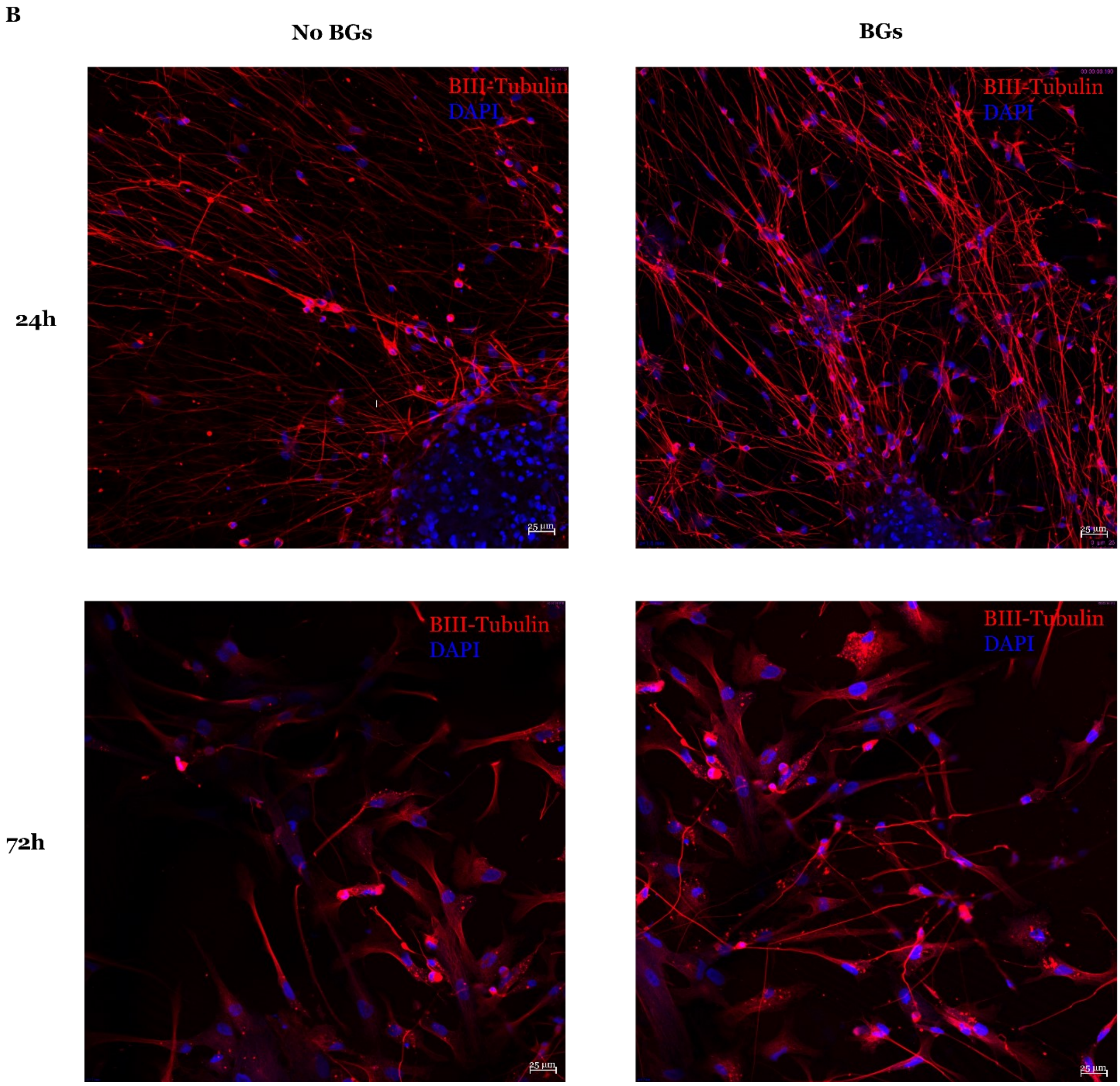
4.5.1 BGs improve survival of iPSC-derived neurons after autophagy inhibition

Both doses of Bafilomycin A1 for three days reduce the number of viable neurons as compared to control [p<0.0001, Two-Way ANOVA; (Figs. 59A-D)]. On the other hand, treatment with 100 nM Bafilomycin A1 for 24h did not induce neuronal mortality as

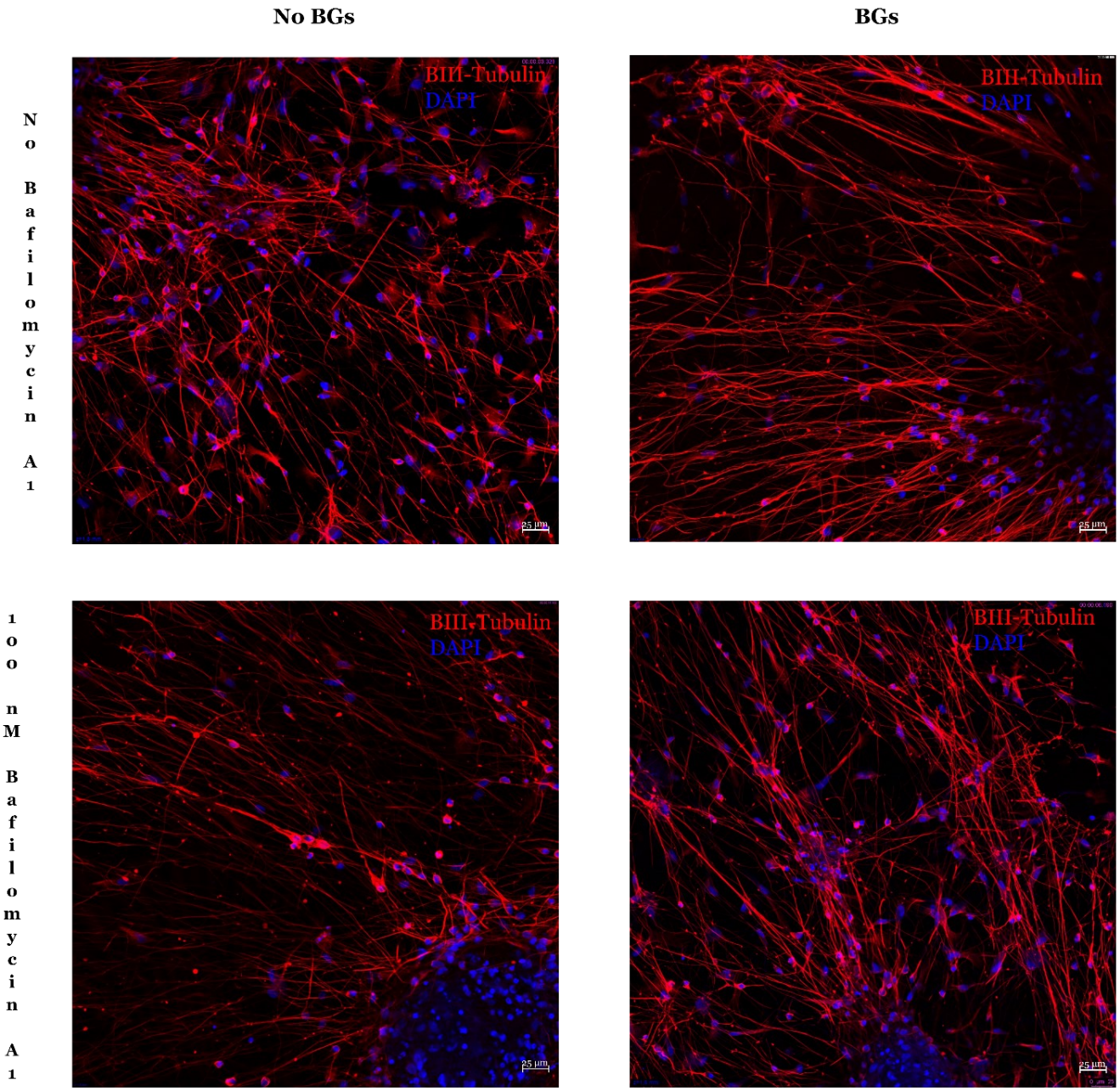
compared to control [p=0.5189, Two-Way ANOVA; (Figs. 59B-D)]. BGs significantly reduce neuronal mortality in neurons treated for 3 days with 10 nm (p=0.037, Two-Way ANOVA), but not 100 nm Bafilomycin A1 (p=0.3644). In addition, BGs did not improve neuronal survival in conditions where mortality is not increase, such as control condition (p=0.7417, Two-Way ANOVA) and during the treatment of Bafilomycin A1 at 100 nm for 24h (p=0.9573, Two-Way ANOVA).

A


No BGs
BG
No Bafilomycin A1
10 nM Bafilomycin A1
100 nM Bafilomycin A1
BIII-Tubulin
DAPI
25 µm


B
No BGs
BGs
24h
72h
BIII-Tubulin
DAPI
25 µm

C


No BGs
BGs
No Bafilomycin A1
100 nM Bafilomycin A1
BIII-Tubulin
DAPI
25 µm

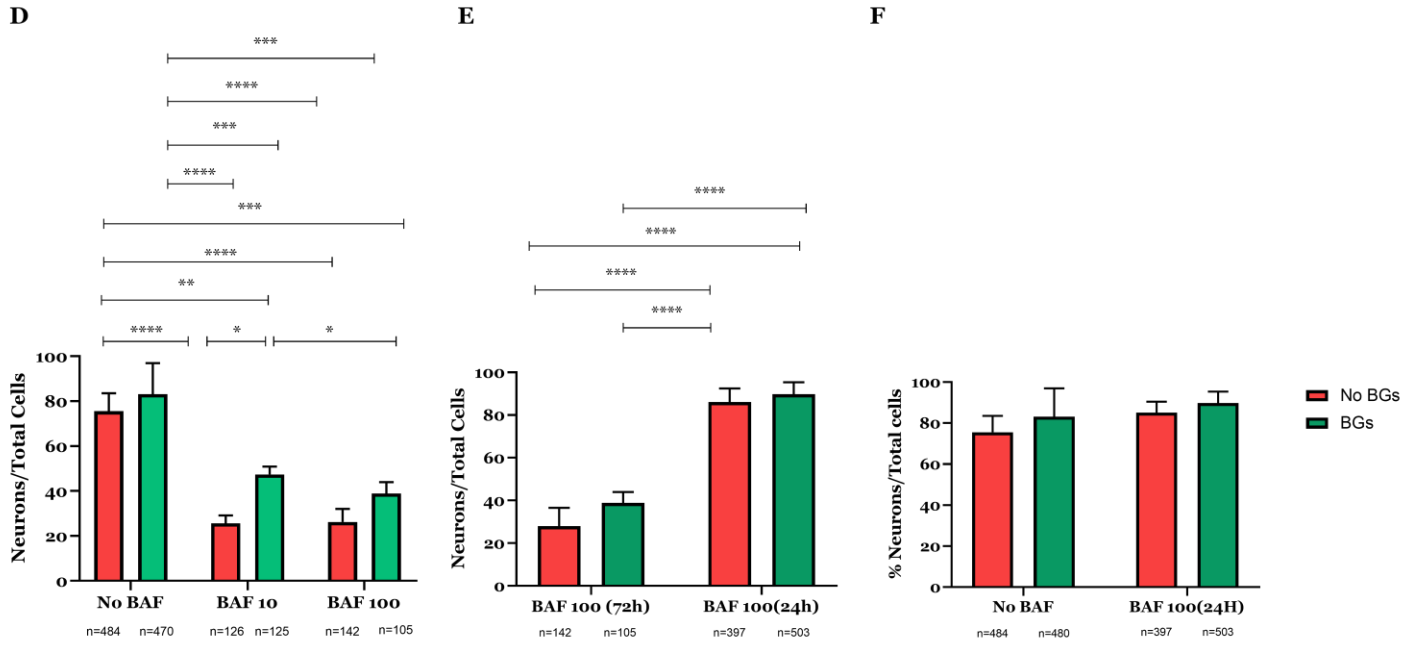
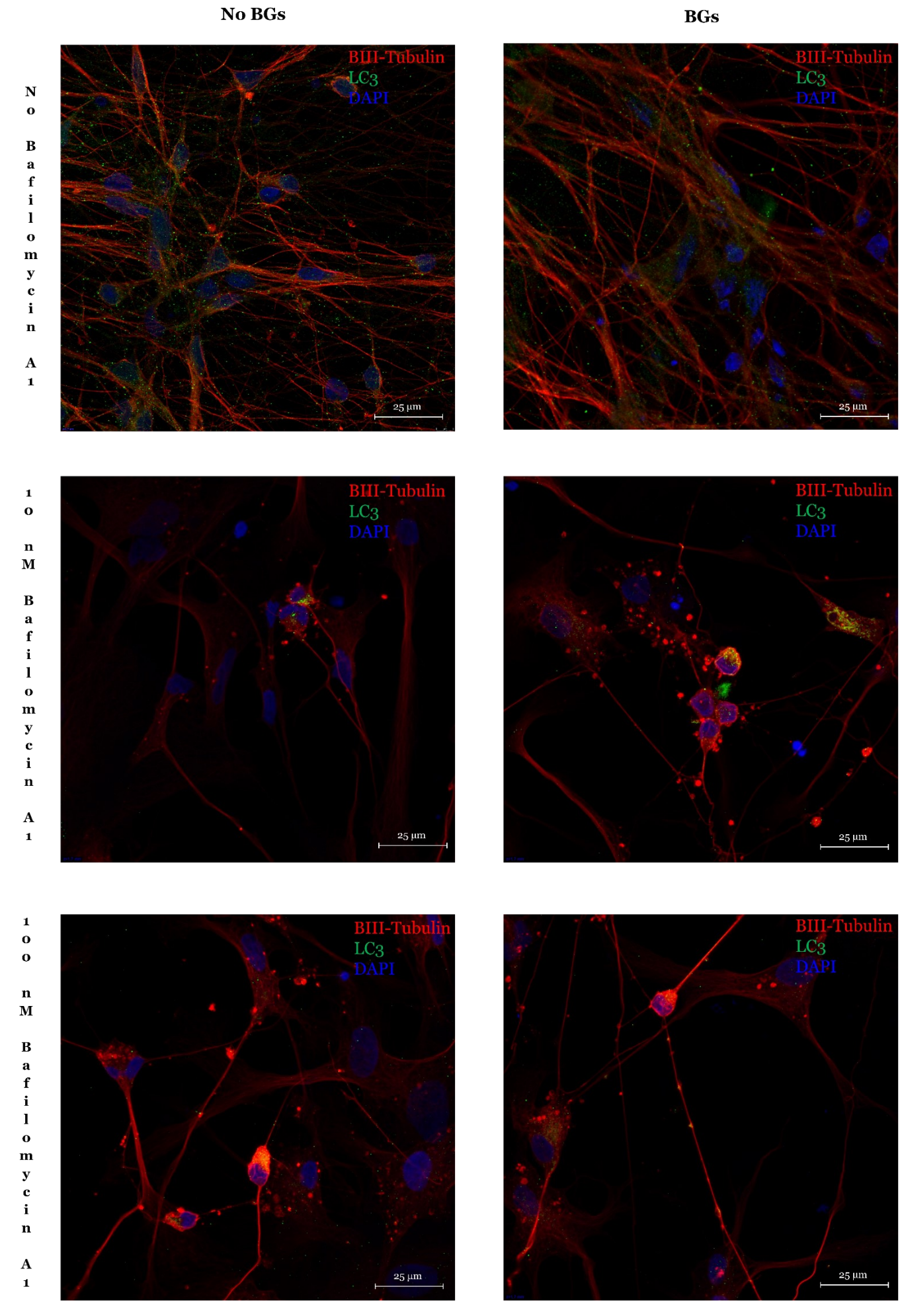


Figure 59: Effects of Bafilomycin and or BGs on neuronal mortality. A) Representative figure of 72 hours of treatments, B) Representative figure of comparison between treatment of 100 nM Bafilomycin A1 and BGs 72 and 24 hours, C) Representative figure 24 hours of treatments, D) Quantification of 72 hours of treatments, E) Quantification of comparison between treatment of 100 nM Bafilomycin A1 and BGs 72 and 24 hours, F) Quantification of 24 hours of treatments. BAF indicates Bafilomycin A1. Numbers below of graphs indicate total number of cells

4.5.2 BGs counteract impaired autophagy in iPSC-derived neurons

BGs do not induce autophagy process under normal condition control unperturbed neurons (p=0.9918), consistently with the observation in *Nfu* that BGs act only when autophagy is impaired. Previous studies showed that blockade of autophagy for 48h by Bafilomycin A1 in iPSC involves mortality and a reduction of LC3 (Sotthibundhu et al., 2016). Consistently, both doses of Bafilomycin A1 caused a reduction of Number LC3/area of Beta III tubulin compared to control [p=0.0102; p=0.0482 at increasing doses respectively, Two-Way ANOVA; (Figs. 60A-D)]. BGs did not increase autophagy after treatment with Bafilomycin A1 10 and 100 nM for 3 days (p=0.6801 and p<0.9999, respectively, Two-Way ANOVA). On the contrary, acute treatment with Bafilomycin A1 at 100 nM for 24 hours increased the number LC3/area of Beta III tubulin as compared to control unperturbed neurons (p=0.0210). Combined treatment with Bafilomycin A1 and BGs increased the Number of LC3 / Area of Beta III Tubulin as compared to Bafilomycin A1 alone (p=0.0343, Two-Way ANOVA). These results indicate that BGs increase the formation of autophagosomes in human neurons in conditions when autophagy is impaired, similarly to what observed in the *Nfu*.

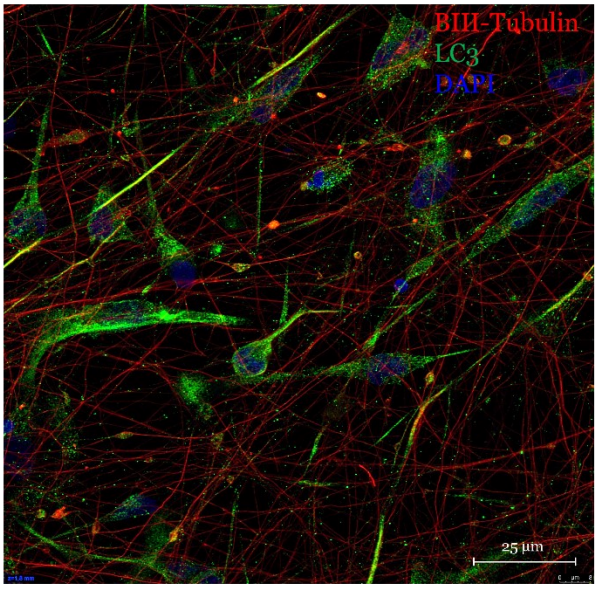
A

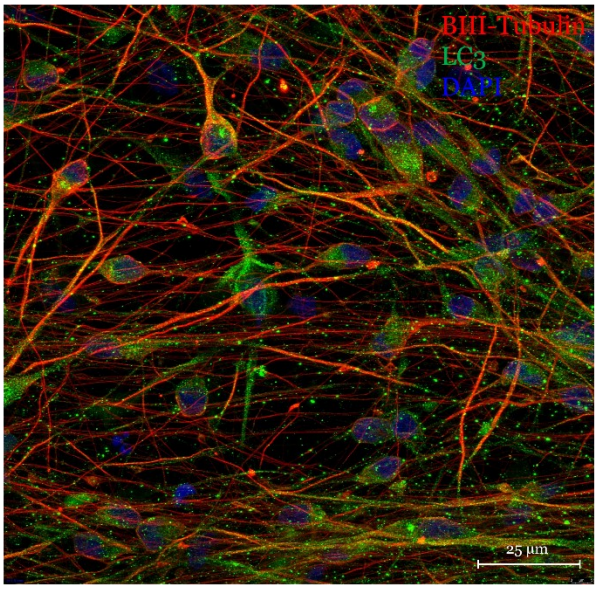

No BGs
BGs
No Bafilomycin A1
10 nM Bafilomycin A1
100 nM Bafilomycin A1
BIII-Tubulin
LC3
DAPI
25 µm

B

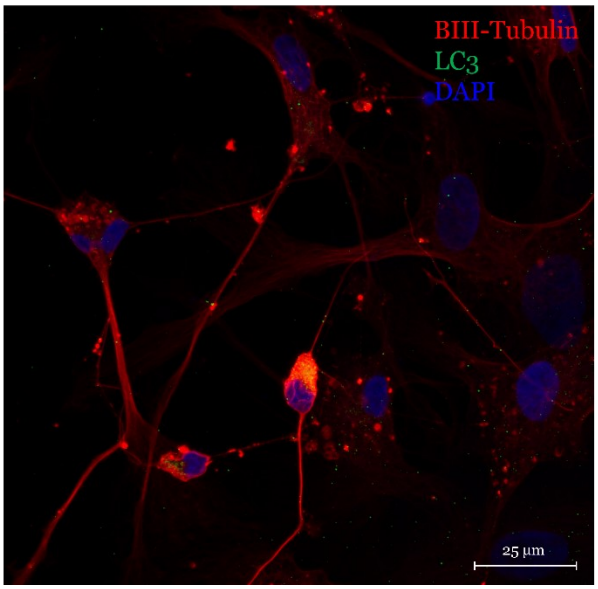
No BGs **BGs**

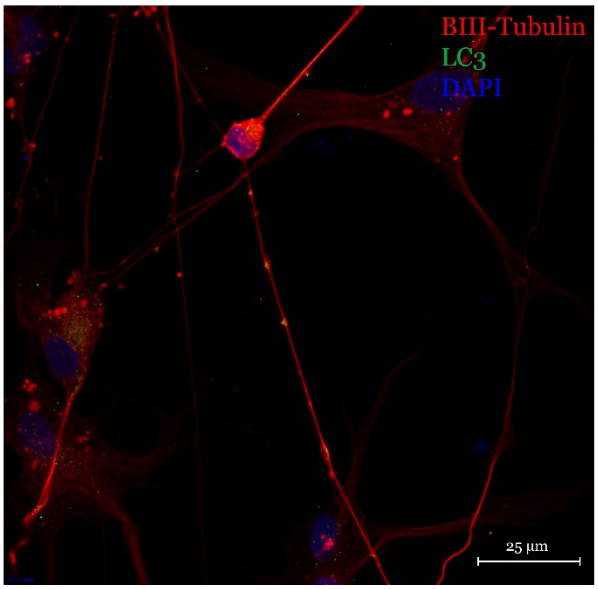
24h



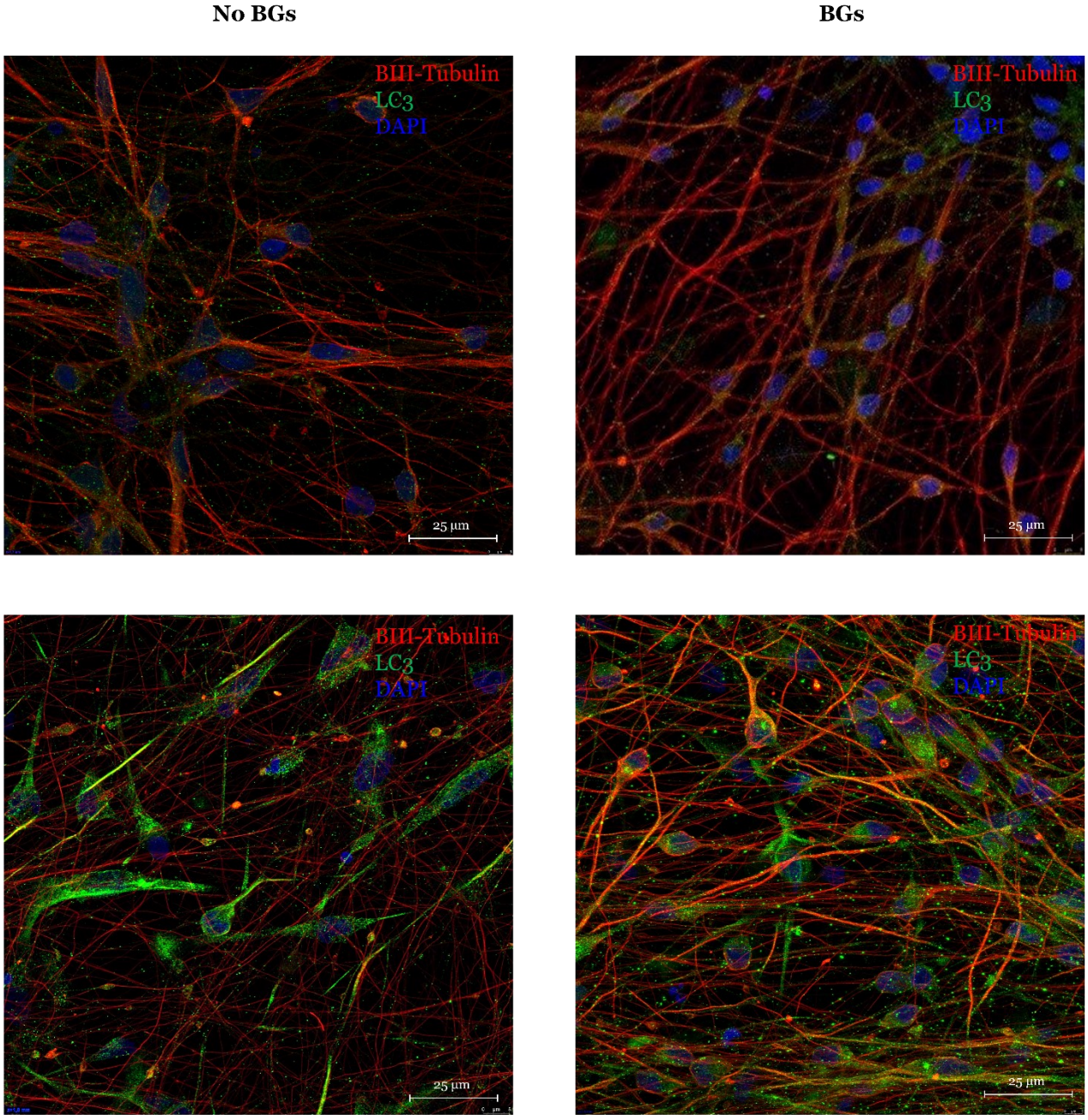


72h





C


No BGs
BGs
No Bafilomycin A1
100 nM Bafilomycin A1
BIII-Tubulin
LC3
DAPI
25 µm

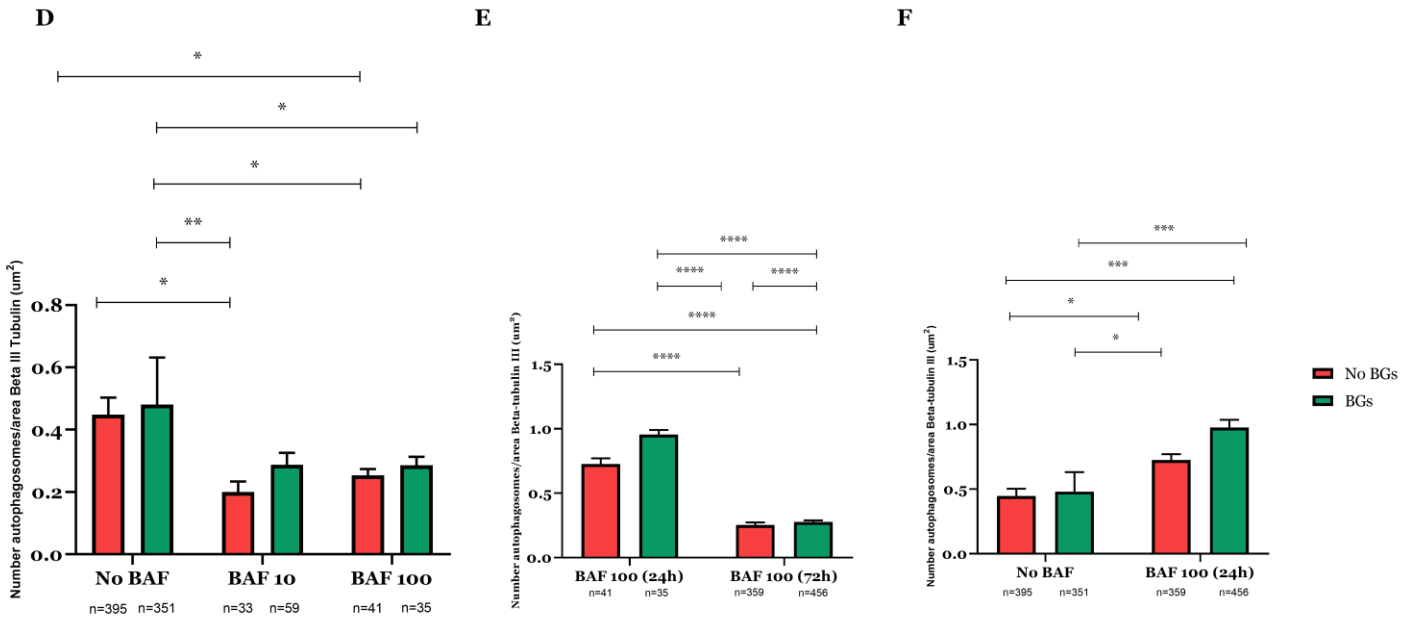


Figure 60: Effects of BGs on neuronal autophagy impaired by Bafilomycin A1. A) Representative figure of 72 hours of treatments, B) Representative figure of comparison between treatment of 100 nM Bafilomycin A1 and BGs 72 and 24 hours, C) Representative figure 24 hours of treatments, D) Quantification of 72 hours of treatments, E) Quantification of comparison between treatment of 100 nM Bafilomycin A1 and BGs 72 and 24 hours, F) Quantification of 24 hours of treatments. BAF indicates Bafilomycin A1. Numbers below of graphs indicate total number of neurons

4.6 Chapter 6 Proteomics analysis of ex-vivo model

Identification of differentially expressed proteins in the *ex-vivo* model from young, adult and aged brains treated with BGs

In order to evaluate the global effect of ages and BGs on proteins of cultured brain slices, the dimensionality of the data was reduced by principal component analysis (PCA). Overall, the PC1 axis (29.7% of variance explained) didn't show strong dissimilarity, although BGs at 27 wph (BG27) shows dissimilarity compared other groups (Fig. 61). Moreover, control at 27 wph show dissimilarity compared control at 5 and 10 wph.

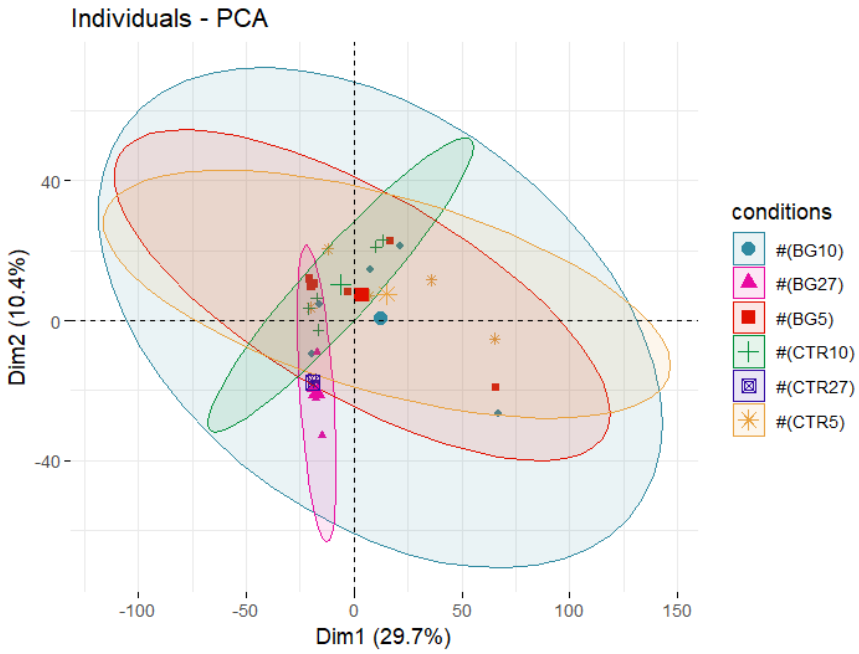


Figure 61: Principal components analysis (PCA) of proteomics data of cultured brain slices with difference ages and treatment of BGs

Mass-spectroscopy based proteomics was used to evaluate the effects of acute ex-vivo treatment with BGs on global protein regulation at 3 different ages: 5, 10 and 27 wph. The number of total detected proteins ranged around 4000 and was not significant different ($\chi 2=123$) in the three groups (Fig. 62A), nor was the total number of differentially expressed proteins (DEPs). However, with age a prevalence of up-regulated proteins was detected (Fig. 62C).

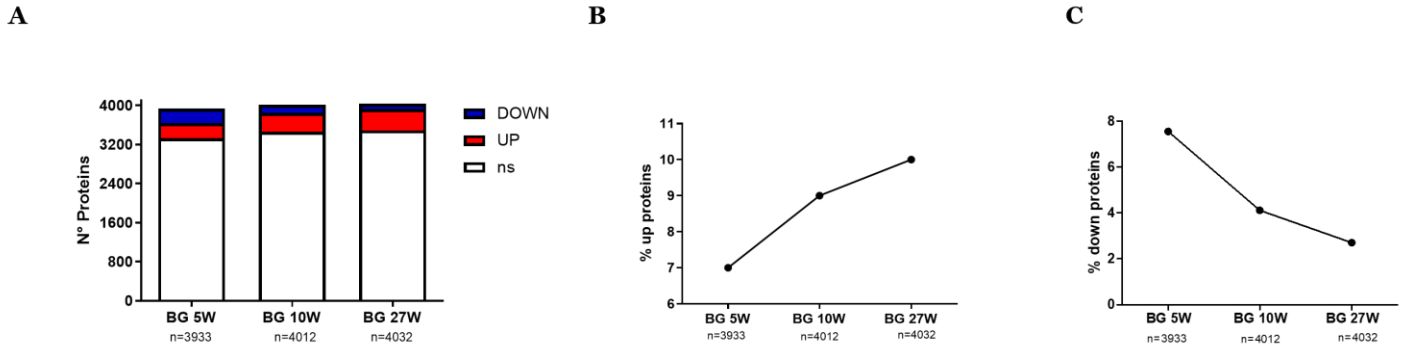


Figure 62: Differentially expressed proteins in ex-vivo model treated with BGs. A) Graph show the number of totals, upregulated and downregulated proteins in 5, 10, and 27 wph treated with BGs, B) Trend of upregulated proteins during aging, C) Trend of down-regulated proteins during aging

Only five proteins are significantly upregulated at all ages: Aconitase 2 (ACO2), Myosin 10 (MYH10), Voltage-dependent anion-selective channel protein 1 (VDAC1), Pyruvate

Carboxylase (PC) and Thioredoxin domain containing 2 (TXNDC2) (Fig. 63). The latter human protein is sperm specific, so I realigned the *Nfu* protein with the human one and found that TXN, or Thioredoxin isoform 1, has greater similarity than other proteins, including TXNDC2. ACO2 and PC are enzymes the TCA cycle. MYH10 is a conventional non-muscle myosin that plays an important role in cytoskeleton reorganization enriched in neurons and specifically in dendritic spines (Korobova and Svitkina, 2010), but it is also involved in neuronal autophagy (Jun et al., 2023). MYH10 also supports mitochondrial homeostasis in situations of cellular stress. VDAC1 encodes a voltage-dependent anion channel protein that is a major component of the outer mitochondrial membrane. The proteins described above are all associated with mitochondrial respiration and ATP production. TXN is mitochondrial protein that plays an important role in diverse cellular functions such as maintaining redox homeostasis, proliferation, and DNA synthesis, but also modulating transcription factors and controlling cell death (Oberacker et al., 2023). Specifically, TXN has a neuroprotective role from oxidative stress (Wang et al., 2016; Guo et al., 2021; Cha et al., 2023).

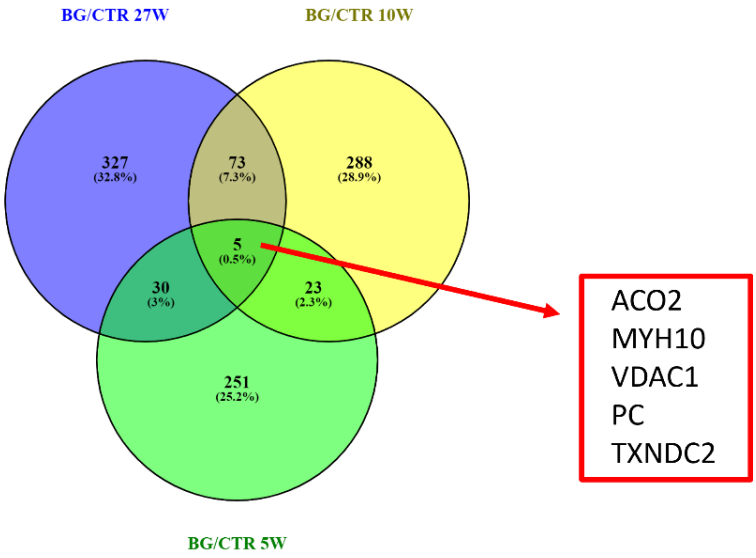


Figure 63: Upregulated proteins in common between different ages in ex-vivo model treated with BGs.

No downregulated proteins are common between different ages in ex-vivo model treated with BGs (Fig. 64).

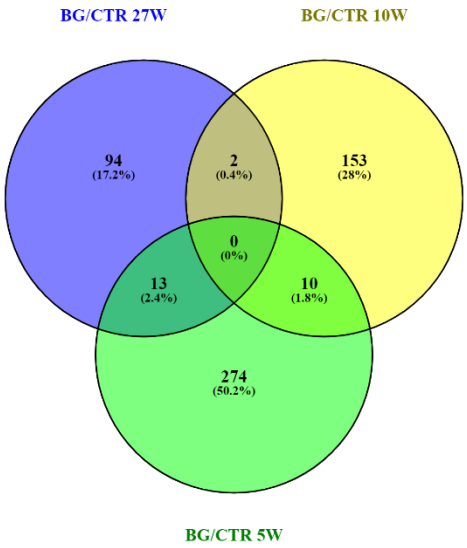


Figure 64: Down-regulated proteins in common between different ages in ex-vivo model treated with BGs.

After observing the upregulated proteins in common by the treatment of BGs at different ages, I analysed the Cellular Components (CCs) in common (Fig. 65). About 23% of CCs are in common at different ages after BGs treatment. In detail, CCs in common are associated with the cytoskeleton, mitochondria, and synapses.

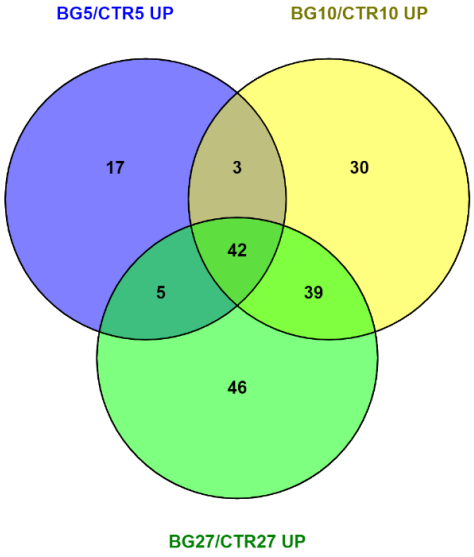


Figure 65: Up-regulated CCs in common between different ages in ex-vivo model treated with BGs.

For contrary, 7.46% of Cellular components are in common between downregulated protein by BGs in different ages (Fig. 66). CCs in common are mainly associated with extracellular region and cytoplasm.

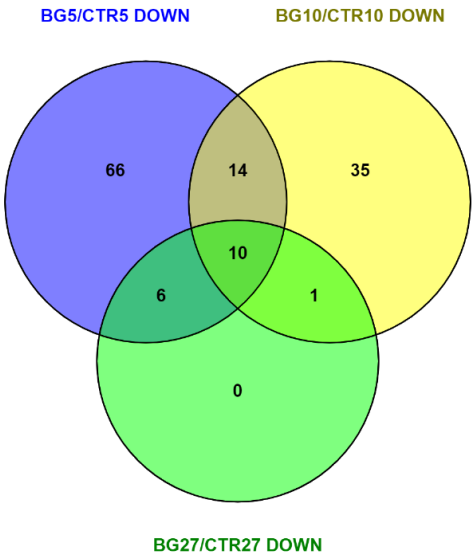


Figure 66: Down-regulated CCs in common between different ages in ex-vivo model treated with BGs.

At this point, I observed the differently expressed proteins in common between normal aging (CTR27/5) and the treatment of BGs at different ages, specifically BG5/CTR5 or BG10/CTR10 or BG27/CTR27.

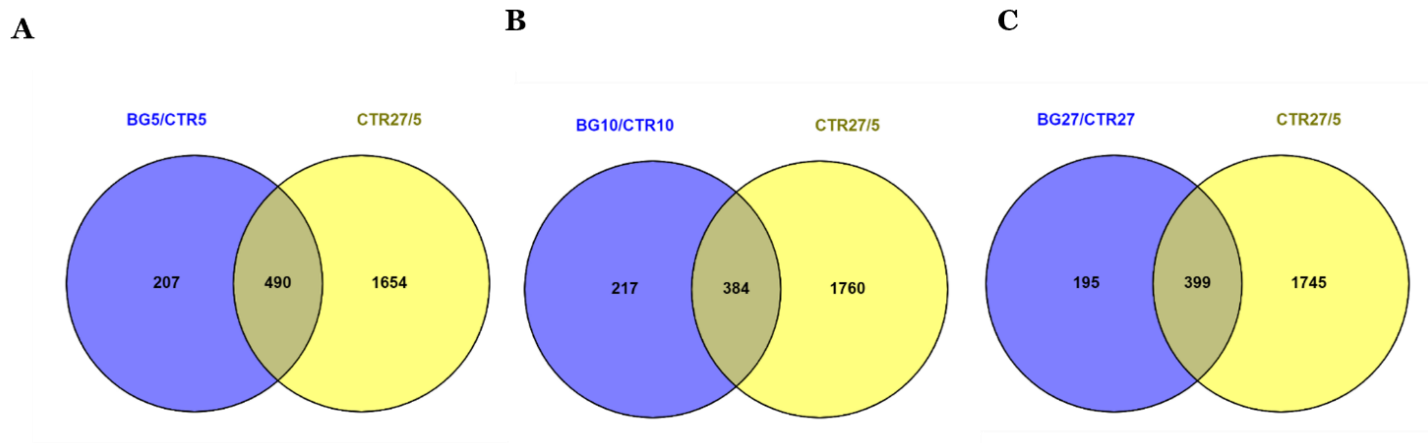


Figure 67: DEPs in common between normal aging and treatment of BGs in different ages. A) 5wph, B) 10 wph, C) 27 wph

The DEPs in common between BGs at 5 wph and normal aging are 22%. The DEPs in common with BGs at 10 wph are 17.9% and those between BG 27 wph and normal aging are 18.6% (Fig. 67). I observed at the direction (up or downregulation) of DEPs for each age treated with BGs and aging (Fig. 68).

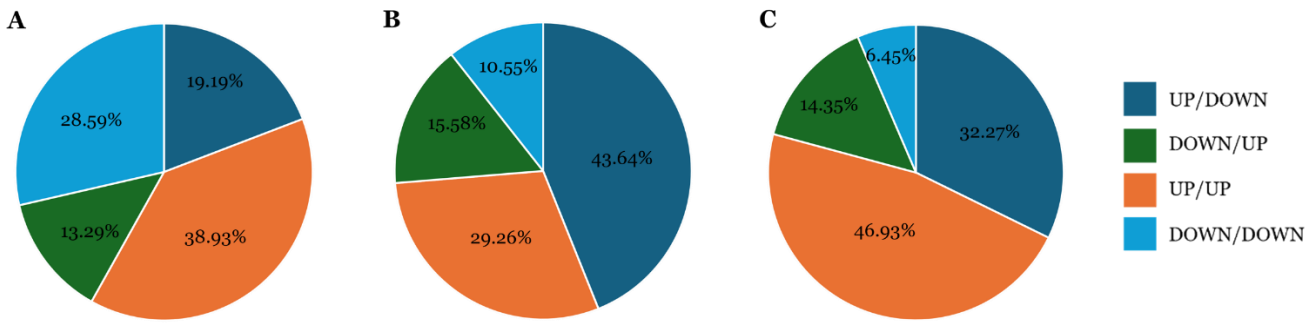


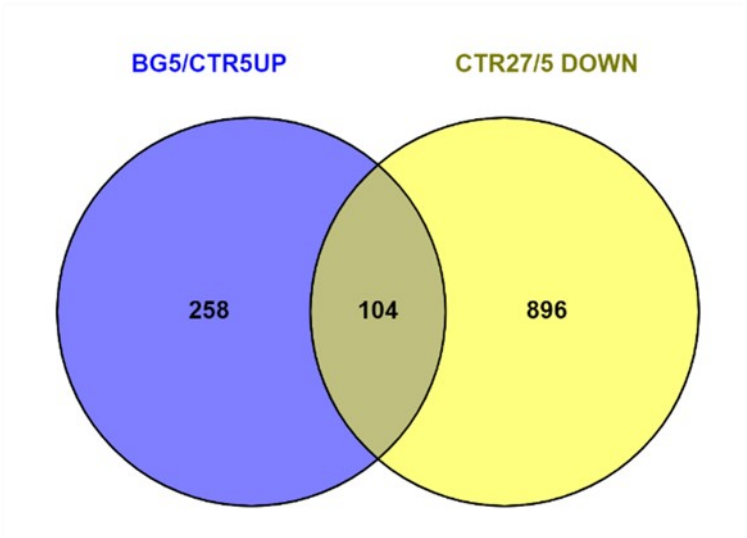
Figure 68: Direction of DEPs for each age treated with BGs and normal aging. A) 5wph, B) 10 wph, C) 27 wph

At all 3 ages we observe a greater reverse direction with normal aging. I focus on DEPs downregulated with aging are upregulated by BGs. At 5 wph 19.19%, at 10 wph 43.64% and at 27 wph 32.27% are upregulated by BGs and downregulated by normal aging. DEPs upregulated with aging and downregulated by BGs are 13.29% at 5 wph, 15.58% at 10 wph and 14.35% at 27 wph. For contrary, I observed that 38.93% at 5 wph, 29.26% at 10 wph and 46.93% at 27 wph are both upregulated by BGs and in normal aging. In addition, 28.59% at 5 wph, 10.55% at 10 wph and 6.45% at 27 wph are both downregulated by BGs and in normal aging.

4.6.1 Restoration of DEPs during normal aging by treatment with BGs

I focused on DEPs with reverse direction to know which proteins or CCs dysregulated with aging were restored by BGs at different ages. I clustered proteins downregulated during normal aging and upregulated by BGs at 5 wph using protein-protein interaction (PPI) analyses performed in STRING. These proteins formed a structure network with significantly non-random connectivity (PPI entrenchment $p<1\ e^{-6}$). At 5 wph BGs restore 28.7% of proteins associated with Mitochondria, Protein-Folding and Synapses (Fig. 69). Over Expression Analyses (ORA) didn't find any about Biological Processes (BPs) regulated by BGs. Furthermore, CCs not dysregulated by aging but upregulated by BGs at 5 wph (71% upregulated DEPs) are predominantly associated with Ribosomes (Translation) and Cytoskeleton.

A


BG5/CTR5UP
CTR27/5 DOWN
258
104
896

B

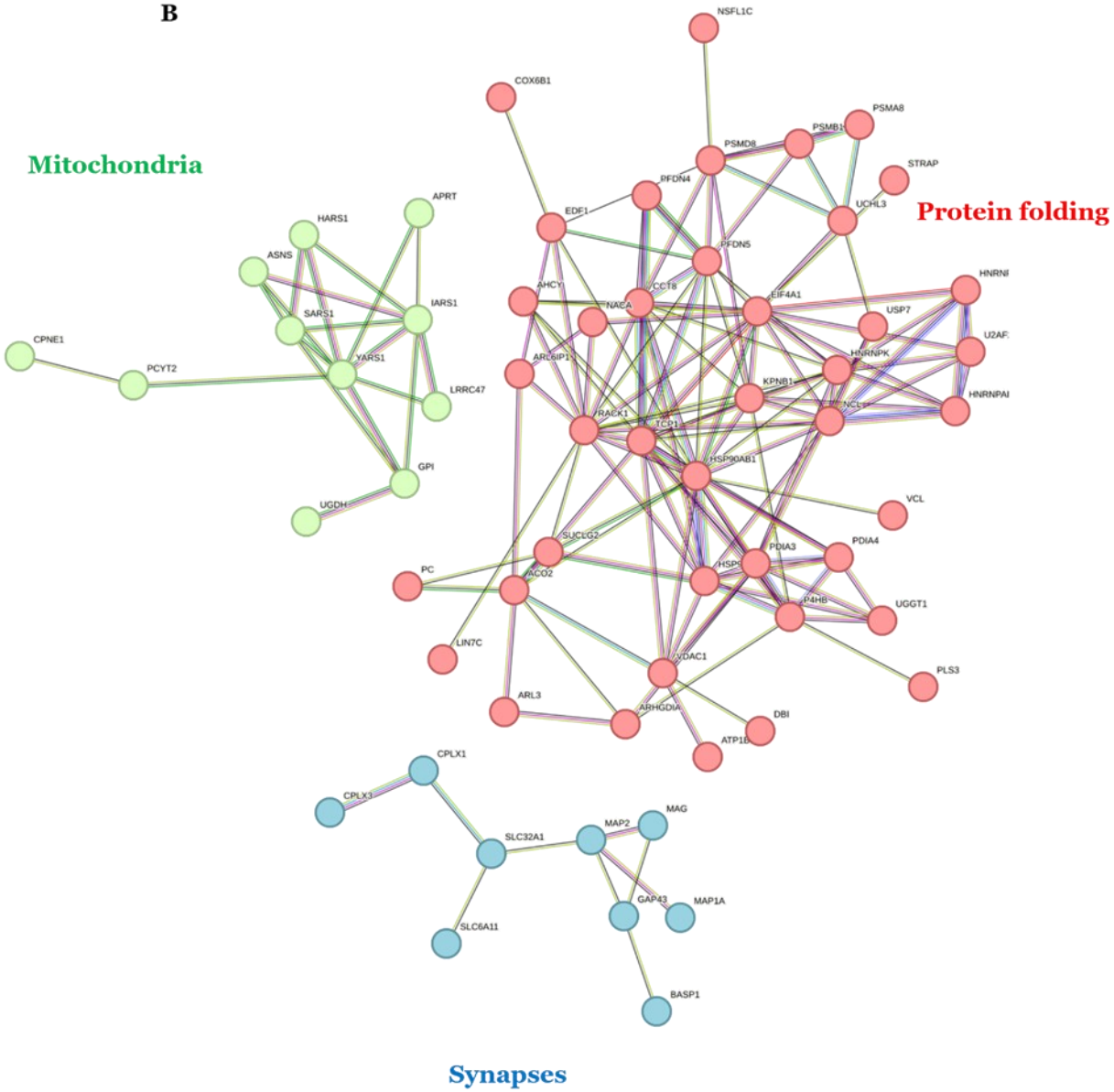
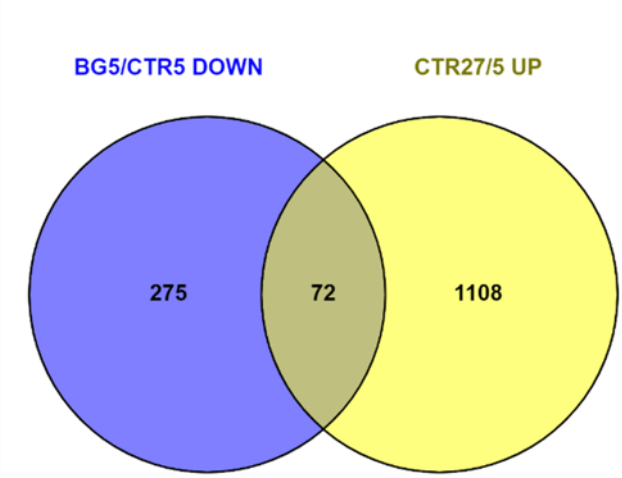

Mitochondria
Protein folding
Synapses
NSFL1C
COX6B1
PSMA8
PSMB1
PSMD8
STRAP
PFDN4
UCHL3
EDF1
PFDN5
HNRNI
AHCY
CCT8
NACA
EIF4A1
USP7
U2AF
ARC6IP1
HNRNPK
KPNB1
HNRNPAI
RACK1
TCP1
HSP90AB1
VCL
SUCLG2
PDIA3
PDIA4
PC
ACO2
HSP
P4HB
UGGT1
LIN7C
VDAC1
PLS3
ARL3
ARHGDIA
DBI
ATP1B
APRT
HARS1
ASNS
IARS1
SARS1
CPNE1
YARS1
PCYT2
LRRC47
GPI
UGDH
CPLX1
CPLX3
MAG
MAP2
SLC32A1
GAP43
MAP1A
SLC6A11
BASP1

Figure 69: DEPs downregulated in aging and upregulated by BGs at 5 wph. A) Venn diagram of DEPs, B) PPI related to CCs in common between aging and BGs treatment at wph

I clustered proteins upregulated during normal aging and downregulated by BGs at 5 wph using PPI performed in STRING (PPI entrenchment $p=2.31e^{-9}$). At 5 wph BGs had effects on 5.85% of proteins associated with Synapses and Endoplasmic Reticulum (ER). Furthermore, CCs not dysregulated by aging but downregulated by BGs at 5 wph (78.39% downregulated DEPs) are predominantly associated with neuronal component, such as GABA receptor complex (Fig. 70).

A



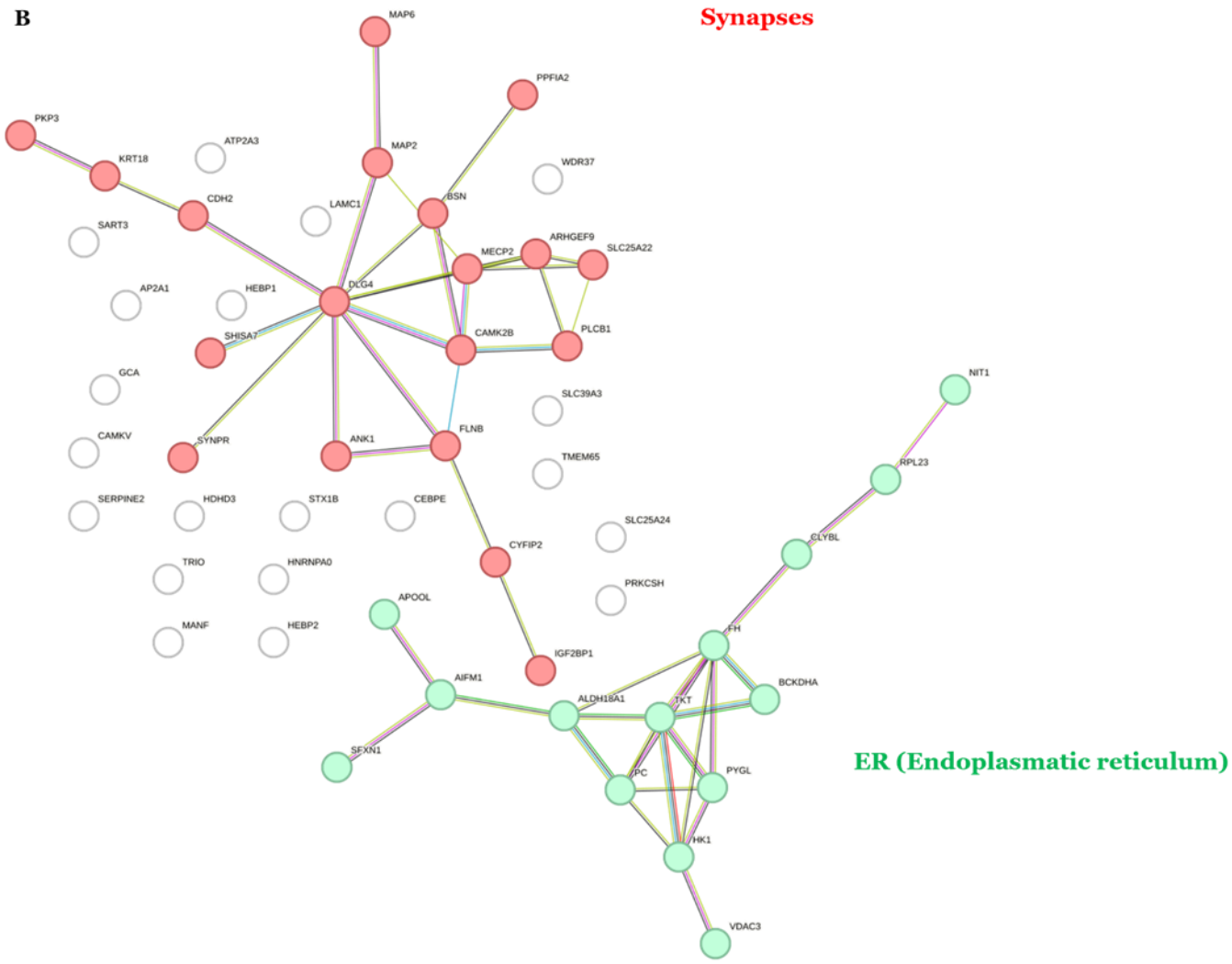
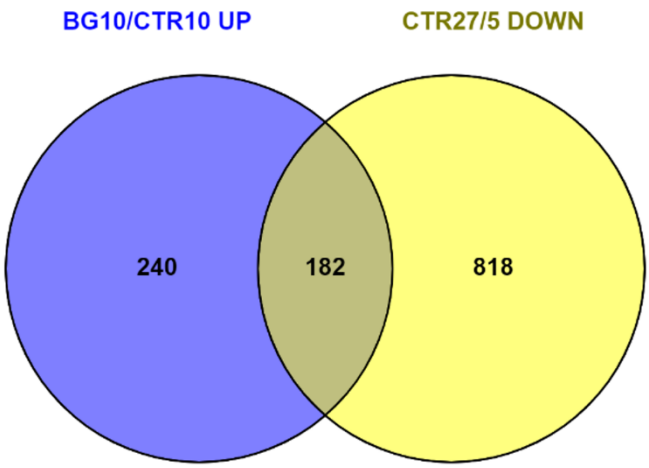


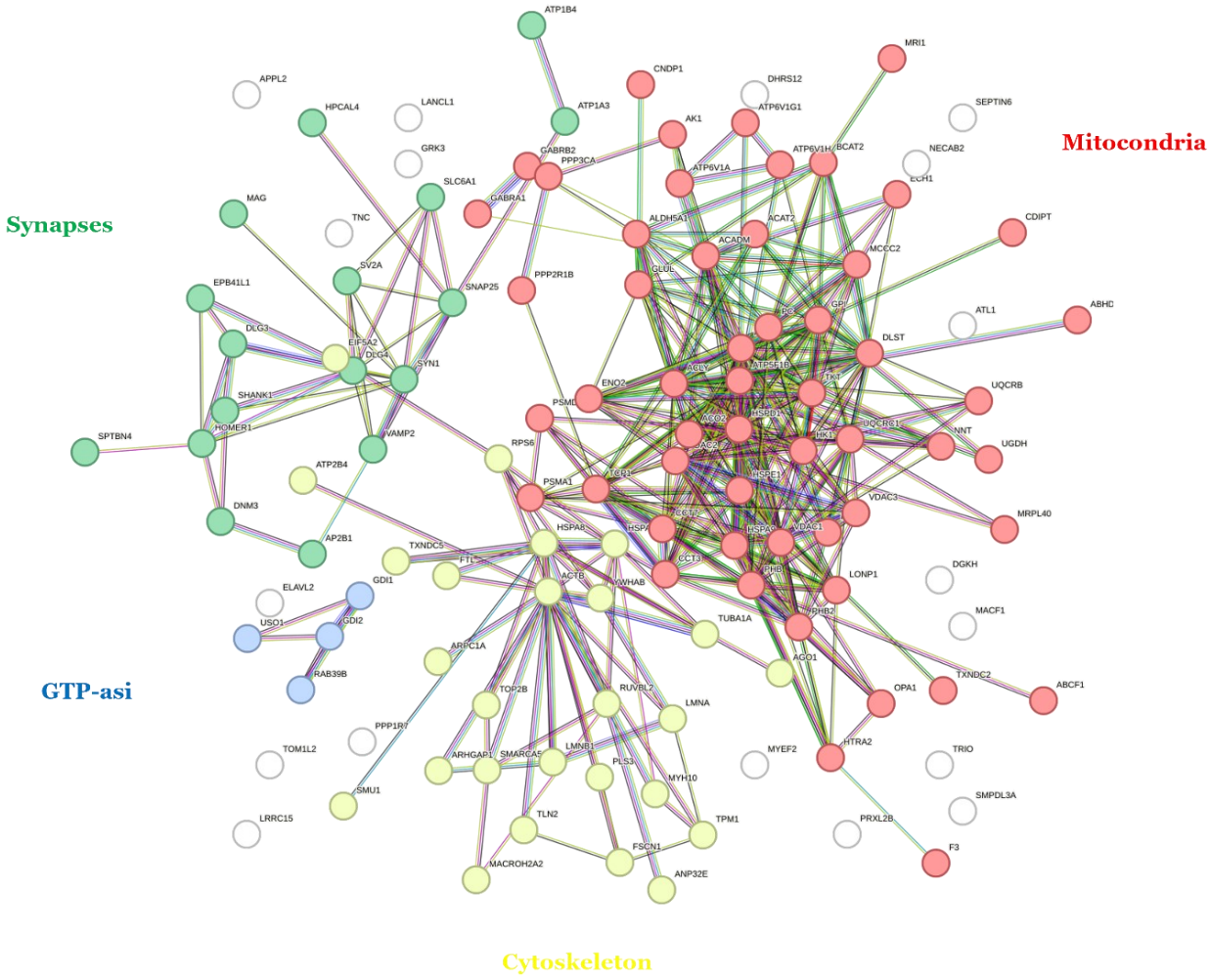
Figure 70: DEPs upregulated in aging and downregulated by BGs at 5 wph. A) Venn diagram of DEPs, B) PPI related to CCs in common between aging and BGs treatment at 5 wph

I clustered proteins downregulated during normal aging and upregulated by BGs at 10 wph using PPI (PPI entrenchment $p<1\ e^{-6}$). At 10 wph BGs restore 18.2% of proteins associated with Mitochondria, Cytoskeleton, Synapses and GTP-asi (Fig. 71). Moreover, ORA shown BPs regulated by BGs, such as protein-folding, synapses organization, maintenance local of cell and transport (Fig. 67C). Furthermore, CCs not dysregulated by aging but upregulated by BGs at 10 wph (56.87% upregulated DEPs) are again associated with Mitochondria, Synapses, and Spliceosome.

A


BG10/CTR10 UP
CTR27/5 DOWN
240
182
818

B


Synapses
Mitocondria
GTP-asi
Cytoskeleton
ATP1B4
MRI1
CNDP1
APPL2
DHRS12
HPCAL4
LANCL1
ATP1A3
ATP6V1G1
SEPTIN6
AK1
GRK3
GABRB2
PPP3CA
ATP6V1H
BCAT2
NECAB2
ATP6V1A
SLC6A1
ECH1
MAG
GABRA1
TNC
ALDH5A1
ACAT2
CDIPT
ACADM
MCCC2
SV2A
GLUL
PPP2R1B
SNAP25
EPB41L1
ATL1
ABHD
DLG3
EIF5A2
DLG4
SYN1
DLST
ACLY
ATP5F1B
TKT
ENO2
UQCRB
SHANK1
PSMD1
HSD17B4
ACO2
UQCRC1
NNT
HOMER1
SPTBN4
VAMP2
ACO1
HK1
UGDH
RPS6
ATP2B4
TCP1
HSPE1
PSMA1
VDAC3
DNM3
MRPL40
HSPA8
HSPA5
VDAC1
AP2B1
TXNDC5
FTL
CCT3
PHB
DGKH
LONP1
ELAVL2
GDI1
ACTB
WHAB
PHB2
MACF1
USO1
GDI2
TUBA1A
ARPC1A
AGO1
TXNDC2
ABCF1
RAB39B
OPA1
TOP2B
RUVBL2
LMNA
PPP1R7
HTRA2
TOM1L2
ARHGAP1
SMARCA5
LMNB1
MYEF2
TRIO
PLS3
MYH10
SMU1
SMPDL3A
LRRC15
TLN2
TPM1
PRXL2B
FSCN1
F3
MACROH2A2
ANP32E

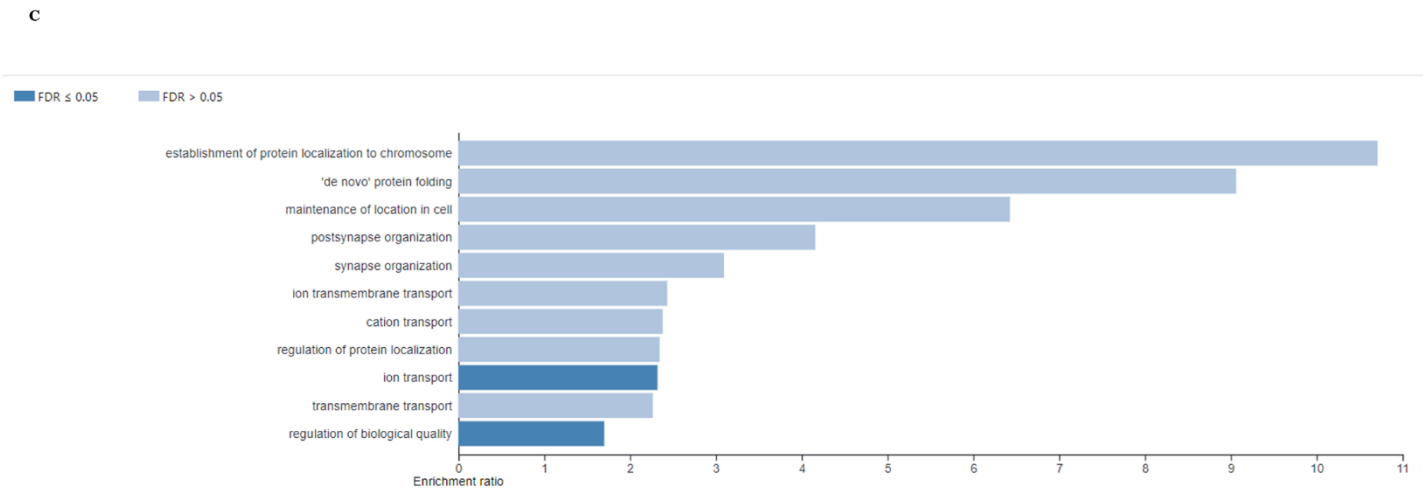
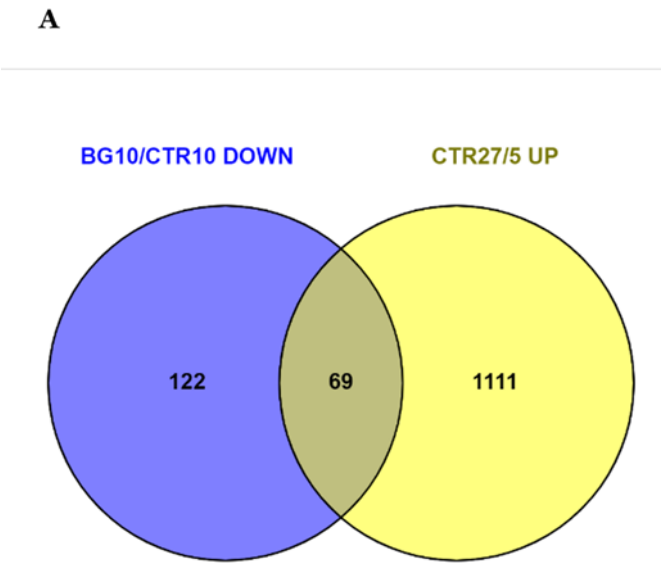


Figure 71: DEPs downregulated in aging and upregulated by BGs at 10 wph. A) Venn diagram of DEPs, B) PPI related to CCs, C) ORA of BPs in common between aging and BGs treatment at 10 wph

I clustered proteins upregulated during normal aging and downregulated by BGs at 10 wph using PPI (PPI entrenchment p=4.5 e^{-5}). At 10 wph BGs affected on 6.1% of proteins associated mainly with Spectrin associated cytoskeleton and Mitochondrial cristae. In detail, β-spectrin accumulation has been observed in amyloid beta plaques in AD (Sihang and Cataldo, 1996). In addition, it was observed an increase of swelling and rupture of mitochondria cristae during aging (Terman and Brunk., 2004). Furthermore, CCs not dysregulated by aging but downregulated by BGs at 10 wph (63.87% downregulated DEPs) are predominantly associated with Supramolecular complex and Collagen (Fig. 72).



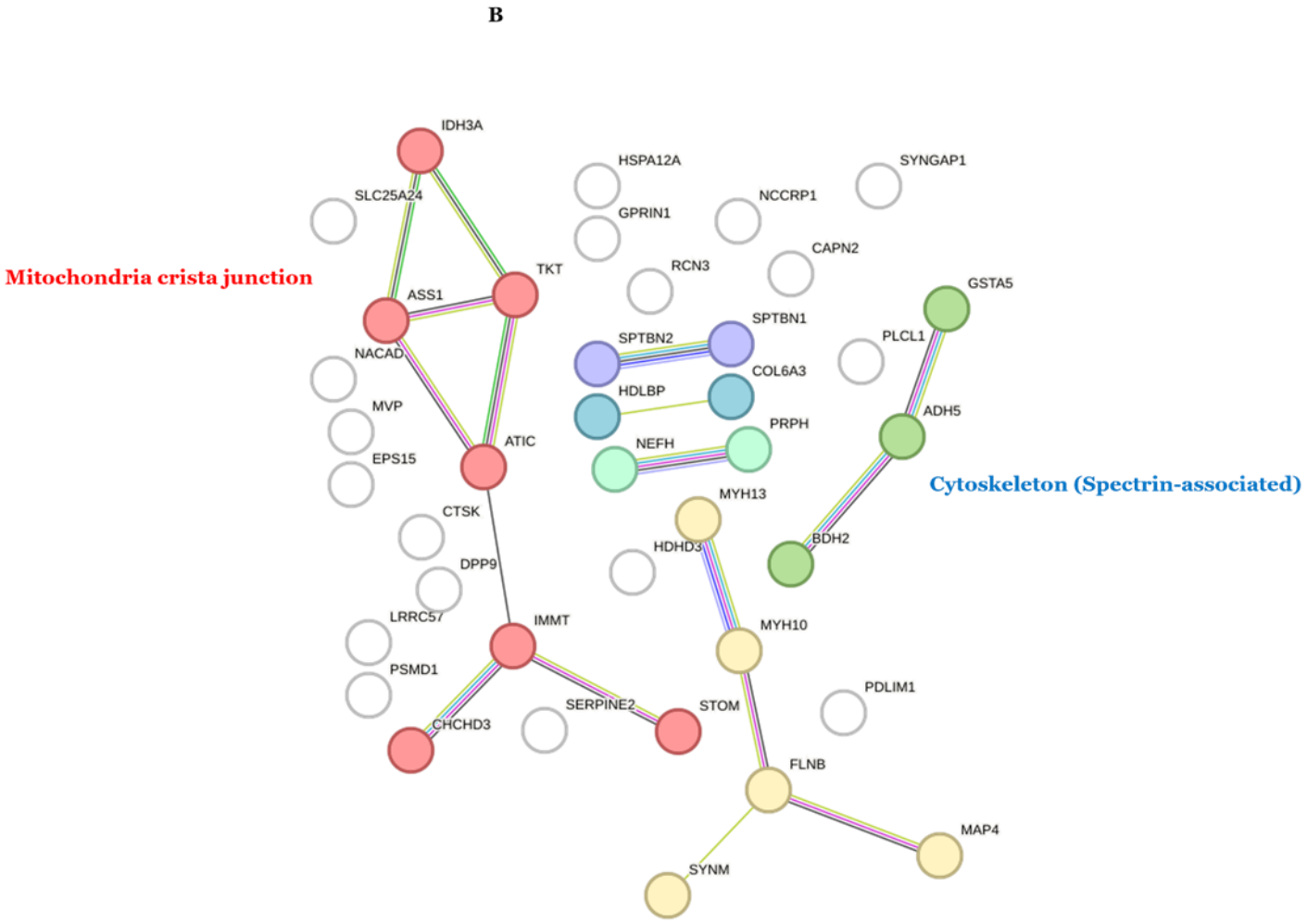
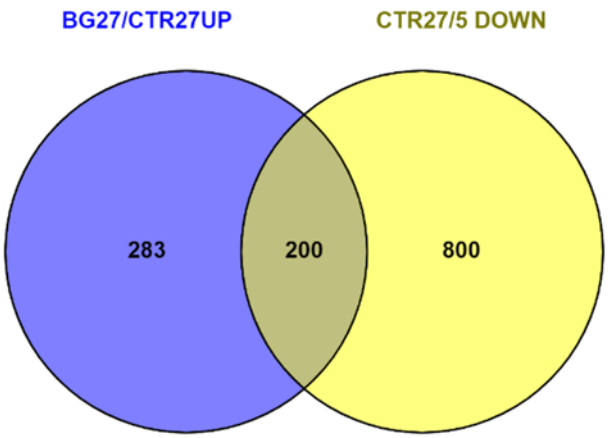


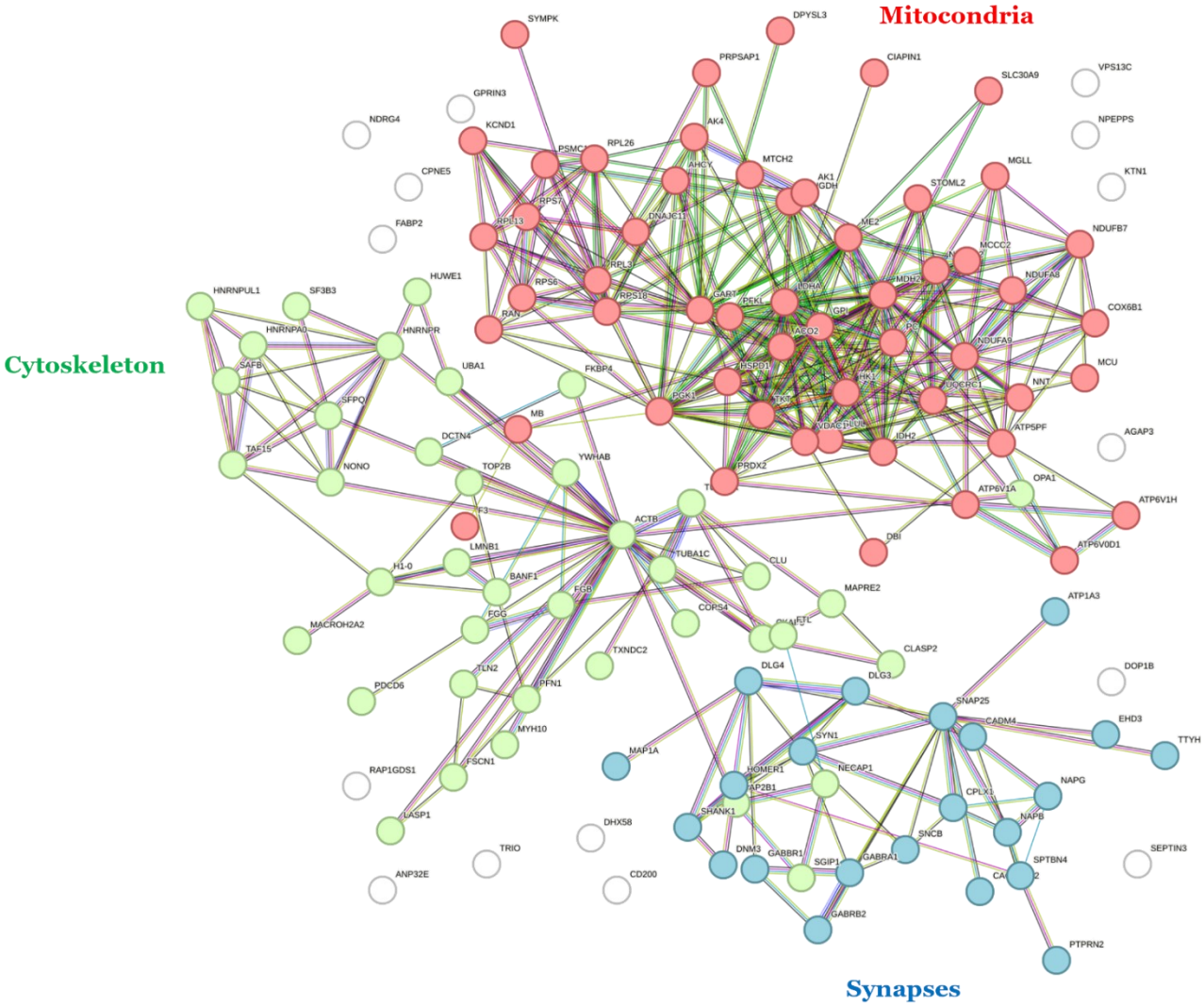
Figure 72: DEPs upregulated in aging and downregulated by BGs at 10 wph. A) Venn diagram of DEPs, B) PPI related to CCs in common between aging and BGs treatment at 10 wph

I clustered proteins downregulated during normal aging and upregulated by BGs at 17 wph using PPI performed in STRING. These proteins formed a structure network with significantly non-random connectivity (PPI entrenchment $p<1\ e^{-6}$). At 27 wph BGs restore 20% of proteins associated with Mitochondria, Synapses and Cytoskeleton (Fig. 73). Moreover, ORA shown BPs regulated by BGs are mainly associated to mitochondrial respiration, such as ATP hydrolysis coupled transmembrane transport, synapses organization, and transport etc (Fig. 73C). Furthermore, CCs not dysregulated by aging but upregulated by BGs at 27wph (41.4% upregulated DEPs) are again associated with Mitochondria, Cytoskeleton and also Ribosomes (Translation).

A


BG27/CTR27UP
CTR27/5 DOWN
283
200
800

B


Mitocondria
Cytoskeleton
Synapses
SYMPK
DPYSL3
PRPSAP1
CIAPIN1
SLC30A9
VPS13C
GPRIN3
NDRG4
KCND1
AK4
NPEPPS
PSMC1
RPL26
MTCH2
CPNE5
AHCY
AK1
MGLL
KTN1
RPS7
STOML2
FABP2
RPL13
DNAJC11
ME2
NDUFB7
MCCC2
HUWE1
RPL3
HNRNPUL1
SF3B3
RPS6
RPS18
NDUFA8
COX6B1
RAN
HNRNPA0
HNRNPR
ACO2
NDUFA9
SAFB
UBA1
FKBP4
MCU
NNT
SFPQ
MB
PGK1
ATP5PF
AGAP3
DCTN4
TAF15
NONO
TOP2B
YWHAB
PRDX2
OPA1
ATP6V1A
ATP6V1H
ACTB
DBI
ATP6V0D1
LMNB1
TUBA1C
CLU
H1-0
BANF1
MAPRE2
ATP1A3
MACROH2A2
CORO1C
CLASP2
TXNDC2
DLG4
DLG3
DOP1B
PDCD6
TLN2
PFN1
SNAP25
CADM4
EHD3
MYH10
SYN1
TTYH
MAP1A
RAP1GDS1
FSCN1
HOMER1
NECAP1
NAPG
AP2B1
CPLX1
LASP1
SHANK1
NAPB
DHX58
SNCB
SEPTIN3
TRIO
DNM3
GABBR1
SGIP1
GABRA1
SPTBN4
ANP32E
CD200
GABRB2
PTPRN2

C

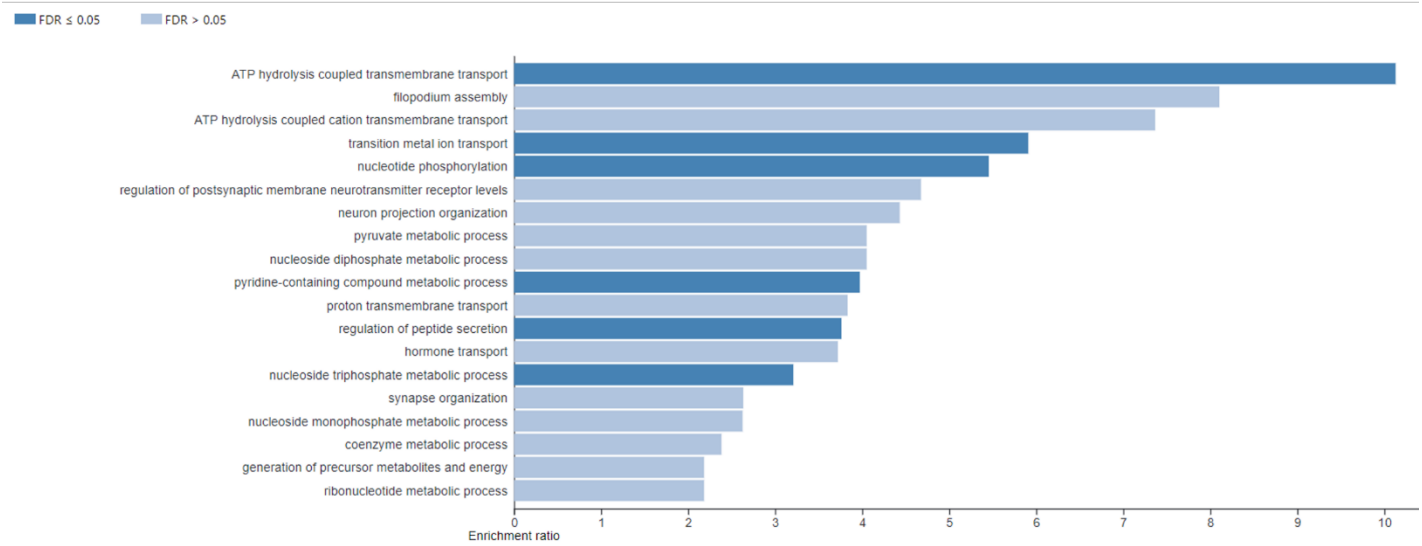
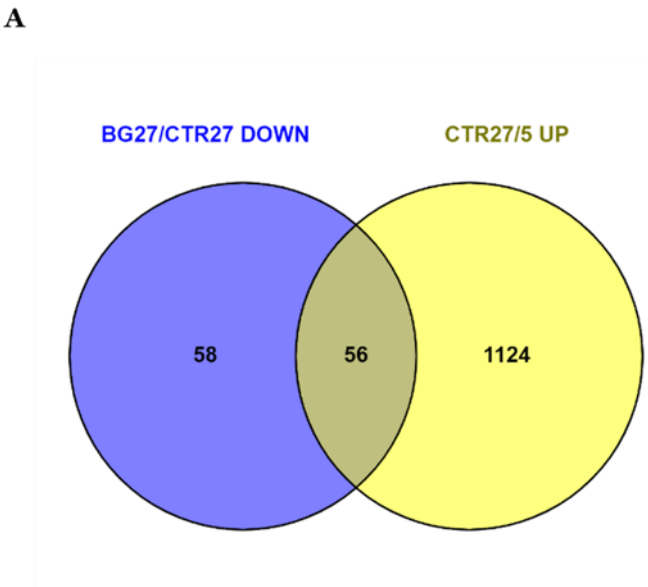


Figure 73: DEPs downregulated in aging and upregulated by BGs at 27 wph. A) Venn diagram of DEPs, B) PPI related to CCs, C) ORA of BPs in common between aging and BGs treatment at 27 wph

I clustered proteins upregulated during normal aging and downregulated by BGs at 27 wph PPI (PPI entrenchment p=0.0125). At 27 wph BGs restore 4.75% of DEPs upregulated during normal aging. These proteins are very heterogeneous even if they are mainly associated with Spectrin-associated cytoskeleton and Extracellular space (Fig. 74). As I observed at 10 wph, BGs downregulated β-spectrin, upregulated during aging. In addition, it is interesting to describe some individual proteins restored by BGs during aging, such as PCNA (Proliferating Nuclear Cell Antigen), DDC (DOPA decarboxylase), SNCA (α-sinuclein), AK2 (Adenylate kinase 2), NT5E (5'-Nucleotidase Ecto). PCNA can also be expressed during repair synthesis. For example, the reactive cell cycle of glial cells is important for the pathogenesis of neurodegenerative diseases related to aging. In fact, it has been observed that PCNA increases during aging and AD mainly in glia (Wharton et al., 2005). I observed that BGs restore the upregulation of PCNA during aging. DDC has been observed to be upregulated in patients with Lewy body disease (LBD). The upregulation of DDC appears to serve compensation for low dopaminergic levels, as in the case of lesions to dopaminergic neurons (Hadjiconstantinou and Neff, 2008). Furthermore, increased levels of DDC in PD could be an indicator of reduced dopamine signalling in the brain, a key feature of both LBD and atypical parkinsonian disorders. Another potential explanation for increased levels of DDC in the CSF could be increased peripheral DDC in parkinsonian disorders with loss of peripheral DDC in the CSF (Pereira et al., 2023). In fact, DDC could have a future role in clinical practice as a biomarker of dopaminergic dysfunction to detect parkinsonian disorders even during the preclinical stages of the disease and predict their progression towards clinical LBD (Pereira et al., 2023). Along with the upregulation of DDC,

I observed elevated expression of SNCA during aging. Accumulation of SNCA is the hallmark pathological lesion in brains of patients with PD and related neurological disorders characterized as synucleinopathies (Reyes et al., 2021). Then, BGs reduce these two proteins related to PD during aging. In addition, I observed downregulation by BGs of oncogenic proteins, such as AK2 and NT5E. In detail, AK2 is a biomarker related to the prognosis of glioma and the immune microenvironment (Liu et al., 2023). And NT5E is overexpressed in glioblastoma (GBM), where it contributes to the tumour's pathophysiology via the generation of immunosuppressive adenosines (Wang and Matosevic, 2019). Furthermore, CCs not dysregulated by aging but downregulated by BGs at 27 wph (50.86% downregulated DEPs) are predominantly associated with extracellular region (Fig. 74).



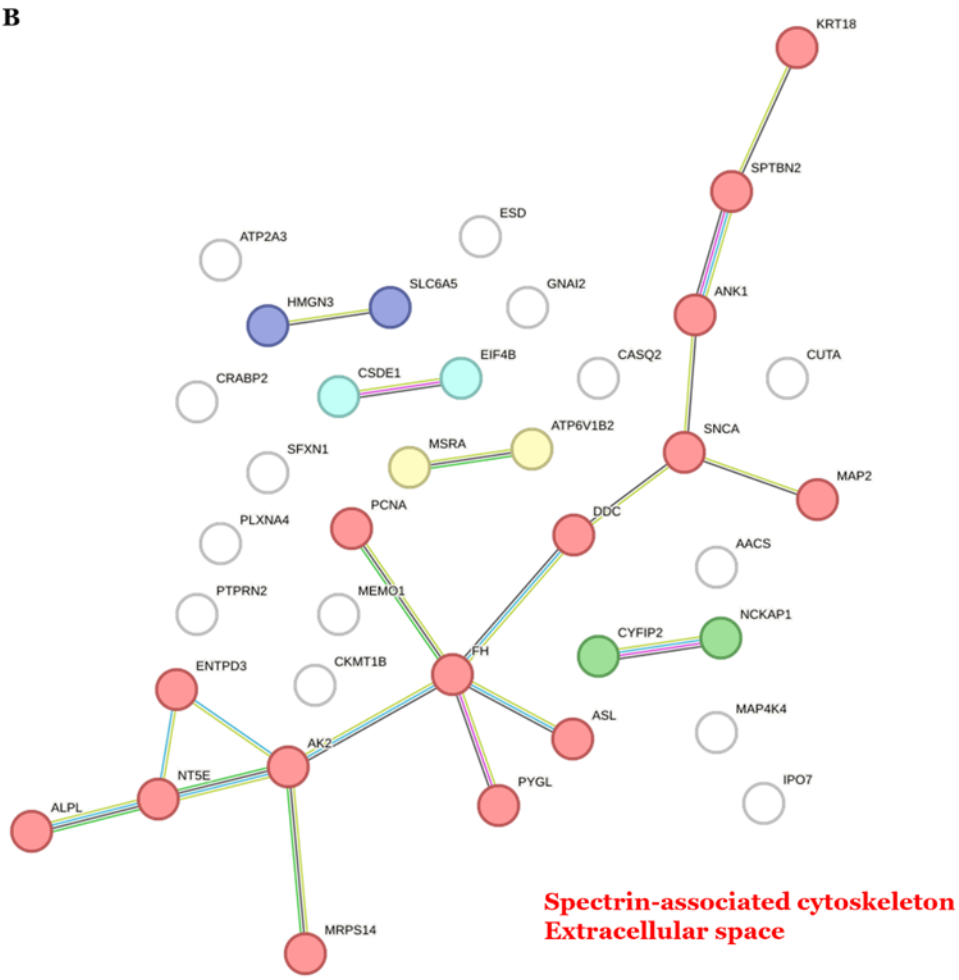
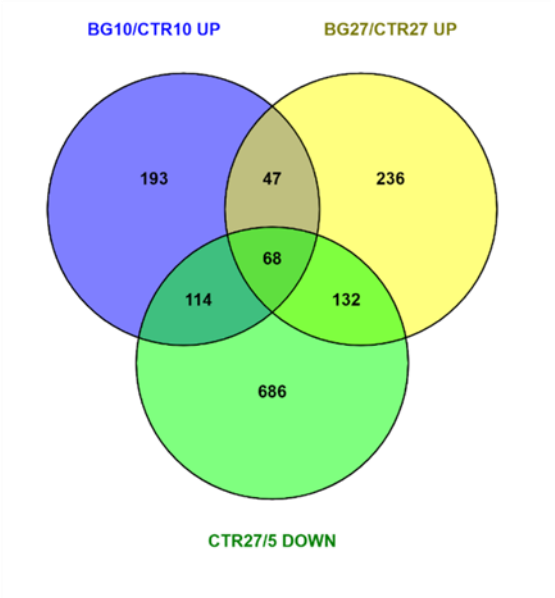


Figure 74: DEPs upregulated in aging and downregulated by BGs at 27 wph. A) Venn diagram of DEPs, B) PPI related to CCs in common between aging and BGs treatment at 27 wph

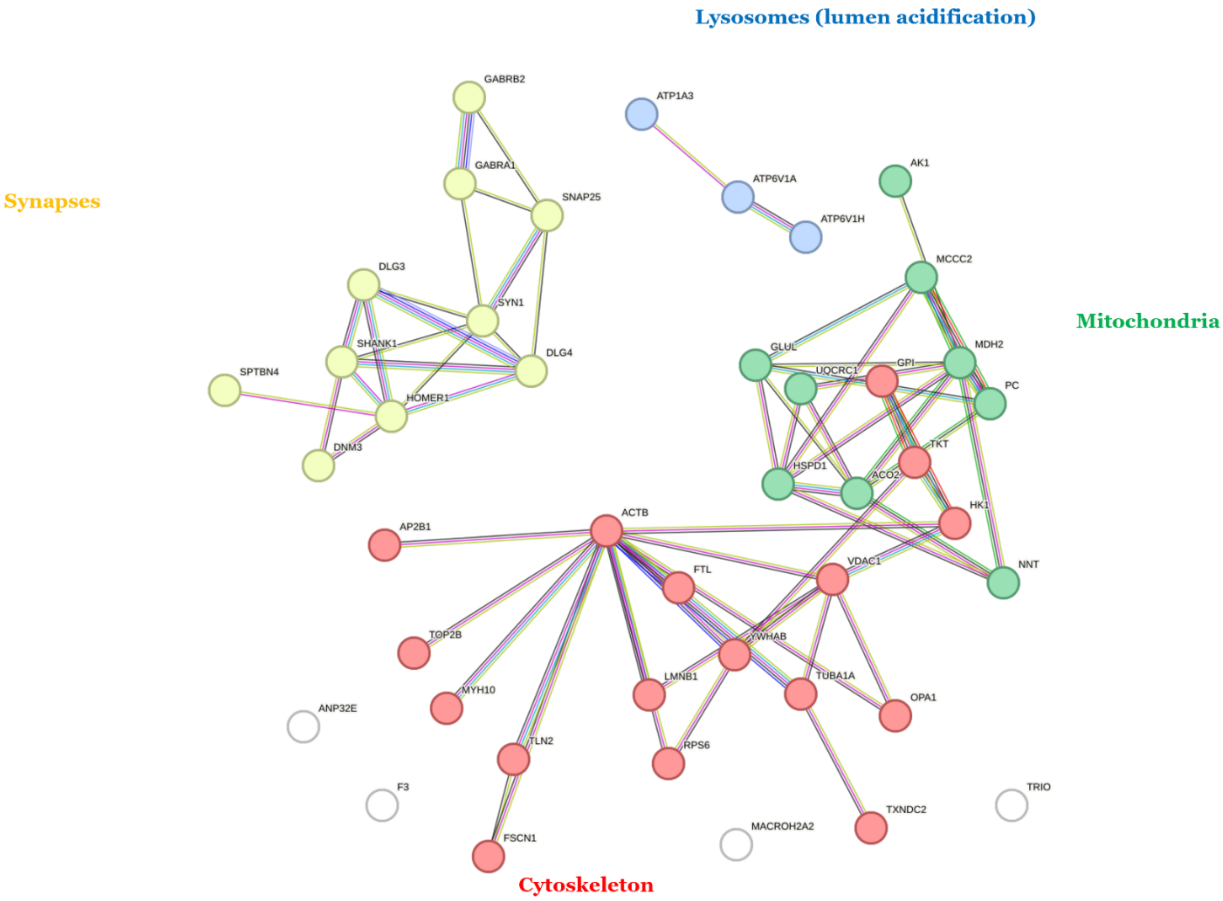
Furthermore, it is interesting to note that proteins downregulated in aging involved in mitochondrial respiration (V-ATPase) are associated in phagocytosis and autophagy, particularly with the acidification of lysosomes. V-ATPase are downregulated during aging (Aufschnaiter and Buttiner, 2018; Song et al., 2020) and this involves in impairment impair the uncoupling of oxidative phosphorylation (reduction of ATP) and reduction of autophagy (Colaciurcio and Nixon, 2016; Aufschnaiter and Buttiner, 2018). In fact, V-ATPase is a master regulator of cellular homeostasis and lifespan (Aufschnaiter and Buttiner, 2018). Interestingly, downregulation of ATP6V1A, one of the proteins observed in this analysis, is involved in Alzheimer's disease through synaptic vesicle cycling, phagosome, and oxidative phosphorylation (Zhou et al., 2021). BGs at 10 and 27 wph upregulated the downregulated expression of these proteins during aging. For contrary, at 5 wph BGs didn't affect on these proteins. These results confirm what I was observed in the quantification of autophagy via immunofluorescence analysis. I clustered proteins downregulated during normal aging and upregulated by BGs at 10 and 27 wph using PPI. These proteins formed a structure network with significantly non-random connectivity (PPI entrenchment $p<1\ e^{-6}$). CCs are associated

with Mitochondrial, Synapses, Cytoskeleton and Lysosomes (Fig. 75). Specifically, BGs caused acidification of lumen's lysosomes. Lysosomes generate and maintain their pH gradients by using the activity of a proton-pumping V-type ATPase, which uses metabolic energy in the form of ATP to pump protons into the lysosome lumen. In fact, ORA show an important CC involved in this process, or Proton transporting two sector ATPase complexes (Fig. 75C).

A



B


Lysosomes (lumen acidification)
Synapses
Mitochondria
Cytoskeleton
GABRB2
GABRA1
SNAP25
DLG3
SYN1
SHANK1
DLG4
SPTBN4
HOMER1
DNM3
ATP1A3
ATP6V1A
ATP6V1H
AK1
MCCC2
GLUL
MDH2
UQCRC1
GPI
PC
TKT
HSPD1
ACO2
HK1
NNT
ACTB
AP2B1
FTL
VDAC1
TOP2B
YWHAB
LMNB1
TUBA1A
OPA1
MYH10
ANP32E
TLN2
RPS6
F3
FSCN1
MACROH2A2
TXNDC2
TRIO

C

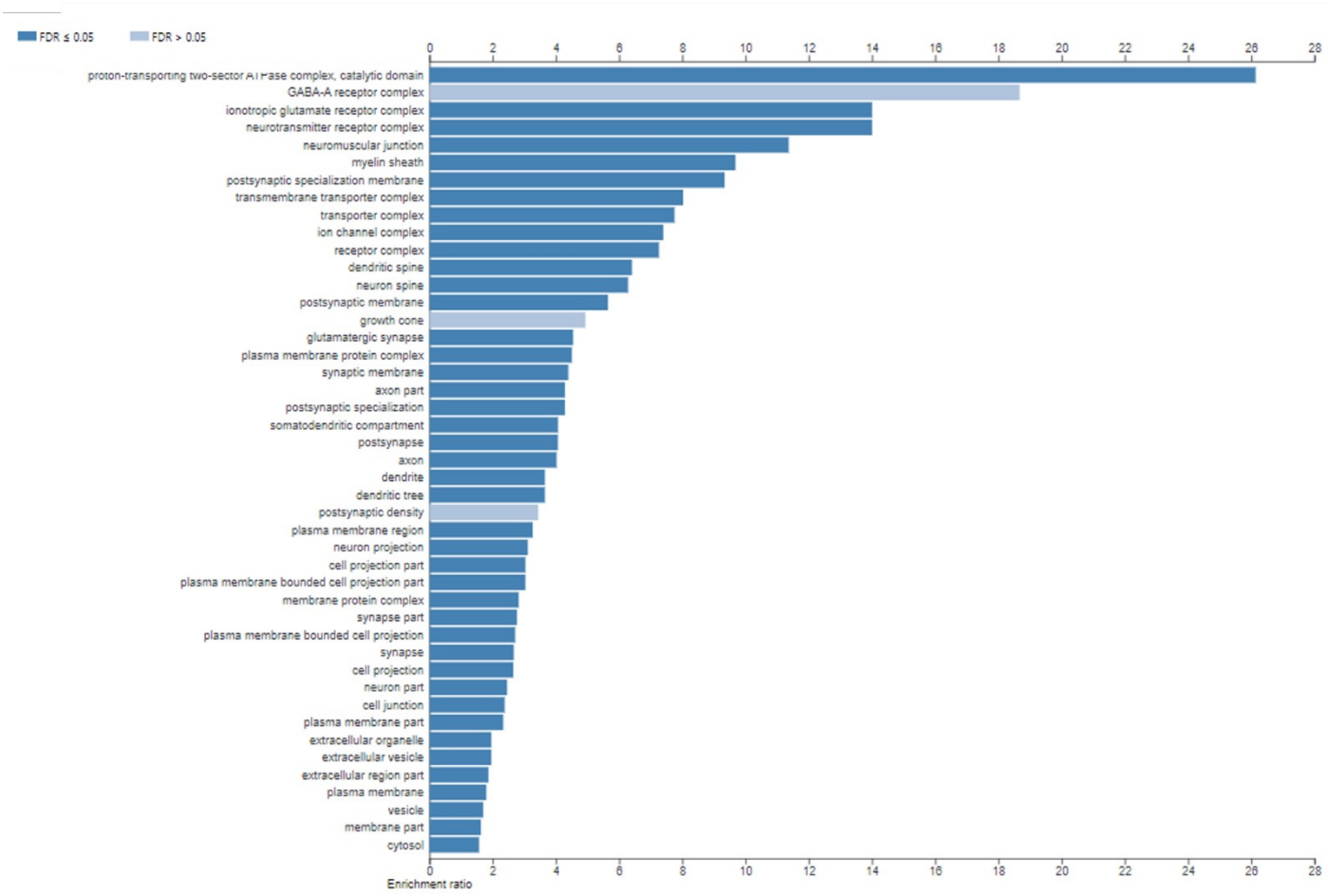


Figure 75: DEPs downregulated in aging and upregulated by BGs at 10 and 27 wph. A) Venn diagram of DEPs, B) PPI related to CCs, C) ORA of CCs in common between aging and BGs treatment at 10 wph

7 Discussion

Overall, the results obtained from my analyses show that BGs are able to partially ameliorate phenotypes compromised with aging. Next, I will discuss separately each set of results I reported.

7.1 BGs can be safely supplemented in a chronic diet

BGs allow the extension of lifespan as described in the *Nothobranchius guentheri* model (Song et al., 2020), in Caenorhabditis *elegans* (Aranaz et al., 2021) and in the PD model of Drosophila *melanogaster* (Tripodi et al., 2022). I did not observe differences in the lifespan of *Nfu* chronically treated with BGs (Fig. 35). These results appear to be in contrast with what was observed in my study. In addition to the different Nothobranchius species used in my study (*Nothobranchius furzeri*) and in that of Song et al., (*Nothobranchius guentheri*), it is possible that the origin of the BGs, in my study *Saccaromyces cerevisiae*, may have determined different effects on the same parameters analysed. For contrary, Song et al (2020) used 1.3 BGs derived from alga *Euglena gracilis* at a concentration (1.25 g/Kg BW), 10 and 100 times higher than that used in my study. In detail, BGs were included in the worms in the study of Song et al. (2020), while I included them in the Zebrafeed feed. Additionally, my experimental design differed from that of Song et al. (2020). In their study, BG administration began at the 36th week post-hatching (wph) and continued until the spontaneous death of the fish. In contrast, I started administering BGs from the 2nd wph and sacrificed all Nfu at a specific time point, which was 27 wph. Therefore, the number of *Nfu* that I used for this study is under power for survival analyses and the result may be a false negative. For comparison, studies of pharmacological interventions on lifespan by the Intervention Testing Program of the National Institute on Aging require ~ 300 control mice and ~ 150 mice for each dose (e.g. Miller et al., 2014). For this reason, I did not expect to observe differences regarding extension of lifespan. Likewise, this analysis confirms there is no toxic effect on the health of *Nfu* chronically fed with a concentration of BGs that is compatible with the dosages used for animal feeds. It is possible that higher concentrations may also increase survival but would not be of practical implementation. BGs, in addition to not being toxic to *Nfu*, do not cause any increase in body weight (Fig. 36). Moreover, BGs have no effect on kidney histology (Fig. 48), which indicates that BGs are not nephrotoxic but safe for kidney health. These findings suggest the safe consumption of BGs as dietary supplements for chronic use.

7.2 BGs reduce aging phenotypes in multiple organs

Overall, the chronic BGs-fortified diet reduces aging phenotypes in a dose dependent manner (Fig. 37; Tab. 4). In detail, I have observed that 125 mg/Kg BW of BGs has a more powerful anti-aging action than 12.5 mg/Kg BGs. In any case, it is essential to specify that

even the highest dose is considered a low dose as dietary supplement. BGs specifically act on mechanisms related to autophagy (e.g. LC3 brain and liver), protein peroxidation (e.g. nitrotyrosine heart and liver, lipofuscin in brain and liver) and inflammation (e.g. LCP1 brain).

7.2.1 BGs reduce peroxidation of proteins in aging

Immunomodulatory function of BGs also determines its antioxidant properties (Kofuji et al., 2012). Aging of Nfu is characterized by accumulation of oxidative stress on protein, and lipids (Hsu et al., 2008; de Costa et al., 2020) that is detectable, for example, as increased nitrosylation of tyrosines in the heart (Heid et al., 2017). BGs are also able to reduce the peroxidation of proteins (nitrotyrosine) altered in dysfunctional processes, such as excessive blood platelet activation (Saluk-Juszcak et al., 2010). Furthermore, the antioxidant function of BGs in aging has been recently suggested by the observation of a reduced oxidative stress on protein oxidation and lipids in the muscles of aged Nothobranchius *guenteri* treated with BGs (Song et al., 2020). The mitigation of oxidative stress during aging, such as protein peroxidation in the heart and liver of *Nfu*, observed in my study confirms its antioxidant properties. Moreover, I have also observed the antioxidant property of BGs on the effect of the accumulation of lipofuscin, a formation derived from an oxidative alteration of macromolecules by ROS. As I have demonstrated in my study, BGs prevent accumulation of lipofuscin in the livers, hearts and brains of old *Nfu* (Fig. 40). Additionally, Song et al. reported a decrease in lipofuscin in the gills of old Nothobranchius *guenteri* (Song et al., 2020). These data suggest that the antioxidant function of BGs during aging contributes to the mitigation of age-associated phenotypes.

7.2.2 BGs reduce inflammation in aging

BGs are immunomodulators capable of attenuating the inflammatory response. In fact, BGs can reduce inflammation by modulating inflammatory cytokines, such as Nitric Oxide (NO), Interleukins (ILs), Tumor Necrotic Factor a (TNF-a), Interferon Gamma γ (INF-γ) (Miest et al., 2006; de O. Silvia et al., 2007; Brogi et al., 2021; No et al., 2021; Silva et al., 2021). Studies on anti-inflammatory effect of BGs in physiological aging are lacking, but it has been reported that BGs reduce pathological inflammatory response, for example in inflammation stimulated by LPS (No et al., 2021), or infections (Falco et al., 2012), or intestinal pathologies, or in diabetes (Taylor and Vasu, 2021), or in NASH (Huang et al., 2020). Specifically, BGs reduce inflammation by modulating immune cells, such as macrophages and microglia. Indeed, BGs influence the balance of Th1/Th2 cytokines by promoting the secretion of anti-inflammatory cytokines (Chen et al., 2013). Xu et al. observed a reduction in BGs-mediated inflammation with polarization of the M2 phenotype of macrophages (Xu et al., 2022). Cui et al also described a decrease in microglia activation in case of inflammation in the brain infected with Toxoplasma *gondii* (Cui et al., 2023). Anti-

inflammatory property of BGs modulating immune cells described by many studies confirms what I have observed in aging, i.e. a reduction of LCP1+ cells (myeloid cells) in the gut and brain, indicating a reduction in chronic inflammation (Fig. 43) that is a highly conserved hallmark of aging (Baechl et al., 2023). Furthermore, I observed that BGs reduce gliosis (Fig. 44), a reactive response of astrocytes driven by pro-inflammatory cytokines secreted by the activated microglia. Furthermore, the antioxidant action of BGs is reflected in the prevention of sarcopenia, as quantified by Feret's size (Fig. 47A) and the significant reduction in muscle fibrosis (Fig. 47B), as typical sign of chronic inflammatory reactions. Reduction in muscle fibrosis by BGs was also observed by Preethy et al. in DMD mice (Preethy et al., 2023). So, BGs reduce inflammation in aging possibly via a modulation of immune cell activation, mainly macrophages and microglia.

7.2.3 BGs and autophagy in aging

7.2.3.1 Chronic BGs-fortified diet induces autophagy in aging

The effects of BGs on autophagy in the context of physiological aging have not yet been investigated. BGs trigger autophagy in different pathological conditions (Ohman et al., 2014; Fatima et al., 2017; Ding et al., 2019; Li et al., 2019; Kopiasz et al., 2021; Liang et al., 2022; Xu et al., 2023; Kopiasz et al., 2024). I analysed whether BGs normalize impaired autophagy during aging. My results indicate that autophagy, measured by LC3 immunoreactivity, is induced by supplementation with BGs in the brain and liver but not in the muscle of old *Nfu* (Fig. 42) suggesting a tissue-specific dysregulation of autophagy during aging. In line with this hypothesis, Chapin et al. observed tissue-specific autophagic responses to aging in *C. elegans* (Chapin et al., 2015). An alternative hypothesis is that BGs do not enhance muscle autophagy because it is not dysregulated. Ruparelia et al. demonstrated that autophagy is not affected in aging in *Nfu* muscle except at very late age or 52 wph (Ruparelia et al., 2023) and our results (see below) show that BGs do not induce autophagy in young animals where this cellular process is not impaired. Increased autophagy in brain and liver correlates with reduced lipofuscin accumulation in *Nfu* treated chronically with BGs. Indeed, increased autophagy is known to reduce lipofuscin accumulation (Li et al., 2021). Further evidence for the induction of autophagy by BGs in aging is provided by increased colocalization of lysosomal markers with protein aggregates and reduced total protein aggregates in the liver (Fig. 45) in old *Nfu* treated with BGs. These data suggest that induction of autophagy by BGs improves clearance of protein aggregates during aging.

7.2.3.2 Acute BGs-fortified diet induces autophagy in brain aging, but fails to reduce lipofuscin accumulation

Acute (1 week) BGs-fortified diet significantly increased autophagy in the brain (Fig. 50). At the same time, I observed that BGs do not reduce lipofuscin accumulation in the acute diet (Fig. 49), as opposed to what observed in the chronic diet. Taken together, these two findings indicate that acute dietary-induced autophagy fails to reduce accumulated lifetime

lipofuscin in vivo. It seems that the chronic BGs-fortified diet improves aging phenotypes by modulating autophagy, inflammation and oxidative stress, typical processes altered by our lifestyle.

7.3 Ex- rain culture as a model to observe direct effects

Various papers, and my own research, reported positive effects on brain phenotypes after oral administration of BGs *in vivo*. These effects could be mediated by the intestinal microbiome of effects on other peripheral organs. Whether BGs could have a direct effect on the brain remained unknown. To this end, I performed *Nfu* cultured brain slides, as described in the protocol of Bagnoli et al (2022). Using this model, I was able to study their local effect in an isolated system. Moreover, the absence of the blood brain barrier (BBB) allowed the direct exposure of brain cells BGs. In addition to these advantages, it is important to underline that the *ex-vivo* brain model mimics adult neurogenesis and synaptogenesis as observed *in vivo* (Bagnoli et al., 2022). Overall, combined with the general advantages of the *Nfu* model, this ex vivo brain model enabled me to directly observe the effect of BGs on brain aging. On the downside, it remains to be determined whether BGs can cross the BBB *in vivo* or in other ways act on cells facing the brain capillaries.

7.3.1 Autophagy in cultured brain slices

Given the effect of BGs on autophagy in the brain during aging *in vivo*, I quantified for first time autophagy in young, adult, and old *Nfu* ex-vivo brains. As the results in Figure 51 shows, there is reduction in autophagy during aging, particularly from 10 wph. This reduction in autophagy mirrors what as has been observed during aging in other organisms (Cuervo et al., 2010; Cuervo and Wong, 2014; Saha et al., 2018; Barbosa et al., 2019) or in neurodegenerative pathologies (Menzies et al., 2017; Hou et al., 2020; Yuan et al., 2023). It is no coincidence that impaired autophagy is considered a hallmark of aging (Schmauck-Medina et al., 2022). In addition, the age-dependent reduction of autophagy may provide an explanation of why at 27 wph acute BGs treatment boosts autophagy but fails to clear lipofuscin accumulation. In fact, it is possible the dysregulation of autophagy already at 10 wph determines an accelerated accumulation of lipofuscin that cannot be cleared by the acute action of BGs in old *Nfu*. In this regard, it would be interesting in the future to observe the reduction of autophagy at different ages in the in vivo model as observed in the ex vivo model. Furthermore, it would be interesting to compare the reduction of autophagy in vivo and ex vivo. Overall, these findings show that ex-vivo *Nfu* brains are a good model to study the direct effect of compounds on autophagy.

7.3.2 BGs directly act in the brain restoring impaired autophagy

BGs increase autophagy in *ex vivo* model (Fig. 51) demonstrating that the effects observed in vivo does not require peripheral organs and systemic factors. In detail, BGs increased the

number LC3+ puncta/cells and the size of autophagosomes (Volume of LC3) when this mechanism is dysregulated, or at 10 and 27 wph, but not in young animals that are not autophagy-impaired. Moreover, the increment of lysosomes visualized with Lysotracker by BGs in old ex-vivo brains, confirmed what I observed using LC3 (Fig. 54). So, BGs act on impaired autophagy during aging. For contrary, at 5 wph, when autophagy is fully active, BGs do not increase its baseline. Indeed, the level of autophagy in old BGs-treated brains is similar Thus, BGs restore autophagy when the process is dysregulated (i.e. during aging), but not when the process is functioning. Indeed, when autophagy in young *ex-vivo* brains is artificially impaired by Bafilomycin A1 (Fig. 52), BGs increase the number of LC3 puncta/cell and the size of autophagosomes (Volume of LC3). These findings highlight that BGs directly act on the brain restoring autophagy during aging and BGs modulation of autophagy depends on the physiological condition.

7.3.3 BGs as inductor of autophagy

Regulation of autophagy can be either inductive or blocking. Elevated levels of autophagosomes (LC3) indicate either induction of autophagy or block of fusion with lysosomes in late stage (Myerowitz et al., 2020). This can be resolved by artificially blocking the late stage of autophagy. An increase of autophagosomes after inhibition indicate the induction of this process. To observe whether BGs are inducers or blockers, autophagy in ex-vivo brain was blocked by Bafilomycin A1. Bafilomycin A1 disrupts autophagic flux by inhibiting V-ATPase pump, so it inhibits the fusion of autophagosome-lysosome fusion (Yoshimori et al., 1991; Yamamoto et al., 1998; Klionsky et al., 2008). As observed for other autophagy inducers (Lopez et al., 2018), such as Rapamycin (Plaza-Zabala et al., 2021), BGs increase the number and volume of autophagosomes compared to Bafilomycin A1 treatment alone (Figs. 52 and 53). BGs are therefore able to regenerate new autophagosomes. The increase in autophagosomes from BGs in *ex-vivo* brains treated with Bafilomycin A1 compared to Bafilomycin A1 treatment alone indicates the overall number of blocked autophagosomes and those regenerated by BGs. BGs induce of autophagy, which is reduced during aging.

7.3.4 BGs improve morphology of mitochondria

Induction of autophagy induces an increase in the functionality and energy production of mitochondria (Wilson et al., 2023), so I investigated if BGs could influence mitochondrial number and morphology in *old ex-vivo* brains. I show that BGs increased the mass and number of mitochondria during aging (Fig. 55). These results confirm the relationship between mitochondrial- and autophagy-functions, both processes involved in the regulation of longevity (Wong et al., 2023). Furthermore, for the first time, the role of BGs in the mitochondria-autophagy axis is reported. Although the effect of BGs on mitochondria is poorly investigated, it has been observed that BGs regulate mitochondrial respiration under

different conditions (Shaki and Pourahmed, 2012; Brogi et al., 2021; El-Deeb et al., 2022; Wang et al., 2022). It would be of particular interest to investigate the mechanism and the mediators by which BGs regulate the autophagy-mitochondrial axis.

7.3.5 BGs-regulated proteins relate to mitochondrial respiration and autophagy

Mass-spectroscopy based proteomics provided an unbiased and global molecular view of BGs action and pointed to specific proteins that may mediate BGs action. Firstly, proteomic analyzes show that, at each of the three different ages (5, 10 and 27 wph), BGs upregulate the expression of proteins associated with mitochondria, and moreover with the production of ATP (Fig. 63). Specifically at the level of single proteins, ACO2 and PC are enzymes the TCA cycle. MYH10 has role in cytoskeleton reorganization enriched in neurons and specifically in dendritic spines (Korobova and Svitkina, 2010), in neuronal autophagy (Jun et al., 2023) and in mitochondrial homeostasis in situations of cellular stress. VDAC1 is associated with mitochondrial respiration and ATP production. TXN is mitochondrial protein that plays an important role in diverse cellular functions such as maintaining redox homeostasis, proliferation, and DNA synthesis, but also modulating transcription factors and controlling cell death (Oberacker et al., 2023). Specifically, TXN has a neuroprotective role from oxidative stress (Wang et al., 2016; Guo et al., 2021; Cha et al., 2023). At the level of cellular components, I observed that BGs increase proteins of mitochondria, cytoskeleton, and synapses (Figs. 69-71-73). On the contrary, BGs have less action in downregulation of proteins. In any case, BGs at different ages downregulated protein related to extracellular region and cytoplasm (Figs. 70-72-74). Most importantly, BGs in old animals are able to normalize the expression of about 46.62% of dysregulated proteins during normal aging (Fig. 68C). These results indicate that BGs are able to restore a more youthful protein landscape in normal aging. At 5 wph BGs upregulated 28.58 % proteins downregulated in normal aging related to mitochondria, synapse, and protein folding (Fig. 69). It is interesting note that BGs at 5 wph also downregulated proteins upregulated in aging related to synapses and Endoplasmic Reticulum (ER). These results seem to suggest that BGs tend to modulate some processes bidirectionally during young age, that is, when these processes are not dysfunctional. This study suggests that BGs modulate synapse-associated proteins bidirectionally. Moreover, BGs at 5 wph act also in cellular components not dysregulated during aging. Specifically, BGs downregulated neuronal component, such as GABA receptor complex, and upregulated the translation/ribosome e other proteins associated with cytoskeleton. At 10 wph, BGs upregulated 18.2% of proteins downregulated in aging that are associated with Mitochondria, Cytoskeleton, Synapses and GTP-ase (Fig. 71). On the contrary, BGs downregulated proteins associated with Mitochondrial cristae and cytoskeleton, particularly β-Spectrin proteins (Fig. 72). It is interesting to underline that β-Spectrin is integral component of β-amyloid plaques in AD (Sihang and Cataldo, 1996).

Moreover, abnormality in spectrin degradation has been observed during neurodegeneration (Masliah et al., 1990; Yan and Jeromin, 2012; Zhu et al., 2015). This accumulation of spectrin, specifically spectrin breakdown products (SBDPs) may be a consequence of neuronal death during neurodegerative disorders. Overall, these findings may imply that an accumulation of spectrin in aging of *Nfu* is the result of β-amyloid plaque formation and/or neuronal death with release of this protein in the brain. In addition, BGs regulate the possible accumulation of spectrin from neurodegenerative disorders. BGs, in addition to modulating the dysregulation of proteins in aging, upregulate other processes associated with mitochondria and synapses, but also the spliceosome. Furthermore, at 10 wph BGs downregulate Mitochondria cristae and Cytoskeleton-related proteins (Spectrin-associated), these also downregulate BG 5wph. As discussed previously, the accumulation of spectrin in aging *Nfu* may be the result of β-amyloid plaque formation and/or neuronal death with release of this protein in the brain. Taken together these results indicate that BGs, particularly at 5 and 10 wph, prevent β-amyloid plaque formation and/or neuronal death. An increase in collagen has been observed in AD animal models and human patients (Kalaria and Pax, 1995; Farkas et al., 2000; Mehta et al., 2013; Lepelletier et al., 2015; Malgaki et al., 2018). It is very interesting to note the effects on BGs in aging (27 wph). BGs upregulated cellular components related to Mitochondria, Synapses and Cytoskeleton dysregulated during aging (Fig. 73). In detail, BGs restore mitochondrial respiration, such as complexes of OXPHOS. These results are supported by imaging of mitochondria in brain slices treated with BGs. In addition, BGs downregulated proteins upregulated during aging (Fig. 74). These proteins are very heterogeneous and do not create well distinct clusters, although they are mainly associated with the extracellular space and the spectrin-associated cytoskeleton. Downregulation of the latter was also observed at 10 wph. I wish to describe in more detail the function of the most significant proteins that BGs downregulated in aging, specifically PCNA, DDC, SNCA, AK2 and NT5E. The upregulation of PCNA during aging may seem counterintuitive. For contrary, PCNA can also be expressed during repair synthesis, as well as in the case of microglial activation. Not surprisingly, the reactive cell cycle of glial cells is important for the pathogenesis of age-related neurodegenerative diseases. Co-location between PCNA and microglia (but not astrocytes) was observed in AD subjects (Marlatt et al., 2014). This suggests that an increase in PCNA in aging may be determined by the proliferation of microglia. Hence, BGs tend to downregulate the proliferation of cells such as microglia in aging. It is really interesting to have observed accumulation of DDC and SNCA in the aged brain of *Nfu*. These two proteins are associated with PD. In fact, DDC is upregulated in patients with Lewy body disease (LBD). Upregulation of DDC appears to compensate for low dopaminergic levels, as in the case of lesions to dopaminergic neurons (Hadjiconstantinou and Neff, 2008). Furthermore, increased levels of DDC in PD could be an indicator of reduced dopamine signalling in the brain, a key feature of both LBD and atypical parkinsonian disorders. Another potential explanation for increased DDC levels in

cerebrospinal fluid (CSF) could be increased peripheral DDC in parkinsonian disorders with loss of peripheral DDC in CSF (Pereira et al., 2023). It is worth noting that the DCC upregulation was proposed as a biomarker of dopaminergic dysfunction in the preclinical phases of the disease and ´to predict the progression towards clinical LBD (Pereira et al., 2023). In addition to the upregulation of DDC, I observed an increase in SNCA during aging. SNCA accumulation is the characteristic pathological lesion in the brain of patients with PD and related neurological disorders characterized as synucleinopathies (Reyes et al., 2021). The accumulation of α-synuclein or SNCA in *ex-vivo* old brain of *Nfu* agrees with results observed by Matsui et al. in old (20 wph) brain of *Nfu*, in vivo model (Matsui et al., 2019). Overall, these results indicate that the *ex vivo* model mimics what was observed in the *in vivo* model. Specifically, even the ex-vivo model shows spontaneous age-dependent phenotypes are consistent with a degenerative process in the brain. Furthermore, it is important to highlight the reduction by BGs of these 2 proteins associated with the onset of PD. These findings suggest a possible preventive action of BGs in the context of PD. Furthermore, I believe that the downregulation of AK2 and NT5E. These proteins are associated with brain cancer, such as glioblastoma or glioma. AK2 is a biomarker related to glioma prognosis and immune microenvironment (Liu et al., 2023) and NT5E is overexpressed in glioblastoma (GBM), where it contributes to cancer pathophysiology through the generation of immunosuppressive adenosines. (Wang and Matosevic, 2019). Upregulation of proteins associated with brain cancer in this study may lead to the hypothesis and investigation of the onset of brain cancer in the *Nfu* model, as already observed for liver cancer (Di Ciccio et al., 2014). In fact, there is an association between aging and the onset of glioma. Specifically, aging progressively suppresses normal immunosurveillance and thus contributes to the initiation and/or growth of glioblastoma cells (Ladomersky et al., 2019). In addition to these considerations, it is important to highlight and confirm the anti-neoplastic and immunomodulation action of BGs. BGs also acts on proteins not dysregulated during aging such as proteins associated with Mitochondria, Cytoskeleton and Ribosomes (Translation). On the contrary, BGs downregulated proteins related to the extracellular region. Overall, these results indicate that BGs act mainly on the regulation of synapses and mitochondrial respiration. In fact, it seems that memory loss and neurodegeneration during aging are linked to mitochondrial dysfunction. Mitochondria are involved in the regulation of complex synaptic plasticity processes. Mitochondrial motility and re-localization are necessary for the maintenance and strengthening of dendritic spines, but also for the formation of new spines/synapses. In addition, mitochondria are energy suppliers regulating neuronal plasticity processes (Bertholet et al., 2016; Todorova and Blokland, 2017; Kuzniewska et al., 2020; Duarte et al., 2023; Underwood et al., 2023). These results may suggest an action of BGs on synaptic plasticity mediated by regulation of mitochondrial activity. In addition, the convergent results of imaging and proteomic analyzes suggest a role for BGs in preserving mitochondria

during aging. Specifically, aging reduces the functionality of mitochondrial respiration inducing an accumulation of ROS (Sohal and Sohal, 1991; Zorov et al., 2014). The excessive accumulation of ROS reduces biogenesis of mitochondria leading to a decrease in their number and volume (Harman, 2009; Chistiakov et al., 2014). The reduction in the number of mitochondria further increases the dysfunctionality of mitochondrial respiration. Furthermore, I observed crucial proteins involved in mitochondrial respiration (complex V, OXPHOS), V-ATPase, being downregulated in aging and upregulated at both 10 and 27 wph, but not at 5 wph, by BGs. The V-ATPase, in addition to its role in mitochondrial respiration (Jonckheere et al., 2011), also plays a fundamental role in the autophagic flux. V-ATPase are necessary to acidify lysosomes and support the degradation of the internal membranes of autophagosomes and their cargo (Futai et al., 2019; Song et al., 2020; Han et al., 2022). V-ATPase dysfunction contributes to the pathogenesis of multiple neurodegenerative diseases (Song et al., 2020). This finding indicates that BGs at both 10 and 27 wph act not only on proteins associated with mitochondrial respiration, but also with autophagy. Consistent with what was observed in immunofluorescence analyses, BGs normalized dysregulated autophagy at 10 and 27 wph, but not at 5 wph. Furthermore, both analyzes indicate that during aging there is a reduction in the early stage of autophagy, reduction of autophagosome (LC3), and in the late stage, reduction of V-ATPase. BGs are able to restore mitochondrial respiration, autophagy and synapses during aging but also at 10 wph. Overall, a reduction in the autophagy-mitochondrial relationship is observed in aging. This reduction also affects synaptic function. In detail, it is possible that impairment of autophagy during aging causes the accumulation of abnormal mitochondria and ROS (Lee et al., 2012; Barbosa et al., 2019). Accumulation of damaged mitochondria results from the reduction of mitophagy. Furthermore, a reduction of cargo degradation reduces metabolites released from autophagy for resume to fuel for mitochondrial respiration. These altered processes lead to a reduction in both mitochondrial respiration and biogenesis (Kumar et al., 2021; Wilson et al., 2023). Furthermore, mitochondrial respiration, specifically a reduction of OXOPHOS, further leads to a reduction in autophagy, (Thomas et al., 218). Decreases in ATP levels may also block autophagic flux independently of mTORC1 because ATP is required in the sequestration phase as well as to maintain lysosomal function (Sakai et al., 1982; Plomp et al., 1987; Moruno-Manchos et al., 2013; Moran et al., 2014). These findings seem to indicate that the bidirectional connection between autophagy and mitochondria regulates many functions associated with aging, such as synaptic transmission. Overall, BGs modulate bidirectional connection between autophagy and mitochondria thus play a central role for lifespan extension and age-related disorders.

7.3.6 Role of BGs not only in microglia but also in neurons

7.3.6.1 BGs inhibit activation of microglia

BGs are able to reduce the activation on microglia in the *ex-vivo* model. The difficulty in visualizing fine details of microglia morphology in the Nfu model, lent me to the use of the established zebrafish reporter line Tg(mpeg1.1.1:EGFP) to block autophagy by Bafilomycin A1 in ex-vivo zebrafish brain model, a canonical model where microglia activation is induced by impaired of autophagy (Jin et al., 2018; Qin et al., 2021). BGs reduced the canonical proinflammatory morphological transition of microglia (Fig. 56), confirming the role of BGs in polarization of microglia and macrophages. Moreover, this result indicates anti-inflammatory effects of BGs and confirms its action on microglia.

7.3.6.2 BGs act both in microglia and in neurons

The direct effect of BGs on microglia has been discussed in the literature, but also confirmed in my latest experiment. However, the effect of BGs on other brain cells, such as neurons, has not been investigated to date. I therefore designed an experiment to specifically detect a direct action of BGs on neurons. I depleted microglia in the *ex-vivo* Nfu model with plx5622 (Fig. 57). BGs retain the ability to increase autophagy also in brains depleted of microglia, though the magnitude of the effect is smaller (Fig. 58). Furthermore, BGs act with the same magnitude on the number of autophagosomes/cells in brains without or with microglia. For contrast, BGs have a minor effect on the volume of autophagosomes in the brain without microglia. For the first time, I observed that BGs partially restore autophagy through binding to neurons, blocking microglia. In any case, these results suggest the direct effect of BGs on neurons.

In this regard, I investigated the role of BGs in iPSC-derived human neurons.

7.3.6.2.1 BGs have effects on iPSC-derived human neurons

My results indicates that BGs are able to act in impaired autophagy of iPSC-derived human neurons (Fig. 60). Moreover, BGs do not act in non-dysregulated processes, such as autophagy in controls. This confirms that BGs can act also on neurons, specifically when autophagy is impaired. Moreover, prolonged treatment of Bafilomycin A1 (10 nm and 100 nm) induce neuronal mortality in according in the study of Sotthibundhu et al. in iPSC cell (Sotthibundhu et al., 2016). Therefore, the acute treatment of Bafilomycin A1 determines impaired autophagic flux while the prolonged treatment induces the death of neurons. This result indicates that inhibition of the V-ATPase pump induces mortality. BGs reduce neuronal death induced by 10 nm of Bafilomycin A1 (Fig. 59). This may indicate that BGs upregulate V-ATP-associated proteins, as observed in the proteomic data. In addition, at the highest concentration of Bafilomycin A1, or 100 nm, BGs fail to restore this process. Overall, BGs act on neurons by regulating impaired autophagy and therefore reducing neuronal death.

7.4 Hypothesis of BGs' mechanism action

Overall, all results indicate that BGs are immunomodulatory, anti-inflammatory and antioxidant, mainly regulating processes dysregulated in aging such as autophagy, mitochondrial respiration, synaptic transmission and microglial activation. I cannot offer a mechanistic explanation of BGs action, also considering that they generally act as modulators with bidirectional action depending on the cellular and physiological context. My result highlight particularly a bidirectional relationship between autophagy and mitochondrial respiration. This relationship determines secondary effects such as improvement of synaptic function. AMPK signalling appears a possible pathway mediating BGs action. AMPK controls the regulation of cellular homeostasis, metabolism, resistance to stress, mitochondrial respiration, cell survival and growth, cell death, autophagy which are some of the most critical determinants of aging and lifespan (Antero and Kai, 2012; Herzi and Shaw, 2018). Indeed, AMPK inhibits mTOR by inducing autophagy and controls mitochondrial biogenesis and metabolism. Furthermore, the upregulation of V-ATPase is mainly dependent on AMPK (Collins and Forgac, 2020). AMPK acts in the regulation of inflammation and oxidative stress (Carling et al., 2012; Salminen and Kaarnirata, 2012), confirming what I observed in the *in vivo* data. In addition, AMPK promotes the anti-inflammatory polarization (M2) of macrophages or microglia (Cui et al., 2023). Activation of AMPK delays aging and extends lifespan (Stancu, 2015; Burkewitz et al., 2016). Indeed, indirect AMPK activators, such as resveratrol, metformin, exercise, caloric restriction, increase longevity (Sifani et al., 2011; Sifani et al., 2012; Lundberg et al., 2014; Duca et al., 2015; Fentz et al., 2015; Ido et al., 2015; Sifani et al., 2015; Weir et al., 2017; Kodali et al., 2021; Cao et al., 2022; Hassani et al., 2022; Suzuta et al. ., 2022; Ameen et al., 2023; Karagoz et al., 2023). Together with these results, some studies on BGs have shown activation of AMPK signalling, mainly during obesity or diabetes (Zhu et al., 2013; Eveline et al., 2015; Tang et al., 2020; Wouk et al., 2021; Chen et al., 2023). Overall, the effects by BGs that I observed could result in activation on AMPK signalling. The specific mechanisms that may regulate AMPK by BGs are unclear. It is possible that BGs fermented by the microbiota produce Short-Chain Fatty Acids, SCFAs, (Jalil et al., 2021; Chen et al., 2022; Petit et al., 2022) which regulate the activation of AMPK (Elamin et al., 2013; Jan et al., 2013; Besten et al., 2015; Sun and Zhu, 2017; He et al., 2020). In addition to this hypothesis, it is possible that BGs modulate immune system regulating AMPK activation. These two hypotheses could coexist, in which case it would be interesting to understand the mechanism of action in detail.

7.5 Future Prospective

The results of this study indicate the anti-aging effect of BG in many impaired processes. First of all, it would be interesting to understand the mechanism of action of BGs during aging, how BGs regulate these processes. After having observed the modulation of the

bidirectional relationship between autophagy and mitochondria, in the future we could investigate the effect of BGs in pathologies with dysregulation of this process. Finally, it would be useful to translate these data into human clinical trial to test whether BGs can reduce cardiometabolic risk factors and improve physiological function in old age.

8 CONCLUSION

To conclude, these results demonstrate an anti-aging effect of BGs. BGs can be used as dietary supplements to prevent certain aging phenotypes. In fact, prolonged use of BGs has been found to be healthy and non-toxic, so BGs can be included in our lifestyle as a preventive anti-aging intervention. Furthermore, I demonstrated the direct effect of BGs in the brain, both on microglia and neurons. The direct effect of BGs predominantly acted in normalizing dysregulated processes, such as autophagy and mitochondrial respiration. Furthermore, these results show the dysregulation of these processes in the *ex-vivo* brain model of *Nfu*. Overall, BGs can be considered valid supplements to decrease aging phenotypes, specifically brain aging phenotypes.

9 LIMITATIONS OF PROJECT

In acknowledging the significance of our research endeavour, it is imperative to address the inherent limitations that shape the scope and generalizability of our findings. Firstly, a major limitation of this study was the insufficient number of fish to accurately determine the survival curve for the chronic diet of BGs. However, despite this limitation, the data allowed us to consider the treatment as non-harmful if taken chronically at a concentration comparable to dietary supplements.

Although *Nfu* is an excellent model for studying aging, it may not accurately replicate all physiological and pathological features of humans, limiting the translation of the results to human medicine. However, in this study we analysed phenotypes conserved between *Nfu* and other mammals, so the observed results can be translated to humans or other mammals. To translate the effect of BGs on human aging, further studies on complementary models in physiological mechanisms not common with Nfu but unique to mammals would be warranted to avoid negative outcomes resulting from chronic BG intake. Furthermore, the observation of the effect of BGs on IPCS derived human neurons lays the foundation to investigate these effects in detail in humans.

Another limitation of this study is the lack of detailed understanding of the mechanisms by which BGs act to modulate and reduce aging. While the study provides evidence that BGs have a beneficial effect, the precise biochemical and molecular pathways involved remain unclear. This gap in knowledge limits our ability to fully comprehend how BGs exert their anti-aging effects and suggests the need for further research to elucidate these mechanisms.

10 REFERENCES

1. Ahnen, R. T., Jonnalagadda, S. S. & Slavin, J. L. Role of plant protein in nutrition, wellness, and health. Nutr. Rev. 77, 735–747 (2019).

2. Ahuja, G. et al. Loss of genomic integrity induced by lysosphingolipid imbalance drives ageing in the heart. EMBO Rep. 20, 1–20 (2019).

3. Al-Edresi, S., Alsalahat, I., Freeman, S., Aojula, H. & Penny, J. Resveratrol-mediated cleavage of amyloid β1–42 peptide: potential relevance to Alzheimer's disease. Neurobiol. Aging 94, 24–33 (2020).

4. Ali, M. A., Poortvliet, E., Strömberg, R. & Yngve, A. Polyamines in foods: Development of a food database. Food Nutr. Res. 55, (2011).

5. Alsaleh, G. et al. Autophagy in t cells from aged donors is maintained by spermidine and correlates with function and vaccine responses. Elife 9, 1–21 (2020).

6. Aman, Y. et al. Autophagy in healthy aging and disease. Nat. Aging 1, 634–650 (2021).

7. Araki, K. et al. mTOR regulates memory CD8 T-cell differentiation. Nature 460, 108–112 (2009).

8. Aramillo Irizar, P. et al. Transcriptomic alterations during ageing reflect the shift from cancer to degenerative diseases in the elderly. Nat. Commun. 9, 1–11 (2018).

9. Aranaz, P. et al. Grifola frondosa (Maitake) Extract Reduces Fat Accumulation and Improves Health Span in C. elegans through the DAF-16/FOXO and SKN-1/NRF2 Signalling Pathways. (2021).

10. Armanios, M. et al. Short Telomeres are Sufficient to Cause the Degenerative Defects Associated with Aging. Am. J. Hum. Genet. 85, 823–832 (2009).

11. Ashkenazi, A. et al. Polyglutamine tracts regulate beclin 1-dependent autophagy. Nature 545, 108–111 (2017).

12. Ashraf Khan, A., Gani, A., Masoodi, F. A., Mushtaq, U. & Silotry Naik, A. Structural, rheological, antioxidant, and functional properties of β–glucan extracted from edible mushrooms Agaricus bisporus, Pleurotus ostreatus and Coprinus attrimentarius. Bioact. Carbohydrates Diet. Fibre 11, 67–74 (2017).

13. Aufschnaiter, A. & Büttner, S. The vacuolar shapes of ageing: From function to morphology. Biochim. Biophys. Acta - Mol. Cell Res. 1866, 957–970 (2019).

14. Bachstetter, A. D. et al. Microglial p38α MAPK is a key regulator of proinflammatory cytokine up-regulation induced by toll-like receptor (TLR) ligands or beta-amyloid (Aβ). J. Neuroinflammation 8, 1–12 (2011).

15. Baechle, J. J. et al. Chronic inflammation and the hallmarks of aging. Mol. Metab. 74, 1–26 (2023).

16. Bagnoli, S., Fronte, B., Bibbiani, C., Terzibasi Tozzini, E. & Cellerino, A. Quantification of noradrenergic-, dopaminergic-, and tectal-neurons during aging in the short-lived killifish Nothobranchius furzeri. Aging Cell 21, 1–12 (2022).

17. Bahour, N. et al. Diabetes mellitus correlates with increased biological age as indicated by clinical biomarkers. GeroScience 44, 415–427 (2022).

18. Bai, J. et al. Source of gut microbiota determines oat β-glucan degradation and short chain fatty acid-producing pathway. Food Biosci. 41, 101010 (2021).

19. Baldwin, K. T., Carbajal, K. S., Segal, B. M. & Giger, R. J. Neuroinflammation triggered by β-glucan/dectin-1 signaling enables CNS axon regeneration. Proc. Natl. Acad. Sci. U. S. A. 112, 2581–2586 (2015).

20. Barbosa, M. C., Grosso, R. A. & Fader, C. M. Hallmarks of aging: An autophagic perspective. Front. Endocrinol. (Lausanne). 10, 1–13 (2019).

21. Barzilai, N., Huffman, D. M., Muzumdar, R. H. & Bartke, A. The critical role of metabolic pathways in aging. Diabetes 61, 1315–1322 (2012).

22. Batbayar, S., Lee, D. H. & Kim, H. W. Immunomodulation of fungal β-glucan in host defense signaling by dectin-1. Biomol. Ther. 20, 433–445 (2012).

23. Baumgart, M. et al. RNA-seq of the aging brain in the short-lived fish N. furzeri - conserved pathways and novel genes associated with neurogenesis. Aging Cell 13, 965–974 (2014).

24. Bejarano, E. & Cuervo, A. M. Chaperone-mediated autophagy. Proc. Am. Thorac. Soc. 7, 29–39 (2010).

25. Bergamini, E., Cavallini, G., Donati, A. & Gori, Z. The role of macroautophagy in the ageing process, anti-ageing intervention and age-associated diseases. Int. J. Biochem. Cell Biol. 36, 2392–2404 (2004).

26. Bergamini, E. et al. A new method for the investigation of endocrine-regulated autophagy and protein degradation in rat liver. Experimental and Molecular Pathology vol. 59 13–26 (1993).

27. Bernardes de Jesus, B. et al. Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer. EMBO Mol. Med. 4, 691–704 (2012).

28. Bernier, M. et al. Resveratrol supplementation confers neuroprotection in cortical brain tissue of nonhuman primates fed a high-fat/sucrose diet. Aging (Albany. NY). 8, 899–916 (2016).

29. Bertholet, A. M. et al. Mitochondrial fusion/fission dynamics in neurodegeneration and neuronal plasticity. Neurobiol. Dis. 90, 3–19 (2016).

30. Bitto, A. et al. Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. Elife 5, 1–17 (2016).

31. Bjedov, I. et al. Mechanisms of Life Span Extension by Rapamycin in the Fruit Fly Drosophila melanogaster. Cell Metab. 11, 35–46 (2010).

32. Blackburn, E. H., Greider, C. W. & Szostak, J. W. Telomeres and telomerase: The path from maize, Tetrahymena and yeast to human cancer and aging. Nat. Med. 12, 1133–1138 (2006).

33. Blasco, M. A. Telomere length, stem cells and aging. Nat. Chem. Biol. 3, 640–649 (2007).

34. Blasco, M. A. et al. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91, 25–34 (1997).

35. Błaszczyk, K. et al. Impact of low and high molecular weight oat beta-glucan on oxidative stress and antioxidant defense in spleen of rats with LPS induced enteritis. Food Hydrocoll. 51, 272–280 (2015).

36. Blažek, R. et al. Repeated intraspecific divergence in life span and aging of African annual fishes along an aridity gradient. Evolution (N. Y). 71, 386–402 (2017).

37. Bodnar, A. G. et al. Extension of life-span by introduction of telomerase into normal human cells. Science (80-.). 279, 349–352 (1998).

38. Boland, B. et al. Autophagy induction and autophagosome clearance in neurons: Relationship to autophagic pathology in Alzheimer's disease. J. Neurosci. 28, 6926–6937 (2008).

39. Bonilla, D. L. et al. Autophagy regulates phagocytosis by modulating the expression of scavenger receptors. Immunity 39, 537–547 (2013).

40. Boonekamp, J. J., Simons, M. J. P., Hemerik, L. & Verhulst, S. Telomere length behaves as biomarker of somatic redundancy rather than biological age. Aging Cell 12, 330–332 (2013).

41. Brogi, L. et al. β-Glucans as Dietary Supplement to Improve Locomotion and Mitochondrial Respiration in a Model of Duchenne Muscular Dystrophy. Nutrients 13, (2021).

42. Brown, G. D. & Gordon, S. Immune recognition of fungal β-glucans. Cell. Microbiol. 7, 471–479 (2005).

43. Brunk, U. T. & Terman, A. Lipofuscin: Mechanisms of age-related accumulation and influence on cell function. Free Radic. Biol. Med. 33, 611–619 (2002).

44. Buffen, K. et al. Autophagy Controls BCG-Induced Trained Immunity and the Response to Intravesical BCG Therapy for Bladder Cancer. PLoS Pathog. 10, (2014).

45. Burkewitz, K., Zhang, Y. & Mair, W. B. AMPK at the nexus of energetics and aging. Cell Metab. 20, 10–25 (2014).

46. Caccamo, A., Majumder, S., Richardson, A., Strong, R. & Oddo, S. Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-β, and Tau: Effects on cognitive impairments. J. Biol. Chem. 285, 13107–13120 (2010).

47. Caccamo, A. et al. Naturally secreted amyloid-β increases mammalian target of rapamycin (mTOR) activity via a PRAS40-mediated mechanism. J. Biol. Chem. 286, 8924–8932 (2011).

48. Cagan, A. et al. Somatic mutation rates scale with lifespan across mammals. Nature 604, 517–524 (2022).

49. Camilli, G. et al. β-Glucan-induced reprogramming of human macrophages inhibits NLRP3 inflammasome activation in cryopyrinopathies. J. Clin. Invest. 130, 4561–4573 (2020).

50. Campisi, J. & D'Adda Di Fagagna, F. Cellular senescence: When bad things happen to good cells. Nat. Rev. Mol. Cell Biol. 8, 729–740 (2007).

51. Cao, B. et al. Neuroprotective effects of liraglutide against inflammation through the AMPK/NF-κB pathway in a mouse model of Parkinson's disease. Metab. Brain Dis. 37, 451–462 (2022).

52. Caponio, D. et al. Compromised autophagy and mitophagy in brain ageing and Alzheimer's diseases. Aging Brain 2, 100056 (2022).

53. Carling, D., Thornton, C., Woods, A. & Sanders, M. J. AMP-activated protein kinase: New regulation, new roles? Biochem. J. 445, 11–27 (2012).

54. Carmona-Gutierrez, D., Hughes, A. L., Madeo, F. & Ruckenstuhl, C. The crucial impact of lysosomes in aging and longevity. Ageing Res. Rev. 32, 2–12 (2016).

55. Carmona-Gutierrez, D., Hughes, A. L., Madeo, F. & Ruckenstuhl, C. The crucial impact of lysosomes in aging and longevity. Ageing Res. Rev. 32, 2–12 (2016).

56. Cellerino, A., Valenzano, D. R. & Reichard, M. From the bush to the bench: The annual Nothobranchius fishes as a new model system in biology. Biol. Rev. 91, 511–533 (2016).

57. Cha, H. J. et al. Tat-Thioredoxin-like protein 1 attenuates ischemic brain injury by regulation of MAPKs and apoptosis signaling. BMB Rep. 56, 234–239 (2023).

58. Chan, G. C.-F., Chan, W. K. & Sze, D. M.-Y. The effects of β-glucan on human immune and cancer cells. J. Hematol. Oncol. 2, (2009).

59. Chang, C. H. et al. Resveratrol-induced autophagy and apoptosis in cisplatin-resistant human oral cancer CAR cells: A key role of AMPK and Akt/mTOR signaling. Int. J. Oncol. 50, 873–882 (2017).

60. Chapin, H. C., Okada, M., Merz, A. J. & Miller, D. L. Tissue-specific autophagy responses to aging and stress in C. elegans. Aging (Albany. NY). 7, 419–434 (2015).

61. Chen, B. et al. β-glucan, a specific immuno-stimulant, produces rapid antidepressant effects by stimulating ERK1/2-dependent synthesis of BDNF in the hippocampus. Eur. J. Pharmacol. 961, (2023).

62. Chen, C., Huang, X., Wang, H., Geng, F. & Nie, S. Effect of β-glucan on metabolic diseases: a review from the gut microbiota perspective. Curr. Opin. Food Sci. 47, 100907 (2022).

63. Chen, H., Liu, N., He, F., Liu, Q. & Xu, X. Specific β-glucans in chain conformations and their biological functions. Polym. J. 54, 427–453 (2022).

64. Chen, J. & Raymond, K. Beta-glucans in the treatment of diabetes and associated cardiovascular risks. Vasc. Health Risk Manag. 4, 1265–1272 (2008).

65. Chen, J. & Seviour, R. Medicinal importance of fungal β-(1→3), (1→6)-glucans. Mycol. Res. 111, 635–652 (2007).

66. Chen, Y. et al. 1,3-β-Glucan affects the balance of Th1/Th2 cytokines by promoting secretion of anti-inflammatory cytokines in vitro. Mol. Med. Rep. 8, 708–712 (2013).

67. Cheung, I. Y. et al. Effect of Oral β-Glucan on Antibody Response to Ganglioside Vaccine in Patients with High-Risk Neuroblastoma: A Phase 2 Randomized Clinical Trial. JAMA Oncol. 9, 242–250 (2023).

68. Chihara, G., Maeda, Y., Hamuro, J., Sasaki, T. & Fukuoka, F. Inhibition of mouse sarcoma 180 by polysaccharides from Lentinus edodes (Berk.) sing. Nature 222, 687–688 (1969).

69. Chinraj, V. & Raman, S. Neuroprotection by resveratrol: A review on brain delivery strategies for Alzheimer's and Parkinson's disease. J. Appl. Pharm. Sci. 12, 1–17 (2022).

70. Chinta, S. J. et al. Cellular senescence and the aging brain. Exp. Gerontol. 68, 3–7 (2015).

71. Chistiakov, D. A., Sobenin, I. A., Revin, V. V., Orekhov, A. N. & Bobryshev, Y. V. Mitochondrial aging and age-related dysfunction of mitochondria. Biomed Res. Int. 2014, (2014).

72. Ciecierska A., Drywień M.E. , Hamulka J., S. T. NUTRACEUTICAL FUNCTIONS OF BETA -GLUCANS. Ann. Natl. Inst. Hyg. 70, 315–324 (2019).

73. Clarke, A. J. & Simon, A. K. Autophagy in the renewal, differentiation and homeostasis of immune cells. Nat. Rev. Immunol. 19, 170–183 (2019).

74. Clarke, L. E. et al. Normal aging induces A1-like astrocyte reactivity. Proc. Natl. Acad. Sci. U. S. A. 115, E1896–E1905 (2018).

75. Clarke, L. E. et al. Normal aging induces A1-like astrocyte reactivity. Proc. Natl. Acad. Sci. U. S. A. 115, E1896–E1905 (2018).

76. Cognigni, V., Ranallo, N., Tronconi, F., Morgese, F. & Berardi, R. Potential benefit of β-glucans as adjuvant therapy in immuno-oncology: a review. Explor. Target. Anti-tumor Ther. 2, 122–138 (2021).

77. Colacurcio, D. J. & Nixon, R. A. Disorders of lysosomal acidification—The emerging role of v-ATPase in aging and neurodegenerative disease. Ageing Res. Rev. 32, 75–88 (2016).

78. Collado, M., Blasco, M. A. & Serrano, M. Cellular Senescence in Cancer and Aging. Cell 130, 223–233 (2007).

79. Collins, M. P., Stransky, L. A. & Forgac, M. AKT Ser/Thr kinase increases V-ATPase-dependent lysosomal acidification in response to amino acid starvation in mammalian cells. J. Biol. Chem. 295, 9433–9444 (2020).

80. Colton, C. A. et al. Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. J. Neuroinflammation 3, 1–12 (2006).

81. Cooney, R. et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat. Med. 16, 90–97 (2010).

82. Coppé, J. P., Desprez, P. Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: The dark side of tumor suppression. Annu. Rev. Pathol. Mech. Dis. 5, 99–118 (2010).

83. Cristofalo, V. J. SA β Gal staining: Biomarker or delusion. Exp. Gerontol. 40, 836–838 (2005).

84. Cuervo, A. M. Chaperone-mediated autophagy: Selectivity pays off. Trends Endocrinol. Metab. 21, 142–150 (2010).

85. Cuervo, A. M. et al. Autophagy and aging: the importance of maintaining 'clean' cells. Autophagy 1, 131–140 (2005).

86. Cuervo, A. M., Stafanis, L., Fredenburg, R., Lansbury, P. T. & Sulzer, D. Impaired degradation of mutant α-synuclein by chaperone-mediated autophagy. Science (80-.). 305, 1292–1295 (2004).

87. Cuervo, A. M. & Wong, E. Chaperone-mediated autophagy: Roles in disease and aging. Cell Res. 24, 92–104 (2014).

88. Cui, Z. et al. β-Glucan alleviates goal-directed behavioral deficits in mice infected with Toxoplasma gondii. Parasites and Vectors 16, 1–15 (2023).

89. Damani, M. R. et al. Age-related alterations in the dynamic behavior of microglia. Aging Cell 10, 263–276 (2011).

90. Daou, C. & Zhang, H. Oat Beta-Glucan: Its Role in Health Promotion and Prevention of Diseases. Compr. Rev. Food Sci. Food Saf. 11, 355–365 (2012).

91. David, J. P. et al. Glial reaction in the hippocampal formation is highly correlated with aging in human brain. Neurosci. Lett. 235, 53–56 (1997).

92. Davis, J. M. et al. Effects of oat β-glucan on innate immunity and infection after exercise stress. Med. Sci. Sports Exerc. 36, 1321–1327 (2004).

93. de Bakker, D. E. M. & Valenzano, D. R. Turquoise killifish: A natural model of age-dependent brain degeneration. Ageing Res. Rev. 90, 102019 (2023).

94. De Biase, D. et al. Amyloid precursor protein, lipofuscin accumulation and expression of autophagy markers in aged bovine brain. BMC Vet. Res. 13, 1–9 (2017).

95. De Costa, J., Barja, G. & Almaida-Pagan, P. F. Membrane peroxidation index and maximum lifespan are negatively correlated in fish of the genus Nothobranchius. J. Exp. Biol. 223, 7–11 (2020).

96. De La Lastra, C. A. & Villegas, I. Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. Mol. Nutr. Food Res. 49, 405–430 (2005).

97. de Magalhães, J. P. Ageing as a software design flaw. Genome Biol. 24, 1–20 (2023).

98. De Marco Castro, E., Calder, P. C. & Roche, H. M. β-1,3/1,6-Glucans and Immunity: State of the Art and Future Directions. Mol. Nutr. Food Res. 65, (2021).

99. Del Roso, A. et al. Ageing-related changes in the in vivo function of rat liver macroautophagy and proteolysis. Exp. Gerontol. 38, 519–527 (2003).

100. Delgado, M. A. & Deretic, V. Toll-like receptors in control of immunological autophagy. Cell Death Differ. 16, 976–983 (2009).

101. Delgado, M. A., Elmaoued, R. A., Davis, A. S., Kyei, G. & Deretic, V. Toll-like receptors control autophagy. EMBO J. 27, 1110–1121 (2008).

102. den Besten G., Bleeker A., Gerding A., van Eunen K., Havinga R., van Dijk T.H., Oosterveer M.H., Jonker J.W., Groen A.K., Reijngoud D.-J., B. B. M. Short-Chain Fatty Acids Protect Against High-Fat Diet–Induced Obesity via a PPARg-Dependent Switch From Lipogenesis to Fat Oxidation. Diabetes 64, 2398–2408 (2015).

103. Den Besten, G. et al. Short-chain fatty acids protect against high-fat diet-induced obesity via a pparg-dependent switch from lipogenesis to fat oxidation. Diabetes 64, 2398–2408 (2015).

104. Deretic, V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. Immunol. Rev. 240, 92–104 (2011).

105. Deretic, V. & Levine, B. Autophagy, Immunity, and Microbial Adaptations. Cell Host Microbe 5, 527–549 (2009).

106. Deretic, V. et al. Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cell. Microbiol. 8, 719–727 (2006).

107. Dhanya, T. et al. Influence of age on the pathology of hippocampus and variation in the number of neurons of the hilar region in the brain of dogs. J. Vet. Anim. Sci. 53, (2022).

108. Di Cicco, E., Tozzini, E. T., Rossi, G. & Cellerino, A. The short-lived annual fish Nothobranchius furzeri shows a typical teleost aging process reinforced by high incidence of age-dependent neoplasias. Exp. Gerontol. 46, 249–256 (2011).

109. Di Luzio N.R. and Riggi S.J. The effects of laminarin, sulfated glucan and oligosaccharides of glucan on reticuloendothelial activity. J Reticuloendothel Soc . 8, 465–473 (1970).

110. Di Luzio, N. R. Update on the immunomodulating activities of glucans. Springer Semin. Immunopathol. 8, 387–400 (1985).

111. Dimri, G. P. et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. U. S. A. 92, 9363–9367 (1995).

112. Ding, B. et al. Effects of dietary yeast beta-1,3-1,6-glucan on growth performance, intestinal morphology and chosen immunity parameters changes in Haidong chicks. Asian-Australasian J. Anim. Sci. 32, 1558–1564 (2019).

113. Ding, J. et al. β-Glucan induces autophagy in dendritic cells and influences T-cell differentiation. Med. Microbiol. Immunol. 208, 39–48 (2019).

114. Ding, L., Kuhne, W. W., Hinton, D. E., Song, J. & Dynan, W. S. Quantifiable biomarkers of normal aging in the Japanese Medaka fish (Oryzias latipes). PLoS One 5, (2010).

115. Ding, S. et al. Resveratrol and caloric restriction prevent hepatic steatosis by regulating SIRT1-autophagy pathway and alleviating endoplasmic reticulum stress in high-fat diet-fed rats. PLoS One 12, 1–16 (2017).

116. Dodzian, J., Kean, S., Seidel, J. & Valenzano, D. R. A protocol for laboratory housing of Turquoise killifish (Nothobranchius furzeri). J. Vis. Exp. 2018, 1–9 (2018).

117. Donati, A. et al. Age-related changes in the regulation of autophagic proteolysis in rat isolated hepatocytes. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 56, B288–B293 (2001).

118. dos Santos, M. G. et al. Neuroprotective Effects of Resveratrol in In vivo and In vitro Experimental Models of Parkinson's Disease: a Systematic Review. Neurotox. Res. 40, 319–345 (2022).

119. Du, B., Meenu, M., Liu, H. & Xu, B. A concise review on the molecular structure and function relationship of β-glucan. Int. J. Mol. Sci. 20, (2019).

120. Duarte, F. V., Ciampi, D. & Duarte, C. B. Mitochondria as central hubs in synaptic modulation. Cell. Mol. Life Sci. 80, (2023).

121. Duca, F. A. et al. Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. Nat. Med. 21, 506–511 (2015).

122. Dunlop, E. A. & Tee, A. R. MTOR and autophagy: A dynamic relationship governed by nutrients and energy. Semin. Cell Dev. Biol. 36, 121–129 (2014).

123. Durgan, J. et al. Non-canonical autophagy drives alternative ATG8 conjugation to phosphatidylserine. Mol. Cell 81, 2031-2040.e8 (2021).

124. Duysburgh, C. et al. In vitro–in vivo validation of stimulatory effect of oat ingredients on lactobacilli. Pathogens 10, 1–18 (2021).

125. Edelmann, K. et al. Increased radial glia quiescence, decreased reactivation upon injury and unaltered neuroblast behavior underlie decreased neurogenesis in the aging zebrafish telencephalon. J. Comp. Neurol. 521, 3099–3115 (2013).

126. Edirisinghe, S. L. et al. Dietary supplementation with a multi-strain yeast fraction enhances skin wound healing in zebrafish (Danio rerio). Aquac. Reports 32, 101741 (2023).

127. Eichhoff, G., Busche, M. A. & Garaschuk, O. In vivo calcium imaging of the aging and diseased brain. Eur. J. Nucl. Med. Mol. Imaging 35, (2008).

128. Eisenberg, T. et al. Cardioprotection and lifespan extension by the natural polyamine spermidine. Nat. Med. 22, 1428–1438 (2016).

129. El Khoury, D., Cuda, C., Luhovyy, B. L. & Anderson, G. H. Beta glucan: Health benefits in obesity and metabolic syndrome. J. Nutr. Metab. 2012, (2012).

130. Elamin, E. E., Masclee, A. A., Dekker, J., Pieters, H. J. & Jonkers, D. M. Short-chain fatty acids activate AMP-activated protein kinase and ameliorate ethanol-induced intestinal barrier dysfunction in Caco-2 cell monolayers. J. Nutr. 143, 1872–1881 (2013).

131. Ellett, F., Pase, L., Hayman, J. W., Andrianopoulos, A. & Lieschke, G. J. Mpeg1 Promoter Transgenes Direct Macrophage-Lineage Expression in Zebrafish. Blood 117, 49–56 (2011).

132. Endo, F. et al. Molecular basis of astrocyte diversity and morphology across the CNS in health and disease. Science (80-.). 378, (2022).

133. Evsiukova, V. S., Kulikova, E. A. & Kulikov, A. V. Age-related alterations in the behavior and serotonin-related gene mRNA levels in the brain of males and females of short-lived turquoise killifish (Nothobranchius furzeri). Biomolecules 11, (2021).

134. Falco, A. et al. Reduced inflammatory response to Aeromonas salmonicida infection in common carp (Cyprinus carpio L.) fed with β-glucan supplements. Fish Shellfish Immunol. 32, 1051–1057 (2012).

135. Fang, E. F. et al. Mitophagy inhibits amyloid-β and tau pathology and reverses cognitive deficits in models of Alzheimer's disease. Nat. Neurosci. 22, 401–412 (2019).

136. Fang, E. F. et al. Nuclear DNA damage signalling to mitochondria in ageing. Nat. Rev. Mol. Cell Biol. 17, 308–321 (2016).

137. Fang, J. et al. Structure of a β-glucan from Grifola frondosa and its antitumor effect by activating Dectin-1/Syk/NF-κB signaling. Glycoconj. J. 29, 365–377 (2012).

138. Farkas, E., De Jong, G. I., De Vos, R. A. I., Jansen Steur, E. N. H. & Luiten, P. G. M. Pathological features of cerebral cortical capillaries are doubled in Alzheimer's disease and Parkinson's disease. Acta Neuropathol. 100, 395–402 (2000).

139. Fatima, N. et al. Particulate β-glucan activates early and delayed phagosomal maturation and autophagy within macrophage in a NOX-2 dependent manner. Life Sci. 266, 118851 (2021).

140. Fatima, N., Upadhyay, T., Sharma, D. & Sharma, R. Particulate beta-glucan induces early and late phagosomal maturation in murine macrophages. Front. Biosci. - Elit. 9, 129–140 (2017).

141. Fentz, J. et al. AMPKα is critical for enhancing skeletal muscle fatty acid utilization during in vivo exercise in mice. FASEB J. 29, 1725–1738 (2015).

142. Fernández, Á. F. et al. Regulatory Complex Promotes Longevity in Mice. Nature 558, 136–140 (2018).

143. Ferraresi, A. et al. Resveratrol inhibits IL-6-induced ovarian cancer cell migration through epigenetic up-regulation of autophagy. Mol. Carcinog. 56, 1164–1181 (2017).

144. Ferrari, R. Mitochondria and the heart. Eur. Heart J. 32, 2911–2918 (2011).

145. Ferreira, C. S. et al. Metabolic variables of obese dogs with insulin resistance supplemented with yeast beta-glucan. BMC Vet. Res. 18, 1–10 (2022).

146. Ferrucci, L. & Fabbri, E. Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nat. Rev. Cardiol. 15, 505–522 (2018).

147. Flanary, B. E. & Streit, W. J. Progressive Telomere Shortening Occurs in Cultured Rat Microglia, but Not Astrocytes. Glia 45, 75–88 (2004).

148. Florea, M. Aging and immortality in unicellular species. Mech. Ageing Dev. 167, 5–15 (2017).

149. Fonken, L. K. et al. The Alarmin HMGB1 Mediates Age-Induced Neuroinflammatory Priming. 36, 7946–7956 (2016).

150. Fonseca-Santos, B. et al. Intranasal in situ gelling liquid crystal for delivery of resveratrol ameliorates memory and neuroinflammation in Alzheimer's disease. Nanomedicine Nanotechnology, Biol. Med. 51, (2023).

151. Fontana, L., Partridge, L. & Longo, V. D. Extending healthy life span-from yeast to humans. Science (80-.). 328, 321–326 (2010).

152. Frake, R. A., Ricketts, T., Menzies, F. M. & Rubinsztein, D. C. Autophagy and neurodegeneration. J. Clin. Invest. 125, 65–74 (2015).

153. Fronte, B., Kim, C., Bagliacca, M., Casini, L. & De Zoysa, M. 1,3-1-6 ß-glucans enhance tissue regeneration in zebrafish (Danio rerio): Potential advantages for aquaculture applications. Aquac. Res. 50, (2019).

154. Fuller, R. et al. Influence of yeast-derived 1,3/1,6 glucopolysaccharide on circulating cytokines and chemokines with respect to upper respiratory tract infections. Nutrition 28, 665–669 (2012).

155. Fumagalli, M. et al. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nat. Cell Biol. 14, 355–365 (2012).

156. Futai, M., Sun-Wada, G. H., Wada, Y., Matsumoto, N. & Nakanishi-Matsui, M. Vacuolar-type ATPase: A proton pump to lysosomal trafficking. Proc. Japan Acad. Ser. B Phys. Biol. Sci. 95, 261–277 (2019).

157. Garc, J., Alonso-bellido, I. M. & Burguillos, M. A. Reformulating Pro-Oxidant Microglia in Neurodegeneration.

158. Gasser, T. Mendelian forms of Parkinson's disease. Biochim. Biophys. Acta - Mol. Basis Dis. 1792, 587–596 (2009).

159. Ge, X., Huang, S., Ren, C. & Zhao, L. Taurocholic Acid and Glycocholic Acid Inhibit Inflammation and Activate Farnesoid X Receptor Expression in LPS-Stimulated Zebrafish and Macrophages. Molecules 28, (2023).

160. Genade, T. et al. Annual fishes of the genus Nothobarnchius as a model system for aging research. Aging Cell 4, 223–233 (2005).

161. Gerhard, G. S. et al. Life spans and senescent phenotypes in two strains of Zebrafish (Danio rerio). Exp. Gerontol. 37, 1055–1068 (2002).

162. Germic, N., Frangez, Z., Yousefi, S. & Simon, H. U. Regulation of the innate immune system by autophagy: neutrophils, eosinophils, mast cells, NK cells. Cell Death Differ. 26, 703–714 (2019).

163. Geyfman, M. & Andersen, B. Clock genes, hair growth and aging. Aging (Albany. NY). 2, 122–128 (2010).

164. Gharagozloo, M. et al. Effects of beta-glucan on protection of young and aged rats from renal ischemia and reperfusion injury. Bratislava Med. J. 116, 296–301 (2015).

165. Giese, E. C. et al. Free-radical scavenging properties and antioxidant activities of botryosphaeran and some other β-D-glucans. Int. J. Biol. Macromol. 72, 125–130 (2015).

166. Ginhoux, F., Lim, S., Hoeffel, G., Low, D. & Huber, T. Origin and differentiation of microglia. Front. Cell. Neurosci. 7, 1–14 (2013).

167. Gkikas, I., Palikaras, K. & Tavernarakis, N. The role of mitophagy in innate immunity. Front. Immunol. 9, 1–15 (2018).

168. Glatigny, M. et al. Autophagy Is Required for Memory Formation and Reverses Age-Related Memory Decline. Curr. Biol. 29, 435-448.e8 (2019).

169. Goel, S. et al. Metformin: Activation of 5′ AMP-activated protein kinase and its emerging potential beyond anti-hyperglycemic action. Front. Genet. 13, 1–15 (2022).

170. Golisch, B., Lei, Z., Tamura, K. & Brumer, H. Configured for the Human Gut Microbiota: Molecular Mechanisms of Dietary β-Glucan Utilization. ACS Chem. Biol. 16, 2087–2102 (2021).

171. Gomez-Nicola, D. & Perry, V. H. Microglial dynamics and role in the healthy and diseased brain: A paradigm of functional plasticity. Neuroscientist 21, 169–184 (2015).

172. Gómez-Sintes, R., Ledesma, M. D. & Boya, P. Lysosomal cell death mechanisms in aging. Ageing Res. Rev. 32, 150–168 (2016).

173. Gou, M. et al. Reprogramming of ovarian aging epigenome by resveratrol. PNAS Nexus 2, 1–16 (2023).

174. Goyal, V. K. Lipofuscin pigment accumulation in human brain during aging. Exp. Gerontol. 17, 481–487 (1982).

175. Graf, M., Hartmann, N., Reichwald, K. & Englert, C. Absence of replicative senescence in cultured cells from the short-lived killifish Nothobranchius furzeri. Exp. Gerontol. 48, 17–28 (2013).

176. Gray, D. A. & Woulfe, J. Lipofuscin and aging: a matter of toxic waste. Sci. Aging Knowledge Environ. 2005, 1–5 (2005).

177. Greenwood, E. K. & Brown, D. R. Senescent microglia: The key to the ageing brain? Int. J. Mol. Sci. 22, (2021).

178. Griffin, R. et al. The age-related attenuation in long-term potentiation is associated with microglial activation. J. Neurochem. 99, 1263–1272 (2006).

179. Guebel, D. V. & Torres, N. V. Sexual dimorphism and aging in the human hyppocampus: Identification, validation, and impact of differentially expressed genes by factorial microarray and network analysis. Front. Aging Neurosci. 8, 1–22 (2016).

180. Guerrero-Navarro, L., Jansen-Dürr, P. & Cavinato, M. Age-Related Lysosomal Dysfunctions. Cells 11, 1–20 (2022).

181. Guo, Y. et al. Mitochondrial dysfunction in aging. Ageing Res. Rev. 88, 101955 (2023).

182. Guo, Y. et al. Thioredoxin-1 Is a Target to Attenuate Alzheimer-Like Pathology in Diabetic Encephalopathy by Alleviating Endoplasmic Reticulum Stress and Oxidative Stress. Front. Physiol. 12, 1–14 (2021).

183. Gustafsson, T. & Ulfhake, B. Sarcopenia: What Is the Origin of This Aging-Induced Disorder? Front. Genet. 12, 1–16 (2021).

184. Hadjiconstantinou, M. & Neff, N. H. Enhancing aromatic L-amino acid decarboxylase activity: Implications for L-DOPA treatment in Parkinson's disease. CNS Neurosci. Ther. 14, 340–351 (2008).

185. Halloran, J. et al. Chronic inhibition of mammalian target of rapamycin by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice. Neuroscience 223, 102–113 (2012).

186. Han, B. et al. Beta-glucan's varying structure characteristics modulate survival and immune-related genes expression from Vibrio harveyi-infected Artemia franciscana in gnotobiotic conditions. Fish Shellfish Immunol. 102, 307–315 (2020).

187. Han, X. et al. Research progress on natural β-glucan in intestinal diseases. Int. J. Biol. Macromol. 219, 1244–1260 (2022).

188. Han, X. et al. Impaired V-ATPase leads to increased lysosomal pH, results in disrupted lysosomal degradation and autophagic flux blockage, contributes to fluoride-induced developmental neurotoxicity. Ecotoxicol. Environ. Saf. 236, 113500 (2022).

189. Hand, S. C. Division of Comparative Physiology and Biochemistry , Society for Integrative and Comparative Biology Depression of Protein Synthesis during Diapause in Embryos of the Annual Killifish Austrofundulus limnaeus. (2016).

190. Hansen, M. et al. A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS Genet. 4, (2008).

191. Hansen, M., Rubinsztein, D. C. & Walker, D. W. Autophagy as a promoter of longevity: insights from model organisms. Nat. Rev. Mol. Cell Biol. 19, 579–593 (2018).

192. Harel, I. et al. A platform for rapid exploration of aging and diseases in a naturally short-lived vertebrate. Cell 160, 1013–1026 (2015).

193. Harman. The biologic clock: the mitochondria? J Am Geriatr Soc. 4, 145–147 (1972).

194. Harrison, D. E. et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 460, 392–395 (2009).

195. Hartl, F. U., Bracher, A. & Hayer-Hartl, M. Molecular chaperones in protein folding and proteostasis. Nature 475, 324–332 (2011).

196. Hartmann, N. et al. Telomeres shorten while Tert expression increases during ageing of the short-lived fish Nothobranchius furzeri. Mech. Ageing Dev. 130, 290–296 (2009).

197. Hartmann, N. et al. Mitochondrial DNA copy number and function decrease with age in the short-lived fish Nothobranchius furzeri. Aging Cell 10, 824–831 (2011).

198. Hashimoto, S., Gon, Y., Matsumoto, K., Takeshita, I. & Horie, T. N-acetylcysteine attenuates TNF-α-induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. Br. J. Pharmacol. 132, 270–276 (2001).

199. Hassani, B., Goshtasbi, G., Nooraddini, S. & Firouzabadi, N. Pharmacological Approaches to Decelerate Aging: A Promising Path. Oxid. Med. Cell. Longev. 2022, (2022).

200. Hatakeyama, H. et al. The teleost Oryzias latipes shows telomere shortening with age despite considerable telomerase activity throughout life. Mech. Ageing Dev. 129, 550–557 (2008).

201. Hayashi, Y. et al. Reverse of age-dependent memory impairment and mitochondrial DNA damage in microglia by an overexpression of human mitochondrial transcription factor A in mice. J. Neurosci. 28, 8624–8634 (2008).

202. He, J. et al. Short-chain fatty acids and their association with signalling pathways in inflammation, glucose and lipid metabolism. Int. J. Mol. Sci. 21, 1–16 (2020).

203. Heid, J. et al. Age-dependent increase of oxidative stress regulates microRNA-29 family preserving cardiac health. Sci. Rep. 7, 1–15 (2017).

204. Heng, Y. et al. Systemic administration of β-glucan induces immune training in microglia. J. Neuroinflammation 18, 1–15 (2021).

205. Henry, C. J., Huang, Y., Wynne, A. M. & Godbout, J. P. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. Brain. Behav. Immun. 23, 309–317 (2009).

206. Herbert W Virgin and Beth Levine. Autophagy genes in immunity. Nat. Immunol. 10, 461–470 (2009).

207. Herrera, E. et al. Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J. 18, 2950–2960 (1999).

208. Hewitt, G. et al. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. Nat. Commun. 3, (2012).

209. Hickman, E. et al. Expanded characterization of in vitro polarized M0, M1, and M2 human monocyte-derived macrophages: Bioenergetic and secreted mediator profiles. PLoS One 18, 1–19 (2023).

210. Hilbig, H., Bidmon, H. J., Steingrüber, S., Reinke, H. & Dinse, H. R. Enriched environmental conditions reverse age-dependent gliosis and losses of neurofilaments and extracellular matrix components but do not alter lipofuscin accumulation in the hindlimb area of the aging rat brain. J. Chem. Neuroanat. 23, 199–209 (2002).

211. Hoeijmakers, J. H. J. DNA Damage, Aging, and Cancer. Mol. Orig. CANCER 361, (2009).

212. Hofer, S. J. et al. Mechanisms of spermidine-induced autophagy and geroprotection. Nat. Aging 2, 1112–1129 (2022).

213. Holly, A. C. et al. Changes in splicing factor expression are associated with advancing age in man. Mech. Ageing Dev. 134, 356–366 (2013).

214. Holmes, C. Inflammation in Alzheimer's disease. Dementia, Fifth Ed. 14, 508–518 (2017).

215. Holtman, I. R. et al. Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis. Acta Neuropathol. Commun. 3, 31 (2015).

216. Hong F., Hansen R.D., Yan J., Allendorf D.J., Baran J.T., Ostroff G.R., R. G. D. Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. Cancer Res. 63, 9023–9031 (2003).

217. Hong, F. et al. Mechanism by Which Orally Administered. 3, (2022).

218. Hou, X., Watzlawik, J. O., Fiesel, F. C. & Springer, W. Autophagy in Parkinson's Disease. J. Mol. Biol. 432, 2651–2672 (2020).

219. Hsu, C. Y., Chiu, Y. C., Hsu, W. L. & Chan, Y. P. Age-related markers assayed at different developmental stages of the annual fish nothobranchius rachovii. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 63, 1267–1276 (2008).

220. Hu, M. et al. Three Different Types of β-Glucans Enhance Cognition: The Role of the Gut-Brain Axis. Front. Nutr. 9, 1–14 (2022).

221. Huang, T. et al. β-Glucan ameliorates nonalcoholic steatohepatitis induced by methionine and choline-deficient diet in mice. J. Food Biochem. 44, 1–12 (2020).

222. Hubbard, V. M. et al. Macroautophagy Regulates Energy Metabolism during Effector T Cell Activation. J. Immunol. 185, 7349–7357 (2010).

223. Ido, Y. et al. Resveratrol prevents oxidative stress-induced senescence and proliferative dysfunction by activating the AMPK-FOXO3 cascade in cultured primary human keratinocytes. PLoS One 10, 1–18 (2015).

224. Ikewaki, N. et al. β-glucan vaccine adjuvant approach for cancer treatment through immune enhancement (B-VACCIEN) in specific immunocompromised populations (Review). Oncol. Rep. 47, 1–9 (2022).

225. Ilie, O. D. et al. Mini-review on lipofuscin and aging: Focusing on the molecular interface, the biological recycling mechanism, oxidative stress, and the gut-brain axis functionality. Med. 56, 1–22 (2020).

226. Imler, E. et al. A drosophila model of neuronal ceroid lipofuscinosis CLN4 reveals a hypermorphic gain of function mechanism. Elife 8, 1–34 (2019).

227. Inoue, M. Free radical theory of aging. Nippon Ronen Igakkai zasshi. Japanese J. Geriatr. 39, 36–38 (2002).

228. Isakson, P., Holland, P. & Simonsen, A. The role of ALFY in selective autophagy. Cell Death Differ. 20, 12–20 (2013).

229. Islam, F. et al. Resveratrol and neuroprotection: an insight into prospective therapeutic approaches against Alzheimer's disease from bench to bedside. Mol. Neurobiol. 59, 4384–4404 (2022).

230. Jain, N. & Vogel, V. Spatial confinement downsizes the inflammatory response of macrophages. Nat. Mater. 17, 1134–1144 (2018).

231. Janes, M. R. & Fruman, D. A. Immune regulation by rapamycin: Moving beyond T cells. Sci. Signal. 2, 1–4 (2009).

232. Jang, S. A. et al. The comparative immunomodulatory Effects of β-glucans from yeast, bacteria, and mushroom on the function of macrophages. J. Food Sci. Nutr. 14, 102–108 (2009).

233. Jeong, H. et al. Acetylation Targets Mutant Huntingtin to Autophagosomes for Degradation. Cell 137, 60–72 (2009).

234. Jia, Y., Wang, N. & Liu, X. Resveratrol and amyloid-beta: Mechanistic insights. Nutrients 9, 1–13 (2017).

235. Jin, M. M. et al. A Critical Role of Autophagy in Regulating Microglia Polarization in Neurodegeneration. Front. Aging Neurosci. 10, 1–13 (2018).

236. Jo, M. et al. The role of TDP-43 propagation in neurodegenerative diseases: integrating insights from clinical and experimental studies. Exp. Mol. Med. 52, 1652–1662 (2020).

237. Johansson, L., Tuomainen, P., Ylinen, M., Ekholm, P. & Virkki, L. Structural analysis of water-soluble and -insoluble β-glucans of whole-grain oats and barley. Carbohydr. Polym. 58, 267–274 (2004).

238. Johnson, A. A. et al. The role of DNA methylation in aging, rejuvenation, and age-related disease. Rejuvenation Res. 15, 483–494 (2012).

239. Jonckheere, A. I., Smeitink, J. A. M. & Rodenburg, R. J. T. Mitochondrial ATP synthase: Architecture, function and pathology. J. Inherit. Metab. Dis. 35, 211–225 (2012).

240. Jounai, N. et al. The Atg5-Atg12 conjugate associates with innate antiviral immune responses. Proc. Natl. Acad. Sci. U. S. A. 104, 14050–14055 (2007).

241. Jun, Y. W., Lee, S., Ban, B. K., Lee, J. A. & Gao, F. B. Non-muscle MYH10/myosin IIB recruits ESCRT-III to participate in autophagosome closure to maintain neuronal homeostasis. Autophagy 19, 2045–2061 (2023).

242. Jung, M. & Pfeifer, G. P. Aging and DNA methylation. BMC Biol. 13, 1–8 (2015).

243. Jung, T., Bader, N. & Grune, T. Lipofuscin: Formation, distribution, and metabolic consequences. Ann. N. Y. Acad. Sci. 1119, 97–111 (2007).

244. Kalafatakis, I. & Karagogeos, D. Oligodendrocytes and microglia: Key players in myelin development, damage and repair. Biomolecules 11, (2021).

245. Kalaria, R. N. & Pax, A. B. Increased collagen content of cerebral microvessels in Alzheimer's disease. Brain Res. 705, 349–352 (1995).

246. Kanfi, Y. et al. The sirtuin SIRT6 regulates lifespan in male mice. Nature 483, 218–221 (2012).

247. Karagöz, M. F. & Gülçin Sağdıçoğlu Celep, A. The effect of caloric restriction on genetical pathways. Food Sci. Hum. Wellness 12, 1450–1457 (2023).

248. Katrachanca, S. M. & Koleske, A. J. 乳鼠心肌提取 HHS Public Access. Physiol. Behav. 176, 139–148 (2017).

249. Kaur R., Sharma M., Ji D., Xu M., A. D. Structural Features, Modification, and Functionalities of Beta-Glucan. 1–29 (2020).

250. Kaushik, S. et al. Autophagy and the hallmarks of aging. Ageing Res. Rev. 72, 101468 (2021).

251. Kaya, K. Beta-glucan attenuates cerebral ischemia / reperfusion-induced neuronal injury in a C57BL / J6 mouse model. 1–7 (2016).

252. Kelmer Sacramento, E. et al. Reduced proteasome activity in the aging brain results in ribosome stoichiometry loss and aggregation. Mol. Syst. Biol. 16, 1–22 (2020).

253. Kent S.A. and Miron V.E. Microglia regulation of central nervous system myelin health and regeneration. Nat. Rev. Immunol. 24, 49–63 (2023).

254. Kenyon, C. J. The genetics of ageing. Nature 464, 504–512 (2010).

255. Khokhlov, A. N. Impairment of regeneration in aging: Appropriateness or stochastics? Biogerontology 14, 703–708 (2013).

256. Kim, J. et al. (1-3)(1-6)-β-Glucan-enriched materials from Lentinus edodes mushroom as a high-fibre and low-calorie flour substitute for baked foods. J. Sci. Food Agric. 91, 1915–1919 (2011).

257. Kim, Y., Nam, H. G. & Valenzano, D. R. The short-lived African turquoise killifish: An emerging experimental model for ageing. DMM Dis. Model. Mech. 9, 115–129 (2016).

258. King, K. E., Losier, T. T. & Russell, R. C. Regulation of Autophagy Enzymes by Nutrient Signaling. Trends Biochem. Sci. 46, 687–700 (2021).

259. Kishi, S. et al. The zebrafish as a vertebrate model of functional aging and very gradual senescence. Exp. Gerontol. 38, 777–786 (2003).

260. Klionsky, D. J. et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4, 151–175 (2008).

261. Klionsky, D. J. et al. Autophagy in major human diseases. EMBO J. 40, 1–64 (2021).

262. Kodali, M. et al. Metformin treatment in late middle age improves cognitive function with alleviation of microglial activation and enhancement of autophagy in the hippocampus. Aging Cell 20, 1–19 (2021).

263. Koellhoffer, E. C., McCullough, L. D. & Ritzel, R. M. Old maids: Aging and its impact on microglia function. Int. J. Mol. Sci. 18, 1–25 (2017).

264. Kofuji, K. et al. Antioxidant Activity of β -Glucan. ISRN Pharm. 2012, (2012).

265. Koga, H., Kaushik, S. & Cuervo, A. M. Protein homeostasis and aging: The importance of exquisite quality control. Ageing Res. Rev. 10, 205–215 (2011).

266. Kogan, G. et al. Yeast cell wall polysaccharides as antioxidants and antimutagens: Can they fight cancer? Neoplasma 55, 387–393 (2008).

267. Kohama, S. G., Goss, J. R., Finch, C. E. & McNeill, T. H. Increases of glial fibrillary acidic protein in the aging female mouse brain. Neurobiol. Aging 16, 59–67 (1995).

268. Kopiasz, Ł. et al. Effects of dietary oat beta-glucans on colon apoptosis and autophagy through tlrs and dectin-1 signaling pathways— crohn's disease model study. Nutrients 13, 1–25 (2021).

269. Kopiasz, Ł., Dziendzikowska, K., Oczkowski, M., Harasym, J. & Gromadzka-Ostrowska, J. Low-molar-mass oat beta-glucan impacts autophagy and apoptosis in early stages of induced colorectal carcinogenesis in rats. Int. J. Biol. Macromol. 254, (2024).

270. Kotob, M., Hussein, A. & Abd-Elkareem, M. Histopathological changes of kidney tissue during aging. SVU-International J. Vet. Sci. 4, 54–65 (2021).

271. Kou, X. & Chen, N. Resveratrol as a natural autophagy regulator for prevention and treatment of Alzheimer's disease. Nutrients 9, (2017).

272. Krishnan, R., Jang, Y. S. & Oh, M. J. Beta glucan induced immune priming protects against nervous necrosis virus infection in sevenband grouper. Fish Shellfish Immunol. 121, 163–171 (2022).

273. Kuilman, T., Michaloglou, C., Mooi, W. J. & Peeper, D. S. The essence of senescence Thomas. GENES Dev. 1–17 (2010) doi:10.1101/gad.1971610.by.

274. Kujoth, G. C., Leeuwenburgh, C. & Prolla, T. A. Mitochondrial DNA mutations and apoptosis in mammalian aging. Cancer Res. 66, 7386–7389 (2006).

275. Kumar, A. V. & Mills, J. Non-canonical autophagy in aging and age-related diseases. Front. Cell Dev. Biol. 11, 1–7 (2023).

276. Kumar, B., Iqbal, M. A., Singh, R. K. & Bamezai, R. N. K. Resveratrol inhibits TIGAR to promote ROS induced apoptosis and autophagy. Biochimie 118, 26–35 (2015).

277. Kumar, D. et al. RTN4B interacting protein FAM134C promotes ER membrane curvature and has a functional role in autophagy. Mol. Biol. Cell 32, 1158–1170 (2021).

278. Kumar, R. & Reichert, A. S. Autophagy promotes mitochondrial respiration by providing serine for one-carbon-metabolism. Autophagy 17, 4480–4483 (2021).

279. Kursvietiene, L. et al. Anti-Cancer Properties of Resveratrol: A Focus on Its Impact on Mitochondrial Functions. Antioxidants 12, 1–24 (2023).

280. Kurz, D. J., Decary, S., Hong, Y. & Erusalimsky, J. D. Senescence-associated β-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. J. Cell Sci. 113, 3613–3622 (2000).

281. Kuzniewska, B. et al. Mitochondrial protein biogenesis in the synapse is supported by local translation. EMBO Rep. 21, 1–15 (2020).

282. Ladomersky, E. et al. The coincidence between increasing age, immunosuppression, and the incidence of patients with glioblastoma. Front. Pharmacol. 10, 1–13 (2019).

283. Lahiri, V., Hawkins, W. D. & Klionsky, D. J. Watch What You (Self-) Eat: Autophagic Mechanisms that Modulate Metabolism. Cell Metab. 29, 803–826 (2019).

284. Lan, F., Weikel, K. A., Cacicedo, J. M. & Ido, Y. Resveratrol-induced AMP-activated protein kinase activation is cell-type dependent: Lessons from basic research for clinical application. Nutrients 9, (2017).

285. Laplante, M. & Sabatini, D. M. MTOR signaling in growth control and disease. Cell 149, 274–293 (2012).

286. Latorre, E. et al. Small molecule modulation of splicing factor expression is associated with rescue from cellular senescence. BMC Cell Biol. 18, 1–15 (2017).

287. Lawrence, C. The husbandry of zebrafish (Danio rerio): A review. Aquaculture 269, (2007).

288. Lawrence, J. M., Schardien, K., Wigdahl, B. & Nonnemacher, M. R. Roles of neuropathology-associated reactive astrocytes: a systematic review. Acta Neuropathol. Commun. 11, 1–28 (2023).

289. Lee, C. et al. Structural specificities of cell surface β-glucan polysaccharides determine commensal yeast mediated immuno-modulatory activities. Nat. Commun. 12, (2021).

290. Lee, D. C. et al. Aging enhances classical activation but mitigates alternative activation in the central nervous system. Neurobiol. Aging 34, 1610–1620 (2013).

291. Lee, H. K., Lund, J. M., Ramanathan, B., Mizushima, N. & Iwasaki, A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science (80-.). 315, 1398–1401 (2007).

292. Lee, J.-A. Neuronal Autophagy: A Housekeeper or a Fighter in Neuronal Cell Survival? Exp. Neurobiol. 21, 1–8 (2012).

293. Lee, J., Giordano, S. & Zhang, J. Autophagy, mitochondria and oxidative stress: Cross-talk and redox signalling. Biochem. J. 441, 523–540 (2012).

294. Lee, J. Y. et al. HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. EMBO J. 29, 969–980 (2010).

295. Lee, J. H. et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell 141, 1146–1158 (2010).

296. Lee, J. H. et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell 141, 1146–1158 (2010).

297. Leonardi, G. C., Accardi, G., Monastero, R., Nicoletti, F. & Libra, M. Ageing: From inflammation to cancer. Immun. Ageing 15, 1–7 (2018).

298. Lepelletier, F. X., Mann, D. M. A., Robinson, A. C., Pinteaux, E. & Boutin, H. Early changes in extracellular matrix in Alzheimer's disease. Neuropathol. Appl. Neurobiol. 43, 167–182 (2017).

299. Letiembre, M. et al. Innate immune receptor expression in normal brain aging. Neuroscience 146, 248–254 (2007).

300. Levels, P. J., Gubbels, R. E. M. B. & Denucé, J. M. Oxygen consumption during embryonic development of the annual fish. Comp. Biochem. Physiol. 84A, 767–770 (1986).

301. Levine, B. Eating oneself and uninvited guests: Autophagy-related pathways in cellular defense. Cell 120, 159–162 (2005).

302. Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. Nature 469, 323–335 (2011).

303. Li, C. et al. Autophagy Is Induced in CD4+ T Cells and Important for the Growth Factor-Withdrawal Cell Death. J. Immunol. 177, 5163–5168 (2006).

304. Li, W. W., Li, J. & Bao, J. K. Microautophagy: Lesser-known self-eating. Cell. Mol. Life Sci. 69, 1125–1136 (2012).

305. Li, W. wen et al. Reducing lipofuscin accumulation and cardiomyocytic senescence of aging heart by enhancing autophagy. Exp. Cell Res. 403, 112585 (2021).

306. Li, X. et al. β-glucan, a dectin-1 ligand, promotes macrophage M1 polarization via NF-κB/autophagy pathway. Int. J. Oncol. 54, 271–282 (2019).

307. Li, Y. C. et al. Preparation and characterization of feruloylated oat β-glucan with antioxidant activity and colon-targeted delivery. Carbohydr. Polym. 279, 119002 (2022).

308. Liang, H. et al. The effect and underlying mechanism of yeast β-glucan on antiviral resistance of zebrafish against spring viremia of carp virus infection. Front. Immunol. 13, 1–13 (2022).

309. Liang, X. H. et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402, 672–676 (1999).

310. Liatis, S. et al. The consumption of bread enriched with betaglucan reduces LDL-cholesterol and improves insulin resistance in patients with type 2 diabetes. Diabetes Metab. 35, 115–120 (2009).

311. Liddelow, S. A. et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541, 481–487 (2017).

312. Lim, J. et al. Proteotoxic Stress Induces Phosphorylation of p62/SQSTM1 by ULK1 to Regulate Selective Autophagic Clearance of Protein Aggregates. PLoS Genet. 11, 1–28 (2015).

313. Lindner, A. B. & Demarez, A. Protein aggregation as a paradigm of aging. Biochim. Biophys. Acta - Gen. Subj. 1790, 980–996 (2009).

314. Lipinski, M. M. et al. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 107, 14164–14169 (2010).

315. Liu, B. et al. Oat β-glucan inhibits adipogenesis and hepatic steatosis in high fat diet-induced hyperlipidemic mice via AMPK signaling. J. Funct. Foods 41, 72–82 (2018).

316. Liu, C. et al. Differential expression of aging biomarkers at different life stages of the annual fish Nothobranchius guentheri. Biogerontology 13, 501–510 (2012).

317. Liu, L. R., Liu, J. C., Bao, J. S., Bai, Q. Q. & Wang, G. Q. Interaction of Microglia and Astrocytes in the Neurovascular Unit. Front. Immunol. 11, 1–11 (2020).

318. Liu, Z., Tang, C., Teng, X., Mohamed, Z. A. & Fan, J. Adenylate kinase 2 is a biomarker related to the prognosis of glioma and the immune microenvironment. J. Clin. Lab. Anal. 37, 1–15 (2023).

319. Lopez, A., Fleming, A. & Rubinsztein, D. C. Seeing is believing: Methods to monitor vertebrate autophagy in vivo. Open Biol. 8, (2018).

320. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The Hallmarks of Aging Europe PMC Funders Group. Cell 153, 1194–1217 (2013).

321. Lord, C. J. & Ashworth, A. The DNA damage response and cancer therapy. Nature 481, 287–294 (2012).

322. Lou, G. et al. Mitophagy and Neuroprotection. Trends Mol. Med. 26, 8–20 (2020).

323. Louka, A. et al. New lessons on TDP-43 from old N. furzeri killifish. Aging Cell 21, 1–18 (2022).

324. Lucas-Sánchez, A., Almaida-Pagán, P. F., Madrid, J. A., de Costa, J. & Mendiola, P. Age-related changes in fatty acid profile and locomotor activity rhythms in Nothobranchius korthausae. Exp. Gerontol. 46, 970–978 (2011).

325. Lucas-Sánchez, A., Almaida-Pagán, P. F., Tocher, D. R., Mendiola, P. & De Costa, J. Age-related changes in mitochondrial membrane composition of Nothobranchius rachovii. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 69 A, 142–151 (2014).

326. Lundberg, T. R., Fernandez-Gonzalo, R. & Tesch, P. A. Exercise-induced AMPK activation does not interfere with muscle hypertrophy in response to resistance training in men. J. Appl. Physiol. 116, 611–620 (2014).

327. Luyten, T. et al. Resveratrol-induced autophagy is dependent on IP3Rs and on cytosolic Ca2 +. Biochim. Biophys. Acta - Mol. Cell Res. 1864, 947–956 (2017).

328. M. Medina-Gali, R. et al. Beta-glucan enhances the response to SVCV infection in zebrafish. Dev. Comp. Immunol. 84, 307–314 (2018).

329. Ma, J. & Underhill, D. M. B-glucan signaling connects phagocytosis to autophagy. Glycobiology 23, (2013).

330. Macian, F. Autophagy in T Cell Function and Aging. Front. Cell Dev. Biol. 7, 1–9 (2019).

331. Madeo, F. et al. Spermidine : a physiological autophagy inducer acting as an anti-aging vitamin in humans ? Autophagy 15, 165–168 (2019).

332. Maglione, M. et al. Spermidine protects from age-related synaptic alterations at hippocampal mossy fiber-CA3 synapses. Sci. Rep. 9, 1–12 (2019).

333. Majtan, J. & Jesenak, M. β-Glucans: Multi-functional modulator of wound healing. Molecules 23, 1–15 (2018).

334. Majumder, S. et al. Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1β and enhancing NMDA signaling. Aging Cell 11, 326–335 (2012).

335. Mariën, V., Piskin, I., Zandecki, C., Van houcke, J. & Arckens, L. Age-related alterations in the behavioral response to a novel environment in the African turquoise killifish (Nothobranchius furzeri). Front. Behav. Neurosci. 17, 1–14 (2023).

336. Markus, M. A. & Morris, B. J. Resveratrol in prevention and treatment of common clinical conditions of aging. Clin. Interv. Aging 3, 331–339 (2008).

337. Marlatt, M. W. et al. Proliferation in the alzheimer hippocampus is due to microglia, not astroglia, and occurs at sites of amyloid deposition. Neural Plast. 2014, (2014).

338. Martinez, P. & Blasco, M. A. Role of shelterin in cancer and aging. Aging Cell 9, 653–666 (2010).

339. Martinez-Vicente, M. Autophagy in neurodegenerative diseases: From pathogenic dysfunction to therapeutic modulation. Semin. Cell Dev. Biol. 40, 115–126 (2015).

340. Masahito P., Aoki K., Egami N., Ishikawa T., S. H. Life-span studies on spontaneous tumor development in the Medaka (Oryzias latipes). 1058–1065 (1989).

341. Masliah, E., Iimoto, D. S., Saitoh, T., Hansen, L. A. & Terry, R. D. Increased immunoreactivity of brain spectrin in Alzheimer disease: a marker for synapse loss? Brain Res. 531, 36–44 (1990).

342. Masuda, T. et al. Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. Nature 566, 388–392 (2019).

343. Matsui, H., Kenmochi, N. & Namikawa, K. Age- and α-Synuclein-Dependent Degeneration of Dopamine and Noradrenaline Neurons in the Annual Killifish Nothobranchius furzeri. Cell Rep. 26, 1727-1733.e6 (2019).

344. Matsumoto, G., Wada, K., Okuno, M., Kurosawa, M. & Nukina, N. Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. Mol. Cell 44, 279–289 (2011).

345. McIntosh, M., Stone, B. A. & Stanisich, V. A. Curdlan and other bacterial (1→3)-β-D-glucans. Appl. Microbiol. Biotechnol. 68, 163–173 (2005).

346. McKay, A. et al. An automated feeding system for the African killifish reveals the impact of diet on lifespan and allows scalable assessment of associative learning. Elife 11, 1–33 (2022).

347. Mehta, D. C., Short, J. L. & Nicolazzo, J. A. Altered brain uptake of therapeutics in a triple transgenic mouse model of alzheimer's disease. Pharm. Res. 30, 2868–2879 (2013).

348. Meléndez, A. et al. Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science (80-.). 301, 1387–1391 (2003).

349. Menzies, F. M. et al. Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. Neuron 93, 1015–1034 (2017).

350. Menzies, F. M., Fleming, A. & Rubinsztein, D. C. Compromised autophagy and neurodegenerative diseases. Nat. Rev. Neurosci. 16, 345–357 (2015).

351. Metur, S. P. & Klionsky, D. J. Adaptive immunity at the crossroads of autophagy and metabolism. Cell. Mol. Immunol. 18, 1096–1105 (2021).

352. Michelucci, A., Heurtaux, T., Grandbarbe, L., Morga, E. & Heuschling, P. Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-β. J. Neuroimmunol. 210, 3–12 (2009).

353. Miest, J. J., Arndt, C., Adamek, M., Steinhagen, D. & Reusch, T. B. H. Dietary β-glucan (MacroGard®) enhances survival of first feeding turbot (Scophthalmus maximus) larvae by altering immunity, metabolism and microbiota. Fish Shellfish Immunol. 48, 94–104 (2016).

354. Miller, R. A. et al. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 66 A, 191–201 (2011).

355. Miller, R. A. et al. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging Cell 13, 468–477 (2014).

356. Mitchelson, K. A. J. et al. Yeast β-Glucan Improves Insulin Sensitivity and Hepatic Lipid Metabolism in Mice Humanized with Obese Type 2 Diabetic Gut Microbiota. Mol. Nutr. Food Res. 66, 1–13 (2022).

357. Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. Nature 451, 1069–1075 (2008).

358. Molina-Martínez, P. et al. Microglial Hyperreactivity Evolved to Immunosuppression in the Hippocampus of a Mouse Model of Accelerated Aging and Alzheimer's Disease Traits. Front. Aging Neurosci. 12, (2021).

359. Moqri, M. et al. Biomarkers of aging for the identification and evaluation of longevity interventions. Cell 186, 3758–3775 (2023).

360. Morán, M. et al. Bulk autophagy, but not mitophagy, is increased in cellular model of mitochondrial disease. Biochim. Biophys. Acta - Mol. Basis Dis. 1842, 1059–1070 (2014).

361. Moreau, K. et al. PICALM modulates autophagy activity and tau accumulation. Nat. Commun. 5, (2014).

362. Moreno-García, A., Kun, A., Calero, O., Medina, M. & Calero, M. An overview of the role of lipofuscin in age-related neurodegeneration. Front. Neurosci. 12, 1–13 (2018).

363. Morselli, E. et al. Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. J. Cell Biol. 192, 615–629 (2011).

364. Mortensen, M. et al. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo. Proc. Natl. Acad. Sci. U. S. A. 107, 832–837 (2010).

365. Mortensen, M. et al. The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance. J. Exp. Med. 208, 455–467 (2011).

366. Moruno-Manchón, J. F., Peŕez-Jiménez, E. & Knecht, E. Glucose induces autophagy under starvation conditions by a p38 MAPK-dependent pathway. Biochem. J. 449, 497–506 (2013).

367. Mosikanon, K., Arthan, D., Kettawan, A., Tungtrongchitr, R. & Prangthip, P. Yeast β–Glucan Modulates Inflammation and Waist Circumference in Overweight and Obese Subjects. J. Diet. Suppl. 14, 173–185 (2017).

368. Moskalev, A. A. et al. The role of DNA damage and repair in aging through the prism of Koch-like criteria. Ageing Res. Rev. 12, 661–684 (2013).

369. Mostoslavsky, R. et al. Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124, 315–329 (2006).

370. Münz, C. Enhancing immunity through autophagy. Annu. Rev. Immunol. 27, 423–449 (2009).

371. Murphy, E. J., Rezoagli, E., Major, I., Rowan, N. J. & Laffey, J. G. B-Glucan Metabolic and Immunomodulatory Properties and Potential for Clinical Application. J. Fungi 6, 1–33 (2020).

372. Myerowitz, R., Puertollano, R. & Raben, N. Impaired autophagy: The collateral damage of lysosomal storage disorders. EBioMedicine 63, 103166 (2021).

373. Nakamura, S., Shioda, T. & Yoshimori, T. Autophagy in aging and longevity. Aging Mech. II Longevity, Metab. Brain Aging 189–208 (2022) doi:10.1007/978-981-16-7977-3_11.

374. Nakashima, A. et al. β-Glucan in Foods and Its Physiological Functions. J. Nutr. Sci. Vitaminol. (Tokyo). 64, 8–17 (2018).

375. Nath, R. D., Bedbrook, C. N., Nagvekar, R. & Brunet, A. Husbandry of the African Turquoise Killifish Nothobranchius furzeri. Cold Spring Harb. Protoc. 2023, 422–429 (2023).

376. Ng'oma, E. et al. The age related markers lipofuscin and apoptosis show different genetic architecture by QTL mapping in short-lived Nothobranchius fish. Na 6, 468–480 (2014).

377. Nguyen, P. et al. Elimination of Age-Associated Hepatic Steatosis and Correction of Aging Phenotype by Inhibition of cdk4-C/EBPα-p300 Axis. 24, 1597–1609 (2021).

378. Niccoli, T. & Partridge, L. Ageing as a risk factor for disease. Curr. Biol. 22, R741–R752 (2012).

379. Nicole Jung-Eun Kim Elizabeth Torrese, R. M., Bud Nicola, and Catherine Karr., V. B. V. Yeast-derived Particulate β-Glucan Treatment Subverts the Suppression of Myeloid-derived Suppressor Cells by Inducing PMN-MDSC Apoptosis and M-MDSC Differentiation to APC in Cancer. Physiol. Behav. 176, 139–148 (2017).

380. Nixon, R. A. The aging lysosome: An essential catalyst for late-onset neurodegenerative diseases. Biochim. Biophys. Acta - Proteins Proteomics 1868, 140443 (2020).

381. Nixon, R. A. & Yang, D. S. Autophagy and neuronal cell death in neurological disorders. Cold Spring Harb. Perspect. Biol. 4, 1–23 (2012).

382. No, H. et al. Anti-inflammatory effects of β-1,3-1,6-glucan derived from black yeast Aureobasidium pullulans in RAW264.7 cells. Int. J. Biol. Macromol. 193, 592–600 (2021).

383. No, H. et al. Anti-inflammatory effects of β-1,3-1,6-glucan derived from black yeast Aureobasidium pullulans in RAW264.7 cells. Int. J. Biol. Macromol. 193, 592–600 (2021).

384. Norden, D. M., Muccigrosso, M. M. & Godbout, J. P. Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. Neuropharmacology 96, 29–41 (2015).

385. Nordenstedt, H., White, D. L. & El-Serag, H. B. The changing pattern of epidemiology in hepatocellular carcinoma. Dig. Liver Dis. 42, S206–S214 (2010).

386. Novak, M. & Vetvicka, V. β-glucans, history, and the present: Immunomodulatory aspects and mechanisms of action. J. Immunotoxicol. 5, 47–57 (2008).

387. Novakovic, B. et al. β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. Cell 167, 1354-1368.e14 (2016).

388. Oberacker, T., Kraft, L., Schanz, M., Latus, J. & Schricker, S. The Importance of Thioredoxin-1 in Health and Disease. Antioxidants 12, (2023).

389. Ogrodnik, M. et al. Cellular senescence drives age-dependent hepatic steatosis. Nat. Commun. 8, (2017).

390. Oh, J. E. & Lee, H. K. Pattern recognition receptors and autophagy. Front. Immunol. 5, 1–7 (2014).

391. Öhman, T. et al. Dectin-1 Pathway Activates Robust Autophagy-Dependent Unconventional Protein Secretion in Human Macrophages. J. Immunol. 192, 5952–5962 (2014).

392. Olovnikov, A. M. Telomeres, telomerase, and aging: Origin of the theory. Exp. Gerontol. 31, 443–448 (1996).

393. Ott, C., König, J., Höhn, A., Jung, T. & Grune, T. Macroautophagy is impaired in old murine brain tissue as well as in senescent human fibroblasts. Redox Biol. 10, 266–273 (2016).

394. Palm, W. & De Lange, T. How shelterin protects mammalian telomeres. Annu. Rev. Genet. 42, 301–334 (2008).

395. Paolicelli, R. C. et al. Microglia states and nomenclature: A field at its crossroads. Neuron 110, 3458–3483 (2022).

396. Park, D. et al. Resveratrol induces autophagy by directly inhibiting mTOR through ATP competition. Nat. Publ. Gr. 1–11 (2016) doi:10.1038/srep21772.

397. Pawate, S., Shen, Q., Fan, F. & Bhat, N. R. Redox regulation of glial inflammatory response to lipopolysaccharide and interferonγ. J. Neurosci. Res. 77, 540–551 (2004).

398. Pereira, J. B. et al. DOPA decarboxylase is an emerging biomarker for Parkinsonian disorders including preclinical Lewy body disease. Nat. Aging 3, 1201–1209 (2023).

399. Petit, J. et al. β-Glucan-Induced Immuno-Modulation: A Role for the Intestinal Microbiota and Short-Chain Fatty Acids in Common Carp. Front. Immunol. 12, 1–17 (2022).

400. Phadwal, K. et al. A novel method for autophagy detection in primary cells: Impaired levels of macroautophagy in immunosenescent T cells. Autophagy 8, 677–689 (2012).

401. Pillemer, L. & Ecker, E. E. Anticomplementary Factor in Fresh Yeast. J. Biol. Chem. 137, 139–142 (1941).

402. Pincus, Z., Mazer, T. C. & Slack, F. J. Autofluorescence as a measure of senescence in C. elegans: Look to red, not blue or green. Aging (Albany. NY). 8, 889–898 (2016).

403. Plaza-Zabala, A., Sierra-Torre, V. & Sierra, A. Assessing Autophagy in Microglia: A Two-Step Model to Determine Autophagosome Formation, Degradation, and Net Turnover. Front. Immunol. 11, 1–11 (2021).

404. PLOMP, P. J. A. M. et al. Energy dependence of autophagic protein degradation in isolated rat hepatocytes. Eur. J. Biochem. 164, 197–203 (1987).

405. Podrabsky, J. E. & Hand, S. C. The Bioenergtics of Embryonic Diapause in an anual Austrofundulus limnaeus. J. Exp. Biol. 202, 2567–2580 (1999).

406. Poeschla, M. & Valenzano, D. R. The turquoise killifish: A genetically tractable model for the study of aging. J. Exp. Biol. 223, 1–8 (2020).

407. Polačik, M., Blažek, R. & Reichard, M. Laboratory breeding of the short-lived annual killifish Nothobranchius furzeri. Nat. Protoc. 11, 1396–1413 (2016).

408. Pompei, F. Age distribution of cancer: The Incidence turnover at old age. Hum. Ecol. Risk Assess. 7, 1619–1650 (2001).

409. Porter, C. et al. Mitochondrial respiratory capacity and coupling control decline with age in human skeletal muscle. Am. J. Physiol. - Endocrinol. Metab. 309, E224–E232 (2015).

410. Powers, E. T., Morimoto, R. I., Dillin, A., Kelly, J. W. & Balch, W. E. Biological and chemical approaches to diseases of proteostasis deficiency. Annu. Rev. Biochem. 78, 959–991 (2009).

411. Preethy, S. et al. Resolution of fibrosis in mdx dystrophic mouse after oral consumption of N-163 strain of Aureobasidium pullulans produced β-glucan. Sci. Rep. 13, 1–10 (2023).

412. Priller, J. et al. Targeting gene-modified hematopoietic cells to the central nervous system: Use of green fluorescent protein uncovers microglial engraftment. Nat. Med. 7, 1356–1361 (2001).

413. Prinz, M. & Priller, J. Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease. Nat. Rev. Neurosci. 15, 300–312 (2014).

414. Pua, H. H., Dzhagalov, I., Chuck, M., Mizushima, N. & He, Y. W. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J. Exp. Med. 204, 25–31 (2007).

415. Pua, H. H., Guo, J., Komatsu, M. & He, Y.-W. Autophagy Is Essential for Mitochondrial Clearance in Mature T Lymphocytes. J. Immunol. 182, 4046–4055 (2009).

416. Puleston, D. J. & Simon, A. K. Autophagy in the immune system. Immunology 141, 1–8 (2014).

417. Puleston, D. J. et al. Autophagy is a critical regulator of memory CD8+ T cell formation. Elife 3, 1–21 (2014).

418. Pyo, J. O. et al. Overexpression of Atg5 in mice activates autophagy and extends lifespan. Nat. Commun. 4, 1–9 (2013).

419. Qian, J. et al. Protective effects of betaglucin on myocardial tissue during myocardial infarction in rats and dogs. Acta Pharmacol. Sin. 30, 1092–1098 (2009).

420. Qin, N. et al. Local intra-articular injection of resveratrol delays cartilage degeneration in C57BL/6 mice by inducing autophagy via AMPK/mTOR pathway. J. Pharmacol. Sci. 134, 166–174 (2017).

421. Qin, Y. et al. Impaired autophagy in microglia aggravates dopaminergic neurodegeneration by regulating NLRP3 inflammasome activation in experimental models of Parkinson's disease. Brain. Behav. Immun. 91, 324–338 (2021).

422. Qu, D. et al. BAG2 gene-mediated regulation of PINK1 protein is critical for mitochondrial translocation of PARKIN and neuronal survival. J. Biol. Chem. 290, 30441–30452 (2015).

423. Queiroz, E. A. I. F. et al. Antiproliferative and pro-apoptotic effects of three fungal exocellular β-glucans in MCF-7 breast cancer cells is mediated by oxidative stress, AMP-activated protein kinase (AMPK) and the Forkhead transcription factor, FOXO3a. Int. J. Biochem. Cell Biol. 67, 14–24 (2015).

424. Ramirez, A. et al. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat. Genet. 38, 1184–1191 (2006).

425. Ranawat, N. & Masai, I. Mechanisms underlying microglial colonization of developing neural retina in zebrafish. Elife 10, 1–28 (2021).

426. Rando, T. A. & Chang, H. Y. Aging, rejuvenation, and epigenetic reprogramming: Resetting the aging clock. Cell 148, 46–57 (2012).

427. Ransohoff, R. M. A polarizing question: Do M1 and M2 microglia exist. Nat. Neurosci. 19, 987–991 (2016).

428. Rao, A. N., Sivasankar, B. & Sadasivam, V. Photo-oxidative degradation of an azo dye direct red 31 in the presence of ZnS catalyst. Indian J. Chem. - Sect. A Inorganic, Phys. Theor. Anal. Chem. 49, 901–905 (2010).

429. Ravikumar, B. et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. 36, 585–595 (2004).

430. Reddy, P. H. Mitochondrial medicine for aging and neurodegenerative diseases. NeuroMolecular Med. 10, 291–315 (2008).

431. Release, A. I.- et al. Microglia but not Astrocytes Across Central Nervous System Regions for. 1–9 (2014) doi:10.1038/srep06824.

432. Reyes, J. F. et al. Accumulation of alpha-synuclein within the liver, potential role in the clearance of brain pathology associated with Parkinson's disease. Acta Neuropathol. Commun. 9, 1–20 (2021).

433. Riederer, F. Donanemab in early Alzheimer's Disease. J. fur Neurol. Neurochir. und Psychiatr. 22, 142–143 (2021).

434. Riffelmacher, T. et al. Autophagy-Dependent Generation of Free Fatty Acids Is Critical for Normal Neutrophil Differentiation. Immunity 47, 466-480.e5 (2017).

435. Riley, J. S. & Tait, S. W. Mitochondrial DNA in inflammation and immunity . EMBO Rep. 21, 1–17 (2020).

436. Ripa, R. et al. Refeeding-associated AMPKγ1 complex activity is a hallmark of health and longevity. Nat. Aging 3, 1544–1560 (2023).

437. Rodríguez, J. J. & Verkhratsky, A. Neurogenesis in Alzheimer's disease. J. Anat. 219, 78–89 (2011).

438. Rubinsztein, D. C., Mariño, G. & Kroemer, G. Autophagy and aging. Cell 146, 682–695 (2011).

439. Rudolph, K. L. et al. Longevity, Stress Response, and Cancer in Aging Telomerase-Deficient Mice. 96, 701–712 (1999).

440. Runwal, G. et al. LC3-positive structures are prominent in autophagy-deficient cells. Sci. Rep. 9, 1–14 (2019).

441. Ruparelia, A. A. et al. The African killifish: A short-lived vertebrate model to study the biology of sarcopenia and longevity. Aging Cell 23, 1–22 (2024).

442. Russell and Kahn. Endocrine regulation of ageing. Nat. Rev. Mol. Cell Biol. 8, 681–691 (2007).

443. Säemann, M. D., Haidinger, M., Hecking, M., Hörl, W. H. & Weichhart, T. The multifunctional role of mTOR in innate immunity: Implications for transplant immunity. Am. J. Transplant. 9, 2655–2661 (2009).

444. Safwat El-Deeb, O., El-Esawy, R. O., Al-Shenawy, H. A. & Ghanem, H. B. Modulating gut dysbiosis and mitochondrial dysfunction in oxazolone-induced ulcerative colitis: the restorative effects of β-glucan and/or celastrol. Redox Rep. 27, 60–69 (2022).

445. Saha, S., Panigrahi, D. P., Patil, S. & Bhutia, S. K. Autophagy in health and disease: A comprehensive review. Biomed. Pharmacother. 104, 485–495 (2018).

446. Sahasrabudhe, N. M., Dokter-Fokkens, J. & de Vos, P. Particulate β-glucans synergistically activate TLR4 and Dectin-1 in human dendritic cells. Mol. Nutr. Food Res. 60, 2514–2522 (2016).

447. Saitoh, T. et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production. Nature 456, 264–268 (2008).

448. Sakai, M. & Ogawa, K. Energy-dependent lysosomal wrapping mechanism (LWM) during autophagolysosome formation. Histochemistry 76, 479–488 (1982).

449. Salameh, Y., Bejaoui, Y. & El Hajj, N. DNA Methylation Biomarkers in Aging and Age-Related Diseases. Front. Genet. 11, 1–11 (2020).

450. Salminen, A., Kaarniranta, K. & Kauppinen, A. Inflammaging: Disturbed interplay between autophagy and inflammasomes. Aging (Albany. NY). 4, 166–175 (2012).

451. Saluk-Juszczak, J., Krolewska, K. & Wachowicz, B. β-glucan from Saccharomyces cerevisiae as a blood platelet antioxidant. Platelets 21, 451–459 (2010).

452. Sambasivarao, S. V. Aging of the circadian system in zebrafish and the effects of melatonin on sleep and cognitive performance. 18, 1199–1216 (2013).

453. Sanjuan, M. A. et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature 450, 1253–1257 (2007).

454. Schmauck-Medina, T. et al. New hallmarks of ageing: a 2022 Copenhagen ageing meeting summary. Aging (Albany. NY). 14, 6829–6839 (2022).

455. Schroeder, S. et al. Dietary spermidine improves cognitive function. Cell Rep. 35, (2021).

456. Schwartz, B. & Vetvicka, V. Review: β-glucans as effective antibiotic alternatives in poultry. Molecules 26, 1–12 (2021).

457. Schwarz, C. et al. Effects of Spermidine Supplementation on Cognition and Biomarkers in Older Adults with Subjective Cognitive Decline: A Randomized Clinical Trial. JAMA Netw. Open 5, E2213875 (2022).

458. Seale K., Horvath S., Teschendorff A., Eynon N., V. S. Making sense of the ageing methylome. Nat Rev Genet 10, 585–605 (2022).

459. Selkoe, D. J. Treatments for Alzheimer's disease emerge. Science (80-.). 373, 624–626 (2021).

460. Selman, M. & Pardo, A. Fibroageing: An ageing pathological feature driven by dysregulated extracellular matrix-cell mechanobiology. Ageing Res. Rev. 70, (2021).

461. Seo, G., Hyun, C., Choi, S., Kim, Y. M. & Cho, M. The wound healing effect of four types of beta-glucan. Appl. Biol. Chem. 62, (2019).

462. Shah, V. B. et al. β-Glucan Activates Microglia without Inducing Cytokine Production in Dectin-1-Dependent Manner. J. Immunol. 180, 2777–2785 (2008).

463. Shaki, F. & Pourahmad, J. Mitochondrial toxicity of depleted uranium: Protection by beta-glucan. Iran. J. Pharm. Res. 12, 131–140 (2013).

464. Sharma, S., Goyal, H., Joshi, S., Nehru, B. & Saini, A. Molecular interactions of resveratrol with Aβ 42 peptide and fibril during in-vitro Aβ 42 aggregation. Adv. Redox Res. 7, 100060 (2023).

465. Sheldrake, A. R. The ageing, growth and death of cells. Nature 250, 381–385 (1974).

466. Shen, T. et al. Alternative polyadenylation dependent function of splicing factor SRSF3 contributes to cellular senescence. 11, (2019).

467. Sheng, J. G., Mrak, R. E. & Griffin, W. S. T. Enlarged and phagocytic, but not primed, interleukin-1α-immunoreactive microglia increase with age in normal human brain. Acta Neuropathol. 95, 229–234 (1998).

468. Shi, C. S. & Kehrl, J. H. TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced Autophagy. Sci. Signal. 3, 1–10 (2010).

469. Shi, H. et al. B-Glucan Attenuates Cognitive Impairment Via the Gut-Brain Axis in Diet-Induced Obese Mice. Microbiome 8, 1–21 (2020).

470. Shibata, M. et al. Regulation of intracellular accumulation of mutant huntingtin by beclin 1. J. Biol. Chem. 281, 14474–14485 (2006).

471. SIERRA A., GOTTFRIED-BLACKMORE A.C., MCEWEN B.S., B. K. Advancing equity, equality and non-discrimination in food systems : Pathways to reform. Glia 479, 132 (2015).

472. Sierra, A. et al. The “Big-Bang” for modern glial biology: Translation and comments on Pío del Río-Hortega 1919 series of papers on microglia. Glia 64, 1801–1840 (2016).

473. Sierra, A., Paolicelli, R. C. & Kettenmann, H. Cien Años de Microglía: Milestones in a Century of Microglial Research. Trends Neurosci. 42, 778–792 (2019).

474. Sihag, R. K. & Cataldo, A. M. Brain β-spectrin is a component of senile plaques in Alzheimer's disease. Brain Res. 743, 249–257 (1996).

475. Silva, G. C., Costa, E. D., Lemos, V. S., Queiroz-Junior, C. M. & Pereira, L. J. Experimental Periodontal Disease Triggers Coronary Endothelial Dysfunction in Middle-Aged Rats: Preventive Effect of a Prebiotic β-Glucan. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 76, 1398–1406 (2021).

476. Silva, V. D. O. et al. Effects of β-glucans ingestion on alveolar bone loss, intestinal morphology, systemic inflammatory profile, and pancreatic β-cell function in rats with periodontitis and diabetes. Nutrients 9, 1–13 (2017).

477. Singh Kushwaha, S., Patro, N. & Kumar Patro, I. A Sequential Study of Age-Related Lipofuscin Accumulation in Hippocampus and Striate Cortex of Rats. Ann. Neurosci. 25, 223–233 (2019).

478. Singh, R. P. & Bhardwaj, A. B-Glucans: a Potential Source for Maintaining Gut Microbiota and the Immune System. Front. Nutr. 10, 1–22 (2023).

479. Sohal, R. S. & Sohal, B. H. Hydrogen peroxide release by mitochondria increases during aging. Mech. Ageing Dev. 57, 187–202 (1991).

480. Son, J. H., Shim, J. H., Kim, K. H., Ha, J. Y. & Han, J. Y. Neuronal autophagy and neurodegenerative diseases. Exp. Mol. Med. 44, 89–98 (2012).

481. Son, J. H., Shim, J. H., Kim, K. H., Ha, J. Y. & Han, J. Y. Neuronal autophagy and neurodegenerative diseases. Exp. Mol. Med. 44, 89–98 (2012).

482. Song, G. & Du, Q. Structure characterization and antitumor activity of an α β-glucan polysaccharide from Auricularia polytricha. Food Res. Int. 45, 381–387 (2012).

483. Song, G. J. & Suk, K. Pharmacological modulation of functional phenotypes of microglia in neurodegenerative diseases. Front. Aging Neurosci. 9, (2017).

484. Song, L. et al. Dietary Intake of β-Glucans Can Prolong Lifespan and Exert an Antioxidant Action on Aged Fish Nothobranchius guentheri. Rejuvenation Res. 23, 293–301 (2020).

485. Song, Q., Meng, B., Xu, H. & Mao, Z. The emerging roles of vacuolar-type ATPase-dependent Lysosomal acidification in neurodegenerative diseases. Transl. Neurodegener. 9, 1–14 (2020).

486. Sotthibundhu, A. et al. Rapamycin regulates autophagy and cell adhesion in induced pluripotent stem cells. Stem Cell Res. Ther. 7, 1–16 (2016).

487. Spilman, P. et al. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-β levels in a mouse model of alzheimer's disease. PLoS One 5, 1–8 (2010).

488. Spittau, B. Aging microglia-phenotypes, functions and implications for age-related neurodegenerative diseases. Front. Aging Neurosci. 9, 1–9 (2017).

489. Stancu, A. L. AMPK activation can delay aging. Discoveries 3, e53 (2015).

490. Steenwijk, H. P. Van & Bast, A. Immunomodulating Effects of Fungal Beta-Glucans : Nutrients 13, 1–20 (2021).

491. Stefanatos, R. & Sanz, A. The role of mitochondrial ROS in the aging brain. FEBS Lett. 592, 743–758 (2018).

492. Stefanis, L. a -Synuclein in Parkinson ' s Disease. 1–24 (2024).

493. Streit, W. J., Braak, H., Xue, Q. S. & Bechmann, I. Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. Acta Neuropathol. 118, 475–485 (2009).

494. Streit, W. J., Sammons, N. W., Kuhns, A. J. & Sparks, D. L. Dystrophic Microglia in the Aging Human Brain. Glia 45, 208–212 (2004).

495. Streit, W. J., Xue, Q. S., Tischer, J. & Bechmann, I. Microglial pathology. Acta Neuropathol. Commun. 2, 1–17 (2014).

496. Sujana, K. M., Lakshmi, M. S., Ramayya, P. J. & Naik, B. R. Age Related Histomorphological Changes in Cerebral Neocortex of Pig (Sus scrofa). 32, 49–51 (2020).

497. Sun, N. et al. Measuring In Vivo Mitophagy. Mol. Cell 60, 685–696 (2015).

498. Sun, X., Yang, Q., Rogers, C. J., Du, M. & Zhu, M. J. AMPK improves gut epithelial differentiation and barrier function via regulating Cdx2 expression. Cell Death Differ. 24, 819–831 (2017).

499. Sung, M., Yoon, Y. & Lee, J. The Immunomodulatory Effect of β-Glucan Depends on the Composition of the Gut Microbiota. Foods 12, 1–14 (2023).

500. Suzuta, S. et al. Metformin suppresses progression of muscle aging via activation of the AMP kinase-mediated pathways in Drosophila adults. Eur. Rev. Med. Pharmacol. Sci. 26, 8039–8056 (2022).

501. Synytsya, A. & Novák, M. Structural diversity of fungal glucans. Carbohydr. Polym. 92, 792–809 (2013).

502. Tan, J. X. & Finkel, T. Lysosomes in senescence and aging. EMBO Rep. 24, 1–20 (2023).

503. Tang, T. et al. A synbiotic consisting of Lactobacillus plantarum S58 and hull-less barley β-glucan ameliorates lipid accumulation in mice fed with a high-fat diet by activating AMPK signaling and modulating the gut microbiota. Carbohydr. Polym. 243, 116398 (2020).

504. Tanji, K. et al. Phosphorylation of serine 349 of p62 in Alzheimer's disease brain. Acta Neuropathol. Commun. 2, 1–14 (2014).

505. Tartiere, A. G., Freije, J. M. P. & López-Otín, C. The hallmarks of aging as a conceptual framework for health and longevity research. Front. Aging 5, 1–12 (2024).

506. Taylor, H. B. & Vasu, C. Impact of Prebiotic β-glucan Treatment at Juvenile Age on the Gut Microbiota Composition and the Eventual Type 1 Diabetes Onset in Non-obese Diabetic Mice. Front. Nutr. 8, 1–12 (2021).

507. Terman, A. & Brunk, U. T. Aging as a catabolic malfunction. Int. J. Biochem. Cell Biol. 36, 2365–2375 (2004).

508. Terman, A., Gustafsson, B. & Brunk, U. T. Mitochondrial damage and intralysosomal degradation in cellular aging. Mol. Aspects Med. 27, 471–482 (2006).

509. Terzibasi, E. et al. Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish Nothobranchius furzeri. Aging Cell 8, 88–99 (2009).

510. Terzibasi, E. et al. Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish Nothobranchius furzeri. Aging Cell 8, 88–99 (2009).

511. Terzibasi, E. et al. Large differences in aging phenotype between strains of the short-lived annual fish Nothobranchius furzeri. PLoS One 3, (2008).

512. Terzibasi, E., Valenzano, D. R. & Cellerino, A. The short-lived fish Nothobranchius furzeri as a new model system for aging studies. Exp. Gerontol. 42, 81–89 (2007).

513. Thomas, H. E. et al. Mitochondrial Complex I Activity Is Required for Maximal Autophagy. Cell Rep. 24, 2404-2417.e8 (2018).

514. Thomas, S. et al. β-Glucans from Yeast—Immunomodulators from Novel Waste Resources. Appl. Sci. 12, (2022).

515. Tian, J. et al. β-Glucan enhances antitumor immune responses by regulating differentiation and function of monocytic myeloid-derived suppressor cells. Eur. J. Immunol. 43, 1220–1230 (2013).

516. Tian, X. et al. SIRT6 Is Responsible for More Efficient DNA Double-Strand Break Repair in Long-Lived Species. Cell 177, 622-638.e22 (2019).

517. Tichauer, J. E. et al. Age-dependent changes on TGFβ1 Smad3 pathway modify the pattern of microglial cell activation. Brain. Behav. Immun. 37, 187–196 (2014).

518. Tivey, H. S. E., Brook, A. J. C., Rokicki, M. J., Kipling, D. & Davis, T. P38 MAPK stress signalling in replicative senescence in fibroblasts from progeroid and genomic instability syndromes. Biogerontology 14, 47–62 (2013).

519. Todorova, V. & Blokland, A. Mitochondria and Synaptic Plasticity in the Mature and Aging Nervous System. Curr Neuropharmacol 15, 166–173 (2017).

520. Tomás-Loba, A. et al. Telomerase Reverse Transcriptase Delays Aging in Cancer-Resistant Mice. Cell 135, 609–622 (2008).

521. Toren, D. et al. Gray whale transcriptome reveals longevity adaptations associated with DNA repair and ubiquitination. Aging Cell 19, 1–11 (2020).

522. Tozzini, E. T., Baumgart, M., Battistoni, G. & Cellerino, A. Adult neurogenesis in the short-lived teleost Nothobranchius furzeri: Localization of neurogenic niches, molecular characterization and effects of aging. Aging Cell 11, 241–251 (2012).

523. Travassos, L. H. et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat. Immunol. 11, 55–62 (2010).

524. Trifunovic, A. et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429, 417–423 (2004).

525. Tripodi, F. et al. Anti-Aging and Neuroprotective Properties of Grifola frondosa and Hericium erinaceus Extracts. Nutrients 14, (2022).

526. Underwood, E. L. et al. Enhanced presynaptic mitochondrial energy production is required for memory formation. Sci. Rep. 13, 1–13 (2023).

527. Untergasser, G. et al. TGF-β cytokines increase senescence-associated beta-galactosidase activity in human prostate basal cells by supporting differentiation processes, but not cellular senescence. Exp. Gerontol. 38, 1179–1188 (2003).

528. Usoltseva, R. V. et al. Structure and anticancer activity of native and modified polysaccharides from brown alga Dictyota dichotoma. Carbohydr. Polym. 180, 21–28 (2018).

529. Valdesalici, S. & Cellerino, A. Extremely short lifespan in the annual fish Nothobranchius furzeri. Proc. R. Soc. B Biol. Sci. 270, 189–191 (2003).

530. Valenzano, D. R., Terzibasi, E., Cattaneo, A., Domenici, L. & Cellerino, A. Temperature affects longevity and age-related locomotor and cognitive decay in the short-lived fish: Nothobranchius furzeri. Aging Cell 5, 275–278 (2006).

531. Valenzano, D. R. et al. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr. Biol. 16, 296–300 (2006).

532. Valenzano, D. R. & Cellerino, A. Resveratrol and the pharmacology of aging: A new vertebrate model to validate an old molecule. Cell Cycle 5, 1027–1032 (2006).

533. Van houcke, J. et al. A short dasatinib and quercetin treatment is sufficient to reinstate potent adult neuroregenesis in the aged killifish. npj Regen. Med. 8, 1–13 (2023).

534. Van houcke, J. et al. Aging impairs the essential contributions of non-glial progenitors to neurorepair in the dorsal telencephalon of the Killifish Nothobranchius furzeri. Aging Cell 20, 1–18 (2021).

535. VanGuilder, H. D. et al. Concurrent hippocampal induction of MHC II pathway components and glial activation with advanced aging is not correlated with cognitive impairment. J. Neuroinflammation 8, 1–21 (2011).

536. Vanhunsel, S. et al. The killifish visual system as an in vivo model to study brain aging and rejuvenation. npj Aging Mech. Dis. 7, (2021).

537. Vanhunsel, S. et al. The age factor in optic nerve regeneration: Intrinsic and extrinsic barriers hinder successful recovery in the short-living killifish. Aging Cell 21, 1–24 (2022).

538. Vanhunsel, S., Bergmans, S. & Moons, L. Killifish switch towards mammalian-like regeneration upon aging. 14, 6–7 (2022).

539. Varnum, M. M. & Ikezu, T. The classification of microglial activation phenotypes on neurodegeneration and regeneration in alzheimer's disease brain. Arch. Immunol. Ther. Exp. (Warsz). 60, 251–266 (2012).

540. Vellai, T. et al. Influence of TOR kinase on lifespan in C. elegans. Nature 426, 620 (2003).

541. Vermulst, M. et al. DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. Nat. Genet. 40, 392–394 (2008).

542. Vetvicka, V., Vannucci, L. & Sima, P. B-Glucan As a New Tool in Vaccine Development. Scand. J. Immunol. 91, 1–10 (2020).

543. Vetvicka, V., Vannucci, L., Sima, P. & Richter, J. Beta glucan: Supplement or drug? From laboratory to clinical trials. Molecules 24, (2019).

544. Vizioli, M. G. et al. Mitochondria-to-nucleus retrograde signaling drives formation of cytoplasmic chromatin and inflammation in senescence. Genes Dev. 34, 428–445 (2020).

545. von Bernhardi, R., Eugenín-von Bernhardi, L. & Eugenín, J. Microglial cell dysregulation in brain aging and neurodegeneration. Front. Aging Neurosci. 7, 1–21 (2015).

546. Walters, H. E., Deneka-Hannemann, S. & Cox, L. S. Reversal of phenotypes of cellular senescence by pan-mTOR inhibition. Aging (Albany. NY). 8, 231–244 (2016).

547. Wang, C. et al. The effects of microglia-associated neuroinflammation on Alzheimer's disease. Front. Immunol. 14, 1–10 (2023).

548. Wang, J. & Matosevic, S. NT5E/CD73 as correlative factor of patient survival and natural killer cell infiltration in glioblastoma. J. Clin. Med. 8, (2019).

549. Wang, J. & Matosevic, S. NT5E/CD73 as correlative factor of patient survival and natural killer cell infiltration in glioblastoma. J. Clin. Med. 8, (2019).

550. Wang, M., Zhu, K., Zhang, L., Li, L. & Zhao, J. Thioredoxin 1 protects astrocytes from oxidative stress by maintaining peroxiredoxin activity. Mol. Med. Rep. 13, 2864–2870 (2016).

551. Wang, X. et al. Natural Polysaccharide β-Glucan Protects against Doxorubicin-Induced Cardiotoxicity by Suppressing Oxidative Stress. Nutrients 14, (2022).

552. Wang, Y. et al. High-molecular-weight β-glucan decreases serum cholesterol differentially based on the CYP7A1 rs3808607 polymorphism in mildly hypercholesterolemic adults. J. Nutr. 146, 720–727 (2016).

553. Wang, Y. et al. Barley β-glucan reduces blood cholesterol levels via interrupting bile acid metabolism. Br. J. Nutr. 118, 822–829 (2017).

554. Wang, Y., Yuan, Q. & Xie, L. Histone Modifications in Aging: The Underlying Mechanisms and Implications. Curr. Stem Cell Res. Ther. 13, 125–135 (2018).

555. Wang, Z. et al. Functional tumor specific CD8 + T cells in spleen express a high level of PD-1. Int. Immunopharmacol. 80, 106242 (2020).

556. Wareham, L. K., Baratta, R. O., Del Buono, B. J., Schlumpf, E. & Calkins, D. J. Collagen in the central nervous system: contributions to neurodegeneration and promise as a therapeutic target. Mol. Neurodegener. 19, 1–12 (2024).

557. Webb, M. & Sideris, D. P. Intimate Relations — Mitochondria and Ageing. (2020).

558. Weir, H. J. et al. Dietary Restriction and AMPK Increase Lifespan via Mitochondrial Network and Peroxisome Remodeling. Cell Metab. 26, 884-896.e5 (2017).

559. Wendler, S., Hartmann, N., Hoppe, B. & Englert, C. Age-dependent decline in fin regenerative capacity in the short-lived fish Nothobranchius furzeri. Aging Cell 14, 857–866 (2015).

560. Wensink, M. J. & Cohen, A. A. The Danaid Theory of Aging. Front. Cell Dev. Biol. 9, 1–16 (2022).

561. Wharton, S. B. et al. Expression of Ki67, PCNA and the chromosome replication licensing protein Mcm2 in glial cells of the ageing human hippocampus increases with the burden of Alzheimer-type pathology. Neurosci. Lett. 383, 33–38 (2005).

562. Wilkinson, J. E. et al. Rapamycin slows aging in mice. Aging Cell 11, 675–682 (2012).

563. Wilmanski, T. et al. Gut microbiome pattern reflects healthy ageing and predicts survival in humans. Nat. Metab. 3, 274–286 (2021).

564. Wilson Z.N., West M., English A.M., Odorizzi G., H. A. L. Mitochondrial-Derived Compartments are Multilamellar Domains that Encase Membrane Cargo and Cytosol. bioRxiv 68, 83–94 (2023).

565. Wilson, N. et al. The autophagy–NAD axis in longevity and disease. Trends Cell Biol. 33, 788–802 (2023).

566. Winner, B. et al. In vivo demonstration that α-synuclein oligomers are toxic. Proc. Natl. Acad. Sci. U. S. A. 108, 4194–4199 (2011).

567. Wolf, A. J. & Underhill, D. M. Peptidoglycan recognition by the innate immune system. Nat. Rev. Immunol. 18, 243–254 (2018).

568. Wolfe, D. M. et al. Autophagy failure in Alzheimer's disease and the role of defective lysosomal acidification. Eur. J. Neurosci. 37, 1949–1961 (2013).

569. Wong, A. M. et al. Macrosialin increases during normal brain aging are attenuated by caloric restriction. Neurosci. Lett. 390, 76–80 (2005).

570. Wong, S. Q., Kumar, A. V., Mills, J. & Lapierre, L. R. Autophagy in aging and longevity. Hum. Genet. 139, 277–290 (2020).

571. Woodhead A. D., Pond V., D. K. Aging changes in the kidneys of two poeciliid fishes, the guppy Poecilia reticulata and the Amazon molly P. formosa. Exp. Gerontol. 18, 211–221 (1983).

572. Woodhead, A. D. Aging changes in the heart of a poeciliid fish, the guppy Poecilia reticulatus. Exp. Gerontol. 19, 383–391 (1984).

573. Wouk, J., Dekker, R. F. H., Queiroz, E. A. I. F. & Barbosa-Dekker, A. M. β-Glucans as a panacea for a healthy heart? Their roles in preventing and treating cardiovascular diseases. Int. J. Biol. Macromol. 177, 176–203 (2021).

574. Wzorek-Łyczko K., Woźniak W., Piwowarczyk A., and K. E. The anti-infective effect of β-glucans in children. Int. J. Vitam. Nutr. Res. (2023).

575. Xiao, F. H., Wang, H. T. & Kong, Q. P. Dynamic DNA methylation during aging: A “prophet” of age-related outcomes. Front. Genet. 10, 1–8 (2019).

576. Xilouri, M., Brekk, O. R. & Stefanis, L. Autophagy and Alpha-Synuclein: Relevance to Parkinson’s Disease and Related Synucleopathies. Mov. Disord. 31, 178–192 (2016).

577. Xilouri, M., Vogiatzi, T., Vekrellis, K. & Stefanis, L. α-synuclein degradation by autophagic pathways: A potential key to Parkinson’s disease pathogenesis. Autophagy 4, 917–919 (2008).

578. Xu, H., Chen, M., Manivannan, A., Lois, N. & Forrester, J. V. Age-dependent accumulation of lipofuscin in perivascular and subretinal microglia in experimental mice. Aging Cell 7, 58–68 (2008).

579. Xu, M. et al. Yeast β-glucan alleviates cognitive deficit by regulating gut microbiota and metabolites in Aβ1–42-induced AD-like mice. Int. J. Biol. Macromol. 161, 258–270 (2020).

580. Xu, M. et al. Oat beta-glucan reduces colitis by promoting autophagy flux in intestinal epithelial cells via EPHB6-TFEB axis. Front. Pharmacol. 14, 1–10 (2023).

581. Xu, T. T. et al. Spermidine and spermine delay brain aging by inducing autophagy in SAMP8 mice. Aging (Albany. NY). 12, 6401–6414 (2020).

582. Xu, Y. et al. Toll-like Receptor 4 Is a Sensor for Autophagy Associated with Innate Immunity. Immunity 27, 135–144 (2007).

583. Xu, Y. et al. Macrophage-Targeted Berberine-Loaded β-Glucan Nanoparticles Enhance the Treatment of Ulcerative Colitis. Int. J. Nanomedicine 17, 5303–5314 (2022).

584. Yamada, J., Hamuro, J., Hatanaka, H., Hamabata, K. & Kinoshita, S. Alleviation of seasonal allergic symptoms with superfine β-1,3-glucan: A randomized study. J. Allergy Clin. Immunol. 119, 1119–1126 (2007).

585. Yamamoto, A., Tagawa, Y., Yoshimori, T. & Moriyama, Y. Bafilomycin Ai Prevents Maturation of Autophagic Vacuoles by Inhibiting Fusion between Autophagosomesand Lysosomesin Rat HepatomaCell Line, H-4-II-E Cells. 42, 1–10 (2006).

586. Yan X.X and Jeromin A. Spectrin Breakdown Products (SBDPs) as Potential Biomarkers for Neurodegenerative Diseases. Curr Transl Geriatr Exp Gerontol Rep. 23, 1–7 (2013).

587. Yano, T. et al. Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat. Immunol. 9, 908–916 (2008).

588. Yegorov, Y. E., Akimov, S. S., Hass, R., Zelenin, A. V. & Prudovsky, I. A. Endogenous β-galactosidase activity in continuously nonproliferating cells. Exp. Cell Res. 243, 207–211 (1998).

589. Yim, W. W. Y. & Mizushima, N. Lysosome biology in autophagy. Cell Discov. 6, (2020).

590. Yonashiro, R. et al. Mutant SOD1 and Attenuates Mutant SOD1-induced Reactive Oxygen Species Generation. Mol. Biol. Cell 20, 4524–4530 (2009).

591. Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. & Tashiro, Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J. Biol. Chem. 266, 17707–17712 (1991).

592. Yu, X. & Li, G. Effects of resveratrol on longevity, cognitive ability and aging-related histological markers in the annual fish Nothobranchius guentheri. Exp. Gerontol. 47, 940–949 (2012).

593. Yu, Y. et al. The alteration of autophagy and apoptosis in the hippocampus of rats with natural aging-dependent cognitive deficits. Behav. Brain Res. 334, 155–162 (2017).

594. Yuan, J. et al. Acupuncture for autophagy in animal models of middle cerebral artery occlusion: A systematic review and meta-analysis protocol. PLoS One 18, 1–8 (2023).

595. Yuan, Y. et al. Mitochondrial ROS-induced lysosomal dysfunction impairs autophagic flux and contributes to M1 macrophage polarization in a diabetic condition. Clin. Sci. 133, 1759–1777 (2019).

596. Žák, J., Dyková, I. & Reichard, M. Good performance of turquoise killifish (Nothobranchius furzeri) on pelleted diet as a step towards husbandry standardization. Sci. Rep. 10, 1–11 (2020).

597. Zhang, C. S. et al. Metformin Activates AMPK through the Lysosomal Pathway. Cell Metab. 24, 521–522 (2016).

598. Zhang, C. & Cuervo, A. M. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. Nat. Med. 14, 959–965 (2008).

599. Zhang, G. et al. Hypothalamic programming of systemic ageing involving IKK-β, NF-κB and GnRH. Nature 497, 211–216 (2013).

600. Zhang, H., Puleston, D. J. & Simon, A. K. Autophagy and Immune Senescence. Trends Mol. Med. 22, 671–686 (2016).

601. Zhang, J. et al. Autophagy and mitochondrial dysfunction in adjuvant-arthritis rats treatment with resveratrol. Sci. Rep. 6, 1–10 (2016).

602. Zhang, Y. et al. Resveratrol improves hepatic steatosis by inducing autophagy through the cAMP signaling pathway. Mol. Nutr. Food Res. 59, 1443–1457 (2015).

603. Zhang, Y., Liang, X., Bao, X., Xiao, W. & Chen, G. Toll-like receptor 4 (TLR4) inhibitors: Current research and prospective. Eur. J. Med. Chem. 235, 114291 (2022).

604. Zhao, C. et al. Obligatory role of microglia-mobilized hippocampal CREB-BDNF signaling in the prophylactic effect of β-glucan on chronic stress-induced depression-like behaviors in mice. Eur. J. Pharmacol. 964, (2024).

605. Zhao, Y. et al. The Beneficial Effects of Quercetin, Curcumin, and Resveratrol in Obesity. Oxid. Med. Cell. Longev. 2017, (2017).

606. Zhen, W. et al. Yeast β-Glucan Altered Intestinal Microbiome and Metabolome in Older Hens. Front. Microbiol. 12, (2021).

607. Zhong, X. et al. Immunomodulatory Effect and Biological Significance of β-Glucans. Pharmaceutics 15, 1–16 (2023).

608. Zhong, Y., Marungruang, N., Fåk, F. & Nyman, M. Effects of two whole-grain barley varieties on caecal SCFA, gut microbiota and plasma inflammatory markers in rats consuming low- and high-fat diets. Br. J. Nutr. 113, 1558–1570 (2015).

609. Zhou, D. D. et al. Effects and Mechanisms of Resveratrol on Aging and Age-Related Diseases. Oxid. Med. Cell. Longev. 2021, (2021).

610. Zhou, T. X., Jung, J. H., Zhang, Z. F. & Kim, I. H. Effect of dietary β-glucan on growth performance, fecal microbial shedding and immunological responses after lipopolysaccharide challenge in weaned pigs. Anim. Feed Sci. Technol. 179, 85–92 (2013).

611. Zhou, Z. et al. Downregulation of ATP6V1A Involved in Alzheimer's Disease via Synaptic Vesicle Cycle, Phagosome, and Oxidative Phosphorylation. Oxid. Med. Cell. Longev. 2021, (2021).

612. Zhu, H. X. et al. Age-related intraneuronal accumulation of αII-spectrin breakdown product SBDP120 in the human cerebrum is enhanced in Alzheimer's disease. Exp. Gerontol. 69, 43–52 (2015).

613. Zhu, L. et al. Telomere length regulation in mice is linked to a novel chromosome locus. Proc. Natl. Acad. Sci. U. S. A. 95, 8648–8653 (1998).

614. Zorov, D. B., Juhaszova, M. & Sollott, S. J. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol. Rev. 94, 909–950 (2014).