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Review

Autophagy-related proteins: Potential diagnostic and prognostic biomarkers of aging-related diseases

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ABSTRACT

Autophagy plays a key role in cellular, tissue and organismal homeostasis and in the production of the energy load needed at critical times during development and in response to nutrient shortage. Autophagy is generally considered as a pro-survival mechanism, although its deregulation has been linked to non-apoptotic cell death. Autophagy efficiency declines with age, thus contributing to many different pathophysiological conditions, such as cancer, cardiomyopathy, diabetes, liver disease, autoimmune diseases, infections, and neurodegeneration. Accordingly, it has been proposed that the maintenance of a proper autophagic activity contributes to the extension of the lifespan in different organisms. A better understanding of the interplay between autophagy and risk of age-related pathologies is important to propose nutritional and life-style habits favouring disease prevention as well as possible clinical applications aimed at promoting long-term health.

1. Introduction

"Pugnandum, tamquam contra morbum sic contra senectutem" (we have to fight against disease, as we do against aging) is argued by Cicero in De Senectute, revealing that the question of aging as a real disease was discussed since the Romans time. Nowadays, the relation between longevity of population and onset of aging-related diseases is investigated in depth by the World Health Organization (WHO). Given the increasing lifetime of the populations worldwide and particularly in the developed countries, it is more crucial than ever to identify determinants of healthy aging that can be applicable to build the path to a better health status. In particular, the WHO gives some shocking predictions: "By 2030, 1 in 6 people in the world will be aged 60 years or over, such that the share of the population aged 60 years and over will increase from 1 billion in 2020–1.4 billion, and by 2050, it will double (2.1 billion). Moreover, the number of persons aged 80 years or older is expected to triple in the 2020–2050 period, when it will reach 426 million." (https://www.who.int/news-room/fact-sheets/detail/ageing-and-health). These figures are even more worrying considering that the Institute for Health Metrics and Evaluation (IHME) estimated that, by 2050, 153 million people globally will be living with age-related pathological conditions such as Alzheimer's disease (AD) (https://www.alzint.org/news-events/news/new-data-predicts-the-number-of-people-living-with-alzheimers-disease-to-triple-by-2050). Taken together, these numbers highlight the importance to improve the knowledge about aging-related diseases, their onset, molecular determinants, and treatments.

At the present, aging is the predominant risk factor for most diseases and conditions that limit health span. Common negative conditions in older age include hearing loss, cataracts and refractive errors, back and neck pain and osteoarthritis, chronic obstructive pulmonary disease, type-2 diabetes (T2DM), depression and dementia (Jaul and Barron, 2017). Older age is also characterized by the emergence of several complex health states, commonly referred to as geriatric syndromes. Often, the latter are the result of multiple underlying factors and include frailty, urinary incontinence, falls, delirium and pressure ulcers.

Several studies have highlighted different factors that could link aging with life-threatening disorders in people aged over 60 years, including ischemia, stroke, and cognitive disorders. There is considerable evidence indicating the existence of a link between poorer health outcomes, early morbidity, early mortality and a lower socio-economic status (Braveman et al., 2010; Zimmer et al., 2016). The factors

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experienced during the prenatal intrauterine life as well as environment where people live and grow during the childhood, combined with their personal characteristics, exert long-term effects on the path and the mode of aging. Physical and social environments can affect health directly or through barriers or incentives that affect opportunities, decisions, and healthy habits; being able to maintain the latter throughout life, particularly eating a balanced diet, performing regular physical activity, and limiting from/refraining the use of alcohol and tobacco, all contribute to reduce the risk of the occurrence of non-communicable diseases, improve the physical and mental capacity and delay care dependency. Supportive physical and social environments also enable aged people to do what is really important to them, in spite of the loss of their capacities. The availability of safe and accessible public offices, buildings and transport, and of places where it is easy to walk around, are examples of supportive environments. In developing a public-health response to aging, it is also important to consider not only individual and environmental approaches aimed at ameliorating the physical/functional losses associated with older age, but also those that may reinforce recovery, adaptation, and psychosocial growth.

In addition to the considerations reported above, not only lifestyle, but also personal genetic, epigenetic and physiological predisposition may either delay the onset and/or affect the severity of aging-associated conditions. Indeed, at the biological level, aging results from the impact of deterioration of the cell functioning due the accumulation of cell damage over time (Vellai et al., 2009). In spite of the generally accepted concept that aging is a multifactorial process, several theories have emerged to describe it in terms of a single predominant age-related change. A popular aging theory, the "Stochastic Theory," suggests that aging results both from a random accumulation of cell damage produced by external and internal sources over time and from some failure of the cell repairing capacity. On the other hand, other theories support the idea that aging is a process regulated mainly by the genetic code, that includes telomeres length, number of divisions a somatic cell can go through (the "Hayflick limit") and the spatiotemporal regulation of gene expression (Kamel et al., 2015; Wahab Pathath, 2017).

One of the most popular theories of aging is the "Free Radicals (or Oxidative Stress) Theory"; the latter hypothesizes that an accumulation of Reactive Oxygen Species (hereafter ROS) results in progressive oxidative damage of biomolecules (nucleic acids, proteins, lipids), with the consequent decline of cell functioning in terms of deregulation of homeostatic systems (Kamel et al., 2015; Harman, 2003). The theory is supported by a considerable body of evidence; it points to an age-associated increase of the cellular levels of ROS, due both to their increased production and to the failure of the cellular antioxidant systems (Finkel and Holbrook, 2000; Bokov et al., 2004). Many studies carried out with cell and animal models have provided a solid contribution to our knowledge of the interplay between autophagy and redox homeostasis and to the understanding of how the impairment of the latter is involved in age-related diseases. Particularly, a wealth of studies carried out with genetic ablation or induction of autophagic genes have revealed the importance of autophagy in the aging process of yeast, nematodes, flies, and mammals (Markaki et al., 2017).

In consideration of the central role of autophagy in aging, in this review we will summarize the results of the studies that have focussed the relationship between autophagy impairment and the onset of aging-related diseases.

2. Autophagy in cell survival and in cell death

Two distinct self-destructive processes, autophagy and apoptosis, ensure the physiological turnover of cell organelles and the survival/death of whole cells, respectively, and the loss of their balance promotes the development of many pathological processes. Autophagy and apoptosis often occur in the same cell, mostly in a sequence whereby apoptosis is preceded by autophagy (Maiuri et al., 2007); in fact, while a specific non-lethal stressful stimulus triggers the autophagic response, under stressful stimuli exceeding cell tolerance, apoptosis and non-apoptotic death programmes are activated (Kroemer et al., 2010). In such a case, the autophagic response is inactivated due, at least in part, to the cleavage of autophagic proteins by caspases.

In 2016, the research on autophagy and its importance to human health and disease enabled Yoshinori Ohsumi to be awarded with the Nobel Prize for Physiology or Medicine (Assembly TN. The Nobel Assembly at Karolinska Institutet has today decided to award the 2016 Nobel Prize in Physiology or Medicine to Yoshinori Ohsumi. 2016). Autophagy, or the cell's self-digestion mechanism, is an evolutionarily conserved self-degradative pathway originally identified from genetic screens in yeast. Autophagy is carried out by a molecular machinery encoded by autophagy-related genes (*Atg*) and is pivotal to maintain cellular homeostasis in terms of protein synthesis/degradation (proteostasis), proper metabolic energy and redox status. Autophagy is exploited by cells to eliminate, by degradation through the lysosomal apparatus, senescent or damaged cell organelles, mutated/chemically modified or misfolded proteins or their aggregates, and is mainly aimed at molecular recycling (Mizushima and Komatsu, 2011). Autophagy occurs at basal levels in all cell types and is upregulated under conditions that modify cellular environment, such as oxidative stress, nutrient shortage, hypoxia, chemotherapy, and others (Levine and Kroemer, 2008). The identified canonical forms of autophagy are indicated as microautophagy, macroautophagy and chaperone-mediated autophagy (CMA) (Figs. 1 and 2). Microautophagy indicates the direct uptake of cytosolic components by lysosomal enzymes that occurs by encircling cytoplasmic components/organelles at the lysosomal membrane (Li et al., 2017; Li et al., 2012; Wu and Lu, 2020). Macroautophagy, simply referred to as autophagy, is the most widespread and investigated form of autophagy involved in the degradation of long-lived proteins, aggregate-prone proteins, protein complexes, and defective organelles. The mammalian autophagic flux can be divided into five steps: initiation, nucleation, elongation and closure, fusion, and degradation. Briefly, upon autophagy induction, a double membrane structure, the phagophore or pre-autophagosome, is generated (nucleation). The next step, the phagophore elongation, invaginates and encloses the cargo, forming an autophagosome (elongation and closure). Once formed, the autophagosome fuses with the lysosome generating an "autolysosome" and the cytoplasmic material is degraded and/or recycled. All these steps are closely associated with the activity of autophagy-related proteins (ATG). At the molecular level, the initiation of autophagy consists of vesicle nucleation around the cargo material induced by the ULK/-FIP200/ATG13 complex, followed by the involvement of the Beclin1 complex, including VPS34. Vesicle expansion and elongation require the presence of ubiquitin-like conjugation systems, whereby phosphatidylethanolamine conjugation to LC3II is favoured by ATG4B, a protease, and ATG7, an E1-like enzyme. The LC3II incorporation into the growing membrane results in autophagosome-lysosome fusion (Antonioli et al., 2017; Yu et al., 2018).

The cellular regulation of the autophagy flux is under the control of the two key metabolic players, the mammalian target of rapamycin (mTOR) and the adenosine 5′ monophosphate-activated protein kinase (AMPK) complexes (Kim et al., 2011). Under normal conditions, the nutrient sensor mTOR interacts with the ULK1/2 complex, consisting of the ULK1/2 kinases, Atg13, Atg101, and FIP200 and phosphorylates ULK1/2, thus inhibiting its kinase activity. However, under stress conditions such as starvation, oxidative stress or hypoxia, ULK1/2 is activated following phosphorylation by AMPK, a sensor of the cell energy levels in terms of the AMP/ATP ratio, such that, during starvation, the intracellular levels of AMP increase leading to AMPK activation (Hardie et al., 2012). Macroautophagy can also be activated by stress stimuli resulting from medical treatments such as chemotherapy and radiotherapy (see later) (Pérez-Hernández et al., 2019).

Transcriptionally, macroautophagy is under the control of the transcription factor EB (TFEB), a member of the microphthalmia family of transcription factors (MiT-TFE family) that regulate autophagy and

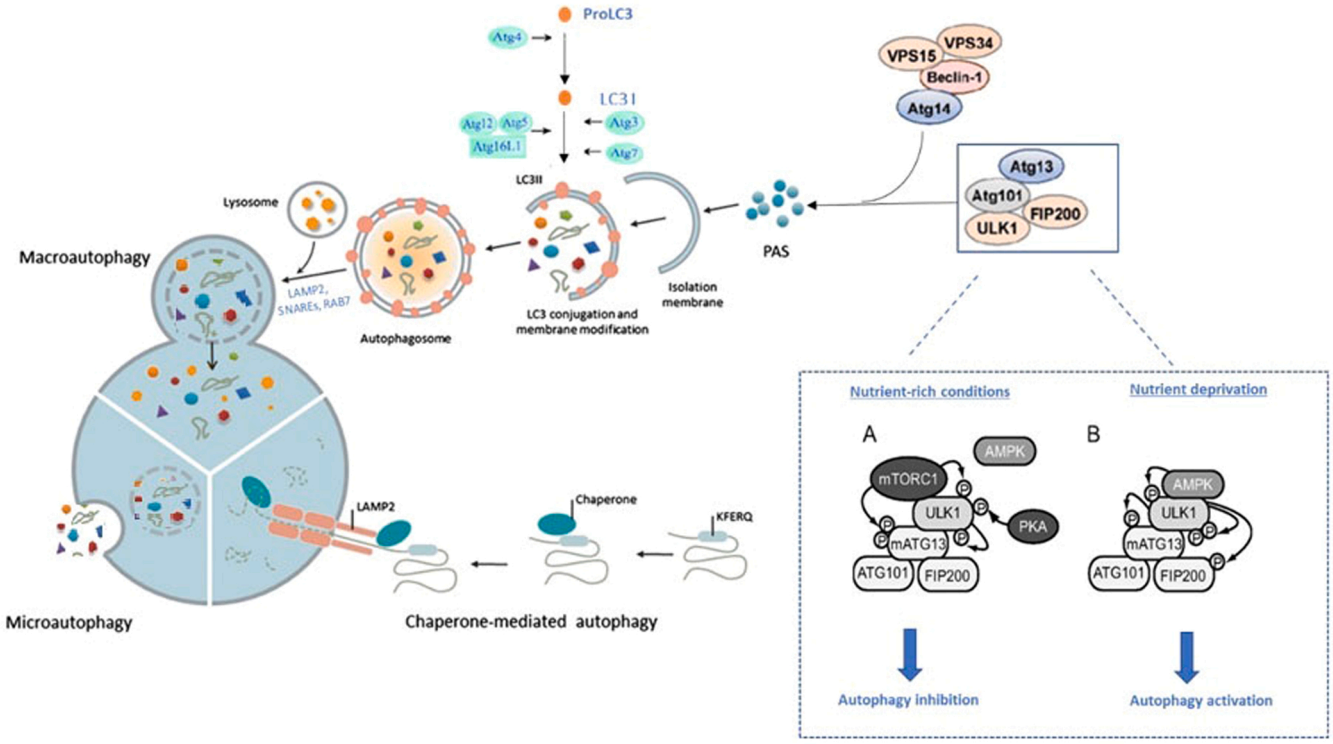

Fig. 1. Autophagy machinery and its regulation. The figure shows a schematic representation of the three types of autophagy: macroautophagy, microautophagy, and chaperone mediated autophagy. Autophagy, notably macroautophagy, is upregulated in mammalian cells when nutrient supplies are limited. The mammalian target of rapamycin (mTOR) is a key regulator of autophagy induction. Under nutrient-rich conditions, mTORC1 phosphorylates ULK1 at Ser 757, thus destabilising the Ulk1-AMPK-ATG13 complex and inhibiting autophagy. Under starvation conditions, mTORC1 dissociates from the complex allowing adenosine 5′ monophosphate-activated protein kinase (AMPK) to bind and to phosphorylate ULK1 at Ser 317 and Ser 777. Next, the autophosphorylation of ULK1 increases its kinase activity, phosphorylating other components of the complex. The final effect is the activation of autophagy (Modified by Xu et al., 2021; Mao and Klionsky, 2011).

lysosomal biogenesis (Zhang et al., 2020). TFEB activity is dependent on nutrient availability via protein phosphorylation. In the presence of nutrients, mTORC1 is active and phosphorylates TFEB, determining its cytoplasmic localization and inactivation. When mTORC1 is inactivated, TFEB is dephosphorylated and can translocate to the nucleus, where it exerts its transcriptional activity (Sardiello et al., 2009; Settembre et al., 2011; Puertollano et al., 2018). In spite of the fact that nuclear TFEB translocation is usually a protective event, a costitutive activation of TFEB and its excessive transcriptional activity can be tumorigenic (see Section 4.1 Autophagy and cancer) (Sardiello et al., 2009; Napolitano et al., 2020; Kauffman et al., 2014; Calcagnì et al., 2016).

Unlike macroautophagy, which delivers proteins and organelles in bulk to the lysosome for degradation, CMA is characterized by the chaperone-mediated selective delivery of intracellular proteins to lysosomes for degradation. CMA occurs when cytosolic proteins harbouring motifs related to the KFERQ sequence are recognized by the HSC70 protein, thus forming a complex that translocates to the lysosome via the lysosomal-associated membrane protein 2 A (LAMP2A) (Arias and Cuervo, 2011; Kaushik et al., 2011). CMA does not require the presence of autophagic vesicles since, during the process, the substrate to be degraded reaches the lysosomes via a protein translocation complex located at the lysosomal membrane (Wu and Lu, 2020; Hao et al., 2019).

Under some conditions, autophagy, besides being a resource for cells to survive in the presence of unfavourable environmental cues, can also be involved in cell death in the absence of apoptosis; however, the relation between autophagy and cell death is very complex and not completely understood (Mariño et al., 2014, 2014). Autophagy is induced in response to multiple signals triggered by several key regulators, some of which can be similar for both cell survival/death outcomes. Moreover, distinct, and diversely integrated, regulatory mechanisms of autophagy-dependent cell death are at work in different cells types or tissues, where they may determine the cellular fate. The internal/external stimuli that determine the modes/times/types of autophagic response and the molecular mechanisms leading to cell demise during autophagy-dependent cell death are poorly understood (Denton and Kumar, 2019). The autophagic flux is dependent on lysosomal function and, possibly, the lysosome can contribute to the autophagy-dependent cell death (Karch et al., 2017). However, the molecular machinery required for autophagy to mediate cell death may differ from that promoting cell survival in terms of rate of autophagic flux, duration of its activation, recycling or degradation of the lysosomal contents. All these differences may contribute to distinct requirements for specific components of the autophagic machinery. The latter conclusion stems from a number of studies in midgut cells of Drosophila, where only a subset of the multi-subunit complexes of the ATG machinery were required for autophagy-dependent cell death respect to those needed for starvation-induced autophagy (Xu et al., 2015). In particular, the most convincing evidence for the physiological role of autophagy as a cell death mechanism in Drosophila comes from several studies showing that, in specific tissues such as salivary glands, autophagy-dependent cell death, coupled in some cases with apoptosis, does occur (Denton et al., 2013; Berry and Baehrecke, 2007).

In higher organisms displaying an intact apoptotic machinery, a complex crosstalk between autophagy and apoptosis does exist, and research has focused such a relation to understand the connection between these two types of cell death (Green et al., 2011). Autophagy inhibition by treatment with specific siRNAs targeting the expression of autophagic proteins (ATG5, ATG6/Beclin1, ATG10, ATG12) or with chemical/pharmacological agents (3-methyladenine, hydroxychloroquine and others) destabilizes the mitochondrial membrane

and/or inhibits caspases, with the ensuing enhancement of apoptosis-induced cell death (Boya et al., 2005). ATG proteins have also been described as regulators of apoptosis, as it has been reported for calpain-mediated cleavage of ATG5, a component of the autophagic flux that switches autophagy to apoptosis (Rubinstein et al., 2011).

Some caspases can act as molecular brakes by degrading ATGs and Beclin1 leading to inhibition of autophagy during extrinsic apoptosis (Tsapras and Nezis, 2017; Mariño et al., 2014). Furthermore, an autophagy regulator, ATG4D, has been reported to be an important factor in the regulatory interface between autophagy and apoptosis, since it acquires affinity for damaged mitochondria in hydrogen peroxide-treated cells and induces apoptosis (Betin et al., 2012). On the other hand, the autophagy machinery can control regulators of apoptosis; a reciprocal regulation between ATG7 and caspase 9 has been proposed as a possible mechanism of choice between participation of caspase 9 in autophagy or in the apoptotic process (Han et al., 2014).

Finally, it has been reported that the absence of ATG12, a positive mediator of mitochondrial apoptosis that binds and inactivates pro-survival BCL-2 and BCL-2 family members, results in the inhibition of mitochondria-mediated cell death (Radoshevich et al., 2010). In turn, in the context of cancer, autophagy inhibition by many oncogene products (PI3K, AKT, BCL-2, mutant p53) is a potentially oncogenic event (White, 2012). These and other findings strongly suggest that mitochondria are not only key players of the autophagic flux and the apoptotic pathway but also represent a molecular platform where the cross-regulation between autophagic versus apoptotic cell death can occur (Green et al., 2011; Noguchi et al., 2015).

In the light of the findings reported above and many other studies, the intricate cross-talk between apoptosis and autophagy can have an important biological significance as it would allow the cell to coordinate these two processes through many "moonlighting" genes to achieve a finely-tuned and a flexible utilization of the autophagy machinery in the cell survival/death decision.

3. Autophagy in age-related diseases

Aging is a complex biological condition characterized by the accumulation of damaged molecules, organelles, cells, tissues, and organs eventually leading to increased risk of developing "age related diseases" such as neurodegenerative and cardiovascular diseases, cancer, immune system disorders, osteoporosis, T2DM/metabolic syndrome, and others (Li et al., 2021, 2021; He et al., 2013, 2020). The impairment of the autophagic process is a major aging-associated molecular modification playing a crucial role in disease onset and progression (Aman et al., 2021).

Given the essential role of autophagy in maintaining cellular homeostasis, it is not surprising that a defect in the autophagic machinery, leading to the accumulation of mutated proteins and damaged organelles, has been implicated in a wide range of age-related pathologies. Actually, dysfunctional autophagy is one of the major causes of the loss of cellular function, an event commonly associated to aging. (Yen and Klionsky, 2008; Martinez-Lopez et al., 2015). In support of this theory, Menzies has shown that the knock-out of specific autophagy genes in the nervous system of murine models leads to the accumulation of damaged proteins and to an accelerated aging phenotype (Menzies et al., 2017; in turn, autophagy stimulation, either by genetic manipulation or by administration of autophagy inducers (resveratrol and other plant polyphenols, rapamycin, spermidine) has been associated to lifespan extension in different model organisms such as yeasts, mice and worms (Hansen et al., 2018). The most significant study that associates the overexpression of a specific *Atg* with an increment in mammal lifespan was carried out by Pyo and co-workers. The authors overexpressed ATG5 in mice and found that the autophagy process was enhanced in these animals, that also displayed anti-aging features, as compared to the wild type littermates. The mean lifespan was also incremented in the transgenic mice, suggesting the importance of autophagy in the longevity of these animals (Pyo et al., 2013). Generally, the KO of essential *Atg* genes is lethal in mice, whereas their tissue-specific ablation determines a less-dramatic phenotype, with the appearance of signs of premature aging (Rubinsztein et al., 2011). For example, in most of the reported studies, the KO of *Atg5* or *Atg7* results in neurodegeneration or in tissue abnormalities [for a more detailed summary see reference (Rubinsztein et al., 2011). Furthermore, knockdown of genes encoding transcription factors that modulate autophagy, such as TFEB and FOXO (encoding forkhead box O) shortened lifespan in both wild-type worms and long-lived daf-2 (insulin/insulin-like growth factor-1 (IGF-1) receptor) mutants. (Aman et al., 2021).

A significant relationship between autophagy augmentation and extended lifespan has been reported in exceptionally healthy centenarians, who showed increased levels of the autophagy biomarker Beclin1 as compared to young people (Emanuele et al., 2014).

It can be expected that, hopefully, in the near future these preliminary studies in humans will be more advanced and that clinical case studies will provide insights into the mechanisms underlying the longevity of our species.

Finally, the incidence of cancer rises along with aging, probably because of the decline of homeostatic processes and of the increase of the accumulation of DNA mutations and of potentially harmful molecules such as ROS and protein aggregates. Autophagy has been proposed to play a dual role in tumorigenesis, being important both in suppression as well as in tumour progression and surveillance (Jiang et al., 2015; Taji et al., 2017).

3.1. Autophagy in cancer

Aging is a main risk factor for cancer in adulthood. It is widely accepted that autophagy is important in many diseases, particularly in cancer therapy, even though, at the present, most of the clinical studies focusing autophagy manipulation for cancer therapy have been carried out mostly with patients with cancer in advanced stage (Pérez-Hernández et al., 2019). Nevertheless, it is generally agreed that autophagy is important to prevent cancer development in several ways; in particular, it maintains cellular homeostasis by removing oncogenic protein substrates, damaged organelles and unfolded proteins with toxic potential, thus preventing enduring cell damage and pro-inflammatory responses with cell transition to a cancer-initiating stage (White, 2015). To date, the role of autophagy in cancer is still controversial. Genetic deficiency of several autophagy genes (*Becn1, Atg2, Atg5, Atg9, Atg12, Uvrag*) leading to the accumulation of defective organelles and mutated proteins inside the cells has been associated to various types of cancers. Moreover, autophagy can also involve the immune system in terms of immunosurveillance. For example, the suppression of the immune system with the ensuing decrease of efficient immunosurveillance that promotes tumorigenesis is favoured by the infiltration of regulatory T cells that can result from defective autophagy (Rao et al., 2014). However, it has also been reported that in many types of fully established cancers, survival and growth of tumour cells can be favoured by increased autophagy (White, 2012; Amaravadi et al., 2016).

The data reported above support the idea that the role of autophagy may be very different, depending on the type and stage of a tumour. In some cases, the enhancement of the autophagic flux can prevent cancer in premalignant lesions (Galluzzi et al., 2015) even though, in several cancers at advanced stages, both the enhancement and the inhibition of autophagy have been proposed as therapeutic strategies (Amaravadi et al., 2016; Levy and Thorburn, 2011; Towers and Thorburn, 2016).

MiT/TFE proteins have recently been associated with several human tumors. MIT/TFE genomic amplifications are present in 5–20% of melanoma, and chromosomal translocations involving the MiT/TFE family have also been reported in pediatric renal cell carcinoma and alveolar soft part sarcoma (ASPS) (Perera et al., 2019; Tan et al., 2022). Overexpression of MIT-TFE family members has been observed in pancreatic ductal adenocarcinoma (PDA) (Perera et al., 2015). In 2020, Ballabio

and colleagues indicated TFEB overactivation as the main driver of kidney cysts and tumours in a mouse model of Birt-H-ogg-Dubé syndrome (BHD), genetic disease caused by mutations in the RagC and RagD activator folliculin (FLCN) and characterized by benign skin tumours, kidney cysts and renal cell carcinoma. Depletion of TFEB in kidneys of these mice rescued the kidney phenotype and associated lethality (Napolitano et al., 2020).

In light of the studies reported above, clinical investigations aimed at manipulating, mostly at inhibiting, the autophagy flux for cancer therapy purposes are underway (Towers and Thorburn, 2016). In particular, these studies have been aimed at blocking autophagy thus hindering the recycling and renewal of cellular molecules, particularly specific proteins, involved in cancer cells survival under stress conditions (Blagosklonny, 2013; Wu et al., 2015; Guo et al., 2018). For example, recycling through autophagy of mutant CDC48/p97, an essential segregase that extracts proteins from membranes or multi-subunit complexes involved in several diseases such as Familial Amyotrophic Lateral Sclerosis (FALS), Charcot-Marie-Tooth Disease, Type 2Y (CMT2Y) and Multisystem Proteinopathy (MSP) (Tang and Xia, 2016) can affect the development and severity of these pathologies (Marshall et al., 2019). The same treatments can also be exploited to improve standard cancer treatments such as chemotherapy (Barnard et al., 2014) and radiation (Briceno et al., 2003).

At the present, we have a good mechanistic understanding of how autophagy interacts with cell death pathways, notably apoptosis, particularly the apoptotic machinery, with the ensuing modification of the response to cancer therapy. The inactivation of apoptosis is central to carcinogenesis, and the disassembly of the apoptotic responses might be a major contributor both to cancer resistance to therapeutic treatments and to the observation that, in many tumours, the main mechanism for cancer cell death in response to chemotherapy or radiotherapy does not proceed through apoptosis. This consideration suggests that other mechanisms of cell death, possibly involving autophagy, must be involved in the response to cancer therapy. Considering the different effects of autophagy in different tumours at different pathogenic stages, targeting the apoptosis-autophagy relation for medical purposes can be an important tool in cancer therapy.

Two anticancer drugs, 6-hydroxychlorochine and 2-deoxyglucose (2-DG) have been investigated as an example of this relation. Most cancer cells obtain energy prevalently from glucose by glycolysis, whereas normal cells produce ATP mostly through the oxidative phosphorylation; accordingly, suppression of glycolysis can be a possible therapeutic strategy for several tumours (Choe et al., 2015). 2-DG suppresses glycolysis and can also interfere with N-linked glycosylation (Pajak et al., 2020) inducing the continuous accumulation of unfolded/misfolded proteins in the endoplasmic reticulum (ER), with ensuing ER stress and subsequent cell death. These effects make 2-DG a useful tool in antitumor research; however, the possibility of clinical application of 2-DG monotherapy is limited, due to its side effects and to the possibility that cancer cells survive by exploiting protective autophagy following ER stress that mediates the elimination of defective proteins and organelles (Wu et al., 2009). Chloroquine and hydroxychloroquine (HCQ) are the sole autophagy inhibitors approved by FDA for cancer therapy (Pérez-Hernández et al., 2019). A recent study has checked whether the combination of HCQ and 2-DG can provide a positive therapeutic perspective. The authors found that the HCQ-2-DG combination can better impair the viability and migration of breast tumour cells, as compared with other individual drugs, by triggering the apoptotic response. This effect was achieved by suppression of the formation of autolysosomes and the termination of the autophagic response triggered by 2-DG-mediated ER stress resulting from the continuous accumulation of misfolded proteins in the ER (Zhou et al., 2022).

Another recent study concerning chronic myeloid leukaemia (CML) confirmed the possible role of autophagy in cancer therapy. The authors found that inhibition of glucose metabolism by 2-DG significantly impaired the viability of CML cells and that co-treatment with 2-DG and imatinib, a tyrosine kinase inhibitor used for CML treatment, induced a synergistic inhibition of CML cells. 2-DG did not induce cell death by apoptosis, rather, the latter proceeded by autophagy, as supported by the increased expression of Beclin1 and LC3II and by the lack of annexin V/PI-positive cells. At the biochemical level, 2-DG produced a severe metabolic stress by inhibiting glycolysis and mitochondrial oxygen consumption, with ATP depletion, resulting in cell death by autophagy, suggesting the anticancer potential of the 2-DG-imatinib combination in imatinib-resistant CML (Li et al., 2022).

The importance of autophagy modulation in cancer was recently reported by the studies on the effect of a novel anticancer small molecule Pevonedistat (MLN4924), currently in phase I trial, that inhibits tumor cell growth by inducing all three common types of cell death: apoptosis, autophagy, and senescence (Luo et al., 2012). All three types of death were correlated to the neddylation, an important post-translational modification that adds to substrate proteins the ubiquitin-like molecule NEDD8, a pivotal regulator of the Cullin Ring Ligases E3 (CRL), which has been implicated in the regulation of cell-cycle arrest, apoptosis, and senescence in cancer cells (Liang et al., 2020; Li et al., 2021).

An example of autophagy as an enhancer of tumour cell survival and of the opportunity to block it in these cells, is provided by a recent study on the therapeutic resistance of glioblastoma cells. Several studies have reported that autophagy is upregulated in cancer cells displaying radio resistance, and that the latter is acquired following autophagy enhancement via radiotherapy (Firat et al., 2012). The diverse metabolic fuel sources produced by autophagy and the autophagy-induced metabolic plasticity are known to trigger drug or radio resistance in glioblastoma. The study showed that autophagy was upregulated upon treatment of glioblastoma cells with ionizing radiation through upregulation of nuclear receptor binding factor 2 (NRBF2), a positive regulator of the autophagy initiation step, and the ensuing increase of ATP production and oxygen consumption rate following autophagy activation (Kim et al., 2022).

Taken together, the studies reported above support the notion that autophagy in cancer can be both tumour suppressive and tumour promotional, depending on several factors (type of cancer cell, stage of its transformation, interactions with the surrounding environment, etc.) (Marinkovic et al., 2018) and that its activation can also trigger cell death in many cancers. In fact, in addition to its role as a recognized mechanism of cell survival that can promote tumour development, autophagy can also be involved in a caspase-independent type of programmed cell death, as it happens with some anticancer agents, highlighting the possibility to exploit this process in cancer therapy. In this case the cross-talk between autophagy and autophagy-promoted cell death is also finely tuned by the modulation of the expression of common proteins (ATG5, ATG6, and ATG7) and the formation of autophagosomes (Shimizu et al., 2004; Yu et al., 2004). A recent study reported that autophagy activation is needed for cell death and, thereby, for the elimination of precancerous cells during replicative crisis and that tumorigenesis can be initiated by impaired autophagic cell death in fibroblasts and epithelial cells (Nassour et al., 2019). Another study showed that melanoma cells resistant to apoptosis-inducing drugs can undergo autophagy-dependent death following treatment with compounds favouring the translocation to mitochondria of the orphan nuclear receptor TR358 by interaction with Nix, a protein in the mitochondrial outer membrane, dissipation of the mitochondrial membrane potential, mitochondrial clearance, and loss of ATP production (Wang et al., 2014).

Overall, the autophagy-tumorigenesis relation is complex, and autophagy displays different effects in cancer development and treatment. It may act as a tumour-suppressor mechanism by maintaining genomic stability, by digesting defective cellular organelles, notably depolarized dysfunctional mitochondria, thus removing the main sources of cellular oxidative stress, and by regulating the inflammatory process, all important factors involved in the prevention of cancer

development. However, the opposite survival-favouring or death-promoting functions of autophagy make very complex the association of the latter with cancer treatment. In fact, along with its antitumor role that favours cancer cells eradication by cell death, in some cases autophagy can also favour the viability of cancer cells treated with chemotherapeutic drugs or with irradiation under starvation/nutrient-limited conditions by providing them metabolic substrates. Under these stressful microenvironments, the onset of apoptosis can be retarded, and cancer cell survival enhanced (Levine, 2007).

In conclusion, in many cases autophagy, as a key player of cellular quality control, appears an essential mechanism for protection against the initiation of tumorigenesis. Chemotherapeutic drugs, such as tamoxifen, have been found to act, at least in part, through modulation of autophagy. In fact, the impairment of the autophagic flux can promote cell necrosis with release of the cell content to the extracellular space and stimulation of an inflammatory response that can support the onset of carcinogenesis. Accordingly, autophagy stimulation in most cases appears protective against carcinogenesis and is considered a promising therapeutic strategy against cancer. However, beside the protective effects, under certain conditions, notably in advanced stages of tumours, autophagy can promote tumorigenesis due to its ability to sustain cancer viability in stressful microenvironments (Chavez-Dominguez et al., 2020). Further studies are needed to determine whether autophagy should be activated or inhibited depending on tumour type and the stage of the carcinogenic process as well as to individuate the exact timing of treatment with autophagy activators or inhibitors (He et al., 2013).

3.2. Autophagy in neurodegenerative diseases

Autophagy and the associated lysosomal function are considered determinants for the maintenance of neuronal health. Under normal conditions, two major intracellular protein degradation systems, the autophagy machinery and the ubiquitin-proteasome system (UPS) are in charge to maintain cellular proteostasis; however, both machineries decline with aging and in neurodegenerative diseases (including AD, Parkinson (PD), Huntington diseases (HD), amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia and others) that involve an accumulation of misfolded proteins or damaged mitochondria, eventually causing alteration of normal cell function and/or cell death (Dikic and Elazar, 2018). Mutations in different *Atg* genes associated with distinct steps of the autophagic flux have been linked to the development of many familial neurodegenerative diseases, confirming that autophagy plays a key role in the clearance of altered cellular proteins (Klionsky et al., 2021, 2021). In ALS, a neurodegenerative disease that results in the progressive loss of motor neurons that control voluntary muscles, mutations of several autophagy-related proteins such asp62, ubiquilin, VCP, optineurin, TBK1, CHMP2B, have been reported. Mutations distributed throughout p62 protein, involved both in the UPS and in the autophagic flux, can impair ubiquitin-cargo recognition (UBA-domain mutation), LC3 cargo-recognition (LIR domain mutation) and mutations in the *SQSTM1* promoter region are associated with a reduced p62 protein levels in familial ALS (Chua et al., 2022). Inclusions of mutated p62 have been found in post-mortem brains of subjects with various neurodegenerative disorders such as ALS, HD, PD, AD. In AD, wt or mutated p62 colocalized with neuronal and glial ubiquitin-containing inclusions. In PD, p62 has been detected inside aggregates known as “Lewy bodies”, in PINK1/PARK-positive inclusions as well as in ubiquitin-positive inclusions, even though inclusions containing only p62 were also detected (Ma et al., 2019). In mouse models of HD and in HD patients, p62 levels were decreased in all brain regions in the earlier stage of the disease, whereas at later stages p62 inclusions, co-localized with mutant huntingtin, accumulated in in neuronal nuclei in the striatum and hippocampus (Rué et al., 2013).

In neurons and glial cells of ALS patients, p62 inclusions often overlap with TDP43, ubiquitin and mutant SOD1 (Nakano et al., 2004; Gal et al., 2007). p62 inclusions have been identified also in Mallory bodies in hepatocytes in alcoholic and non-alcoholic steatohepatitis (Zatloukal et al., 2002).

Chen and colleagues reported altered expression of TFEB and Beclin1 both in the spinal cords of transgenic mice as ALS models and in the NSC-34 cell line carrying the SOD1- G93A mutation, an in vitro model of ALS. Overexpression of TFEB in NSC-34 with SOD1-G93A mutation was accompanied by increased survival as well as by an increased expression of autophagy markers as Beclin1 and LC3II. The authors associated the altered TFEB expression to a decline of the autophagy machinery, suggesting that TFEB stimulation could be a promising strategy for ALS therapy (Chen et al., 2015). TFEB activity was also reduced in presenilin (PS) deficient cells, a model of AD.

In mice, neuronal-specific TFEB deletion induced Aβ and tau accumulation in the brain, suggesting that TFEB plays an essential role in the maintenance of neuronal health (Reddy et al., 2016). TFEB overexpression also reduced tau levels in the P301S, a mouse model of tauopathy (Wang et al., 2016).

TBK1 is involved in the recruitment of optineurin (OPTN) and LC3 to damaged mithocondria. TBK1 mutations are present in 1% ALS patients and are associated to defective mitophagy. In mouse model of ALS, ($SOD1^{G93A}$ mice) TBK1 deficiency has opposing effects in early and late stages of ALS: at the early stage, TBK1 deficiency was reported to impair autophagy and to accelerate the build-up of SOD1 in the brain, whereas at late stages of the disease it decreased microglial neuroinflammation and extended mice survival (Brenner et al., 2019; Chua et al., 2022). UBQLN2 (ubiquilin 2) is an adaptor protein involved in the ubiquitin proteasome system (UPS) and in the autophagy process by directly interacting with LC3. *In vivo* overexpression of the ALS mutant UBQLN2 induced p62/SQSTM1 and LC3II accumulation and loss of motoneurons; moreover, in vitro the UBQLN2 mutation disrupted the interaction between UBQLN2 and several ATG proteins, such as ATG9-ATG16L1 (Osaka et al., 2016; Wu et al., 2015; Chua et al., 2022).

Similarly, to UBQLN2, optineurin (OPTN) is involved both in UPS and in autophagy. OPTN plays also a crucial role in mithophagy by targeting damaged mithocondria to the phagophore. OPTN1 mutations have been reported in 1–3% cases of ALS patients and were associated to defective autophagy, inflammation and neuronal loss (Chua et al., 2022; Akizuki et al., 2013).

VCP/p97/Cdc48 (valosin containing protein) is involved in cell cycle regulation, DNA repair and protein clearance. VCP mutations are present in 1% cases of ALS, are accompanied by TARDBP, ubiquitin and SQSTM1-positive inclusions and can impair autophagy at different steps from autophagosomal maturation to autophagosome-lysosome fusion. *In vitro* studies showed that VPS is crucial not only for neuronal health but also for astrocyte functions. In fact, mutant VCP-astrocytes were unable to support motor neuron survival, contributing to disease progression (Custer et al., 2010; Schröder et al., 2005; Hall CE et al., 2017).

CHMP2B (charged multivesicular body protein 2B) is involved in autophagosome initiation and endolysosomal trafficking. Mammalian cell lines carrying CHMP2B mutations resulted in aberrant endosomal structures, defective autophagosome maturation, and accumulation of protein deposits. Finally, CHMP2B-mutant mice displayed progressive neurological defects, early mortality and neuronal loss (Chua et al., 2022).

Dysfunction of selective types of autophagy in specific neurodegenerative diseases has also been reported. Defective chaperone-assisted selective autophagy (CASA) has been associated to motor neuron disorders such as ALS and other neurodegenerative diseases. In this case, small heat shock proteins such as HSPB8 exert a potent pro-degradative activity on misfolded proteins facilitating their removal via autophagy and contributing to the maintenance of the delicate equilibrium that regulates the protein degradative routing to autophagy or to the proteasome (Rusmini et al., 2017). Mutations found in HSPB8 or in BAG3, its co-chaperone, have been associated to motor neuronal or neuromuscular diseases (Adriaenssens et al., 2017; Bouhy et al., 2018). In

Parkinson's disease, mutations in PINK and PARKIN, two essential proteins that regulate mitophagy (a selective form of autophagy involving mitochondrial proteins), have been reported. As a consequence, the accumulation of damaged mitochondria is often present in patients with Parkinson's disease (Dikic and Elazar, 2018). Furthermore, it is worth noting that a failed mithophagy has been associated with increased neurodegeneration in PD. Interestingly, TFEB has recently been shown to play a role in mitophagy. During this process, TFEB translocates to the nucleus and its transcriptional activity is regulated in a PINK-PARKIN-dependent manner. However, the beneficial effects of TFEB stimulation are not only limited to the mitochondrial quality control, but also to the clearance of α synuclein deposits (Martini-Stoica et al., 2016). Post-mortem brain of PD patients showed accumulation of autophagosomes due to defective α-synuclein clearance. In a rat model of PD as well as in human PD midbrain, α-synuclein accumulation was associated to a decline of lysosomal function and cytoplasmic TFEB retention. In a model of α-synuclein overexpression TFEB-induction decreased the deposits of a synuclein and improved cell survival (Decressac et al., 2013). Likewise, treatment with trehalose, a TFEB inducer, resulted in neuronal protection in PD. Similar results were also obtained with 2- hydroxypropyl-β-cyclodextrin, another TFEB activator (Martini-Stoica H et al., 2016). Finally, in HD, a neurodegenerative disorder caused by the expansion of a polyglutamine region within the huntingtin (HTT) protein, TFEB overexpression showed beneficial effects resulting in the clearance of HTT aggregates by PGC1a (Tsunemi et al., 2012; Tan et al., 2022; Sardiello et al., 2009). Taken together, these data indicate that autophagy stimulation has potential beneficial effects to counteract neurodegeneration (Meijer and Codogno, 2006; Dikic and Elazar, 2018).

However, it must be considered that, in neurons, autophagy is regulated differently than in other cell types essentially for two reasons: (1) at the basal level, the number of autophagosomes is reduced in neurons, as compared to other cell types; (2) nutrient starvation, the canonical stimulus that activates autophagy in other tissues/organs, is not able to induce autophagy in the brain. Both findings suggest that the classic autophagy inducers, such as rapamycin, that mimic a starvation-like stimulus may not be fully effective against neurodegeneration and that finding brain-specific autophagy inducers could represent a step forward in treating neurodegenerative diseases. Another consideration is that not all these pathologies are defective for what the onset of autophagy is concerned; in fact, some of these diseases are defective in cargo degradation due to inefficient autophagosome-lysosome fusion or to reduced lysosomal function. For these reasons, the use of drugs that act specifically at these steps could represent a best strategy to treat these conditions (He et al., 2013). Yet, although many autophagy-activating drugs have been approved for use in various neurodegenerative diseases, attention should be paid to the wide spectrum of side effects these drugs can produce. Therefore, it is highly desirable that more selective and safer compounds for use in clinical practice will be introduced in the future.

Recently, some natural compounds such as plant polyphenols have attracted much interest due to their ability to modulate autophagy acting on different intracellular pathways including the mTOR/AMPK platform or the SIRT1/PARP1 complex. In this context, resveratrol, curcumin, and olive-tree polyphenols have been proposed as promising modulators of the autophagy path, in addition to their ability to counteract the growth of amyloid aggregates and their anti-inflammatory properties (Leri et al., 2020). In addition to the beneficial effects of autophagy stemming from its role as a regulator of proteostasis, neuroprotection by autophagy is also due to the involvement of the latter in essential neuronal functions, including regulation of synaptic transmission (Kuijpers et al., 2021), degradation of synaptic vesicles, crosstalk between the cell body and the synaptic terminals, myelination/demyelination events (Hill and Colon-Ramos, 2020; Klionsky et al., 2021), neurogenesis (Klionsky et al., 2021), and others.

In conclusion, the decline in autophagy and neuronal function, found in various forms of neurodegenerative diseases, suggests that the restoration of the autophagic flux will aid the cell to remove accumulated cellular "trash" and to maintain proper functionality. Table 1 reports data on autophagy impairment in various neurodegenerative diseases and the autophagy activators that have been used in clinical trials.

3.3. Autophagy in cardiovascular diseases

It is well-established that aging is characterized by functional and structural alteration of the heart, an event that increases the vulnerability to age-related cardiovascular diseases (CVDs) (stroke, hypertension, atherosclerosis, heart failure etc.) (Bravo-San Pedro et al., 2017). Since autophagy declines with aging, it is reasonable to assume that its decline may contribute to heart diseases. Although it is still not entirely clear how autophagy decreases in the heart with age, hyperactivation of the mTOR pathway with inactivation of the ULK1 complex, beyond the activities of TFEB and FOXO, could play a crucial role in cardiac homeostasis. During aging, mTOR phosphorylates and inactivates TFEB, resulting in inhibition of nuclear translocation and cytoplasmic retention of the latter. TFEB is critical player in maintaining vascular and heart homeostasis. Indeed, TFEB regulates the function of endothelial cells (ECs), vascular smooth muscle cells (VSMCs), macrophages, and cardiomyocytes, thus ensuring protection agains CVDs and regulating cardiovascular homeostasis. In apolipoprotein E (ApoE) knockout (KO) mice, TFEB inhibits atherosclerosis and EC inflammation. In ECs, TFEB plays also a role in post-ischemic angiogenesis through AMPK-mediated autophagy activation. In cultured vascular smooth cells (VSMCs), TFEB inhibited events that occur during atherosclerotic lesion development such as VSMCs migration and proliferation as well as neointima formation. (Wang et al., 2019; Lu et al., 2021). TFEB has also been reported to affect macrophages, where excess of cholesterol accumulation induced alteration of lysosomal function in the ApoE KO mouse. Moreover, macrophage-TFEB overexpression restored normal lysosomal function and activated autophagy in ATG5- and p62-dependent manner. In cardiomyocytes TFEB promoted survival under stress conditions. In ischemia-reperfusion, TFEB restored mithocondrial biogenesis through PGC1α and prevented cardiomyocyte death. In desmin-related cardiomyopathy, TFEB overexpression increased the autophagic flux and reduced the accumulation of protein aggregates (Lu et al., 2021). In a mouse model of heart failure (HF) with monoamine oxidase-A (MAO-A) overexpression, Santin and colleagues reported accumulation of dysfunctional mithocondria and decline of autophagy resulting in cardiomyocyte death. These events were accompanied by cytoplasmic TFEB accumulation and inhibition of its transcriptional activity. TFEB overexpression counteracted the deleterious effects of MAO-A by ameliorating mitochondrial fission, cardiomyocyte survival and HF in transgenic mice (Santin et al., 2016).

FOXO is also negatively regulated during cardiac aging due to reduced AMPK activity, AKT phosphorylation and lysine acetylation, as a consequence of SIRT1 downregulation (Abdellatif et al., 2018).

In murine models, the whole-body KO of PINK1 or the heart-specific deletion of PARK2 (key components of mitophagy) result in a sharp increase of ROS in cardiomyocytes, with ensuing heart failure and premature death (Bravo-San Pedro et al., 2017; Leidal et al., 2018; Billia et al., 2011). In mice, the heart-specific deletion of *Atg5,* that encodes a key autophagy factor, causes severe cardiomyopathy, left ventricular dilation, contractile dysfunction, accompanied by accumulation of damaged mitochondria and a disorganized structure of the sarcomeres (Taneike et al., 2010; Bravo-San Pedro et al., 2017; Leidal et al., 2018). On the same line of evidence, LAMP2-deficient (*Lamp2 -/-*) mice show defective autophagy and a pathology recalling Danon disease b, characterized by heart muscle weakening (cardiomyopathy). Furthermore, mice overexpressing miR212/miR132 (that inhibit the pro-autophagic factor FOXO3) or miR199 (that inhibits autophagy by mTORC1 activation), develop heart failure. Similarly, the deficiency of SIRT6 (a histone

Table 1
Autophagy disfuctions in neurodegenaritve diseases (modified by Pupyshev et al., 2017).

Neurodegenerative disease	Protein pathology	Autophagy impairment and disease-associated genes	Autophagy Activators Inhibiting Neurodegeneration
Alzheimer's disease	Accumulation of extracellular amyloid-β (Aβ) plaques and intracellular tau inclusions (Jiang et al., 2013)	1. Impairment of autophagosome formation and maturation associated to mutation in Presenilin 1 (PS1) (Pupyshev et al., 2016) and PICALM (Menzies et al., 2017). 2. Defect in cargo recognition (PICALM mutation) and lysosomal function (PS1 mutation) (Menzies et al., 2017). 3. Inhibition of autolysosome formation (tau mutation) (Menzies et al., 2017).	mTOR inhibitors: Autophagy induction • Rapamycin (Menzies et al., 2017; Pupyshev et al., 2016). • SMER28 (Pupyshev et al., 2016). • Latrepirdine (HE LQ et al., 2013). • Metformin (Pupyshev et al., 2016). • Resveratrol (Pupyshev et al., 2016). • Arctigenin (Pupyshev et al., 2016). Beclin mimetics: autophagy induction (Pupyshev et al., 2016). Nicotinamide: activation of autophagic degradation (Pupyshev et al., 2016). AMPK activators: • Trehalose (Menzies et al., 2017) • Lithium: AMPK activator, GSK3β inhibition, IMPase inhibition (Motoi et al., 2014; Pupyshev et al., 2016). Gene therapy: Beclin mimetics: autophagy induction (Pupyshev et al., 2016) TFEB gene: induction of autophagosome formation and lysosomal biogenesis (Pupyshev et al., 2016).
Parkinson's disease	Accumulation of intraneuronal protein aggregates (Lewy bodies) resulting from polymerized α-synuclein (Baba et al., 1998).	1. Impaired autophagosome formation (α-synuclein, VPS35 mutation) (Menzies et al., 2017; Zhang et al., 2015). 2. Defective mitophagy with accumulation of damaged mitochondria (Hou et al., 2020). 3. Disrupted lysosomal function: mutation of α − syn, Atp13a2 (lysosomal ATPase), synaptotagmin 11 (syt1), vps 35 (vacuolar protein sorting-associated protein 35) (Menzies et al., 2017).	Gene therapy (autophagy induction): • Beclin gene (Pupyshev et al., 2016). • Rab1A gene (Pupyshev et al., 2016). • HDAC6 gene (Pupyshev et al., 2016). • TFEB gene (Pupyshev et al., 2016). mTOR inhibitors: autophagy induction • Rapamycin (Menzies et al., 2017; Crews et al., 2010; Malagelada et al., 2010; Pupyshev et al., 2016). • Resveratrol (Pupyshev et al., 2016). • Celastrol (Pupyshev et al., 2016). • Curcumin (Pupyshev et al., 2016). • Kaemferol (Pupyshev et al., 2016). • Sperimidine (Pupyshev et al., 2016). AMPK activators: autophagy induction • Lithium: AMPK activation, glycogen synthase kinase (GSK-3β) inhibition, IMPase inhibition (Motoi et al., 2014; Pupyshev et al., 2016). • Trehalose (Menzies et al., 2017). Modulators of the cyclic AMP (cAMP)/inositol triphosphate (IP_3) pathway: autophagy induction • Rilmenidine, clonidine, minoxidil, and verapamil (Rose et al., 2010; Menzies et al., 2017). AMPK activators: autophagy induction • Trehalose (Menzies et al., 2017; Tanaka et al., 2004). • Metformin (Menzies et al., 2017; Ma et al., 2007). • Berberine (Jiang et al., 2015). Histone deacetylase (HDAC) inhibitors: Autophagy induction (Bürli et al., 2013).
Huntington's disease	Accumulation and ensuing aggregation of mutant huntingtin (HTT) (Williams and Paulson, 2008).	Impaired autophagosome formation (Koga et al., 2011; Menzies et al., 2017) and cargo recognition by mutated HTT (Menzies et al., 2017; Martinez-Vicente et al., 2010).	
Amyotrophic lateral sclerosis	Accumulation of protein aggregates composed of wt or mutant FUS, TDP-43, OPTN, UBQLN2, SOD1 (Blokhuis et al., 2013; Brown, Al-Chalabi Oct 19), 2017)	Autophagy can be inhibited at different steps: • Impaired autophagy induction by C9orf72, TBK1 • Inhibition of Pre-autophagosome formation by SOD1 • Impaired autophagosome formation and expansion by VAPB, UBQLN2, VCP, p62, OPTN, TBK1 • Inhibition autophagosome-lysosome fusion by C9orf72, VCP, CHMP2B, ALS2, FIG4 (Amin et al., 2020)	mTOR inhibitors: Autophagy induction • Rapamycin (Staats et al., 2013) AMPK activators: Autophagy induction • Lithium: AMPK activator, glycogen synthase kinase (GSK-3β) inhibition, IMPase inhibition (Motoi et al., 2014; Fornai et al., 2008) • Trehalose (Zhang et al., 2014)

deacetylase that regulates autophagy) in *Sirt6* -/- mice has been associated to cardiac hypertrophy and heart failure. Finally, mice deficient in *Bnip3l* (*Bnip3l* -/-), a component essential for the autophagy machinery, show cardiomegaly and late onset depression (Bravo-San Pedro et al., 2017). In agreement with these findings, autophagy stimulation prevents CVDs. $Becn1^{F121A/F121A}$ mice, showing increased autophagy as a gain of function, display a decrease in age-related genotoxic stress in cardiomyocytes and reduced incidence of cardiac hypertrophy (Leidal et al., 2018). Parkin overexpression affects positively the cardiac function in aged mice, an effect associated to improved elimination of damaged mitochondria (Hoshino et al., 2013).

Taken together, the aforementioned studies and others point out the importance of autophagy to prevent age-related CVDs. However, while basal autophagy has been recognized to be beneficial for the heart, the

role of autophagy under stress conditions has not been completely elucidated. Under acute stress, autophagy is protective favouring the elimination of protein aggregates (during hypertrophy, proteotoxic stress) or damaged organelles (during ischemia, aging), whereas, in chronic stress condition (as in heart failure) it can be also detrimental, due to "autophagic cell death" following its hyperactivation (Mialet-Perez and Vindis, 2017). Moreover, deregulation of mitophagy by oxidative stress and inflammation leads to the accumulation of dysfunctional and damaged mitochondria, resulting in ROS overload, ATP depletion and apoptosis in cardiomyocytes, that can result in CVD, including atherosclerosis (Chistiakov et al., 2018; Morciano et al., 2020; Zhang et al., 2018; Li et al., 2016). Basal and adaptive autophagy may reduce oxidative stress, inflammation and lipid accumulation and can delay plaque formation (Kim and Lee, 2014; Nussenzweig et al., 2015; Kim et al., 2018). On the other hand, hyperactive autophagy may induce autophagy-dependent cell death, further contributing to plaque instability (Kitada et al., 2016; Luo et al., 2016; Zhu et al., 2017; Liu and Levine, 2015).

Considering that in several CVDs autophagy is dysfunctional, its stimulation could have beneficial effects; accordingly, nutritional and pharmacological approaches that activate autophagy have aroused considerable interest. For example, caloric restriction, the most known stress stimulus associated to activated autophagy, reverted cardiac hypertrophy in aged mice (Bravo-San Pedro et al., 2017). Due to the difficulty to maintain long-term caloric restriction in humans, researchers have investigated the benefits of intermittent fasting (IF) that consists in alternate day fasting. The IF protects against cardiac deterioration and its beneficial effects are, at least in part, mediated by the activation of autophagy under these conditions. The ketogenic diet has also displayed heart-protective effects through mTOR inhibition (Zhang et al., 2021). It remains to be investigated whether a diet low in sugar and proteins, but high in unsaturated fatty acids (the "fasting mimicking diet") may offer a similar benefit against age-related CVDs (Abdellatif et al., 2018).

Pharmacological agents that stimulate autophagy such as rapamycin, spermidine, several plant polyphenols, and metformin, an antidiabetic drug, can be exploited as possible treatments in CVDs. For example, oral treatment with rapamycin attenuated inflammation and enhanced plaque stabilization in rabbit models of atherosclerosis, suggesting that the maintenance of the autophagic homeostasis is crucial as a therapeutic strategy for atherosclerosis (Chen et al., 2009). These data agree with the results of a study with old female mice, where a 3-month treatment with rapamycin reverted age-related cardiac deterioration (Flynn et al., 2013), and with similar findings obtained with spermidine (Abdellatif et al., 2018; Bravo-San Pedro et al., 2017). Interestingly, the beneficial effects of spermidine were lost in *Atg5* knockout mice, suggesting that autophagy stimulation is essential for spermidine-mediated cardiac protection (Mialet-Perez and Vindis, 2017).

Resveratrol, a plant polyphenol found in red wine, exerts cardiovascular and antiaging benefits through autophagy stimulation. Its mechanism of action involves Sirtuin (SIRT1, SIRT3) activation and nuclear translocation of FOXO (Ren et al., 2018). Several other plant polyphenols, oleuropein aglycone (OleA), the main polyphenol in olive oil, curcumin, and epigallocathechin 3-gallate have been reported to be cardioprotective by stimulating autophagy. OleA protects cardiomyocytes against MAO-A-Induced autophagy impairment through the activation of TFEB with increased autophagic vacuoles and autophagy-specific markers (Beclin1 and LC3II) (Miceli et al., 2018). Curcumin has been reported to protect cardiomyocytes through inhibition of apoptosis and induction of autophagy via PI3K/AKT/mTOR pathways and enhancement of both p62 expression and mitochondrial membrane potential (Chen et al., 2021). Epigallocathechins are present in several herbs such as green tea; the gallate derivative has been reported to protect against myocardial ischemia/reperfusion injury by inhibition of apoptosis through the PI3K/Akt pathway and restoration of the autophagic flux. The latter proceeded through a decrease of the LC3II/LC3I ratio, the downregulation of Beclin1, ATG5 and p62, the upregulation of active cathepsin D and the increased phosphorylation of mTOR (Xuan and Jian, 2016). Short-term treatment with metformin improves cardiac aging through AMPK activation. Furthermore, in diabetic patients, metformin reduces the risk of stroke and myocardial infarction, as compared to other treatments such as insulin, sulfonylurea, or specific dietetic regimens. (Tzoulaki et al., 2009; Schramm et al., 2011). However, despite the beneficial effects associated to the stimulation of autophagy mentioned above, the nutritional (calorie reduction) and pharmacological (rapamycin, spermidine) treatments have important limitations. In fact, their long-term application can be accompanied to side effects that should be taken into consideration (Ren et al., 2018). These possible warnings appear much less important when plant polyphenols, such as resveratrol and olive polyphenols, are used, due to the substantial lack of side effects associated with their assumption.

3.4. Autophagy in the Metabolic syndrome and related disorders

The metabolic syndrome (MetS) is defined as a complex of chronic metabolic disorders characterized by obesity, glucose intolerance, dyslipidemia, and hypertension (Bonomini et al., 2015). These symptoms may stem from, and contribute to, insulin resistance, oxidative stress, endothelial dysfunction, accumulation of proinflammatory cytokines and other pathological mechanisms associated with the onset of chronic diseases including T2DM, non-alcoholic fatty liver disease (NAFLD), atherosclerosis, heart disease and even several types of cancer. In addition, the MetS, particularly T2DM, is associated to cognitive decline and vascular dementia suggesting that it may be a risk factor for Alzheimer's disease leading to the onset of a "metabolic-cognitive syndrome" (Chatterjee et al., 2016; Schilling, 2016).

The complex role of autophagy in the pathogenesis of metabolic disorders has been investigated in several studies carried out in different model organisms and in various mouse tissues and organs with KO of autophagy genes (Hars et al., 2007; Meléndez et al., 2003; Dwivedi et al., 2009). It resulted that defects in autophagy at the systemic level affects cell adaptation to metabolic stress such as hyperglycaemic glucose intolerance, insulin resistance and decline of insulin production, thus accelerating lifestyle-induced obesity. In turn, the inhibition of autophagy in the liver, muscle and adipose tissue, induces ubiquitinated protein build-up and mitochondrial dysfunction worsening symptomatic features of MetS (Lim et al., 2014; Ren and Zhang, 2018). Conversely, Atg5 overexpression can be observed ubiquitously or in systems with constitutively activated autophagy by mutation in Beclin1, together with improved-cell function, increased insulin sensitivity, loss of weight with aging, resistance to oxidative stress, reduction of ER stress and enhanced motor function, alongside with extended lifespan (Pyo et al., 2018; Fernández et al., 2018).

In aging, as well as under conditions of insulin resistance and obesity, autophagy becomes progressively more dysfunctional in the skeletal muscle, thus contributing to sarcopenic conditions. KO of the gene encoding Atg7 results in increased fat deposition in skeletal muscle and in mitochondrial and ER dysfunction by triggering FGF21 as a mitokine (Kim et al., 2013). Moreover, during aging, the impairment of the autophagic flux is associated with the decline of the regenerative ability of muscle stem cells and with sarcopenia (García-Prat et al., 2016). Autophagy impairment in the hypothalamus results in increased body weight and promotes obesity and hypothalamic inflammation; accordingly, the impaired control of the energy balance by the hypothalamus could contribute to the development of obesity and relative comorbidities (Meng and Cai, 2011).

In the adipose tissue, mitophagy is crucial in the regulation of body lipid stores by determining the balance between white and brown fat and by controlling adipocyte differentiation, in particular by reducing the number of mitochondria in mature adipocytes and their proper functionality (Kovsan et al., 2011; Singh et al., 2009). KO of *Atg7* or the pharmacological inhibition of autophagy in the adipose tissue in

diet-induced obese mice resulted in insulin resistance, reduced adiposity (also in muscle and liver), lower cholesterol and triglyceride levels, increased number of mitochondria and mass of brown adipose tissue, as well as enhanced expression of the mitochondrial uncoupling protein 1 (UCP-1) (Zhang et al., 2009). Recently, it has been reported that, in the obese adipose tissue, the adipogenic differentiation and fat accumulation could also be regulated by the degradation of the peroxisome proliferator-activated receptor-γ (PPARγ) through a crosstalk between the ubiquitin-proteasome and the autophagy-lysosomal systems. Thus, PPARγ stabilization by autophagy can represent a mechanism of pathogenesis of obesity where autophagy plays some role (Zhang et al., 2013). Finally, the specific deletion in mouse white adipose tissue of various autophagic genes, *p62* (Müller et al., 2013), *Bif-1*(Liu et al., 2016), or *Atg161* and *Atg3* (Frendo-Cumbo et al., 2019) induced opposite effects. The mice developed obesity irrespective to their normal or high-fat diet, displayed impaired glucose tolerance, and reduced insulin sensitivity, with increased levels of serum insulin, dysfunctional mitochondria and enhanced accumulation of lipid peroxides in adipose tissue depots, indicating development of peripheral insulin resistance.

Overall, the complexity of the pleiotropic roles of the autophagic pathway also with regard to metabolic syndromes is emerging. Furthermore, studies in obese patients showed a high correlation between induction of the autophagic flux and the hypertrophy and hyperplasia of fat cells. Fat accumulation resulted in elevated oxidative stress, unfolded protein accumulation, and increased ER stress. Moreover, increased autophagy can induce elevated levels of lipolysis, with impairment of triglyceride storage in the adipose tissue of obese people. In addition, the proinflammatory cytokines secreted by macrophages in the adipose tissue can stimulate adipocyte autophagy by TNFα and selective degradation of PLIN1 through SQSTM1, thus underlying the crucial role of autophagy in lipid mobilization mediated by inflammatory cytokines in obese people (Jansen et al., 2012; Ju et al., 2019). On the other hand, a prolonged inflammatory stress can lead to an overall downregulation of autophagy-related genes and may ultimately impair the autophagy/lysosome pathway with further worsening of insulin resistance.

The contribution of autophagy plays a crucial role in liver physiology and metabolic adaptation. Reduced liver autophagic activity, along with lipid accumulation and increased levels of mitochondria-derived reactive oxygen species (mtROS), is reported both in diet-induced and in genetic obese murine models as well as in NAFLD (Carotti et al., 2020). In NAFLD, the ER stress and the consequent impairment of autophagy in the liver may be linked to the upregulation of several negative regulators of autophagy, such as the Rubicon protein (Tanaka et al., 2016), SIRT3 (Li, Y. et al., 2011) and Osteopontin (OPN) (Tang et al., 2020). TFEB is also thought to be involved in the pathogenesis of NAFLD (Settembre et al., 2013). TFEB activation has beneficial effects in metabolic disorders such as hepatic steatosis, obesity, diabetes. Using muscle-specific TFEB gain-and loss-of-function approaches, it was reported that TFEB plays a crucial role in metabolism during exercise, when TFEB moved to the nucleus increasing the expression of genes needed for muscle function such as those involved in glucose metabolism and mitochondrial homeostasis. Given the essential role of TFEB as metabolic regulator, it is possible that TFEB levels are reduced in patients with metabolic disorders (Mansueto et al., 2017). Finally, TFEB was described as a coordinator of lipid metabolism through PGC1α and PPARα activation. Both in diet-induced and in transgenic mouse models of obesity, viral-delivery of TFEB to the liver prevented weight gain and the metabolic syndrome, suggesting it might play a promising role in mebolic disorders (Settembre et al., 2013). In agreement with this conclusion, Ezetimibe, a cholesterol-lowering drug that activates TFEB, was shown to decrease hepatic lipid accumulation and inflammation in a mouse model of steatohepatitis. Moreover, treatment with TFEB activators such as digoxin, alexidine or ikarugamycin counteracted hepatic steatosis, obesity and hyperglycemia in high-fat diet-fed mice. Similar effects of TFEB were observed in diabetic mice, in which MSL inhibited obesity, hyperglycemia, hepatic steatosis, and adipose inflammation directly through TFEB activation (Kim et al., 2017; Wang et al., 2017; Lim et al., 2018; Lu et al., 2021). Taken together, these data point out the decline of autophagy as a crucial event in age-related metabolic disorders, further highlighting the importance of autophagy stimulation as a therapeutical strategy.

In addition, autophagy plays a pivotal role in the protection of pancreatic β-cells by the human islet amyloid polypeptide (hIAPP), an intrinsically disordered protein strongly associated to T2DM.

The autophagy-insulin resistance relationship in MetS is possibly mediated via mTOR which, in turn, is activated by insulin and the insulin-like growth factor 1 (IGF1) with the ensuing impairment of lipophagy (Zhou et al., 2018; Menikdiwela et al., 2020). IGF1 can inhibit autophagy and the ULK complex through the PI3K/Akt or the MAPK/ERK pathway by inhibition of the tumour suppressor complex 1 and 2 (TSC1/TSC2) and activation of mTORC1 also following phosphorylation of other factors, including VPS34 and Ambra1 (Yuan et al., 2013).

In the MetS, elevated insulin levels reduce the autophagy flux also through the Ca^{2+}-dependent protease calpain 2 (Yang et al., 2010) or the FOXO transcriptional factor, suppressing the expression of *Vps34* and *Atg12* autophagy genes (Liu et al., 2009; Martins et al., 2016). In addition, inflammatory cytokines can also activate mTOR via the toll-like receptor (TLR)-mediated PI3K/Akt pathways. Finally, as it happens in the adipose tissue, also in the liver, during the early stages of obesity, an excessive activation of the autophagic flux can be observed as an adaptive response to cell damage that eventually leads to dysregulation of the autophagic flux and can be an indicator of disease development. Therefore, in the MetS-associated chronic obesity, a persistent cellular stress induces the suppression of insulin-mediated autophagy eventually resulting in insulin resistance.

4. Aging: biological and functional biomarkers

Geroscience is a branch of science according to which to prevent the onset or mitigate the severity of chronic diseases, we should target not the disease, but the aging process itself which is common to all aging-related diseases. Assuming that this hypothesis is true, to reduce the incidence of chronic diseases is more urgent than ever to identify specific biomarkers of aging (Le Bourg, 2022). Given the complexity of the molecular and biological mechanisms that regulate aging, to date none of the proposed biomarkers is a valide measure of healthy versus pathological aging. In accordance with Wagner and colleagues the panel of most promising biomarkers of healthy aging should include measures of physical capability, blood and cellular/molecular biomarkers (Wagner et al., 2016). Measures of physical performance, including mobility and body composition changes are useful for defining the biological age. Blood-based biomarkers include lipid and glucose profiles, inflammatory and hormonal markers. One of the most studied aspects of aging is its correlation with the increase in inflammatory peptide biomarkers. Older individuals develop “inflammaging”, a condition characterized by elevated levels of blood inflammatory markers (interleukin (IL)− 6, IL-1β, tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP)) that carries high susceptibility to chronic morbidity, disability, frailty, and premature death (Ferrucci and Fabbri, 2018). Another aging-related change involves the endocrine system with a reduction of insulin-like growth factor-1 (IGF-1) and different growth hormones, including thyroid-stimulating hormone (TSH), free thyroxine (FT4) and triiodothyronine (FT3) (Lobo, 2013; Cunningham, 2013; Junnila et al., 2013; Martin-Ruiz et al., 2011). Aged people also have impaired glucose metabolism (Ravera et al., 2019) and increased levels of glucose correlate with increased levels of Advanced Glycation End products (AGEs), that represent further aging biomarkers (Grillo and Colombatto, 2008).

Aging is not only a physiological process, but at molecular level it is characterize by a set of cellular modifications, widely used to predict the aging state of different tissues or organs. In 2013 Lopez and colleagues identified 9 putative hallmarks of cellular aging, many of which have

been recognized as aging biomarkers (Lopez et al., 2013; Wagner et al., 2016; Aging Biomarker Consortium et al., 2023). These hallmarks are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, resulting in mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. We will report below their relative contribution to aging process (Table 2).

Although cellular-based biomarkers allow us to understand the fundamental molecular basis of aging and the onset of dysfunction at the cellular level, to date they are not considered valid biomarkers in vivo due to their low practicability of measurement. Further studies and more sophisticated approaches will eventually resolve the problem of poor applicability in vivo (Wagner et al., 2016).

The complexity of the biological and molecular mechanisms related to aging makes it currently impossible to measure the extent of biological aging using a single biomarker. Wide relevance should be given to the value of combining these markers with physical and functional parameters. Advances in "omics" and bioinformatics techniques could in the future offer the opportunity to evaluate the interaction between multiple aging factors and provide a measure of ongoing optimal health as well as the knowledge of their modulation in order to develop new therapeutic targets for age-associated diseases. Growing evidence supports that the hallmarks of aging are interconnected and often correlated with the alterations in autophagy machinery. Consequently, autophagy

Table 2
Cellular biomarkers of aging and autophagy involvment.

Hallmark aging	Cellular manifestations	Contribution to aging	Autophagy alterations
Genomic instability	DNA damage (point mutations, translocations, chromosomal gains and losses, telomere shortening, and gene disruption).	• Excessive DNA damage and insufficient DNA repair mechanisms promote aging (Lopez-Otin et al., 2013).	• Upregulation of macroautophagy (p62, AMPK, mTOR and PARP1) (Kaushik et al., 2021). • Upregulation of Chaperone-mediated autophagy (CMA) (Park et al., 2015)
Epigenetic modifications	DNA methylation, aberrant histone modifications, loss of heterochromatin, deregulated RNA modifications.	• Histone methylation increases lifespan, H3K4me3 demethylation boosted the lifespan in C. elegans (Greer et al., 2010). • miR-146a, miR-155 and miR-21 increased in aging (Olivieri et al., 2021; Ekiz et al., 2020)	• Macroautophagy modulation (SIRT1 family) (Kaushik et al., 2021).
Telomeres shortening	Reduced telomere lenght and decline in telomerase activity	• Pathological telomere dysfunction accellerates aging, telomerase stimulation delays aging in mice (Lopez et al., 2013).	• Autophagy can be induced by both telomere loss (Nassour et al., 2019) or telomerase activation (Green et al., 2019). • Telomerase reverse trascriptase (hTERT) regulates PINK1, resulting in increased mitophagy and inhibits mTORC, inducing autophagy (Kaushik et al., 2021) • Modulation of telomere length by autophagy (Taji et al., 2017)
Loss of proteostasis	Decline in cellular proteostasis mechanisms: macroautophagy, chaperone mediated autophagy, ubiquitin proteasome system (UPS)	• Decline in cellular proteostasis increases toxic protein accumulation • Genetic manipulations improving proteostasis delay aging in mammals (Lopez et al., 2013; Zhang and Cuervo, 2008;Aging Biomarker Consortium et al., 2023)	• Failure of macroautophagy and CMA. • Downegulation of SIRT proteins (Xu et al., 2020) and defective PINK1/Parkin-signalling (Roberts et al., 2016) • Deregulation of AMPK in neurons (Ulgherait et al., 2014).
Mitochondrial dysfunction	mtDNA mutations, decreased mitochondrial unfolded protein response (UPRmt), defective mitophagy, destabilization of the electron transport chain (ETC), increase in ROS production	• Mitochondrial dysfunction contributes to aging in mammals (Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008; Aging Biomarker Consortium et al., 2023; Lopez et al., 2013) • It is not clear whether improving mitochondrial function can extend lifespan (Lopez et al., 2013)	• Decline in mitophagy and accumulation of damaged mitochondria (Kaushik et al., 2021) • Mitophagy-receptor loss of function: mithocondrial (Parkin/PINK1, BNIP3/Nix3) and cytosolic (p62, NBR1, OPTN) proteins (Kaushik et al., 2021). • Parkin null flies and PINK1 mutant flies show reduced lifespan (Kaushik et al., 2021).
Cellular senescence	Accumulation of senescent cells in aged tissues and increased b-galactosidase (SABG) levels (Dimri et al., 1995, Lopez et al., 2013).	• Inflammaging: senescent cells induce senescence in neighboring cells through release of inflammatory cytokines (SASP) (Lopez et al., 2013)	• Macroautophagy flux decreases in oxidative stress-induced fibroblasts (Tai et al., 2017) and neurons (Moreno-Blas et al., 2019)
Intercellular communications	Mechanisms of Intercellular communication: 1) Senescence associated secretory phenotype, 2) Gap junctions 3) Long distance communication through Extracellular vesicles (Ribeiro-Rodrigues et al., 2023)	• Modulation of intercellular communication represents a promising tool in aging and age-related disease (Sanz-Ros et al., 2022). • Lifespan-extending manipulations targeting one single tissue can affect other tissues (Lopez et al., 2013; Durieux et al., 2011;Lavasani et al., 2012; Toma´s-Loba et al., 2008).	• Reduced macroautophagy. • ATGs proteins (LC3, ATG16L1) and miRNAs modulation (Kaushik et al., 2021).
Stem cells exaustation	Decline in stem cell- regenerative potential and loss of function	• Reduced number and function of stem cells induces tissue injury (Xu et al., 2019) • Stem cell rejuvenation may reverse aging (Rando and Chang, 2012).	• Decline inmacroautophagy flux and CMA (Kaushik et al., 2021) • Macroautophagy supports stem cells quiescence (Ho et al., 2017), • Loss of autophagy induces decline in stem cells function (Kaushik et al., 2021)
Deregulation of nutrient signalling	Deregulation of insulin/IGF-1 signalling and its downstream targets: FOXO and mTOR(Lopez et al., 2013)	• Caloric restriction, which works through multiple nutrient signaling pathways, increases lifespan (Colman et al., 2009; Fontana et al., 2010; Mattison et al., 2012). • Treatment with rapamycin, inhibitor of mTOR, delays ageing and protects against age-related diseases (Johnson, 2018; Lopez et al., 2013)	• Deficiency in autophagy proteins (ATG7, GABARAPL1) (Kaushik et al., 2021).

is implicated in both aging and age-related disease. Hence the need to improve the measurement of autophagic flux in order to find the optimal manipulation of autophagy is required to achieve benefits and improve lifespan.

5. Autophagy proteins as novel biomarkers for age-related diseases

Autophagy proteins can be used as biomarkers for many diseases, including acute ischemic stroke (AIS) and neurodegenerative diseases. Aging is certainly a risk factor for cerebral ischemia. AIS, a common cause of death in aged people, is a pathological condition caused by decreased blood flow to the brain following the occlusion of a blood artery by a thrombotic or an embolic event. To date, there are no effective treatments to prevent or treat AIS, so the only approved therapeutic approach is the thrombolytic therapy, that presents some limits, such as the narrow therapeutic window and bleeding complications. For these reasons, it is important to find new drugs and intervention strategies for AIS. Recent findings indicate that autophagy is induced during AIS and the research is now aimed at understanding whether this activation exerts a protective or deleterious effect (Sun et al., 2019; Li et al., 2015). AIS can lead to severe brain damage, accompanied by the release of many substances in the serum or the cerebrospinal fluid (CSF). Li and co-workers showed that two autophagy markers, Beclin1 and LC3II, were increased in the serum and the CSF of AIS patients, as compared to controls, and were associated with favourable prognosis and with reduced incidence of neurological deficits (Li et al., 2015). This result suggests that autophagy is an adaptative response with protective effects in AIS. However, this study included only patients with a mild to moderate degree of ischemia. There are no data on patients with severe ischemia (Li et al., 2015). Other studies have shown that acute and severe ischemia may induce excessive activation of autophagy, which could worsen the prognosis of ischemic stroke due to the promotion of neuronal cell death (Morselli et al., 2008; Shi et al., 2012; Ginet et al., 2014). Recently, serum levels of ATG5, apoB-48, malonylaldehyde, total oxidative stress, and total antioxidant capacity have been used as novel biomarkers for diagnosis or treatment of the AIS (Ajoolabady et al., 2022), although further studies are needed to better understand the therapeutic role of autophagy modulation in AIS.

The analysis of autophagy biomarkers results to be useful also for what the prediction and diagnosis of neurodegerative disorders are concerned. Indeed, it is widely reported that autophagy dysregulation occurs in AD patients. In fact, in AD neurons, an abnormal accumulation of autophagosomes (Liang and Jia, 2014) and a significant decrease of BECN1 levels caused by the increased activity of caspase 3 (Rohn et al., 2011), correlated with intracellular Aβ accumulation (Pickford et al., 2008). Moreover, a significant relation between p62 levels in CSF and clinical characteristics of dementia was found in both AD and frontotemporal dementia patients, suggesting a key role of autophagy in these two disorders and the possibility to use p62 levels in CSF as a potential biomarker of neurodegeneration process (Rubino et al., 2022).

Markers of the autophagy levels have also been measured in biological samples of PD patients (Prigione et al., 2010; Haddad et al., 2020). PD, a neurodegenerative disease which affects over 1% of the population over 60, is characterized by cognitive impairment and motor symptoms (slow movement, tremor, rigidity), caused by a loss of dopaminergic neurons of the substantia nigra of the brain. Intracellular protein aggregates of α-synuclein known as “Lewy bodies”, are often found in post-mortem brains of PD patients (Spillantini et al., 1997; El Haddad et al., 2020). A main problem of PD is the late diagnosis, which is made only when the symptoms are evident and most dopaminergic neurons are already lost. However, at the present, no effective therapeutic strategies to relieve disease symptoms have been established; the only available drugs to treat PD are aimed at restoring dopamine stores in the affected brain areas, but this treatment is mostly symptomatic, scarcely effective, and does not lead to substantial recovery. Present research is moving towards the identification of altered molecular pathways that could underlie Parkinson’s damage and whose distinctive molecular signature could be used as early markers of the disease. In particular, disruption of the autophagy flux has been shown in post-mortem brains of PD mouse models (Alvarez-Erviti et al., 2010; Gonzalez-Polo et al., 2013) and autophagy levels were altered in the blood of PD patients (Prigione et al., 2010; Haddad et al., 2020). Particularly, the *Atg8* gene family (*Map1lc3b*, *Gabarap*, *Gabarapl1* and *Gabarapl2*) and the autophagolysosome adapter p62 were significantly increased in transcript levels. On the contrary, both HSPA8 (a protein involved in chaperone-mediated autophagy) and GAPDH (encoded by a housekeeping gene) were decreased. So, if these findings will be confirmed, the signature of autophagy gene expression determined by quantitative PCR could represent a simple and non-invasive method to be used in clinical practice for early PD diagnosis (Haddad et al., 2020). These findings are compatible with the study of Papagiannakis and co-workers, showing a decrease of Hsc70 (a protein that participates to the chaperone-mediated autophagy) and an increase of LC3II (a marker of macroautophagy) in blood samples of PD patients. The authors conclude that lysosomal dysfunction may lead to LC3II accumulation and to the impairment of chaperone-mediated autophagy in PD patients (Papagiannakis et al., 2019). The studies mentioned above measured the mRNA or protein levels of autophagy genes as a prove of efficient or defective autophagy. However, the build-up of autophagosomes (as indicated by an increase of the LC3II levels) is not always indicative of a complete activation of autophagy, rather, it can also be indicative of a block of autophagosome maturation. The best way to distinguish between these two possibilities is to measure the “autophagic flux”, a measure of the degradative completion of autophagy following autophagosome-lysosome fusion and the ensuing substrate degradation.

The levels of the p62 protein in renal glomeruli, as an autophagy activity indicator, has been considered as one of the predictors of the onset of the stage of macroalbuminuria in diabetic kidney disease (Wang et al., 2022). Versaci and co-workers showed that an age-dependent decline in autophagy is detectable in patients with atrial fibrillation. However, its prognostic role requires further investigation (Versaci et al., 2022). Recently, several studies have been performed to assess whether the expression levels of apoptosis and autophagy markers are associated with response to neoadjuvant chemotherapy, a chemotherapy treatment administered before surgical extraction, and are affordable predictors of survival. El Mashed has reported an association between cleaved caspase-3 and LC3II and survival of neoadjuvant treated patients, suggesting that a combination of apoptosis and autophagy markers is likely to be optimal for development of a reliable predictive test in the future (El Mashed et al., 2022). In osteosarcoma, autophagy markers coupled with RNA-dependent protein kinase (PKR) activity could be relevant to the prognosis and targeted treatment of the disease (Ji et al., 2020). The autophagy level is significantly inhibited in septic patients with acute respiratory distress syndrome, and autophagy-associated proteins LC3II, Beclin1, RAB7, LAMP2, and p62 have good predictive value for diagnosis and prognosis of sepsis comorbid with acute respiratory distress syndrome (Xu et al., 2022). LC3 seems to be suitable as a screening marker to recognize an idiopathic inflammatory myopathy. Therefore, could be included as a marker in the routine of neuropathological diagnostics (Drott et al., 2020). In Rheumatoid arthritis (RA) and osteoarthritis (OA), an autophagy related index (ARI) consisting of the autophagy-related genes *CXCR4* and *SERPINA1* resulted to be closely correlated with autophagy scores and immune infiltration in both diseases thus representing a possible aid for differential diagnosis (Huang et al., 2021).

Several studies have shown that the autophagic flux is reduced in cancer, cardiovascular and neurodegenerative diseases (Levine and Kroemer, 2008; Mizushima et al., 2008; Mizushima et al., 2010). On the other hand, pharmacological and nutritional approaches, such as fasting diets and exercise, can regulate the autophagic flux such that their use could be proposed in clinical practice. Accordingly, the need also arises

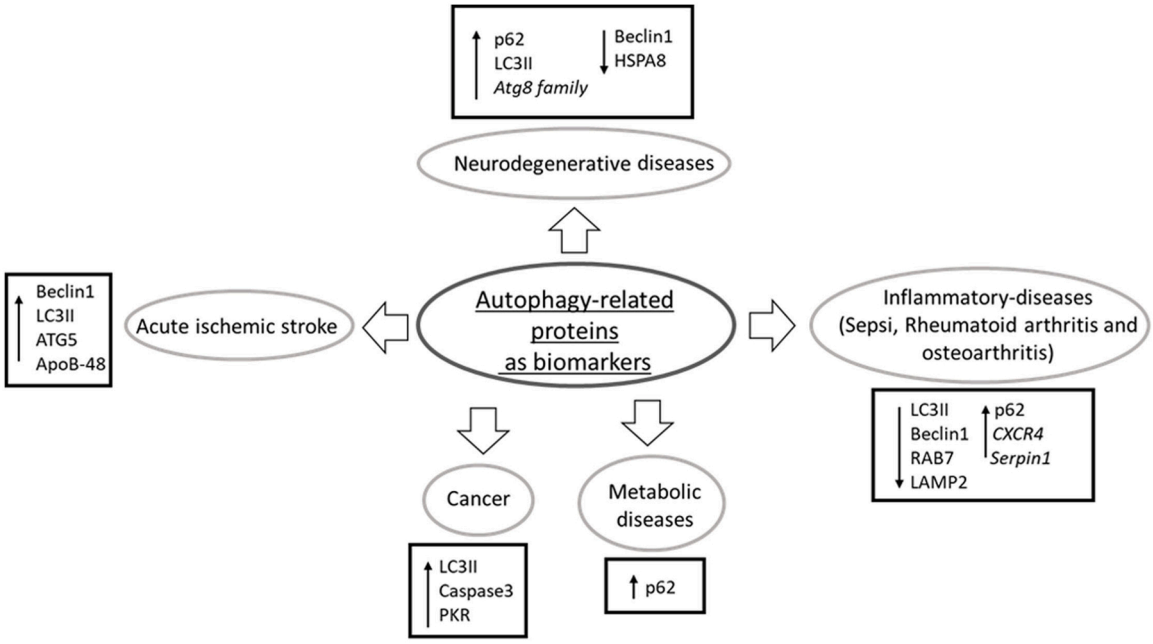

Fig. 2. Autophagy-related proteins and genes used as indicators of aging-related diseases onset.

to follow the possible modulation of the autophagic flux in biological samples of patients (Morselli et al., 2010; He et al., 2012; He et al., 2012). To this purpose, in 2021, Bensalem and co-workers developed a blood test for healthy people, based on the assay of LC3 turnover, to check the the autophagic flux. Based on previous knowledge, the test could definitely answer some unresolved questions such as "what kind of fasting is most effective for people?" or "how much exercise is needed for optimal activation of autophagy?". This research represents the first evidence of how we can translate our knowledge on autophagy into clinical practice (Bensalem et al., 2021).

In the future, assay of autophagy components in biological fluids and/or genetic screening could be added to the standard health check-up tests and be used as a biomarker to diagnose, or to identify a high risk of, age-related diseases or to assess modulation of autophagy as a disease-modifying strategy and to evaluate the therapeutic potential of autophagy modulators Fig. 2.

6. Conclusions

The multifunctionality of autophagy makes it an attractive candidate as a mediator of the interconnections between the hallmarks of aging and as a biomarker of age-related diseases. The growing number of reports highlighting the impairment or imbalance of the autophagic processes with age has supported the concept that (1) autophagy is a crucial determinant of cellular health, longevity and diseases (2). In other terms, we cannot merely describe the autophagy-aging relation with statements such as "decreased autophagy is detrimental" and "increased autophagy is beneficial'. In fact, long-term health benefits arise from the right balance between the molecular machinery involved in the autophagic flux and other cellular processes such as apoptosis, and the homeostatic systems involved in proteostasis, redox and metabolic balance and the inflammatory response that, in turn, depend on the type of tissue/organ and lifestyle as well as on the age. Accordingly, a key goal for health promotion will be finding approaches useful to finely tune autophagy to the right levels, at the right time and in the right tissues. Then, the so established therapeutic interventions could be administered chronically, acutely or in a pulsed fashion, as and when required. The suggestion stemming from the analysis of the cited literature is that the modulation or the evaluation of biomarkers of autophagy, such as autophagy-related proteins, could be useful in disease prevention, or as targets for diagnosis and therapy, to ensure healthier aging, reducing the risk and/or the severity of age-related diseases. Nevertheless, more evident clinical data on the modes and features of the involvement of autophagy deregulation in the different aging-related pathologies are still needed to provide more solid evidence to these possibilities.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Manuela Leri reports financial support was provided by Umberto Veronesi Foundation.

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