Cellular senescence: from mechanisms to current biomarkers and senotherapies

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- List of nonstandard abbreviations used in the paper, with abbreviations listed in alphabetical order:

ADEPT: Antibody-directed enzyme prodrug therapy

AMPK: 5' AMP-activated protein kinase

BCL-2: B-cell lymphoma/leukemia-2

BiP: Binding immunoglobulin protein

C/EBP: CCAAT/enhancer-binding protein

CAR: Chimeric antigen receptor

CDK: Cyclin-dependent kinases

CMA: Chaperone-mediated autophagy

CNNs: Convolutional neural networks

COX-2: Cyclooxygenase 2

CR: Caloric Restriction

DDR: DNA damage response

Deep-SeSMo: Deep Learning-Based Senescence Scoring System by Morphology

ECM: Extracellular matrix

EGCG: Epigallocatechin gallate

ER: Endoplasmic reticulum

ERAD: ER-associated protein degradation

ERK: Extracellular signal-regulated kinase

FAT-ATTAC: Fat apoptosis through targeted activation of caspase

FDA: Food and Drug Administration

FOXO4: Forkhead Box Proteins O 4

GATA4: GATA Binding Protein 4

GFP: Green fluorescent protein

HUVECs: Human umbilical vein endothelial cells

IGFBP: Insulin-like growth factor binding proteins

LBR: Lamin B receptor

LMNB1: Lamin B1 gene

MIDAS: Mitochondrial Dysfunction-Associated Senescence

MTOR: Mammalian target of rapamycin

Nrf2: Nuclear factor-erythroid 2-related factor 2

OIS: Oncogene-induced senescence

PCNA: Proliferating cell nuclear antigen

ROS: Reactive oxygen species

Rb: Rb tumor suppressor protein

SA-β-gal: Senescence-associated beta-galactosidase

SAHF: Senescence-associated heterochromatic foci

SASP: Senescence-associated secretory phenotype

SCAPs: Senescent cell anti-apoptotic pathways

TCA: Tricarboxylic acid

UPR: Unfolded protein response

mRFP: Red fluorescent protein

uPAR: Urokinase-type plasminogen activator receptor

Abstract

An increase in life expectancy in developed countries has led to an insurgency of chronic aging-related diseases. In the last few decades, several studies provided evidence of the prominent role of cellular senescence in many of these pathologies. Key traits of senescent cells include cell cycle arrest, apoptosis resistance, and secretome shift to senescence-associated secretory phenotype (SASP) resulting in increased secretion of various intermediate bioactive factors important for senescence pathophysiology. However, cellular senescence is a highly phenotypically heterogeneous process, hindering the discovery of totally specific and accurate biomarkers. Also, strategies to prevent the pathological effect of senescent cell accumulation during aging by impairing senescence onset or promoting senescent cell clearance have shown great potential during *in vivo* studies and some are already in early stages of clinical translation. The adaptability of these senotherapeutic approaches to human application has been questioned due to the lack of proper senescence targeting and senescence involvement in important physiological functions. In this review, we explore the heterogeneous phenotype of senescent cells and its influence on the expression of biomarkers currently used for senescence detection. We also discuss the current evidence regarding the efficacy, reliability, development stage, and potential for human applicability of the main existing senotherapeutic strategies.

Significance Statement

This manuscript is an extensive review of what is currently known about the complex process of cellular senescence exploring its most defining features. The main body of the discussion focus on how the multi-feature fluctuation of the senescence phenotype and the physiological role of cellular senescence have both caused a limitation in the search for truly reliable senescence biomarkers and the progression in the development of senotherapies.

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I. Introduction

In 1961, Hayflick and Moorhead showed that cultures of fibroblasts had a limited number of cell divisions and therefore could not be maintained indefinitely (Hayflick and Moorhead, 1961). They identified three different phases during human fibroblast embryonic tissue culture. Phase I corresponded to culture establishment where cells present a high growth rate, during Phase II cell division gradually decline until completely stopped, marking the beginning of Phase III where culture cell number started to rapidly decline, leading the culture to no longer be considered viable (Hayflick and Moorhead 1961). It was not known at that time, but this was later explained by the gradual shortening of chromosomes telomeres with each cell division that ultimately lead to chromosomal instability and DNA damage triggering a process now known as replicative senescence, which has been described in many different human and animal cell types (Lundblad and Szostak, 1989; Greider, 1990; Harley, 1991). Replicative senescence belongs to a wide network of cellular mechanisms collectively known as cellular senescence which in its essence is characterized by typical cellular features that include permanent cell cycle arrest, shift in cellular secretome content referred to as senescence-associated secretory phenotype (SASP), morphological alterations and resistance to apoptosis (Hayflick and Moorhead, 1961; Wang, 1995; Campisi, 1996; Coppé *et al.*, 2008). Even though it is thought that cellular senescence only spreads to a small fraction of the total cell population of each tissue, this small percentage can lead to pathological alterations in tissue function due to the continuous pro-inflammatory SASP mediators release which has already been associated with several aging-related pathological processes (Biran *et al.*, 2017). Differently from quiescent or apoptotic cells, senescent cells are highly metabolically active allowing them to sustain the continuous production of SASP mediators (Capasso *et al.*, 2015). SASP mediators generally have pro-inflammatory effects including disrupting progenitor and stem cell function, induction of extracellular matrix rearrangement and even spreading of senescence phenotype (Kumar *et al.*, 1992; Acosta *et al.*, 2008, 2012, 2013; Kuilman *et al.*, 2008; Orjalo *et al.*, 2009). However, they also signal the presence of senescent cells allowing their clearance by the immune cells (Xue *et al.*, 2007; Krizhanovsky *et al.*, 2008; TW Kang *et al.*, 2011a; Sagiv and Krizhanovsky, 2013).

With the aging-related decrease in immune system function called “immunosenescence”, it is thought that the immune system slowly loses the capacity to eliminate senescent cells from tissues, therefore, contributing to the development of age-related chronic diseases (although this is still not entirely accepted) (TW Kang *et al.*, 2011b; Sagiv and Krizhanovsky, 2013; Burton and Stolzing, 2018; Ovadya *et al.*, 2018; Karin *et al.*, 2019).

Nowadays, cellular senescence can be divided into three main categories: **i)** replicative senescence **ii)** developmentally programmed cellular senescence, evidenced to be a crucial process for healthy embryonic development, and **iii)** stress-induced premature senescence (extensively reviewed by (Toussaint *et al.*, 2002; Courtois-Cox *et al.*, 2008; Da Silva-Álvarez *et al.*, 2019)), triggered by a wide range of external and internal by stimuli such as oxidative stress, oncogene expression, DNA damage, and others represented in **Figure 1**. This variety of stimuli can lead to different but intertwined cellular senescence programs that have been individually characterized by specific phenotypic alterations (Wiley *et al.*, 2017) which have not yet made possible the discovery of a single reliable cellular senescence biomarker. This has also been an obstacle in the development of therapeutic strategies targeting senescent cells (in this review we refer to them as senotherapeutics) given that the heterogeneity of the senescence phenotype throughout tissues might mean that some tissues might not be affected by the senotherapy and the lack of a specific biomarker might promote off-target effects. Despite this, pre-clinical and even clinical results have shown some promising results regarding the potential of some senotherapeutic strategies to treat aging-related pathological conditions (Zhu *et al.*, 2016; Yosef *et al.*, 2016; Y Zhu *et al.*, 2017; Munoz-Espin *et al.*, 2018; Amor *et al.*, 2020). With this review, we aim to explain the important aspects of cellular senescence mechanisms that led to the development of current strategies used to detect senescent cells and the potential senotherapeutic approaches that are currently under optimization.

II. Features and Hallmarks of Cellular Senescence

As mentioned earlier, cellular senescence phenotypic manifestations can be highly diverse being mainly influenced by cell type and inductive stimuli (Basistyid *et al.*, n.d.; Kirschner *et al.*,

2020; Tripathi *et al.*, 2021). Some of the senescence-inducing mechanisms have only been reported *in vitro* and remain to be identified *in vivo* studies (**Figure 1**). Also, as previously mentioned, senescence phenotype is a highly heterogeneous and dynamic process, however, there are cellular features and phenotypic traits that are shared by most of the different cellular senescence types.

One defining trait of cellular senescence is cell cycle arrest, which is shared with quiescent cells. Differently from senescent cells that are irreversible arrested in G1, G1/S or G2 cell cycle check points, quiescence cells indulge into a steady cell phase called the G0 phase but retain the ability to re-enter the cell cycle. Although already been proven to have physiological functions (as mentioned earlier), cellular senescence is a degenerative process that is involved in pathological conditions mainly associated with aging and often arises as an alarm response triggered by damaging stimulation or unusual proliferation (Borodkina *et al.*, 2018; Gorgoulis *et al.*, 2019; Fujimaki and Yao, 2020). Differently, quiescence ensues in the absence of appropriate nutrition and growth stimulation (not as a result of cell damage) and is crucial for differentiated tissue homeostasis, regeneration, and repair upon damage and protection of stem and progenitor cells from stressors (Borodkina *et al.*, 2018; Gorgoulis *et al.*, 2019; Fujimaki and Yao, 2020). Further, contrary to senescent cells, given the proper stimuli quiescent cells can return to the cell cycle and proliferate (Borodkina *et al.*, 2018; Gorgoulis *et al.*, 2019; Fujimaki and Yao, 2020). It is also worth mentioning that i) most cells that can enter quiescence are also susceptible to premature and replicative senescence (Borodkina et al. 2018) and ii) despite cellular senescence being considered an irreversible process, recent studies have reported situations where senescent cells (mostly tumor cells) were able to reenter the cell cycle (Milanovic et al., 2018; Patel et al., 2016; Saleh et al., 2019).

Cellular senescence-associated cell cycle arrest is mainly mediated by p53/p21 and p16/Rb pathways occurring in upregulation of p16 and p21 or increased activity of p53 (Childs *et al.*, 2015) (which will be discussed below) (**Figure 1)**. Cells undergoing senescence in *in vitro* cultures, often display macromolecule imbalance such as alterations in protein homeostasis (proteostasis) and intracellular lipidic content along with structural and functional alterations in

cell organelles such as the nucleus (some cells might even display multiple nuclei), mitochondria and lysosomes (often dysfunctional and enlarged and accumulate in abnormally large numbers) (Guerrero *et al.*, 2020) (**Figure 1)**. Alterations in the composition of the cellular membrane such as upregulation of caveolin-1 (shown to promote stress-induced premature senescence through inhibition of Nrf2 (Volonte et al. 2013)) can also be seen which along with cell swelling and flat shape are thought to be caused by cytoskeleton reorganization (Wang and Gundersen 1984, Amaya-Montoya et al. 2020). There is also a senescence-associated tendency towards a metabolic shift favoring glycolysis over fatty acid oxidation (James *et al.*, 2015) and the development of a senescence-specific secretome profile called SASP. In the following chapter we present a detailed characterization of these senescence-associated features.

A. DNA Damage & Epigenomic Alterations

As mentioned earlier, one characteristic of senescent cells is the permanent state of irreversible cell cycle arrest. One of the main cell cycle facilitators is the cyclin-dependent kinases (CDK) which are enzymes responsible for inducing the expression of other enzymes that promote cell cycle progression (Malumbres, 2014). During senescence, upon the activation of the DNA damage response (DDR), a signaling cascade referred to as the p53/p21 and $p16^{INK4A}$ pathways that culminate in CDK inhibition by proteins encoded in the CDKN2A ($p16^{INK4A}$), CDKN2B ($p15^{INK4B}$) and CDKN1A ($p21^{CIP}$) genes (**Figure 1).** The continued inhibition of CDK results in the repression of E2F target genes required for the transition from G1 to S through prevented inactivation of Rb (Salama *et al.*, 2014; Hernandez-Segura *et al.*, 2018) **(Figure 1)**. DDR usually maintains cell cycle arrest until the damage is repaired, however, unlike quiescent cells, senescent cells do not seem to be able to resume the cell cycle due to their incapacity to be reactive to mitogenic or growth factors (Rossiello *et al.*, 2014; Hernandez-Segura *et al.*, 2018). Senescence-inducing stimuli activate the DDR through ATM or ATR serine-protein kinases signaling that initiate cell cycle arrest by promoting p53 stabilization which leads to p21 transcriptional upregulation. p21 inhibits cyclin E-cyclin-dependent kinase2 (cyclin E–Cdk2) preventing Rb inactivation and allowing it to stay bonded to E2F which cannot induce the

expression of essential genes for the transitioning from G1 to S phase (Rossiello *et al.*, 2014; Salama *et al.*, 2014; Herranz and Gil, 2018) (**Figure 1**). ATM or ATR serine-protein kinases signaling can also lead to phosphorylation of the histone H2AX (variant from the H2A family) on Ser 139 residue in the H2AX carboxyterminal tail, forming γH2AX, leading to structural alterations at the damaged site and assembly of specific DNA repair complexes that can promote DNA repair. γH2AX function and mechanisms of action in DDR are reviewed elsewhere (Turinetto and Giachino, 2015; Georgoulis *et al.*, 2017; Hernandez-Segura *et al.*, 2018).

Other epigenetic alterations have also been shown to play a role in cellular senescence, such as H3K9 methylation (H3K9me3) which allows it to serve as a binding site for acetyltransferase Tip60 that can activate ATM through acetylation, triggering the DDR and leading to cell cycle arrest (Wang *et al.*, 2009). H3K9me3 is then reversed through DDR-mediated degradation of G9a/GLP methyltransferase to facilitate the progression of the DNA repairing process.

As will be further discussed below, the CDK4/6 inhibitor p16 is also an essential driver of cell cycle arrest during cellular senescence and currently is one of the most used and more accurate biomarkers for senescence detection both *in vitro* and *in vivo* (Rayess *et al.*, 2012; Hernandez-Segura *et al.*, 2018).

During physiological conditions, p16 remains methylated through the action of DNA (cytosine-5)-methyltransferase 1 (DNMT1) methyltransferase. However, upon cellular senescence DNMT1 inhibition, causes p16 promoter demethylation (BQ Zhu *et al.*, 2017). p16 can also be transcriptionally regulated through the binding of Polycomb repressor complex (PRC) 1 and 2 to its coding locus (*INK4/ARF*) leading to heterochromatin formation. In contrast, detachment of these complexes leads to the formation of euchromatin and restoration of the capacity for p16 transcription (Rayess *et al.*, 2012). JMJD3, ZRF1, and MLL1 are other factors that participate in *INK4* locus epigenetic regulation (Barradas *et al.*, 2009; Kotake *et al.*, 2009; Ribeiro *et al.*, 2013). However, it is still not clear how the recruitment and disassociation of the PRCs and these epigenetic factors from the *INK4/ARF* are mediated (Herranz and Gil, 2018). H3K9me2 is

also reduced in the promoters of IL-6 and IL-8 known as the two main SASP components (Takahashi *et al.*, 2012).

B. Macromolecule imbalance during Cellular Senescence

i. Cellular Senescence induces Protein homeostasis alterations

Endoplasmic reticulum (ER) stress has been proposed as a promoter of senescence-associated oxidative stress (Pluquet *et al.*, 2015). However, at the moment, there is still controversy about whether ER stress causes cellular senescence or it is just one more consequence of this process (Pluquet *et al.*, 2015). The ER is a continuous network of membranes spread throughout the cytoplasm of eukaryotic cells with a connection to the nucleus that plays a huge role in lipid and calcium homeostasis and harbors a highly oxidizing environment that favors the dynamization of crucial processes for protein production such as synthesis, folding, post-translational modification and cytoplasmic protein transport (Pluquet *et al.*, 2015).

During cellular senescence, numerous cellular insults can lead to proteotoxicity through ER stress such as nutrient and energy deprivation, oxidative stress, accumulation of aberrant mutations, and abnormal increase in protein synthesis featuring low chaperone availability. Overall this ultimately can lead to an abnormal accumulation of damaged unfolded/misfolded proteins in the ER lumen (Pluquet *et al.*, 2015). Increased concentration of intracellular oxidative agents such as ROS can lead to the inactivation of a variety of protein tyrosine phosphatases through oxidation of the cysteine residues in their active sites (Deschenes-Simard *et al.*, 2013). This can lead to ERK signaling hyperactivation, which might induce cellular senescence. In fact, high levels of phospho-ERK (ERK activated form) are found throughout senescent abundant pre-neoplastic lesions and therapy-induced senescent cells (Haugstetter *et al.*, 2010; Deschenes-Simard *et al.*, 2013; Gorgoulis *et al.*, 2019). Further, the excess of ROS in conditions where metals are present can lead to carbonylation of many aminoacid residues including arginine, lysine, threonine, and proline which can expose protein hydrophobic surfaces promoting aggregation and unfolding (Nystrom, 2005). Also, other aminoacid groups can react with these carbonyl residues to form Schiff bases that can further promote aggregation

(Gorgoulis *et al.*, 2019). Cross-link between protein aggregates and lipids or sugars can produce insoluble aggregates usually seen in aged tissues and senescent cells called lipofuscin (discussed further below) (Brunk and Terman, 2002; Gorgoulis *et al.*, 2019). To counteract the abnormal intracellular accumulation of protein aggregates, cells are known to possess two systems: the unfolded protein response (UPR) and ER-associated protein degradation (ERAD) (**Figure 2**). ERAD induces unfolded protein transportation from ER to the cytosol for degradation by the proteasome. UPR is a cellular mechanism that promotes the reinstatement of proteostasis (protein homeostasis) by increasing the production of chaperones and components of both autophagy and ERAD systems and promoting a decrease in protein synthesis. UPR can also increase ER capacity in response to increase protein burden through XBP1s and ATF6α, but not ATF4 activation, that increases phospholipid synthesis which promotes ER membranes enlongation (Shaffer *et al.*, 2004; Sriburi *et al.*, 2007; Bommiasamy *et al.*, 2009).

Binding immunoglobulin protein (BiP) is a protein found in ER that can bind and inhibit the UPR key activators IRE1α, ATF6α, and PERK. An increase in unfolded/misfolded protein load in the ER lumen causes Bip to detach from these proteins to bind to unfolded/misfolded proteins leading to UPR activation (**Figure 2**). UPR activation has also been demonstrated to increase Bip translation (Gulow *et al.*, 2002), a phenomenon that is reported to occur during cellular senescence (Hernandez-Segura *et al.*, 2018). In fact, BiP mRNA and protein levels were shown to be upregulated in some models of cellular senescence, however, the same could not be observed in other cellular senescence models (Pluquet *et al.*, 2015). There is also evidence that the UPR activation might vary according to the different cellular senescence mechanisms **(Figure 1)** as replicative senescence of WI38 cells leads to the activation of all UPR inducers (ATF6α, PERK, and IRE1α) while cellular senescence induced by hydrogen peroxide only leads to PERK activation (Matos *et al.*, 2015). More evidence of the correlation between ER stress and cellular senescence can be found throughout the literature (Denoyelle, 2006; Rayess *et al.*, 2012; Dorr *et al.*, 2013) along with indications of the importance of all UPR inducers in cellular senescence which are reviewed by Pluquet et. al (Pluquet *et al.*, 2015).

ii. Autophagy & Cellular Senescence

As shown in **Figure 2**, autophagy is another response mechanism triggered by the cell upon ER proteotoxic stress and other stressing stimuli such as hypoxia, oxidative stress, or nutrient deprivation (Saha *et al.*, 2018). This lysosomal "self-eating" process is essential for preserving the equilibrium between intracellular anabolism and catabolism. While the ERAD system is limited to the degradation of ubiquitin-conjugated protein aggregates, autophagy can be used by the cell to decompose and/or reuse a variety of damaged macromolecules, aggregates, or intracellular organelles including mitochondria, ER, peroxisomes and lipid droplets through lysosome digestion (Saha *et al.*, 2018). The resulting smaller biomolecules can be secreted or used as building blocks for intracellular anabolic reactions. For example, resulting amino acids can be used for protein synthesis, free fatty acids for the tricarboxylic acid (TCA) cycle or β-oxidation, and glucose for ATP production through glycolysis (Gamerdinger *et al.*, 2011; Moreno-Blas *et al.*, 2018). At first, autophagy was thought to be activated non-selectively in response to stress, however recent evidence suggests that some intracellular components or even pathogens can be selectively degraded by this mechanism (Farre and Subramani, 2016). Autophagy is divided into three variants (macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA)) that differ mainly in the process of cargo transportation to the lysosomes. During macroautophagy, cargo to be degraded is sequestered by a double-membrane vesicle structure referred to as autophagosome which is then transported via the dynein machinery on microtubules to the lysosomes to form the autolysosomes where the entire complex is degraded by the lysosomal hydrolytic enzymes. In microautophagy, cargo is directly engulfed by the lysosomes and in CMA protein transportation towards the lysosomes is facilitated through a multi-chaperone complex (Moreno-Blas *et al.*, 2018; Saha *et al.*, 2018).

During aging, macroautophagy (the better-described type of autophagy) is known to decline in various tissues and its stimulation is correlated with an increase in the longevity of model organisms (Madeo *et al.*, 2015; Moreno-Blas *et al.*, 2018). Further, many behavioral and pharmacological interventions that promote longevity in model organisms such as exercise, calorie restriction, target of rapamycin (TOR) or insulin/insulin-like growth factor signaling

inhibition and sirtuins or 5' AMP-activated protein kinase (AMPK) activation are known to induce autophagy (de Cabo *et al.*, 2014; D Zhou *et al.*, 2021). In the context of aging, autophagy has gained more attention than the ERAD mechanism since the proteasome is unable to degrade protein aggregates formed in the pro-oxidant and aggregation-prone milieu seen during aging (Kuwano *et al.*, 2016). Since one known hallmark of cellular senescence is the accumulation of damaged cellular components, it has been proposed that the alterations of autophagy seen during aging can be a factor in the development or maintenance of the senescent phenotype (Rajawat *et al.*, 2009). However, evidence shows that autophagy can have pro and anti-senescent effects depending on the timing and duration along with the type of autophagy (general or specific), cargo and inducing/inhibitory stimuli. Further, this relationship has been shown to play a role in a wide range of pathological conditions including arthritis, atherosclerosis, cancer and renal diseases (Kang and Elledge, 2016; Rajendran *et al.*, 2019).

Evidence suggests that cellular senescence can control macroautophagy, although its effect seems to change according to the context (Kang and Elledge, 2016). Macromolecules recycled during macroautophagy have been shown to be highly important to the synthesis of the SASP factors (Kang and Elledge, 2016). In agreement, inhibition of macroautophagy has been shown to both impair and stimulate $HRAS^{G12V}$ inducing senescence and the formation of SASP (Kang and Elledge, 2016). Downregulation of ATG7 and Atg12 (both indispensable for autophagosome formation) promotes premature senescence in human fibroblasts (HT Kang *et al.*, 2011). In a study performed by Garcia-Prat and colleagues, impaired macroautophagy causes early senescence of young myosatellite cells (crucial for muscle tissue regeneration), through disruption of proteostasis, increased oxidative stress, and mitochondrial dysfunction while re-establishment of autophagy was able to reverse senescence phenotype and restore regenerative functions of old myosatellite cells (Garcia-Prat *et al.*, 2016).

The stability of the transcription factor and major regulator of SASP that accumulates intracellularly during senescence known as GATA4 (GATA Binding Protein 4), is mediated by macroautophagy. GATA4 degradation is induced by a key receptor in the autophagic process named SQSTM1/p62. However, GATA4-SQSTM1 interaction declines upon exposure to

senescence-inducing stimuli leading to abnormal GATA4 accumulation which causes overproduction of SASP mediators through continuous activation of the NF-κB pathway. Further, SASP factors have also been shown to be synthesized from recycled amino acids in a complex formed at the trans-side of the Golgi apparatus during macroautophagy in Oncogene-induced senescence (OIS) known as the TOR-autophagy spatial coupling compartment (Narita *et al.*, 2011). Autolysosomes accumulate in this site forming a pool of reusable amino acids that are then used by MTORC1 to sustain SASP factors production including interleukin-6/8 (IL-6 & IL-8) (Narita *et al.*, 2011).

On the other hand, Zheng and colleagues reported that macroautophagy was increased in senescent mesenchymal stem cells senescence displaying a high number of autophagic vacuoles and upregulation of autophagy-related proteins. Further, pharmacological inhibition using bafilomycin A1 and 3-methyladenine of autophagy in the same type of cells resulted in the downregulation of senescence markers (Zheng *et al.*, 2014). While pharmacological inhibition of autophagy was shown to prevent premature senescent phenotype of human umbilical vein endothelial cells, although increased apoptosis (Patschan *et al.*, 2008). CMA is responsible for the selective degradation of misfolded, oxidized, or damaged proteins which is essential to the maintenance of cellular proteostasis. As such, CMA impairment during aging can lead to intracellular accumulation of protein aggregates which is a known hallmark of cellular senescence that can contribute to the development of age-related diseases (Moreno-Blas *et al.*, 2018). During CMA, proteins set to be degraded display an exposed KFERQ motif which is recognized by HSC70 chaperones and co-chaperones. The formed protein-chaperone complex is later recognized by the lysosomal transmembrane protein LAMP2A that multimerizes into a pore structure allowing for internalization of the now unfolded protein which is then degraded by the lysosomal hydrolases (Moreno-Blas *et al.*, 2018). Evidence suggests that the age-related decline in CMA function is mainly attributed to both LAMP2A downregulation and impaired multimerization which might be associated with the aging increase in cellular senescence since LAMP2A was shown to be reduced in senescent mouse embryonic fibroblasts (Storer *et al.*, 2013). Further, RNase A (CMA target) degradation rate has been shown to be reduced during

cellular senescence (topic more extensively reviewed by Moreno-Blas and colleagues) (Storer *et al.*, 2013). There is still a lot to unveil regarding CMA relationship with cellular senescence, but evidence point to a CMA possessing an anti-senescent effect which is gradually lost during aging.

iii. Klotho, Autophagy and Cellular Senescence

In 1997 Kuro-o et al. reported that the deletion of an unknown gene in mice resulted in a significant decrease in their lifespan as a result of the premature development of various age-related problems (Kuro-o *et al.*, 1997). This gene became to be known as "Klotho" and codes for a protein now known as "α-Klotho". Since then, 3 homologous proteins were discovered including β-Klotho (Ito *et al.*, 2000), KLPH (Ito *et al.*, 2002), and the Klotho-related protein (Klrp) (Hayashi and Ito, 2016) differing in structure, function and localization. α-Klotho and β-Klotho are highly homologous proteins and both regulate important metabolic processes in mammals (Kuro-o, 2019), however, they differ in their specific functions and localization (H Zhou *et al.*, 2021). β-Klotho indeed can be mostly found in the highly metabolic active tissues such as liver, gut, pancreas, yolk sac, and brown and white adipose tissues where it mediates various metabolic processes including fatty acid metabolism, glucose uptake, and bile acid synthesis (Ito *et al.*, 2000). On the other hand, α-Klotho is highly expressed in some brain structures including the epithelial cells of the choroid plexus and at low levels in the pituitary (H Zhou *et al.*, 2021) and is known to participate in homeostasis maintenance-related functions such as prevention of inflammation and ROS-induced oxidative damage, stem cells protection, Ca^{2+} and phosphate homeostasis, myelination induction and long-term enhancement in neurons (Liu *et al.*, 2007; Martin *et al.*, 2012; Chen *et al.*, 2013; Zhou *et al.*, 2018; H Zhou *et al.*, 2021). Interestingly, evidence indicates that α-Klotho seems to influence the autophagic flux which might be correlated with the pathophysiological development of aging-related diseases given that α-Klotho's expression levels decrease during aging in various tissues including brain, liver, kidney, and heart sinoatrial node (Duce *et al.*, 2008; Yamazaki *et al.*, 2010; Semba *et al.*, 2014; Akasaka-Manya *et al.*, 2016; Zhou *et al.*, 2018; H Zhou *et al.*, 2021). In fact, some studies

provide a link between α-Klotho with neurodegenerative disorders, as Alzheimer's disease, whose pathophysiology is characterized by abnormal deposition of amyloid-β and formation of neurofibrillary tangles in the brain which are usually removed through autophagy. whose pathophysiology is characterized by abnormal deposition of amyloid-β and formation of neurofibrillary tangles in the brain which are usually removed through autophagy. Evidence indicates that autophagy is disrupted in the brains of Alzheimer's disease patients and animal models (DS Yang *et al.*, 2011; Castellazzi *et al.*, 2019; Pomilio *et al.*, 2020) and that this impairment is highly influenced by an age-related decrease in α-Klotho 's expression (Zeng *et al.*, 2019). In fact, α-Klotho's overexpression was shown to activate autophagy through inhibition of Akt/mTOR pathway, preventing amyloid-β deposition in amyloid-β1-42 fibrils-treated BV2 cells (Zeng *et al.*, 2019) and decreasing hyperphosphorylated tau protein levels (precursors of neurofibrillary tangles' formation) (Gao *et al.*, 2018; Xin *et al.*, 2018; Zeng *et al.*, 2019). . Further, α-Klotho overexpression in the brain of APP/PS1 mice (Alzheimer's disease mouse model) prevented the abnormal accumulation of lipofuscin which are insoluble autofluorescent aggregates thought to be composed of oxidized proteins that accumulate intracellularly in senescent cells (further discussed below) (Brunk and Terman, 2002; Zeng *et al.*, 2019). This is thought to be mediated by α-Klotho 's autophagy activation capacity (H Zhou *et al.*, 2021). Age-related decrease in α-Klotho's expression in renal tubular epithelial cells has also been correlated with the development of renal problems such as chronic kidney disease. Restoration of α-Klotho levels in cultured human renal tubular epithelial cells (HKC-8) and cells from unilateral ischemia-reperfusion mice had a mitochondrial protective effect mediated by significant inhibition of Wnt1 and Wnt9a-induced mitochondrial injury resulting in decrease in cellular senescence and fibrotic lesions (Miao *et al.*, 2021). In another study, inner medullary collecting duct-3 cells exposed to radiation displayed higher cellular senescence than controls along with decreased α-Klotho's gene expression. The same was further confirmed in the kidney tissues of BALB/c mice and was attributed to radiation's mediated increase in TNF-α expression, which downregulates α-Klotho's expression and prevents the formation of soluble α-Klotho by decreasing the expression of α-Klotho ectodomain shedding enzyme ADAM9/10/17.

This may underlie the development of radiation nephropathy which is a major complication associated with radiotherapy (Kim *et al.*, 2021). Cigarette smoke, one of the major risk factors for the development of COPD, has also been shown to promote a decrease in α-Klotho's expression in lung macrophages of mice and individuals with smoking habits with or without COPD. Moreover, bronchial epithelial cells from COPD patients show lower levels of α-Klotho (Li *et al.*, 2015; Krick *et al.*, 2018) and its overexpression *in vitro* was shown to protect cigarette smoke-induced cell death (Blake *et al.*, 2015). Interestingly, cigarette smoke transiently activates autophagy which leads to cellular senescence (Fujii *et al.*, 2012). Reduction of α-Klotho and other mediators has also been shown to promote vascular dysfunction (precursor to the development of cardiovascular disease) through the induction of endothelial cell senescence (Jia *et al.*, 2019).

iv. Lipidic alterations during cellular senescence

Besides proteins, evidence suggests that lipid macromolecules such as fatty acids, sphingolipids, and glycerolipids might play a role in the development, maintenance and even spreading of senescence phenotype (Saitou *et al.*, 2018). The lipidic amphipathic nature allows for the formation of cellular membranes not only delimiting the cell (plasma membrane) but also the intracellular organelles. The plasma membrane is constituted by phospholipids, glycolipids, and cholesterol and its fluidity depend on the presence of these molecules and the length and unsaturation of the fatty acyl tails (van Meer *et al.*, 2008; Triana-Martinez *et al.*, 2019). As already mentioned, one main hallmark of cellular senescence is remodulation of the plasma membrane (Hernandez-Segura *et al.*, 2018). It is thought that the upregulation of lipid biosynthesis and metabolism during senescence could be prompted by increased lipidic demand to support membrane enlargement (Millner and Atilla-Gokcumen, 2020). There is also solid evidence of various types of lipidic macromolecule's involvement in senescence-related processes including cell cycle arrest, oxidative stress response, inflammation, and SASP production (Millner and Atilla-Gokcumen, 2020). Triacylglycerols and lipid droplet accumulation have been also revealed to be a major lipidic change during fibroblast replicative

senescence (Lizardo *et al.*, 2017). Increased lipid droplets and upregulation of enzymes involved in breaking down reactive lipids were also reported during therapy-induced senescence (Flor *et al.*, 2017). It is thought that lipid droplet accumulation is part of a cell defensive mechanism during senescence that redirects unsaturated fatty acids to triacylglycerols depositing them in lipid droplets preventing their oxidation and formation of lipid peroxides (Flor *et al.*, 2017; Lizardo *et al.*, 2017). Other possible lipidic macromolecules or intervenients in lipid metabolism possibly involved in the process of cellular senescence include ceramides, deoxyceramides sphingosine kinase 1 and 2 (SK1 and SK2, catalyzers of sphingosine phosphorylation for the generation of sphingosine-1-phosphate), CD36 (cluster of differentiation 36, membrane receptor involved in fatty acid metabolism) and carnitine palmitoyl transferase 1 (CPT1, a mitochondrial transporter transmembrane enzyme that converts long-chain fatty acyl-CoA to long-chain acylcarnitine), these are properly reviewed by Millner and Atilla-Gokcumen (Millner and Atilla-Gokcumen, 2020).

C. Structural and functional alterations of cell organelles during Cellular Senescence

i. Cellular senescence-associated nuclear alterations

The development of some cellular features such as transcriptional and proteomic regulation along with cell migration, proliferation, morphology, and lineage specification have been shown to be under the influence of extracellular mechanical stimulation that is converted into biochemical signals (Humphrey *et al.*, 2014; Gilbert and Swift, 2019). Cytoskeleton filaments attached to membrane-anchored receptors such as cell membrane-spanning G-protein-coupled receptors, stretch-activated ion channels, or integrin and cadherin adhesion complexes, can sense mechanical stimuli from the extracellular matrix (ECM) or neighbor cells (Zuidema *et al.*, 2020). These filaments are connected to the nucleus where the mechanical stimuli are mediated by the linker of the nucleoskeleton and cytoskeleton (LINC) complex. This protein structure is formed by 3 main families of conserved proteins which are fairly reviewed elsewhere (Gilbert and Swift, 2019). One of these types is lamins, major components of the nuclear lamina that lines the inside of the nuclear envelope, a structure responsible for containing the nucleus,

composed of the inner, and outer phospholipid bilayer membranes, and a space formed between the two layers referred to as the perinuclear space which is connected to the lumen of the rough ER (Gilbert and Swift, 2019). Lamins, type V intermediate filament proteins, are structural components of the nuclear lamina, a structure that provides a molecular interface between the inner nuclear membrane and chromatin, and that is critical for the definition of the viscoelastic properties and shape of the nucleus. Lamins can be classified into two types: i) A-type lamins encoded by the LMNA gene which can originate both lamins A or C through alternative splicing and are known to be predominantly expressed in differentiated cells and are present in the nuclear lamina and nuclear interior, and ii) B-type lamins composed of lamins B1 and B2 which are respectively encoded by the LMNB1 and LMNB2 genes and are known to be expressed in all mammalian cells and found almost exclusively at the nuclear periphery (Gonzalo *et al.*, 2017).

In the eukaryotic nucleus, while euchromatin is mainly found in the internal nucleus, a great portion of epigenetically silent heterochromatin is distributed in the surrounding areas of the nuclear periphery being anchored to the inner side of the nuclear envelope (Lukasova *et al.*, 2018). These heterochromatin/nuclear bonds are referred to as heterochromatin tethers and have been shown to play a major role in the regulation of the organization and function of chromatin, influencing gene expression (Holmer and Worman, 2001; Lukasova *et al.*, 2018). Heterochromatin tethers are formed by tethering proteins from the inner nuclear membrane. These proteins can recognize lamina-associated heterochromatin domains (LAD) along with nuclear lamins engaging in a bridging role between the heterochromatin and the nuclear envelope. Lamin B receptor (LBR) is part of this group of proteins, it preferably binds to lamin B1 in embryonic and non-differentiated cells and also to chromatin regions marked by specific histone modifications through its Tudor domain resulting in gene silencing (Olins *et al.*, 2010). Mutations in its gene locus are known to be causative of pathological abnormalities such as Pelger–Huet disorder (Lukasova *et al.*, 2018). In fact, two distinguished heterochromatin tethers demark proliferating cells from differentiating cells (Lukasova *et al.*, 2018). In embryonic and non-differentiated cells, LBR heterochromatin tethers promote chromatin structural arrangement

that facilitates the expression of proliferation-inducing genes while heterochromatin tethers formed by lamin A/C and LEM-domain (discussed below) promote chromatin structural arrangement that facilitates the silencing of proliferation-inducing genes and the expression different kinds of differentiation-inducing cells (Brachner and Foisner, 2011; Lukasova *et al.*, 2018). Results from a study performed by Solovei et al. suggest that LEM-domain protein expression is specific to developmental stage and cell type and that they are not detected in every known LBR-non expressing cell type (Solovei *et al.*, 2013).

Mutations in lamin proteins mainly in the *LMNA* gene have been associated with a wide range of degenerative pathologies including syndromes with premature aging phenotypes such as Hutchinson Gilford Progeria Syndrome, restrictive dermopathy, and Atypical Werner Syndrome, peripheral neuropathies such as Charcot-Marie-Tooth-Disease type 2B1, muscular dystrophies such as Emery-Dreyfus Muscular Dystrophy and lipodystrophies (Gonzalo *et al.*, 2017). Studies suggest that during cellular senescence there are alterations in the proteins of the nuclear envelope including downregulation of lamin-B1 (Freund *et al.*, 2012), lamin-A, LEM domain-containing, LBR, and LEM domain-containing protein 3 (LEMD3) and upregulation of SUN1 levels were increased (Lenain *et al.*, 2015). SUN1 belongs to the LINC complex that if altered during senescence could result in aberrant mechanical force transduction to the nucleus which may originate in deviant cellular mechano-response. During cellular senescence, chromatin suffers functional and structural alterations that include the dissociation between the inner nuclear membrane and centromeric repetitive sequences, their relocation to the nucleoplasm, and distension (Lukasova *et al.*, 2018). In particular, decreases in lamin-B1 observed during cellular senescence were shown to contribute to alterations in the organizational structure of heterochromatin promoting the formation of senescence-associated heterochromatic foci (SAHF), (further discussed below) and the loss of integrity of the bond between the nuclear lamina and chromatin. Further studies evidence the importance of both LBR and lamin-B1 in the induction of cellular senescence as their downregulation has resulted in the disassociation of centromeric heterochromatin from the INM to and relocation in an unfolded conformation to the nucleoplasm resulting in alterations in chromatin architectural

structure and gene expression (Lukášová *et al.*, 2017). LBR was also shown to be coordinately expressed with lamin B1 in cancer cells as their downregulation led to cellular senescence while duplication in the lamin B1 gene (*LMNB1)* led to increased LMNB1 expression causing the development of adult-onset autosomal dominant leukodystrophy (Dreesen *et al.*, 2013).

ii. Mitochondrial dysfunction

Mitochondrial dysfunction occurs during aging and several pathological processes and is characterized by mitochondrial swelling, increased mtDNA mutation rate, loss of cristae, and deterioration of the inner membrane resulting in decreased membrane potential. Despite the increased number and size, mitochondria are less functional, with less capacity for ATP production, increased proton leak, reduced fission and fusion rates, and accumulation of TCA cycle metabolites (Kaplon *et al.*, 2013; Gorgoulis *et al.*, 2019). Despite the existence of evidence that dysfunctional mitochondria promote cellular senescence both in *in vitro* cell cultures and *in vivo* models, there is not yet a clear picture of how this occurs (Dai *et al.*, n.d.; Moiseeva *et al.*, 2009; Kang *et al.*, 2012; Ogrodnik *et al.*, 2019).

One major factor thought to potentiate cellular senescence through induction of mitochondrial dysfunction is the rise in ROS caused by stressors (Lee *et al.*, 2002; Passos *et al.*, 2007; Kaplon *et al.*, 2013; Miettinen and Bjorklund, 2017; Gorgoulis *et al.*, 2019; Ogrodnik *et al.*, 2019) (**Figure 1)**. Cellular senescence has been successfully induced in fibroblasts and cancer cells through an intracellular increase in ROS levels after p53 or p21 activation (Macip *et al.*, 2002, 2003). Further, during cellular senescence, $p16^{INK4A}$/Rb-pathway has been shown to cooperate with mitogenic signals to stimulate the rise in ROS intracellular levels triggering the activation of protein kinase C delta, which promotes further generation of ROS (Takahashi *et al.*, 2006). Evidence suggests that an increase in mitochondrial mass and ROS as a result of RAS activation during OIS is dependent on either Rb or p53 signaling (Moiseeva *et al.*, 2009; Ogrodnik *et al.*, 2019). DDR activation during cellular senescence has also been shown to promote mTORC1

and Akt activation leading to mitochondrial dysfunction (Correia-Melo *et al.*, 2016). During senescence, alterations to the intracellular ADP: ATP and AMP: ATP ratios can activate AMPK which contributes to cell-cycle arrest (Birch and Passos, 2017; Gorgoulis *et al.*, 2019). This evidence stands out the relevance of cell cycle arrest factors in the induction of mitochondrial dysfunction during cellular senescence and suggests that the interaction with growth-inducing factors contributes to the process (Correia-Melo *et al.*, 2016; Ogrodnik *et al.*, 2019). Evidence also suggests that cell enlargement during senescence might play a role in the increase in mitochondrial dysfunction during senescence as high cytoplasmic volume has been shown to change mitochondrial dynamics along with the traffic of mitochondrial metabolites (Pezze *et al.*, 2014) and mitochondrial dysfunction could be reversed through inhibition of growth signaling in senescent cells (Passos *et al.*, 2010; Correia-Melo *et al.*, 2016). Also, induction of the autophagic mitochondrial clearance, known as mitophagy, in senescent cells seems to decrease SASP (Correia-Melo *et al.*, 2016; Gorgoulis *et al.*, 2019). Evidence regarding the role of mitochondrial dysfunction in cellular senescence *in vitro* is clear, however characterization of this relationship *in vivo* is still scarce. Also, because mitochondrial dysfunction is associated with many other cellular processes it cannot be used as a reliable cellular senescence biomarker (Gorgoulis *et al.*, 2019).

iii. Mitochondrial dysfunction-associated senescence (MiDAS)

In 2016, Wiley and colleagues described a distinct type of senescence induced by mitochondrial dysfunction which they named mitochondrial dysfunction-associated senescence (MiDAS) (Wiley *et al.*, 2016). This process was not found to be correlated with ROS-related DNA damage but with overactivation of the AMPK-p53 axis due to an increase in the NAD^+/NADH ratio (Wiley *et al.*, 2016). AMPK-p53 activation was shown to promote cell cycle arrest and modeling of SASP profile through decreased activity of both sirtuins and ADP ribose polymerase (PARP), known activators of NF-kB, an important SASP regulator, resulting in the downregulation of the IL-1-dependent SASP arm and the increased expression of TNFα, IL10, and CCL27 (Wiley *et al.*, 2016; Birch and Passos, 2017; Gorgoulis *et al.*, 2019). Further,

MiDAS-associated SASP profile was shown to suppress preadipocyte differentiation and promote keratinocyte differentiation which might suggest a correlation between MiDAS and the development of lipodystrophy and skin problems that tend to develop during aging mice (Wiley *et al.*, 2016).

iv. Lysosomal Dysfunction

As mentioned earlier, dysfunctional organelles such as mitochondria and lysosomes accumulate during cellular senescence since autophagy (their main degradation mechanism) is often impaired (Kang and Elledge, 2016; Doherty and Baehrecke, 2018). As such, in senescent cells, lysosomes often display abnormal size and are unusually increased in number (Robbins *et al.*, 1970). An increase in lysosomal biogenesis might happen as an attempt by senescent cells to compensate for the accumulation of dysfunctional lysosomes (Westermann, 2012; Gorgoulis *et al.*, 2019). Although during senescence, lysosomes increase in size and therefore internal content, their activity is often reduced leading to impairments in mitochondrial clearance mechanism which, as mentioned earlier, might result in increased ROS production that might exacerbate cell damage and therefore promote further lysosome dysfunction (Park *et al.*, 2018; Gorgoulis *et al.*, 2019).

Further, cellular senescence-related enlargement of lysosomal compartments may result from increased content which is related to the accumulation of lipofuscin formed due to the accumulation of ROS and stress (**Figure 1)**. Lipofuscins are insoluble auto fluorescent aggregates visually seen as yellow-brown pigments that accumulate intracellularly and are known to impair the amino acid recycling process by interfering with the lysosomal/autophagic and proteasomal protein degradation pathways (Brunk and Terman, 2002). Curiously, evidence suggests that lipofuscin might contribute to apoptosis resistance characteristic of senescent cells by increasing the expression of Bcl-2(McHugh and Gil, 2018). Using a biotinylated Sudan Black B (SBB) analog (GL13) staining or light microscopy, lipofuscin pigments can be observed in the lysosomes of senescent cells although their presence extends beyond senescent

cells which renders it not specific as a senescence biomarker (Terman and Brunk, 2004; Evangelou and Gorgoulis, 2017; Evangelou *et al.*, 2017).

D. Senescence-associated secretory phenotype (SASP)

Most senescent cells have a profound change in their secretory phenotype to SASP which promotes paracrine and autocrine function through intermediate factors. According to Borodkina and colleagues, SASP factors can be classified into proteases, insoluble extracellular matrix proteins, soluble signaling factors, and non-protein components (Borodkina *et al.*, 2018). They can also be sorted according to their function into 3 main groups: i) factors binding to a receptor; ii) factors acting directly, and iii) regulatory factors (Borodkina *et al.*, 2018). The factors that bind to receptors constitute a class of soluble signaling molecules that interact with specific cell membrane receptors generating a variety of intracellular signaling cascades that result in the development of the mentioned senescent-like alterations. Included in this group are chemokines (CCL-2, CCL-5, CCL-16, CCL-26, CCL-20, GROα, GROβ), interleukins (IL-1a, IL-6, IL-8) and growth factors (FGF, GM-CSF, HGF, TGFβ) (Borodkina *et al.*, 2018). Regarding the factors acting directly, as the name says, these molecules can act directly in the neighboring cells for example by cleaving membrane-bound proteins, destroying signaling molecules, and promoting the restructuring of the surrounding extracellular matrix. In this group are included matrix metalloproteases (MMP-1, MMP-3, MMP-10), serine proteases (tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA)), and free radicals (ROS, reactive nitrogen species) (Borodkina *et al.*, 2018). The regulatory factors are compounds that, although not possessing natural enzymatic activity, exert their function by binding and regulating the activity of the factors that belong to groups I and II. Examples from this group include inhibitors of metalloproteases (TIMP), insulin-like growth factor binding proteins (IGFBP), and the plasminogen activator inhibitor (PAI) (Borodkina *et al.*, 2018). It is also worth mentioning that microRNA containing extracellular vesicles has been found to be part of the SASP secretome and evidence suggests that it might promote important responses for inducing or repressing cellular senescence in nearby cells (Urbanelli *et al.*, 2016; Borodkina *et al.*, 2018).

As already mentioned above, SASP factors can induce restructuring of the surrounding microenvironment through shifting peripheral cells' function and morphology along with tissue biological structure (**Figure 1)** (Wang and Gundersen, 1984; Volonte *et al.*, 2013; James *et al.*, 2015). SASP mediators responsible for inducing these alterations include cell proliferating growth factors that promote an increase in organelle mass to prepare the cell for cell division (Ogrodnik *et al.*, 2019). However, since senescent cells enter cell cycle arrest, overproduction of organelle mass leads instead to dysfunctional organelle accumulation (Ogrodnik *et al.*, 2019). SASP factors are not only important for the maintenance and establishment of cellular senescence, but also for immune signaling, management of neoplastic development, and many other functions (Rodier *et al.*, 2009; Freund *et al.*, 2010; Sagiv and Krizhanovsky, 2013; Childs *et al.*, 2015) (as it well discussed below). However, due to the deactivation of the apoptotic pathways during senescence (Kirkland and Tchkonia, 2017), these cells tend to accumulate in tissues which can lead to the chronic secretion of SASP factors which, over time, may lead to damage in the surrounding cells and tissues (Borodkina *et al.*, 2018) and might promote the development of a low grade and sterile chronic inflammatory profile referred to as inflammaging and thought to be involved in a wide range of aging-related diseases (Franceschi *et al.*, 2018). To fight this, in physiologic conditions, the immune system can recognize and clear senescent cells from tissues (Budamagunta *et al.*, 2021). SASP-mediated senescent cell clearance by immune cells such as macrophages also allows for progenitor cell proliferation and differentiation, regenerating damaged tissue, making cellular senescence a crucial process in tissue repair and remodeling (Prata *et al.*, 2018). Senescent cell burden then determines senescence effect on surrounding tissue is beneficial or detrimental. Continuous SASP signaling-mediated senescence propagation or age-related decrease in immune system function can lead to abnormal accumulation of senescent cells which can impair the repairing effect of cellular senescence and can further exacerbate the damage leading in some cases to the formation of fibrotic scar tissue that can contain senescent cells and inflammatory cells (Sone and Kagawa, 2005).

SASP immunological functions are primarily mediated by the NF-κB and CCAAT/enhancer-binding protein (C/EBP) proinflammatory transcription factor family. Recently the cGAS/STING pathway has been found to regulate the production of SASP during cellular senescence. Cytoplasmic GMP-AMP synthase (cGas) detects and binds to cytoplasmatic chromatin fragments, gaining 2′3′-cyclic GMP-AMP (2′3′-cGAMP) production capacity (from GMP and AMP). 2′3′-cGAMP activates the Stimulator of interferon genes (STING) in TANK-binding kinase 1 (TBK1) in the ER promoting the phosphorylation of IRF3 and the activation of NF-κB. These two transcription factors can then migrate to the nucleus where they can promote the expression of SASP mediators including IL-6, IL-8 and IFN-β. The concrete mechanism of cytoplasmatic chromatin fragment accumulation is still unclear, however, some senescence traits are thought to play a role such as the loss of nuclear integrity related to the senescence-associated disrupted lamin B1 expression and the excessive generation of double-strand DNA fragments as a result of the aberrant nuclear and mitochondrial DNA damage seen during senescence (Baker *et al.*, 2016). NOTCH1 signaling has also been found to be involved in the regulation of SASP. Inhibition of NOTCH1 has been shown to downregulate TGF-β incorporation into the secretome and prevent the inhibition of C/EBPβ, therefore, promoting the SASP secretome (Hoare *et al.*, 2016). Interestingly, during OIS NOTCH1 has been shown to be upregulated (Hoare *et al.*, 2016). This study observed that during OIS, NOTCH1 activity fluctuates dynamically changing the secretory profile accordingly, fluctuating between TGF-β including secretome (shown to promote juxtacrine senescence induction through NOTCH-JAG1 pathway) and pro-inflammatory profile characterized by up-regulation of pro-inflammatory cytokines such as IL-1α respective transcription factor of C/EBPβ (Hoare *et al.*, 2016). Also, a variety of other signaling pathways have been shown to be involved including (but not limited to) PI3K/AKT/mTOR, p38MAPK, and JAK/STAT (Sieben *et al.*, 2018; Sun *et al.*, 2018).

E. Post-mitotic senescence

As discussed above, cellular senescence was originally described as a consequence of the limiting proliferation capacity of mitotic cells (Hayflick and Moorhead, 1961). As the complex machinery behind this process and its correlation with aging and pathology was being unveiled by new studies, the holistic view of cellular senescence shifted towards a cellular stress response mechanism (Von Zglinicki *et al.*, 2021). As such, the question as to whether cellular senescence is indeed restricted to proliferation-competent cells has recently arisen in light of recent evidence indicating the presence of senescence markers in post-mitotic cells from aged mice tissues including mature neurons, cardiomyocytes, skeletal muscle myofibers and osteocytes (D Jurk *et al.*, 2012; Farr *et al.*, 2017; Musi *et al.*, 2018; Anderson *et al.*, 2019; Benkafadar *et al.*, 2019; da Silva *et al.*, 2019). The first evidence of cellular senescence in post-mitotic cells remounts to 2012 when Jurk and colleagues reported the existence of various senescence markers in cerebellar Purkinje neurons, cortical neurons, and neurons of the myenteric plexus from aged mice (Diana Jurk *et al.*, 2012). Jurk and colleagues also showed that the accumulation of senescent neurons was induced by dysfunctional telomere-activated DDR (Diana Jurk *et al.*, 2012). Two more recent independent studies provide evidence regarding the onset of cellular senescence in neurons including the detection of senescence-like traits in the tau neurofibrillary tangles presenting neurons from both post-mortem Alzheimer's disease patients and mouse models (Musi *et al.*, 2018), and senescent mouse retinal ganglial cell layer under ischemia (Oubaha *et al.*, 2016). Particularly, Musi and colleagues were able to detect upregulation of the transcription of genes regulated by p53, p38MAPK, TGFβ, and NF-κB while in the Oubaha et al. study senescence was seen to spread from retinal ganglion neurons to retinal microglial cells and the vasculature through bystander signals (Oubaha *et al.*, 2016).

Growing evidence shows that several post-mitotic cell types undergo cellular senescence during aging; however how post-mitotic senescence impacts tissue integrity and function and weather drives aging is currently ill-defined (reviewed in (Von Zglinicki *et al.*, 2021).

III. Cellular Senescence in physiological processes

Due to its established relationship with aging and related pathological process, for some time, researchers have been looking for therapies to eliminate senescent cells from tissues. However, although chronic deposition of senescence cells in tissues is associated with the pathophysiology of some diseases, acute cellular senescence has been found to play important role in many homeostatic processes (Amaya-Montoya *et al.*, 2020) (**Figure 3**). As mentioned above, cellular senescence is a crucial mechanism for tissue remodeling by promoting damage cell clearance and promoting tissue regeneration. Upon cutaneous wound, cellular senescence is known to stimulate wound closure by the induction of myofibroblasts differentiation through the secretion of platelet-derived growth factor AA (PDGF-AA) by senescent fibroblasts and endothelial cells (Demaria *et al.*, 2014). This acute fibroblasts senescence is induced by the extracellular matrix-associated signaling protein CCN1 which can bind to the cell adhesions receptors, heparan sulfate proteoglycans, and integrin alpha(6)beta(1) (Jun and Lau, 2010).

Also, in liver tissue, senescence of hepatic stellate cells can prevent excessive fibrosis, impair damaged cell proliferation and promote senescent cell clearance by the immune system (Krizhanovsky *et al.*, 2008). Like apoptosis, evidence suggests that cellular senescence can act as a tumor-suppressive mechanism as OIS was proven to impair tumor cell proliferation (Di Micco *et al.*, 2006; Kuilman *et al.*, 2010; Rufini *et al.*, 2013). However, tumor senescent cells are also known to secrete immunosuppressive signals and set a proper microenvironment for tumor cell proliferation and metastasis in the early stages of cancer development (Collado *et al.*, 2005; Ruhland *et al.*, 2016; Demaria *et al.*, 2017; Prieto and Baker, 2019). Cancer's relationship with cellular senescence is complex and is extensively reviewed elsewhere (Campisi, 2013; Liu *et al.*, 2018; Calcinotto *et al.*, 2019; Prieto and Baker, 2019).

During embryonic development, senescence-associated beta-galactosidase $(SA\text{-}\beta\text{-}gal)^{+}$ and proliferation marker protein $Ki\text{-}67^{-}$ senescent cells have been detected in various regions of the mouse embryo such as the neural tube, roof plate, hindbrain, mesonephros, endolymphatic sac, apical ectodermal ridge, gut endoderm, pharyngeal arches, the tip of tail, placental syncytiotrophoblasts and also in the human embryo (mesonephros and endolymphatic

sac)(Munoz-Espin *et al.*, 2013; Storer *et al.*, 2013; Rhinn *et al.*, 2019). These cells showed impaired cell proliferation along with increased marked expression of SA-β-gal, p21, and SASP factors but interestingly did not seem to display DNA damage along with the expression of cell cycle inhibitors p53, $p16^{INK4A}$, $p19^{ARF}$. This indicates that the presence of cellular senescence in embryonic tissues might be of a different type (Rhinn *et al.*, 2019). Moreover, this process was proven to be highly dependent on p21 and TGF-β/PI3K pathways since p21-null mouse embryos are known to develop patterning defects similar to the outcome of chemical or senolytic treatments (Munoz-Espin *et al.*, 2013; Storer *et al.*, 2013; Davaapil *et al.*, 2017; Gibaja *et al.*, 2019; Rhinn *et al.*, 2019). These cells are ultimately cleared by macrophages resulting in tissue remodeling (Munoz-Espin *et al.*, 2013; Rhinn *et al.*, 2019). Natural Killer cells are also highly present in the uterus during early pregnancy and have been shown to promote placental development through secretion of pro-angiogenic factors mediated by the interaction with MHC I expressing trophoblast (Rajagopalan, 2014). On the other hand, senescence natural killer cells were found to mediate embryonic implantation through trophoblast-dependent activation of the p21 signaling pathway through CD158d receptor stimulation, promoting angiogenesis and vascular remodeling (Rajagopalan and Long, 2012; Amaya-Montoya *et al.*, 2020). The information discussed so far has emphasized the detrimental effect of chronic cellular senescence which results from continuous exposure to stress stimuli while recognizing the importance of acute cellular senescence for some homeostatic physiological cellular functions. The same can be said of the SASP as it can vary in content according to cell type, external stimuli, and environmental context (Faget *et al.*, 2019; Amaya-Montoya *et al.*, 2020). This might help explain the variation in senescent cells' features, function, and susceptibility to senotherapeutic treatments (discussed below) found throughout tissues.

IV. Cellular Senescence Detection & Analysis

A. Markers used to evaluate cellular senescence

Beta-galactosidase (SA-β-gal) & p16

A combination of cellular markers is needed to identify senescence in a wide range of cell types (YZ Xiao *et al.*, 2020) as summarized in **Table 1**. The most used combination is the measurement of senescence-associated beta-galactosidase (SA-β-gal) activity detectable at pH 6.0 (Dimri *et al.*, 1996) and the expression of tumor suppressor $p16^{INK4A}$. SA-β-gal is the excessively active form of lysosomal β-D-galactosidase expressed by the GLB1 gene that can catalyze the hydrolysis of β-galactoside into monosaccharides (Piechota *et al.*, 2016). On the other hand, p16 exhibits low expression in healthy cells but has been shown to be phosphorylated during senescence of epithelial cells leading to G1 cell cycle arrest through CDK4/6 augmented affinity which prevents retinoblastoma protein (Rb) phosphorylation perpetuating its cytoplasmic association with the transcription factor E2F1 responsible for positive regulation of transition from G1 to S phase (Rayess *et al.*, 2012) (**Figure 1**). During cellular senescence, SA-β-gal activity at pH 6.0 increases due to augmented lysosomal biogenesis, and p16 phosphorylation is known to contribute to the induction as well as to the maintenance of cellular senescence (Rayess *et al.*, 2012). SA-β-Gal optimal pH for maximal enzymatic activity is between 4.0 and 4.5 which corresponds to lysosomes' intrinsic pH where this enzyme is found. Therefore, when performing SA-β-Gal enzymatic activity testing at pH 6.0 allows for a clear separation between high and normal SA-β-Gal expressing cells given that this pH level is known to reduce enzymatic activity by almost 99% and therefore only cells with augmented lysosomal content possess sufficient amount of enzyme to display some grade of activity (Kurz *et al.*, 2000). SA-β-gal activity at pH6.0 can be measured through a cytochemical assay based on a chromogenic substrate 5-Bromo-4-chloro-3-indolyl b-D-galactosidase (X-gal) conversion to a blue-dyed precipitate while p16 can be detected using RT-PCR, western blot, single-cell RNA sequencing. Also, recent strategies for p16 in vitro detection are currently used including senescent-cell reporters such as luciferase knock-in at the $p16^{INK4A}$ (CDKN2A) locus mouse called p16(LUC) which allows for measurement of $p16^{INK4A}$ promoter activity (Burd *et al.*, 2013) and similarly through p16-3MR or INK-ATTAC systems that promote $p16^{INK4A}$ co-transgene expression of green fluorescent protein (GFP) and monomeric red fluorescent protein (mRFP) respectively allowing senescent cell isolation through flow cytometry (Childs *et al.*,

2015; Khosla *et al.*, 2020). These last methodologies have been implicated in the study and development of potential therapeutic interventions based on the elimination of senescent cells which will be further discussed later in the chapter. High expression of p16 has been detected in various types of senescent cells including senescent fibroblasts (Alcorta *et al.*, 1996; Robles and Adami, 1998) keratinocytes (Loughran *et al.*, 1996), uroepithelial cells (Jarrard *et al.*, 1999), pancreatic β-cells (Krishnamurthy *et al.*, 2006), emerging neoplasms and surrounding stromal cells (Burd *et al.*, 2013). Moreover, p16 expressing cells have been reported to accumulate in mouse tissues during aging, such as in skeletal muscle, eye, adipose tissue (Baker et al. 2011), pancreas (Krishnamurthy *et al.*, 2006), kidney, heart, and blood vessels (Baker *et al.*, 2016; Shimizu and Minamino, 2019). SA-β-gal high activity at pH 6.0 can also be detected in most senescent cells including dermal fibroblasts, epidermal keratinocytes, human umbilical vein endothelial cultures, human mammary epithelial cells, neonatal human melanocytes, and a substantial population of senescent epithelial cells from benign prostatic hyperplasia (Castro *et al.*, 2003). However, specific non-senescent cells such as activated macrophages have been reported to display both high SA-β-gal activity at pH 6.0 and p16 expression (Hall *et al.*, 2016, 2017) while some senescent cells such as senescent geriatric satellite cells display high expression of p16, fail to show increased activity of SA-β-gal at pH 6.0 (Sousa-Victor *et al.*, 2014). Altogether, this evidence show that the combination between these two biomarkers, although can in most cases detect accurately cellular senescence, cannot be considered entirely sensitive or specific, therefore, the additional measurement of other known less specific cellular senescence biomarkers are usually used to confirm the assessment, such as SASP, tumor suppressor genes p53/p21, telomere length, proliferation marker protein Ki-67, clusterin, senescence-associated heterochromatin foci (SAHF), lamin B1 and lamin B receptor (LBR).

Tumor suppressor genes p53/p21, other markers of DDR & Cell cycle arrest

As discussed earlier (**Chapter 1**), abnormal high expression of p53 and p21 is an unquestionable feature of cellular senescence, however not specific since both p53 and p21 seem to be upregulated during apoptosis and transient cell cycle arrest (Wang *et al.*, 2015; YZ

Xiao *et al.*, 2020). In fact, although p21 is a CDKs inhibitor and is upregulated in response to a variety of senescence-inducing stimuli, evidence suggests that it can be activated in a p53-independent manner and is also essential for cell cycle progression (Schwaller *et al.*, 1997; Jung *et al.*, 2010). Further, although p53 and p21 are usually important for the initiation of cell cycle arrest during senescence induction, even if DNA damage continues p53 and consequently p21 signaling may decline over time. This is thought to happen due to the heterochromatinization of E2F targets (part of the SAHF (discussed below)) prolonging the repression of cell cycle progression promoting genes (Narita *et al.*, 2003; He and Sharpless, 2017). As mentioned earlier (**Section 1)**, one of the main indicators of DDR activation is the phosphorylation of histone H2A variant H2AX on serine residue 139 referred to as γH2AX however, it is not specific to cellular senescence as it can be observed in other conditions where the DDR is activated such as apoptotic DNA fragmentation and even in functions not associated with the DDR (Rogakou *et al.*, 1999; Turinetto and Giachino, 2015; Georgoulis *et al.*, 2017). Several other indicators, although used less frequently, can also be measured to detect DNA damage during cellular senescence. This includes ATM, ATR, Tumor suppressor p53 binding protein 1 (53BP1), RAD51 recombinase, and the MRE11/RAD50/NBS1 complex, which can be detected by fluorescence microscopy following co-immunofluorescence staining (Rothkamm *et al.*, 2015). The decreased expression of proliferation marker is also used as a complement biomarker for cellular senescence detection. The protein Ki-67, usually only referred to as Ki-67, is a nuclear protein expressed from the MKI67 gene whose absence has been correlated to an arrest in cell proliferation during cellular senescence (but not exclusively). Ki-67 can be detected through immunostaining where during interphase can be found in the cell nucleus while in mitosis is allocated to the surface of the chromosomes (Cuylen *et al.*, 2016; YZ Xiao *et al.*, 2020). Another proliferation marker often assessed for aiding cellular senescence detection, usually through immunohistochemical staining, is the proliferating cell nuclear antigen (PCNA) (Nagai *et al.*, 2014; El Hasasna *et al.*, 2015). This ring-shaped homotrimer is in the center of a large and complex protein network responsible for regulating and coordinating a wide range of processes important for ensuring correct DNA replication such as mismatch repair, nucleotide excision

repair, error-free damage bypass, Okazaki fragment maturation, translation synthesis, sister chromatid cohesion, chromatin assembly, S-phase specific proteolysis and break-induced replication (Boehm *et al.*, 2016). During cellular senescence, PCNA exhibits low expression although in a non-specific manner as quiescent cells also express the same levels and both normal proliferating and tumor cells display inconsistent PCNA expression. Also, PCNA expression is known to vary during the cell cycle displaying the highest expression during the S phase (Jurikova *et al.*, 2016). Curiously serum treatment induces an increase of PCNA levels in quiescent cells as they resume the cell cycle (Almendral *et al.*, 1987) which highlights its importance for cell cycle progression (Jurikova *et al.*, 2016).

Telomere length

Cell genetic material is continuously subjected to the action of surrounding stimuli which can easily lead to damage and chromosome erosion. To prevent that, chromosomes have nucleoprotein structures called telomeres at the end of each arm (Greider, 1991; Turner *et al.*, 2019). These structures are composed of highly conserved hexameric 5'-TTAGGG- 3' tandem repeats that form specialized T-loop conformation and a G-rich 3'-AATCCC-5' strand called G-strand that protrudes and invades the 5′ double-stranded telomeric duplexes, forming a D-loop (Turner *et al.*, 2019). Also, telomeres are associated with specialized proteins namely proteins that constitute the sheltering complex. The structure promotes the prevention of telomere degradation and regulates the activity of the ribonucleoprotein complex responsible for synthesizing new telomeric repeats known as Telomerase (Turner *et al.*, 2019). This enzymatic complex is composed of the telomerase reverse transcriptase (TERT) subunit responsible for catalyzing the synthesis of new telomeric repeats by copying its telomerase RNA component (TERC) (Greider and Blackburn, 1989). Most mammalian cell types do not express this enzymatic complex in significant amounts or if so, in most cases telomerase activity is repressed by competitive binding of telomeric repeat-containing RNA (TERRA) with TERC, as alternative telomere erosion effect may be contradicted through homologous recombination-mediated alternative lengthening of telomeres (ALT) (Sobinoff and Pickett, 2017; Turner *et al.*,

2019). Both these two mechanisms can delay cell replication-related telomere erosion however, telomeres are still known to shorten with each cell division. Upon reaching critical levels of telomeric length, the sheltering complex is no longer able to perform its functions leaving the chromosome ends vulnerable to destabilizing agents (Turner *et al.*, 2019). One process affected by the compromised sheltering complex function is the activation of DDR. Telomere erosion leaves double-stranded chromosome ends exposed which are recognized by DDR as double-strand breaks (Fumagalli *et al.*, 2012). Two proteins of sheltering complex named POT1 and TRF2 are reported to inhibit ATR kinase and ATM kinase pathways respectively (Sfeir and de Lange, 2012), both of which culminate in the activation of p53 that ultimately may lead to cell cycle arrest **(Figure 1)**. Given that human cells do not express telomerase in sufficient quantities to completely counteract the gradual telomere erosion, the DDR is maintained consistently activated (Engin and Engin, 2021). As already mentioned, this resembles a feature of cellular senescence, in fact, telomere shortening is identified as the definitive cause of the aforementioned replicative-associated senescence and is correlated with aging *in vivo* (Herranz and Gil, 2018). However, there is no convincing evidence to suggest a high specificity of telomere length as a cellular senescence biomarker, although is often used to confirm other markers (YZ Xiao *et al.*, 2020). Measurement of telomere length can be done using Southern Blot (Kimura *et al.*, 2010), flow cytometry with Flow-FISH technique which consists of an *in-situ* hybridization using specific synthetic peptides that mimic the DNA sequences complementary to the telomeres marked with low molecular weight fluorochromes, allowing a quantitative measurement by flow cytometry (Bradford *et al.*, 2009; YZ Xiao *et al.*, 2020) and qPCR (Lin *et al.*, 2019).

Clusterin

Clusterin (also known as Apolipoprotein J) is a chaperone encoded by the CLU gene situated on chromosome 8 and can be found in nuclear, cytoplasmic, or secreted isoforms in a wide range of cell tissues (Poon *et al.*, 2000). This protein has been reported to play a role in several important biological processes such as lipid transport, membrane recycling, cell adhesion, and cell death.

It has an apolipoprotein function in high density-lipoprotein (Desilva *et al.*, 1990) and an extracellular folding function of secreted proteins promoting the prevention of pathological protein aggregation (Poon *et al.*, 2000). As such, clusterin has been correlated with the abnormal proteostasis-related altering of Aβ aggregation/clearance in Alzheimer's Disease (considered the third most significant genetic risk factor for late-onset of Alzheimer's Disease (Foster *et al.*, 2019)). Abnormal high levels of clusterin have also been detected in a wide range of late-stage metastatic cancers (Zhou *et al.*, 2015). It is also known that clusterin engages in other pathophysiological processes such as immune modulation, including mediation of the NF-κB pathway, complement-mediated cell lysis, oxidative stress, programmed cell death, cell survival through modulation of the extracellular signal-regulated kinase (ERK) 1/2 signaling and matrix metallopeptidase-9 overexpression (Koltai, 2014), inhibition of BAX on the mitochondrial membrane (Zhang *et al.*, 2005) and prevention of cellular senescence through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway stimulating cell proliferation (YJ Zhang *et al.*, 2019). High levels of clustering have been observed in some senescent cells including glioblastoma multiforme or WI-38 fibroblasts which have turned it into a recognized cellular senescence biomarker (Matos *et al.*, 2012; Li *et al.*, 2013); however, as described above, it lacks specificity. Its detection can be done by quantitative real-time PCR (qPCR) or western blotting.

Senescence-Associated Heterochromatin Foci (SAHF)

Cells genetic content can be organized into two main types of chromatin: i) euchromatin, where most actively transcribed genes lie; displays a decondensed conformation during interphase and is replicated in early S-phase, and ii) heterochromatin that comparatively displays high condensed conformation during interphase and has a late replication during S-phase and is mostly constituted by repressed genes (Zhang and Adams, 2007). This last type is divided into constitutive heterochromatin mostly composed by repetitive sequences that are constant through cell life and can be found in the telomeres or pericentric satellites present next to the

centromeres, and by facultative heterochromatin which is developmentally regulated and is turned from euchromatin into heterochromatin for permanent silencing of specific genes (Craig, 2005; Zhang and Adams, 2007). SAHFs are specialized domains of facultative heterochromatin formed during cellular senescence and are positive for specific markers of heterochromatin such as di-or trimethylated lysine 9 histone H3 (H3K9Me2/3), heterochromatin protein 1 (HP1), histone H2A variant macroH2A (mH2A), anti-silencing function 1 (ASF1) (Raghuram and Mishra, 2014; YZ Xiao *et al.*, 2020). They were first described by Narita et al in 2003 after noticing that senescent cells displayed 30–50 bright punctate DAPI-stained DNA foci (Narita *et al.*, 2003; Zhang and Adams, 2007). Senescent cells undergo a general change in nuclear architecture which include the formation of SAHF in the promoters of cell proliferation-inducing genes such as E2F target genes as cyclin A which is strictly necessary for S-phase entering (Narita *et al.*, 2003; Zhang *et al.*, 2007) that helps to induce and maintain the cell cycle arrest necessary for cellular senescence. Loss of heterochromatin during cellular senescence has been reported to be correlated with an increase in genetic instability leading to impaired double-strand-break repair capacity (Gorbunova and Seluanov, 2016). As referred above senescence-associated DNA foci SAHF can be observed using DAPI cell staining, usually displaying a more compact structure (YY Xiao *et al.*, 2020). OIS and replicative senescent mouse embryonic fibroblasts did not seem to display SAHF punctate (Kennedy *et al.*, 2010). This recent evidence, put in question SAHF specificity towards cellular senescence, suggesting that this may not be a suitable biomarker for single detection of senescence and instead should be combined with other biomarkers for a more reliable assessment (YY Xiao *et al.*, 2020) **(Figure 1)**.

Nuclear Lamins altered in senescent cells

As already mentioned earlier in **Chapter 1**, during cellular senescence, chromatin undergoes functional and structural alterations that include its distention and relocation to the nucleoplasm along with the dissociation between the inner nuclear membrane and centromeric repetitive sequences (Lukasova *et al.*, 2018). In one study, both OIS and replicative senescence were shown to induce Lamin B1 loss through direct stimulation of either the p53 or pRB pathway

present in primary human and murine cell strains. During apoptosis, lamins are degraded by caspases leading to the formation of lamin B1 degradation end-result products, however in this study, senescent cells did not display these products and caspase inhibition did not seem to influence the reported lamin B1 loss during cellular senescence (Freund *et al.*, 2012). In contrast, a senescence-related decrease in lamin B1 levels was attributed to the decline in lamin B1 mRNA stability (Freund *et al.*, 2012). In another study, Dreesen and colleagues further report a decrease in Lamin B1 protein in human dermal fibroblasts and keratinocytes during cellular senescence (Dreesen *et al.*, 2013). This decrease was shown to be attributed to the negative regulation of LMNB1 transcription and translation by miRNA-23a and was also observed in chronologically aged human skin tissue (Dreesen *et al.*, 2013). Interestingly, both overexpression and depletion of LMNB1 resulted in proliferation impairment but solely LMNB1 overexpression promoted cellular senescence. This could be explained due to the fact that during differentiation LBR and lamin-B1 function is taken over by lamin A/C with specific lamin A/C binding proteins (Lukasova *et al.*, 2018). This effect was further inhibited by telomerase-induced expression or p53 inactivation. In contrast, downregulation of both LMNB1 and LMNA/C aggravates the senescent phenotype (Dreesen *et al.*, 2013). Further, a study performed by Sadaie et al. reports that the induction of cellular senescence led to lamin-B1 specific depletion in the H3K9me3 enriched LADs heterochromatin regions, suggesting the promotion of SAHF formation (Sadaie *et al.*, 2013). Further, Sadaie et al. observed increased lamin-B1 binding to H3K27me3 marked gene-rich regions (Sadaie *et al.*, 2013). This evidence suggests that the spatial organization of chromatin is indeed a major factor not only in the regulation of genome functions but also in the modulation of gene expression according to external mechanical stimuli (Chandra *et al.*, 2015; Gilbert and Swift, 2019). More studies confirming the reduced expression of lamin B1 during cellular senescence as a result of the activation of the p53- and Rb axis include (Shimi *et al.*, 2011; Shah *et al.*, 2013). Despite this evidence, results from two independent studies suggest that lamins can be dispensable in this function given that cell cultures from lamin B1 and lamin B2 depleted mice lacking seemed to display preserved nuclear architecture even in the absence of lamin A/C (Kim *et al.*, 2011; SH

Yang *et al.*, 2011). This was thought to happen due to the fact that LBR can attach to other transmembrane domains of the inner nuclear membrane (Solovei *et al.*, 2013). Further evidence of the relevance of LBR down-regulation for the induction of cellular senescence is presented by a study done by Arai et al. which reports the induction of cellular senescence in HeLa and normal human diploid fibroblast TIG-7 cells was a result of LBR knockdown due to excess thymidine (Arai *et al.*, 2019). However, despite this evidence clearly showing the importance of both lamin-1 and LBR in the development of cellular senescence, there is also evidence that suggests that the down-regulation of these two proteins is not sufficient to induce cellular senescence in every cell types (Arai *et al.*, 2019). Specifically, Lukášová et al (2017) report that small hairpin ribonucleic acid (shRNA) mediated downregulation of lamin-1 and LBR in MCF7 and U2OS cells was not enough to induce senescence despite cells exhibiting a slower proliferation rate after cell cloning compared to the parental cells along with higher nuclear membrane permeability as a result of increased formation of micronuclei suggesting that other factors are required to trigger cellular senescence (Lukášová *et al.*, 2017).

Altogether these studies suggest that lamin B1 and LBR evaluation (e.g. RT-qPCR, Western Blotting, or immunohistochemistry) is a reliable methodology for cellular senescence detection, however, specificity and sensitivity still need improvements.

Senescence-Associated Secretory Phenotype (SASP)

As discussed earlier in **Chapter 1**, SASP mediators are a long list of compounds that mainly include cytokines, chemokines, proteinases, and growth factors (**Chapter 1**) (Ohanna *et al.*, 2011). SASP profile of each senescent cell varies according to cell type, tissue microenvironment, senescent inducing stimuli, and damage extension (**Figure 1**)(Faget *et al.*, 2019). IL-6 and IL-8 are considered to be ubiquitous senescence representative markers (Kuilman *et al.*, 2008; Hernandez-Segura *et al.*, 2018) given their presence in various senescent cell types such as damage-induced senescent fibroblasts, melanocytes, and epithelial cells DNA

(Lu *et al.*, 2006; Green, 2008; YZ Xiao *et al.*, 2020). However, both IL-6 and IL-8 are not senescence-exclusive since they seem to be involved in other physiological and pathological processes such as inflammation and regulation of the immune system (Ramani *et al.*, 2015). Studies reveal IL-6 and IL-8 as essential elements in the NF-κB DDR-induced activation (Takahashi *et al.*, 2012; Hayakawa *et al.*, 2015) and as factors in OIS inflammatory transcriptome (Kuilman *et al.*, 2008). IL-6 interacts with the transcription factor C/EBPβ in a paracrine manner to amplify the activation of the inflammatory network, which includes IL-8 (Kuilman et al., 2008). Besides, IL-6 and IL-8, a small group of mediators including (however not limited) chemokine (C-X-C motif) ligand 1 (CXCL1), Chemokine (C-C motif) ligand 20 (CCL20), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and metalloproteinases have been considered universal SASP representatives as they have been found to be expressed in most senescent conditions (Hernandez-Segura *et al.*, 2017; Wiley *et al.*, 2017; Sun *et al.*, 2018), which begs the question of whether they can be used as reliable senescence detecting biomarkers? Unfortunately, in resemblance with IL-6 and IL-8, evidence of the presence of all of these mediators in non-senescent conditions can be found throughout the literature rendering them not specific (Becher *et al.*, 2016; Wang *et al.*, 2017; Furue *et al.*, 2020). Thus, SASP factors can be detected through a variety of assays including Western blot, ELISA assay using commercial kits even immunostaining with antibodies targeting each SASP factor followed by High Content Microscopy Analysis (Hari and Acosta, 2017). Overall, SASP is seen as a senescence biomarker due to its crucial role in the development and biological impact of cellular senescence, however due to its high heterogeneity, its absence in particular cases and the presence of its mediators in non-senescent conditions (Coppe *et al.*, 2011; Ohanna *et al.*, 2011) renders it not suited to solely be a cellular senescence biomarker. However, it might be used as a confirming biomarker and since its heterogeneity is somewhat originated by its variation according to cellular senescence mechanisms (**Figure 1**), it could also be used as a differential biomarker.

Deep Learning-Based Senescence Scoring System by Morphology (Deep-SeSMo)

Deep learning is a form of Artificial Intelligence that specializes in the development and deployment of a subset of machine learning algorithms referred to as multilayered artificial neural networks that can extract higher-level features and recognize patterns from different kinds of raw input data to make classifications in accordance. Deep learning models are usually trained using supervised learning approaches in which datasets are labeled according to a classification outcome variable. Models learn to associate specific features to each outcome during training that allow subsequent classification of unlabeled data (Esteva *et al.*, 2019). Convolutional neural networks (CNNs) are commonly used models for data that display natural spatial invariance such as images. The deep learning field has been evolving exponentially due to significant increases in computational data processing and manipulation capacity along with more robust and cheaper technology for data generation, seen in the last decades. The ongoing increase in the generation of a diverse range of high throughput biomedical data has already rendered successful applications of Deep learning models in research and the clinic namely medical image-based detection of skin cancer (Esteva *et al.*, 2017), breast lesions and pulmonary nodules (Cheng *et al.*, 2016), early detection of diabetic retinopathy (Gulshan *et al.*, 2016), Alzheimer's Disease diagnosis and many more (Jo *et al.*, 2019). CNN's deep learning models have also been trained to distinguish between different types of cells based on cell feature-specific assessment through microscopy (Buggenthin *et al.*, 2017; Christiansen *et al.*, 2018; Kusumoto *et al.*, 2018; Kusumoto and Yuasa, 2019; Moen *et al.*, 2019).

Kusumoto and colleagues developed a cellular senescence morphology-based classifier algorithm for a convolutional multi-layer neural network system, using input datasets composed of single-cell resolution level 50×50 pixels phase-contrast images of non-senescent and senescent Human umbilical vein endothelial cells (HUVECs) cells induced through H_2O_2 or anti-cancer agent camptothecin exposure or repetitive passage (Kusumoto et al. 2021). Since both senescence rate and the average senescence classification probability were proportional to the intensity of cellular senescence inducting stimulus, authors were able to establish a quantitative senescence score calculated by the pre-trained CNN that was named Deep Learning-Based Senescence Scoring System by Morphology (Deep-SeSMo) (Kusumoto *et al.*,

2021). Pre-trained CNNs were further successfully used to distinguish between senescent and non-senescent cells in HUVEC datasets acquired from two institutes. Authors were also able to train the CNN to efficiently classify senescence in H_2O_2 or camptothecin-treated human diploid fibroblasts. Four anti-senescent candidate compounds (daidzein, PD-98059, Y-27632·2HCl, and terreic acid) were identified using Deep-SeSMo to quantify senolytic activity of individual compounds from a kinase inhibitor screened in HUVEC. Overall, Deep-SeSMo was shown to be a reliable tool for detecting cellular senescence in HUVEC cells and fibroblasts and might also display senescence detection capacity in other cell types. Thus, trained multilayered artificial neural network deep learning algorithms are a promising classification tool for cellular senescence detection and in the near future, we should see further improvements in their classification capacity.

B. Senolytic mouse models developed for cellular senescence study

Although many cell features have been associated with the development of cellular senescence, the majority of the characterization has been made through *in vitro* experiments. Some of these models include high passaged primary cultures of human cells as replicative senescence models, the use of irradiation, chemotherapeutic drugs, or oxidative reagents such as hydrogen peroxide to generate stress-induce senescence culture models or activation of oncogenes such as RAS to create in vitro OIS models (Beck *et al.*, 2020; Yuan *et al.*, 2020). Until very recently, senescence characterization *in vivo* was very limited given the shortage of appropriate tools for *in vivo* accurate senescent cell isolation and identification. Since culture conditions often do not mimic the natural environment where cell behavior and response are subjected to the influence of signals from the rest of the body, *in vitro* studies' findings might not match in vivo observations (Lidzbarsky *et al.*, 2018). As such, efforts have been made to develop *in vivo* models where cellular senescence can be observed and senescent cells' clearance can be controlled to further characterize cellular senescence features, better understand both the pathological and physiological roles of cellular senescence and assess the effect of senescent

cell removal on the overall organism (Pajvani *et al.*, 2005; Demaria *et al.*, 2014; Hashimoto *et al.*, 2016; Omori *et al.*, 2020).

INK-ATTAC

To study the effect of senescence cell clearance in the adipose tissue, Baker and colleagues developed a genetic mouse model based on the earlier produced Fat apoptosis through targeted activation of caspase (FAT-ATTAC) mouse line (Pajvani *et al.*, 2005). This model allowed for selective adipocyte apoptosis upon the administration of the synthetic drug named AP20187 which triggers the dimerization of a membrane caspase named myristoylated FK506-binding-protein–caspase 8 (FKBP–Casp8) fusion protein (Pajvani *et al.*, 2005). This protein is expressed through the *Fabp4* promoter which was replaced by a 2,617-bp fragment of the $p16^{Ink4A}$ gene promoter. As mentioned earlier, $p16^{Ink4A}$ is one of the most specific and sensitive biomarkers of cellular senescence as it is reported to be upregulated in almost all senescent cells. For clear detection and distinction of $p16^{Ink4A}$ senescent cells, it was added an internal ribosome entry site followed by an open reading frame coding for enhanced green fluorescence protein (EGFP) (Baker *et al.*, 2011). This mouse model was named *INK-ATTAC, and to test its effect on age-related comorbidities it was bred with the BubR1* hypomorphic ($BubR1^{H/H}$) cell line a gene known to code for a crucial member of the mitotic checkpoint, that is responsible for ensuring proper chromosome segregation during mitosis. $BubR1^{H/H}$ mice are known to accumulate $p16^{Ink4a}$-positive cells in specific tissues which might be correlated with the early development of age-related pathological features including cardiac arrhythmias, fat loss, sarcopenia, lordokyphosis, arterial wall, and impaired wound healing (Baker *et al.*, 2004, 2011). *INK-ATTAC* was shown to significantly kill $p16^{Ink4a}$-positive senescent cells in adipose tissue, skeletal muscle, and eye *of* $BubR1^{H/H}$ mice and delayed the onset and progression of age-related dysfunctions including such cataracts and lordokyphosis (Baker *et al.*, 2011). This technique has since been used to study the role of senescent cells in various conditions in mice such as senescent glial cells' role in tau-dependent pathology and cognitive decline (Bussian *et al.*,

2018), age-related bone loss (Farr *et al.*, 2017) or in age-dependent hepatic steatosis (Ogrodnik *et al.*, 2017).

p16-3MR (trimodality reporter)

Another $p16^{INK4A}$ targeting senolytic mechanism was developed in 2014 by Demaria and colleagues. Mice were transfected with an engineered bacterial artificial chromosome where the $p16^{INK4A}$ controls the expression of truncated herpes simplex virus 1 thymidine kinase (HSV-TK), the 3MR (trimodality reporter) fusion protein expression (composed of a fluorescence reporter gene mRFP and the functional domains of bioluminescence reporter gene called synthetic *Renilla* luciferase (LUC) that serves as a luminescence detector of 3MR-expressing cells(Ray *et al.*, 2004). The administration of a cytotoxic drug called ganciclovir kills 3MR expressing cells due to its conversion, by the action of HSV-TK, into toxic DNA chain terminator that leads to fragmentation of mitochondrial DNA and consequently apoptosis (Demaria *et al.*, 2014). In this study Demaria et al. used this model to assess the physiological role of senescent cells *in vivo* (which we already discussed in this review), and were able to gather solid evidence that a few days after a cutaneous injury, senescent endothelial cells and fibroblasts can be seen at the wound site (Demaria *et al.*, 2014). These cells were shown to promote wound closure by inducing myofibroblast differentiation through the secretion of the SASP factor PDGF-AA. p16-3MR mice treated with ganciclovir displayed a significant wound closure delay in comparison with the wild-type and vehicle-treated p16-3MR mice supportinga role for SASP and cellular senescence in tissue repair (Demaria *et al.*, 2014).

$p19^{ARF}$ Toxin Receptor-mediated Cell Knockout

More recently, Hashimoto and colleagues produced a mouse model using a different marker called $p19^{ARF}$ ($p14^{ARF}$ mouse homolog) known to be a cell cycle regulator expressed through the *ARF* promoter that plays a crucial role in the induction of cellular senescence in mice. In this study, senescent cells positive for $p19^{ARF}$ were successfully ablated in lung tissue from a transgenic model based on a described cell clearing mechanism using a toxin-mediated cell

knockout system called Toxin Receptor-mediated Cell Knockout (TRECK) improving the age-related decreased lung function and reversing aging-associated gene expression profile (Hashimoto *et al.*, 2016). In this model, expression of a diphtheria toxin receptor along with the bioluminescent enzyme luciferase is under the control of a tissue-specific promoter (in this case of *ARF* promoter), as administration of the diphtheria toxin leads to $p19^{ARF}$ positive senescent cells death (Hashimoto *et al.*, 2016).

p16-Cre^{ERT2}-td Tomato mouse model

In a recent study, Omori and colleagues generated a new mouse model for the study of cellular senescence through single-cell analysis *in vivo called* p16-CreERT2-tdTomato mice. To generate this model, CreERT2 was inserted into the endogenous Ink4a locus (p16 locus) of C57BL/ 6 embryonic stem cells through homologous recombination followed by an SV40 polyadenylation site to exon 1α (first exon of the Ink4a locus) a methodology described by Burd and colleagues (Burd *et al.*, 2013). $p16^{Ink4a}$-CreERT2 mice were then crossed with Rosa26-CAG-lsl-tdTomato (basic orange constitutively fluorescent protein) mice (Iwasaki *et al.*, 2018) originating p16-CreERT2-tdTomato mice (Omori *et al.*, 2020). This model was revealed to be highly efficient in detecting cells with high expression of p16 in combination with tamoxifen administration (controller of Cre activity) (Omori *et al.*, 2020). In this study, cells with p16 high expression, impaired proliferation, and half-lives between 2.6 to 4.2 months were increased in aged mice, being detected in all organs. Single-cell RNA-sequencing of kidney and liver tissues revealed that cells with high expression of p16 displayed widely heterogeneous senescent phenotype and were found in hepatic endothelium, renal proximal and distal tubule epithelial, and manny other cell types (Omori *et al.*, 2020). Also, the elimination of cells with high expression of p16 in a nonalcoholic steatohepatitis mouse model prevented related hepatic lipidosis and ameliorated inflammation (Omori *et al.*, 2020).

V. Senotherapy & Aging-related diseases

As already mentioned, cellular senescence is one of the 9 hallmarks of aging identified in the famous paper by López-Otín and colleagues (Lopez-Otin *et al.*, 2013). Also, age-related

senescent cell accumulation in tissues has been suggested to be a major contributor to the development of several aging-associated pathologies such as cancer, osteoarthritis, Alzheimer's disease type 2 diabetes, atherosclerosis, cataracts, and pulmonary fibrosis (Childs *et al.*, 2015). In the last few years, efforts have been made to take advantage of the current knowledge regarding senescence mechanisms to produce several therapeutic strategies in order to treat aging-related conditions. These strategies can be divided into three main categories (Shetty *et al.*, 2018):

i) preventive non-pharmacological interventions that ameliorate the age-related senescent cell accumulation in tissues (e.g. caloric restriction)

ii) pharmacological interventions for SASP modulation, referred to as senomorphics.

iii) pharmacological interventions, referred to as senolytic therapies, that promote senescent cell death in order to decrease the senescent cell burden in tissues.

While still in early stages, some compounds have already shown senolytic and senomorphic potential in pre-clinical studies and others have already been submitted to clinical experimentation which we are going to be discuss below. **Table 2** summarizes the currently known features of these compounds.

Cellular Senescence & Caloric Restriction (CR)

The first evidence of the longevity and health-promoting effects of caloric restriction (CR) comes from experiments performed on rats in 1935 by McCay et al. (McCay *et al.*, 1935). Since then, CR implementation featuring essential nutrient intake has been shown to extend lifespan and healthspan preventing or delaying the onset of many age-associated chronic diseases in multiple model organisms including fruit flies, worms, yeast, many rodents, and non-human primates (Fontana *et al.*, 2010; Mattison *et al.*, 2017; Fontana, Nehme, *et al.*, 2018). Pioneer randomized clinical trials for the assessment of CR effect in aging and longevity biomarkers in nonobese human subjects were initiated by the US National Institute of Aging as part of a research program called Comprehensive Assessment of the Long-term Effects of Reducing

Intake of Energy 1 (CALERIE-1). These studies showed that 6 months to 1-year Implementation of CR with essential nutrient intake in overweighted (but not obese) men and women was associated with decreased risk for cardiovascular diseases, loss of weight, improved insulin sensitivity, reduced day-time and night-time core body temperature, downregulation of inflammatory markers, alterations in the circulating levels of many hormones including a decrease in the levels of insulin, leptin, testosterone, estradiol, and triiodothyronine and an increase in the levels of adiponectin and cortisol (Most *et al.*, 2017). In following clinical trials (CALERIE-2) healthy non-obese young and middle-aged (mean age 38) men and women submitted to a two-year CR intervention displayed reduced whole-body oxidative stress indicated by the concentration of urinary F2-isoprostanes (a marker of nonenzymatic lipid peroxidation in humans that is formed from polyunsaturated fatty acids) (Yang *et al.*, 2016; Il'yasova *et al.*, 2018). Another CALERIE-2 study revealed a significant increase in serum IGFBP-1 and a reduction IGF-1:IGFBP-1 ration, leptin, and T3, after 2 years of CR (Fontana *et al.*, 2016). Also, the skeletal muscle tissues of healthy and lean men and women (mean age 52.3 ± 11 years) submitted to a CR diet for 3–15 years displayed increased levels of key chaperones for the maintenance of proteostasis levels including HSP70 and Grp78 along with increased protein and mRNA levels of autophagic mediators such as beclin-1 and comparatively to control volunteers (Yang *et al.*, 2016).

Despite all this evidence, the exact mechanisms that underlie the beneficial effects of CR in humans and the conditions under which they are maximized have not been entirely described (Fontana, Nehme, *et al.*, 2018; Austad and Hoffman, 2020). However, contrary to what was previously thought, it is now accepted that the beneficial effects of CR do not only result from the decreased ROS production as a result of low metabolic rate but also from active regulation of defense mechanisms against oxidative stress (Fontana, Nehme, *et al.*, 2018). Interestingly the evidence in both animal models and human studies suggests that CR might be a considerable strategy for preventing abnormal senescent cell formation seen during aging (Fontana, Nehme, *et al.*, 2018). Studies indicate that CR can stimulate the activity of some members of the sirtuin protein family including SIRT1 and SIRT3 which are known to be essential factors in the

antioxidant response (Fontana, Nehme, *et al.*, 2018). CR promotes SIRT3 expression in mice which deacetylates two critical lysine residues on SOD2 and promotes its antioxidative activity (Qiu *et al.*, 2010). Further, downregulation of SIRT3 can cause mitochondrial dysfunction which can lead to cellular senescence (Wiley *et al.*, 2016). This suggests that CR might possess an anti-senescent effect by promoting antioxidant response through sirtuin upregulation (Wiley *et al.*, 2016; Fontana, Nehme, *et al.*, 2018). As previously discussed, in some conditions, autophagy can have an anti-senescent effect by eliminating damaged macromolecules and organelles **(Chapter 1)**. As mentioned above, CR can upregulate several proteins required for this process to function correctly which might be a factor in the CR-mediated prevention of the onset of cellular senescence (Yang *et al.*, 2016; Fontana, Nehme, *et al.*, 2018). CR has also been shown to prevent the age-related decrease in the function of DNA repair mechanisms in model organisms such as the non-homologous end joining, nucleotide excision repair , and base excision repair pathway. This might also contribute to the anti-senescent of CR since it can help prevent DNA damage, a known hallmark of cellular senescence (Fontana, Nehme, *et al.*, 2018). As mentioned above, 2 years of CR implementation in humans has been shown to be associated with an increase in serum IGFBP-1 and a decrease in IGF-1:IGFBP-1 ratio (Fontana *et al.*, 2016). This can downregulate IGF-1 signaling which might produce an anti-senescent effect since chronic exposure to IGF-1 has been shown to induce premature senescence by the regulation of the SIRT1-p53 pathway (Tran *et al.*, 2014; Fontana, Nehme, *et al.*, 2018). CR can also mitigate the activity of mTOR in rodents that, as already mentioned in this review, is a known SASP regulator and its activation is associated with the induction of senescent phenotype (Fontana, Nehme, *et al.*, 2018; Tulsian *et al.*, 2018; Chen *et al.*, 2019). Indeed, obese mice submitted to a 30% CR diet for 2 months displayed significant decreased levels of adipose tissue cytokines and chemokines known as important SASP factors such as IL-1Rα, IL-2, IL-6, CXCL16, and Monocyte Chemoattractant Protein-1 (MCP-1) (Kurki *et al.*, 2012). Further, kidney tissue from aged mice submitted to 10 days of 40 % CR displayed lower levels of pro-inflammatory mediators such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase

(iNOS), IL-1β, IL-6, and NF-κB (Jung *et al.*, 2009; Kim *et al.*, 2020). Lastly, studies suggest that CR can reduce senescence markers in the human colon (Fontana, Mitchell, *et al.*, 2018).

A. Senomorphics

Several senomorphics compounds have been shown to contain the propagation of the senescence phenotype by regulating of SASP content (Martel *et al.*, 2020). As mentioned earlier, SASP is regulated by a wide range of signaling pathways and transcription factors. Pharmacological targeting of these regulating elements can result in a significant disruptive effect in SASP. Due to SASP's high heterogeneity, each compound should be carefully studied taking into account that its effect might vary according to the cellular lineage or environmental context (Sieben *et al.*, 2018; Sun *et al.*, 2018). Chronic secretion of SASP pro-inflammatory mediators is involved in the development of a sterile systemic inflammation environment during aging referred to as inflammaging. Therefore, the pharmacological downregulation of SASP proinflammatory mediators is expected to act as a preventive measure against the development of inflammaging related pathologies. It is worth noting that most of the compounds included in this category have been approved for the treatment of other conditions (Zhu *et al.*, 2020). In this subchapter we discuss what is currently known about each compound that has been proven to possess senomorphic activity.

Metformin

Metformin is a US Food and Drug Administration (FDA)-approved insulin sensitizer administrated orally that has been used for the treatment of type 2 diabetes mellitus for more than 60 years. (Newman *et al.*, 2016). Despite its successful use, metformin's pharmacokinetic properties have not been completely unveiled, however, animal and human studies have shown that metformin can inhibit gluconeogenesis in the liver through impairment of mitochondrial redox shuttle (Flory and Lipska, 2019). Also, it is thought that the metformin effect is also extended to the gut lumen through various mechanisms (Flory and Lipska, 2019). It is worth noting that 25% of patients under metformin treatment have shown some adverse effects such as

nausea and diarrhea although these might be alleviated through careful dose titration (Bonnet and Scheen, 2017; Flory and Lipska, 2019). Also, 60 years of clinical data have not raised any safety concerns for the use of metformin apart from lactic acidosis caused by intake above the recommended dose or by patient's predisposal conditions for lactic acidosis such as chronic kidney disease, hepatic impairment, and heart failure (Crowley *et al.*, 2017; Flory and Lipska, 2019).

A systematic review found that the administration of the insulin sensitizer metformin in diabetic patients was correlated with a decrease in cancer rate and cardiovascular disease onset and overall appeared to extend the life span and health span of patients independently of its effect on diabetes (Campbell *et al.*, 2017). Evidence suggests that these beneficial outcomes might be mediated by an anti-senescence effect. In a recent study, one of the mechanisms that might be behind metformin's prevention of cardiovascular disease through the prevention of cellular senescence was unveiled (Karnewar *et al.*, 2018). In this study, metformin was shown to coordinate the delay of endothelial senescence through mitochondrial function and biogenesis stimulation. The increased expression of AMPK as a result of metformin administration promoted the trimethylation of H3K79 resulting in SIRT3 upregulation. SIRT3 expression is known to promote PGC-1α expression which was shown to enhance the expression of hTERT (telomerase catalytic subunit) delaying endothelial senescence (Karnewar *et al.*, 2018). Metformin has also been shown to inhibit the expression of genes coding for multiple inflammatory cytokines seen during cellular senescence (Moiseeva *et al.*, 2013). In this study, the administration of metformin on prostate cancer cultures prevented the growth-stimulating effect of conditioned medium from senescent cells (Moiseeva *et al.*, 2013). Metformin impaired NF-κB translocation to the nucleus and prevented the activation of the NF-κB pathway by inhibiting the phosphorylation of IκB and IKKα/β (Moiseeva *et al.*, 2013). As already mentioned earlier, NF-κB is one of the main regulators of SASP, which might suggest that metformin administration might cause its disruption leading to SASP downregulation (Moiseeva *et al.*, 2013). Metformin was shown to possess therapeutic potential against intervertebral disc degeneration after its administration to model mice resulted in a reduction in

disc degeneration. *in vitro* experiments revealed that this protective effect was in part mediated by prevention of nucleus pulposus cells senescence or apoptosis through autophagy stimulation (Chen *et al.*, 2016). Another anti-senescence effect of metformin is thought to result from its capacity to upregulate the endoplasmic reticulum-localized glutathione peroxidase which is usually decreased in senescent cells (Fang *et al.*, 2018). This was shown to be mediated by the metformin-induced accumulation of the nuclear factor erythroid 2-related factor 2 which is able to attach to the antioxidant response elements in the GPX7 gene promoter promoting its expression (Fang *et al.*, 2018).

Mammalian target of rapamycin (mTOR) suppressant rapamycin

MTOR is a sensor of nutrient and growth signals whose relationship with aging and cellular senescence is widely described in the literature (Weichhart, 2018) however here we are only focusing on evidence regarding MTOR influence over the SASP. mTOR inhibition is known to increase the lifespan of most model organisms, although the responsible mechanism is not yet entirely clear (Weichhart, 2018). However, mTORC1 (mTOR complex) inhibition namely through rapamycin has been shown to downregulate SASP through a variety of mutually non-exclusive mechanisms (Weichhart, 2018). The RNA-binding protein ZFP36L1 can inhibit the transcription of many SASP mediators, however, during senescence, mTORC1 upregulation promotes the translation of MAP kinase-activated protein kinase 2 (MAPKAPK2) that phosphorylates ZFP36L1 leading to SASP expression which is, therefore, subjected to inhibition by rapamycin administration (Herranz *et al.*, 2015; Weichhart, 2018). Further, rapamycin has also been shown to prevent the secretion of pro-inflammatory SASP mediators by inhibiting IL-1A translation resulting in the downregulation of pro-inflammatory genes regulated by NF-κB (Laberge *et al.*, 2015; Weichhart, 2018). In another study, rapamycin was shown to downregulate SASP independently from its Nrf2 (a pro-longevity signaling pathway shown to be decreased during cellular senescence-mediated cell cycle arrest) (Volonte *et al.*, 2013; Wang *et al.*, 2017; Weichhart, 2018).

Rapamycin is a macrolide known to inhibit mTOR that is produced by *Streptomyces hygroscopicu* bacterium, originally discovered in soil samples from Easter island (Vézina and Kudelski, 1975). It has been shown to impair protein translation in T-cells through the inhibition of the incorporation of amino acids into proteins leading to a decrease in their proliferation rate. Also, rapamycin has a similar structure to that of FK506 which is another macrolide known to inhibit IL-2 production and calcineurin activity in T cells (Martel *et al.*, 1977; Bierer *et al.*, 1991; Arriola Apelo and Lamming, 2016). in fact, the FDA has already approved rapamycin for immunosuppressive treatment during solid organ transplantation and its analogs are referred to as "rapalogs" temsirolimus and everolimus for the treatment of advanced renal cancer carcinoma. In 2011, everolimus was also approved for the treatment of progressive neuroendocrine tumors of pancreatic origin (Li *et al.*, 2014). However, despite the pro-longevity effects shown in model organisms, it has shown some side effects in rodents including increased rates of kyphosis and cataracts. In humans, rapamycin and rapalogs administration has been associated with the development of aphthous ulcers and metabolic dysfunction along with impaired wound healing (Newman *et al.*, 2016). As such, possible future rapamycin-based semomorphic therapeutics should be designed to circumvent these adversities.

JAK inhibitors

Evidence suggests that the JAK pathway is one of the main regulators of SASP-associated pro-inflammatory cytokine production. This pathway has been found to be highly activated in aged adipocytes and its inhibition with a 3-day ruxolitinib treatment could prevent proinflammatory SASP secretion from preadipocytes and endothelial cells by 40–60% while at 10-day treatment aged mice showed reduced frailty and inflammation (Xu, Tchkonia, *et al.*, 2015). Ruxolitinib is a JAK1/2 inhibitor that was approved by the FDA and European Medicines Agency (EMA) for the treatment of myelofibrosis in 2011 and 2012 respectively and later in 2014 for the treatment of hydroxyurea (HU)-resistant or -intolerant polycythemia vera. Both these pathologies are myeloproliferative neoplasms characterized by the abnormal activation of the JAK-STAT pathway (Ajayi *et al.*, 2018). It has also shown great results *in vitro* and *in vivo* research

revealing a great senotherapeutic potential (Zhu *et al.*, 2020) In human progerin expressing fibroblasts, ruxolitinib was shown to reverse cellular senescence traits including cell cycle arrest aberrant nuclei shape and in progeric mice delayed the onset of aging phenotypes, including bone mineral content, bone fractures and loss of grip strength (Griveau *et al.*, 2020). Further, in another study, aged mice (22-month-old) submitted to a ruxolitinib 8-week treatment displayed increased insulin sensitivity, reduced circulating activin A (adipogenesis downregulating hormone shown to be highly secreted by primary human senescent fat progenitors), reduced lipotoxicity and preserved fat mass (Xu, Palmer, *et al.*, 2015). However, a clinical study that aimed to assess the therapeutic effect of ruxolitinib in myelofibrosis patients, despite promoting a reduction in associated frailty also reported some side effects such as thrombocytopenia and anemia (Verstovsek *et al.*, 2012). Also, since ruxolitinib is metabolized by Cytochrome P450 Family 3 Subfamily A Member 4 (CYP3A4) its administration also limits the simultaneous use of potent CYP3A4 inhibitors with the risk of side effect aggravation (Ajayi *et al.*, 2018). Therefore, despite the great potential displayed by this SASP inhibiting drug, translation to a clinical application should require great optimization in treatment strategy.

B. Senolytic treatments

For years now, researchers have been looking for ways to disrupt the senescence process in order to delay aging and treat aging-related diseases. However, as further discussed in previous chapters, senescent cells do not always have nefarious effects on tissues, in fact, it is well known that transient senescence plays a role in some physiological processes (**Figure 2**). Also, induction of cellular senescence can prevent dysfunctional cells from proliferating which could potentially be harmful to the organism, as with the case of tumor cells (Di Micco *et al.*, 2006; Kuilman *et al.*, 2010; Rufini *et al.*, 2013). So, to circumvent this conundrum, more advanced senolytic therapies were developed with the intent of killing the already existing senescent cells instead of preventing their formation. However, there are still challenges that need to be addressed to translate these therapeutic strategies to the clinic (Palmer *et al.*, 2019). These include: i) the already mentioned need for stablisment specific and reliable senescence

biomarkers in order to unmistakably identify senescent cells; ii) to investigate possible variations of the senolytic therapies effect in the various diseases stages along with the fluctuation of the formation/reestablishment of cellular senescence according to the variation of possible age-related insults; iii) the long term safety of these therapies.

In this subchapter, we discuss what is currently known about each compound that has been proven to possess senolytic activity.

B-cell lymphoma/leukemia-2 (BCL-2) protein family inhibitors

To prevent cell death as a result of external or their apoptotic SASP signaling, senescent cells use different but inter-related senescent cell anti-apoptotic pathways (SCAPs) to maintain their viability which has been shown to include the p53-p21 and PI3K-Akt pathways as well as other intervenient proteins such as tyrosine kinases, ephrins, and serpine (Zhu *et al.*, 2015; Hu *et al.*, 2022). Other important regulators in apoptosis resistance in cellular senescence are the B-cell lymphoma/leukemia-2 (BCL-2) protein family including BCL-2 (the first element of this family to be discovered), BCL-XL, BCL-W, MCL-1, and A1 (Wang, 1995). Proteins from this family are characterized by a conserved BH3 region (an interactive site composed of 16–25 amino acids) that has since been targeted by a variety of inhibiting compounds, some displaying senolytic activity which we will further discuss below (Anantram and Degani, 2019).

ABT-737

In 2005, Abbott laboratories developed the first BH3 mimetic, a synthetic small-molecule called ABT-737 (Oltersdorf *et al.*, 2005). This compound can bind with high affinity ($K_i < 1$ nM) to Bcl-2, Bcl-w, and Bcl-xL but with low affinity ($K_i > 460$ nM) while binding to other members of the antiapoptotic BCL-2 family members, and was proven to display effective antitumorigenic activity in cancer cell lines (Anantram and Degani, 2019). Its senolytic capacities were indicated by a study where it was able to induce lung and epidermis senescent cell apoptosis by Bcl-xL and Bcl-W inhibition leading to an increase in hair-follicle stem cell proliferation (Yosef *et al.*, 2016). Further, a recent study found that ABT-737 treatment was

able to significantly eliminate Cox2-expressing senescent cells in mice reducing tumor development (Kolodkin-Gal *et al.*, 2022). However, ABT-737 showed poor oral bioavailability in animal models and its low aqueous solubility prevents intravenous delivery.

Navitoclax (ABT-263)

Navitoclax is an orally active homolog of ABT-737 that was generated through modifications in 3 important backbone regions (Anantram and Degani, 2019). Due to its low aqueous solubility, Navitoclax displays a prolonged dissolution rate-limited oral absorption. This compound was shown to possess low plasma clearance values, low volumes of distribution in mice, rats, monkeys, and dogs (bioavailability after oral gavage of 20%), and plasma elimination half-lives after i.v. dose of 4.6 to 8.4 hours. bioavailability and oral elimination are increased up to 50% and 8.9 hours respectively in dogs if the compound is orally administered in lipid-based formulations (Tse *et al.*, 2008). This anti-cancer drug is also an inhibitor of the BCL-2 protein family including Bcl-2, Bcl-xL, and Bcl-w (Zhu *et al.*, 2016). Together, in 2016 two independent studies showed that this compound was able to induce apoptosis in cultured senescent human umbilical vein epithelial cells, IMR90 human lung fibroblasts, murine embryonic fibroblasts and reduce the levels of senescent bone marrow hematopoietic stem cells and senescent muscle stem cells in normally aged and sublethally irradiated mice, however, failed to induce apoptosis in human senescent preadipocytes (Chang *et al.*, 2016; Zhu *et al.*, 2016). In a more recent studies, the administration of Navitoclax attenuated the post-traumatic osteoarthritis incidence through selective senescent cell clearance in the cartilage tissue along with the decrease in the production and secretion of SASP cytokines (Yang *et al.*, 2020) and ameliorated osteoarthritis thermal and mechanical hyperalgesia and peripheral sensitization in a mouse model of osteoarthritis aged mice through reduction in nociceptive neuron projection to the synovium, expression of axon guidance proteins such as nerve growth factor NGF/TrkA, decrease in nociceptive neuron projection to the synovium and knee joint angiogenesis along with reduction in dorsal root ganglion and subchondral bone marrow (Gil *et al.,* 2022). Also, Navitoclax administration to osteoarthritis chondrocyte monolayer and 3D pellet culture

triggered senescent cells' apoptosis resulting in the stimulation of cartilage matrix aggregation along with the decrease in the expression of inflammatory cytokines (Yang et al., 2020). Post-traumatic osteoarthritis-related subchondral bone and cartilage deformations were alleviated by ABT263 intra-articular injection (Yang *et al.*, 2020). Navitoclax administration was also found to deplete the senescent cell burden from atherogenic plaques of atherosclerotic mice (Childs *et al.*, 2016). Despite these beneficial effects, toxic side effects such as transient neutropenia and thrombocytopenia have been associated with pre-clinical and clinical navitoclax administration (Rudin *et al.*, 2012; Chang *et al.*, 2016). However, a recent study by Lim et al. reports promissing results about a new strategy that could significantly reduce these toxic side effets (Lim *et al.*, 2022). In this study, navitoclax was loaded into poly(lactic-co-glycolic acid) nanoparticles and intradiscally administered into injury-induced intervertebral disc degeneration rat models promoting Intervertebral disc navitoclax local delivery. This strategy promoted selective amelioration of senescent cell burden and downregulation of pro-inflammatory cytokines and matrix proteases from degenerative mouse intervertebral disc leading to retardation of progression of intervertebral disc degeneration, and even restructuring of the intervertebral disc structure (Lim *et al.*, 2022). Further, a drug combinatorial strategy involving the simultaneous administration of navitoclax and pan-mTOR inhibitors such as PP242 and AZD8055 was shown to decrease the requeired dosage or timespan of navitoclax needed for reaching IC50 and LT50 in senescent cells while extending the lifespan of premature-aged Drosophila and delaying the onset of aging-related phenotype (Xu *et al.*, 2022).

A1331852 & A1155463

In the earlier mentioned study, the BCL-XL-selective inhibitors A1331852 and A1155463 also displayed senolytic activity in umbilical vein epithelial cells and IMR90 human lung fibroblasts (Y Zhu *et al.*, 2017). Given that these compounds only inhibit BCL-XL, they might benefit from the lack of neutrophil toxicity that was generated by navitoclax-associated BCL-2 inhibition.

Dasatinib & Quercetin

This drug combination is one of the most widely used senolytic therapy for cellular senescence study in model mice and in fact, human clinical trials are currently taking place to assess its potential for the treatment of age-related diseases associated with increased senescent cell burden. Dasatinib (D) is a tyrosine kinase inhibitor that has been previously used for cancer treatment and is known to target the EFNB-dependent suppression of apoptosis which lead to cell apoptosis of particularly senescent human adipocyte progenitors. In contrast, Quercetin (Q) is a plant flavonol, that targets mostly the SCAP present in human endothelial cells and mouse bone marrow-derived mesenchymal stem cells which include the ones mediated by BCL-2 protein family, HIF-1α, PI3-kinase or p21. Further, its administration on HFD submitted mice was proven to decrease fibrosis of renal tissue along with cellular senescence markers and to promote the increase in renal oxygenation and creatinine levels (Kim *et al.*, 2019). Given that neither Q targets senescent human adipocyte progenitors efficiently nor D targets senescent human endothelial cells, a combination of the two drugs (D + Q) was established as a potential senolytic treatment by Zhu and colleagues in 2015 (Zhu *et al.*, 2015). More studies since then have been published, proving the senolytic effect of this treatment in many more mouse cells and tissues such as senescent embryonic fibroblasts, Aβ-induced senescent oligodendrocyte progenitor cells, skeletal muscle tissue of old mice, articular cartilage and synovium from osteoarthritis mouse models, in the liver of hepatocellular carcinoma mouse models and in in ovaries of obese mice (Demaria *et al.*, 2017; Nath *et al.*, 2018; Xu *et al.*, 2018; PS Zhang *et al.*, 2019; Dungan *et al.*, 2022; Gil *et al.*, 2022; Hense *et al.*, 2022; Thadathil *et al.*, 2022).

D + Q was submitted to its first clinical trial in January 2019 where 14 patients with idiopathic pulmonary fibrosis (fatal senescence-associated disease) showed improved physical function along with decreased levels of SASP (Justice *et al.*, 2019). Later in September 2019, another clinical trial revealed decreased SASP factors in the skin biopsy specimens of patients suffering from systemic sclerosis, upon continuous D treatment (Martyanov *et al.*, 2019). In the same month, for the first time, it was shown that D + Q decrease senescent cell burden in human subjects, through an open-label Phase 1 pilot study. Subjects with diabetic kidney disease (the most common cause of end-stage kidney failure) which is characterized by increased cellular

senescence, displayed decreased adipose tissue senescent cell burden, increased SA-β-gal activity, and high $p16^{INK4A}$ and $p21^{CIP1}$ expression, 11 days after a 3-day oral D + Q treatment course. Further, skin senescent cell burden was also alleviated, and SASP factors which included the Metalloproteases MMP9 and MMP12 along with the interleukins IL-6 and IL-1α were reported to be decreased in circulation (Hickson *et al.*, 2019). Oral D + Q treatment has elimination half-life of <11 h in humans and according to this study, a "hit-and-run" treatment regimen is enough to alleviate the senescent cell burden in humans maintaining low levels of senescent cells from days to weeks coinciding with the average time (>2 weeks) for the development of new senescent cells in in vitro conditions (Hickson *et al.*, 2019).

Fisetin

This quercetin-related natural flavonoid can be found in a variety of fruits including strawberries, grapes, apples, and persimmon, and in some vegetables such as onions and cucumbers (Khan *et al.*, 2013). A flavonoid-rich diet is correlated with a reduced risk of coronary heart disease and cardiovascular disease (Terao 2017, Kim and Je 2017) and is thought to protect brain function during aging-associated neurodegenerative diseases (Maher, 2015). Despite poor solubility (10.45 μg/mL) and relatively low oral bioavailability (44%), fisetin was firstly found to selectively induce apoptosis in senescent cells but not proliferating human umbilical vein endothelial cells while failing to kill senescent human lung fibroblast IMR90 cells or primary human preadipocytes (Y Zhu *et al.*, 2017). In a following senolytic activity *in vitro* screen assessment of 10 natural flavonoid compounds (quercetin included) in senescent induced human and murine fibroblasts, fisetin was revealed to possess the highest senolytic potency. After *in vitro* assays, acute or intermittent fisetin administration to progeroid model mice carrying a $p16^{INK4a}$-luciferase reporter caused a reduction in $p16^{Ink4A}$ and p21 expression along with impairments in the secretion of SASP mediators in multiple tissues. Fisetin reduced senescence in a subset of cells in human and adipose tissue explants while its administration to old wild-type mice reduced age-related pathology, restored tissue homeostasis, and extended median and maximum lifespan, causing fewer adverse reactions in mice than quercetin

(Yousefzadeh *et al.*, 2018). Fisetin capacity for PI3K/AKT pathway inhibition is thought to be behind its apoptosis-promoting effect on senescent cells (Syed *et al.*, 2013; Zhu *et al.*, 2020). Fisetin-based treatments have not yet been proven to have senotherapeutic effects in humans, however according to clinicaltrials.gov, as of 2020 clinical testing is ongoing.

Piperlongumine

Piperlongumine is a natural compound that can be found in long pepper and is thought to have anti-cancer properties. Piperlongumine has a solubility of ± 26 μg/ml in water (27 fold higher with the addition of 10% Tween 80), however, it dissolves better in organic solvents including dimethyl sulfoxide (20 mg/ml), ethanol (0.150 mg/ml) and dimethylformamide (20 mg/ml) (Tripathi and Biswal, 2020). One study revealed that this compound was able to induce apoptosis (independently of ROS generation) of senescent human WI-38 fibroblasts generated by replicative exhaustion, ectopic expression of the oncogene *Ras or* exposure to ionizing radiation. Piperlongumine's senolytic capacity was shown to be synergistically augmented through simultaneous navitoclax administration (Wang *et al.*, 2016).

FOXO4-DRI Peptide

Forkhead box O4 protein (FOXO4) is a transcription factor that jointly with p53 can regulate a wide range of pathways involved in metabolism, cell cycle, and apoptosis making them important regulators of cellular senescence (Bourgeois and Madl, 2018). Administration of a cell-permeable D-Retro Inverso (DRI)-isoform-FOXO4 Peptide (FOXO4-DRI) capable of disrupting p53-FOXO4 interaction was shown to cause p53 nuclear exclusion and activation of the intrinsic apoptotic arm in senescent human and mouse cell cultures. It also alleviated the senescent cell burden of doxorubicin exposed mice and preserved renal function, mice fitness, and fur density in old wild-type and accelerated-aging model progeroid mice ($Xpd^{TTD/TTD}$ mice) (Baar *et al.*, 2017). FOXO4-DRI is fused with a basic and hydrophilic sequence named HIV-TAT to enhance its cellular uptake which has been shown to happen around 2-4h after being administrated (remained detected after a least 72h) (Baar *et al.*, 2017).

Epigallocatechin gallate

Epigallocatechin gallate (EGCG) is a compound found in green tea whose study has revealed possible therapeutic effects in age-associated immune disorders and organ dysfunction (Kumar *et al.*, 2019). EGCG is absorbed by paracellular diffusion without hydrolysis or de-conjugation mainly through the epithelial lining of the ileum and jejunum. It then undergoes a series of metabolic reactions including sulfation, methylation, and glucuronidation in erythrocytes and liver cells (Cione *et al.*, 2020). Due to its capacity to activate the antioxidant gene regulator the nuclear factor-erythroid 2-related factor 2 (Nrf2), a 2016 study sought to assess a possible association between EGCG's antioxidant promoting role and cellular senescence in human mesenchymal stem cells. Indeed, senescence-induced cells through H_2O_2 exposure displayed decreased levels of acetyl-p53 and p21 upon EGCG treatment and Nrf2-knockdown leading to EGCG loss of antioxidant effect maintaining acetyl-p53 and p21 high levels. This suggests that EGCG possess an oxidative stress-induced senescence prevention function (Shin *et al.*, 2016).

Further, EGCG applied treatment to U251 glioblastoma cells caused telomere shortening and disruption of genome integrity, which led to cellular senescence. Given that the DNA damage marker phosphorylation of γ-H2AX histone and micronuclei was upregulated during specific timepoints and treatment doses displaying no telomere shortening, the authors of this study concluded that EGCG treatment induced both telomere-shortening-cellular senescence and genotoxicity independently (Udroiu *et al.*, 2019). More recent experiments in 3T3-L1 preadipocytes with prematurely induced cellular senescence through exposure to Hydrogen peroxide at a sub-lethal concentration (150 μM), revealed another possible mechanism behind the therapeutic effect of EGCG as its administration resulted in significant downregulation of mTOR/PI3K/Akt/ and AMPK signaling, the suppression of SASP, Cox-2, ROS, iNOS and NF-κB along with cell cycle inhibition through p53 signaling. Also, EGCG prevented the accumulation of anti-apoptotic protein Bcl-2 in senescent cells leading to cellular apoptosis (Kumar *et al.*, 2019).

Gingerenone A

As a result of a drug screen performed on in radiation-induced WI-38 senescent human fibroblasts in a recent study by Moaddel et al. extract from Zingiber officinale Rosc. (ginger) (known to have anti-inflammatory effects) revealed senomorphic and senolytic potential (Moaddel *et al.*, 2022). The main active compound was identified as gingerenone A and it displayed higher selectivity for senescent cells clearance compared to the known and already mentioned senolytic cocktail (D+Q). Gingerenone A, was shown to decrease senescent cell viability and SA-β-Gal activity, increase the expression of the anti-inflammatory cytokines IL-10 and IL-13 and reduce the secretion of pro-inflammatory factors, such as IP-10, MCP-1, IL-6 as well increasing the pro-inflammatory cytokines IL-1B and IL-8 (Moaddel *et al.*, 2022).

The distinct effect of gingerenone A in the expression of IL-6 and IL-8 suggests that the mechanism of action is probably independent of IL-1A which is known to mediate the activation of both IL-6 and IL-8 through amplification of C/EBPβ activation (Moaddel *et al.*, 2022). Gingerenone A treatment was not able to increase p53 levels either in senescent or proliferating cells, however, it downregulated the expression of Bcl-XL in senescent cells leading to an increase in caspase-3, which Is a strong indicator that gingerenone A senolytic effect is p53-independent and mediated by caspase-3 cleavage (Moaddel *et al.*, 2022).

Cardiac glycosides

Cardiac glycosides are a family of organic compounds used in the treatment of congestive heart failure and cardiac arrhythmias although commonly associated with secondary toxicity. They are Na^+, K^+-ATPase pump inhibitors, however, evidence suggests that they might have other targets (Prassas and Diamandis, 2008). Na^+/K^+ ATPase inhibition can lead to membrane depolarization and intracellular acidification through disruption of cells' natural electrochemical gradient. As previously mentioned, abnormal intracellular acidic pH and depolarized plasma membrane are both typical traits of senescent cells which in theory might increase their susceptibility to the effects of this family of compounds. Following this line of thought, two

recent studies (Guerrero *et al.*, 2019; Triana-Martinez *et al.*, 2019) sought to explore this vulnerability in order to assess if cardiac glycosides could be used for future senotherapeutic strategies. Indeed, drug screens revealed a senolytic activity by some of this family members in oncogene- and therapy-induced senescence cells (IMR90 ER: RAS, etoposide/doxorubicin-IMR90, human lung adenocarcinoma cell line, Bleomycin-A549, Palbociclib-SK-MEL-103 melanoma cell line) (Guerrero *et al.*, 2019; Triana-Martinez *et al.*, 2019). Senolytic activity of identified elements of the cardiac glycoside family including digoxin, digitoxin, CGP-74514A (a CDK1 inhibitor), and ouabain was confirmed in the following *in vitro* assays. Digoxin and digitoxin, often used in the treatment of atrial fibrillation and heart failure showed senolytic activity, the last at a concentration similar to the found in the plasma of treated cardiac patients (20–33nM), which is a good indicator of safe clinical senotherapeutic application in the future (Guerrero *et al.*, 2019). Further, Digoxin treatment was shown to reduce cell viability of SA-β-gal expressing primary chondrocytes from osteoarthritic donors compared to healthy controls (Triana-Martinez *et al.*, 2019). Digoxin senolytic activity was not only extended to A549 cells treated with Bleomycin but as well to cells treated with another senescence-inducing chemotherapeutic drug including Doxorubicin, Gemcitabine, Palbociclib, and Etoposide (Triana-Martinez *et al.*, 2019). Curiously, Guerrero and colleagues verified that both ouabain and digoxin promoted the expression of several pro-apoptotic Bcl2 family proteins mainly NOXA which seemed to mediate its senolytic effects. In addition, ouabain was also shown to induce the activation of JNK, GSK3-ß, and p38 in senescent which was shown to be involved in the upregulation of NOXA (Guerrero *et al.*, 2019).

Ouabain administration to mid-passage cultures of primary bronchial epithelial cells led to a decrease in senescent $p16^{INK4a}$-positive-cells while showing no effect in normal ($p16^{INK4a}$-negative) cell population while also revealing cytotoxic effect in high expressing RAS non-senescent cells (Guerrero *et al.*, 2019). Both ouabain and digoxin showed potential in preventing secondary tumors with increased aggressiveness as a result of the accumulation of senescent cancer cells after chemotherapeutic treatment. Triana-Martínez and colleagues showed that simultaneous gemcitabine (chemotherapeutic drug) and digoxin treatment reduced

tumor mass formed as a result of subcutaneous injection of luciferase-expressing A549 cells in Immunodeficient nude mice compared to controls (Triana-Martinez *et al.*, 2019). Also, *ex vivo* patient-derived xenograft obtained from a breast tumor patient was shown to be susceptible to the simultaneous treatment of Digoxin and Doxorubicin (senescent inducing chemotherapy agent), and the same was verified when transplanted to nude mice submitted to the same dose adapted pharmacological intervention (Triana-Martinez *et al.*, 2019). In addition, Guerrero and colleagues showed that ouabain or digoxin significantly decreased cell viability of cancer cell lines (SKHep1, HuH7, and HLF liver cancer, A549 lung cancer, SK-Mel-5 melanoma, MCF7 breast cancer, HCT116 colon cancer) submitted to a variety of senescent inducing chemotherapeutic treatments, while showing no significant effects in cells treated with chemotherapeutic drugs which do not induce senescence, revealing high senescence specificity (Guerrero *et al.*, 2019). Further, OIS cell burden in the liver triggered the transposon-mediated transfer of oncogenic NRAS ($NRAS^{G12V}$) to hepatocytes in immunosuppressed mice was significantly alleviated by ouabain treatment (Guerrero *et al.*, 2019). Also, ouabain treatment was able to selectively induce apoptosis of senescent β-catenin positive cells in preneoplastic clusters formed as a result of adamantinomatous craniopharyngioma in embryonic pituitaries dissected and cultured *ex vivo* without affecting other cell types (Guerrero *et al.*, 2019). Ouabain treatment was also able to minimize the expression of inflammatory cytokines including Il1α or Il6 and the accumulation of bystander senescent cells in the lung of irradiated mice (where the accumulation of these cells after irradiation are often first seen) (Guerrero *et al.*, 2019). Similarly, in Triana-Martínez study, a ten-day digoxin treatment to immunodeficient mice, three weeks after intratracheal instillation of gamma-irradiated senescent human fibroblasts IMR90 to the lungs led to a decrease in fibrosis and senescence markers in lung tissue (Triana-Martinez *et al.*, 2019). Altogether, this evidence stands out the potential that cardiac glycosides might possess as synergetic anti-cancer agents as they seem to promote cancer cell death and eliminate bystander senescent cells from tissues that are correlated with the development of side effects associated with current anti-cancer treatments.

In the Guerrero study, old female mice subjected to 5 rounds of intermittent ouabain treatment showed improvements in motor condition, displayed an increment in low phosphate and albumin levels, and a decrease in abnormally high levels of amylase in the blood observed during aging and related pathology (Guerrero *et al.*, 2019). Further, comparatively, to their age-matched counterparts, old mice treated with ouabain, showed a significant reduction in $p16^{INK4a}$ levels in many tissues including heart, kidney, and liver where there was also a significant reduction in SA-ß-Gal activity, immune infiltration, and markers of inflammation. Upon ouabain treatment, there was not any significant alteration in blood immune composition nor increased infiltration of granulocytes ($Ly6G^{+}$) and platelets ($CD42b^{+}$) suggesting that ouabain treatment might have an effect on cell-specific local immune infiltration (Guerrero *et al.*, 2019). Results from these *in vitro*, *in vivo, and ex vivo* experiments suggest that cardiac glycosides, mainly ouabain and digoxin, are promising compounds for the development of future senolytic approaches for the treatment of age-related conditions. However, some obstacles still need to be surpassed, such as toxicity considerations and the current restriction of mice studies to the use of immunodeficient mice due to the known insensitivity of rodents to cardiac glycosides (Mijatovic *et al.*, 2007), which is thought to be due to differences in the ATP1A1 protein (alpha subunit of the Na+/K+ATPase pump) (Price and Lingrel, 1988).

Autophagy modulation to promote senolysis

Some piece of evidence suggests that modulation of autophagy can induce apoptosis of senescent cells. However, autophagy-induced senolysis has been shown to be achieved by both inhibiting and activating autophagy (Dörr *et al.*, 2013; Wakita *et al.*, 2020; L'Hôte *et al.*, 2021, 2022). A recent study performed by Hôte et al., found that compounds from the previously mentioned cardioglycoside family, specially ouabain, have a potent senolytic effect on OIS-fibroblast model through expression of BRAF-V600E (involved in development of melanoma and other types of cancer) (this model was named BRafSen cells). Cardioglycosides are known Na,K-ATPase pump inhibitors, however, the senolytic effect of oubain was shown to be mediated by Na,K-ATPase pump signaling rather than inhibition of ion transport. The

expression of BRAF-V600E in BRafSen cells promoted ER stress and autophagy suggesting that these cells require an elevated autophagy flux for survival. Authors concluded that this autophagy dependence by BRafSen cells makes them susceptible to ouabain and other cardioglycosides which were shown to inhibit autophagy flux through Na,K-ATPase pump signaling (L'Hôte *et al.*, 2021). The most recent evidence that autophagy activation leads to senolysis comes from a study by Wakita, et al., which after performing an unbiased high-throughput screening of a 47,000 small molecule chemical compound library, found that a BET family protein degrader (BETd), also known as ARV825, a small molecule developed by Lu J, et al, displayed senolytic effect (Lu *et al.*, 2015; Wakita *et al.*, 2020). BETd is able to block BRD4, a protein from the BET family that protects cells from onset of cellular senescence through activation of the non-homologous end joining (NHEJ) repair and the downregulation of autophagy promoting genes (Sakamaki *et al.*, 2017). BETd was able to promote the elimination of senescent hepatic stellate cells in obese mouse livers while reducing liver cancer development, and the elimination of chemotherapy-induced senescent cells by BETd which led to increased efficacy of chemotherapy against xenograft tumors in immunocompromised mice (Wakita *et al.*, 2020). This was thought to be mediated by the inhibition of BRD4 that led to a decrease in the non-homologous end joining (NHEJ) repair resulting DNA double-strand breaks (DSBs) that ultimately along with the up-regulation of autophagy-related genes led to autophagy-induced apoptosis in senescent cells (Wakita *et al.*, 2020). BETd displayed more robust senolytic activity than senolytic drugs already discussed in this review including D + Q, ABT26319 and 17-DMAG25 (Wakita *et al.*, 2020). This was verified in every type of a cellular senescence induction and at a concentration of 5-10 nM. It is however worth standing out some still limitations pointed out by the authors regarding this early stage senolytic therapy (Wakita *et al.*, 2020). Firstly, authors were not entirely sure of every mechanism behind the senolytic effect mediated by BRD4 inhibition. This in particular is suggested by the fact that BET inhibitors have been shown to downregulate SASP factor expression in senescent cells (Tasdemir *et al.*, 2016) including senescent cell surviving promoting factors such as GM-CSF8 or PDGF (Demaria *et al.*, 2014) which leads the author to suspect that the senolytic effect of BETd might

also be mediated through SASP factor downregulation depending on the biological context. Further, more studies are still needed to assess whether BETd can have senolytic effect in every kind of senescent cells *in vivo*. Given that BETd high concentration treatment (≤ 50 nM) resulted in a reduction in the proliferation of control cells, the authors stand out the importance of determining an optimal concentration for *in vivo* treatment (Wakita *et al.*, 2020).

HSP90 Inhibitors

Hsp90 is a 90 kDa Heat shock protein with a variety of various isoforms, that is involved in a series of physiological processes including ensuring correct protein folding and stabilization against heat stress, chaperoning the irreversibly misfolded proteins to proteasome degradation preventing them from aggregating in large numbers. Some proteins stabilized by Hsp90 are essential for tumor growth of various cancer cell types which has made HSP90 a potential drug target, for cancer treatment (Fuhrmann-Stroissnigg *et al.*, 2018). In a 2017 study, several autophagic regulators were submitted to a senescence associated β-galactosidase assay as a screening platform using progeroid $Ercc1^{-/-}$ primary murine embryonic fibroblasts with defective DNA repairing capacity, resulting in the discovery of the senolytic properties of the chaperone HSP90 inhibitors Geldanamycin and 17-AAG (tanespimycin), displaying low toxicity for heathy cells. These two compounds were also able to significantly induce cell death in senescent human cells in culture (vascular endothelial cells, WI-38 and IMR90 human lung fibroblasts and mesenchymal stem cells) (Fuhrmann-Stroissnigg *et al.*, 2017). In addition, a third HSP90 inhibitor, known as 17-DMAG (alvespimycin) was able to extend lifespan of $Ercc1^{-/-}$ progeroid mice, downregulating $p16^{INK4A}$ expression in the kidney tissue (although no significant change in liver tissue), improving overall body condition and delaying the onset of age-related problems such as loss of forelimb grip strength, kyphosis, coat condition, ataxia, dystonia, gait disorder and tremor (Fuhrmann-Stroissnigg *et al.*, 2017). This study also suggested that the senolytic effect of these HSP90 inhibitors might result from the impaired HSP90 mediated AKT and p-AKT (S473) stabilization (regulators of the anti-apoptotic PI3K-Akt pathway) (Fuhrmann-Stroissnigg *et al.*, 2017). Due to this selective inhibition, treatments

based on these compounds might be more efficient in combination with inhibitors of other anti-apoptotic pathways such as D + Q or navitoclax, although clinical testing should be cautiously prepared.

C. Alternative Senolytic Strategies

*Senolytic CAR-T-Cells (*Immunotherapy*)*

Despite the major advances the clinical application of the diferent senolytic stategies, there has been consistent association with some significant grade of side effect toxicity. This might have to do with the lack of specificity given that the targeted pathways are also present in non-senesent cells. Recently, Amor and colleagues were able to design a new senolytic strategy based on the anti-tumor Chimeric antigen receptor (CAR) T cells genetically modified cell-based therapy, previously used for treating certain haematological malignancies (Amor *et al.*, 2020). CARs proteins are synthetically made receptors that prompt T-cells to recognize and kill cells expressing a specific target antigen independently of MHC-mediated antigen presentation. These receptors are composed of four subunits that vary accordingly to the target protein (the extracellular antigen-binding domain, a hinge bound to a intracellular signaling subunit through a transmembrane domain) (Rafiq *et al.*, 2020). This therapeutic strategy showed very promising results in tumor treatment field as autologous CD19-targeting CAR T cells for treatment of pediatric and adult B-cell malignancies were the first therapeutic mechanism featuring a genetic engineering component to obtain FDA approval (Park *et al.*, 2016).

RNA-sequencing results from three senescent models (therapy-induced senescence in mouse lung adenocarcinoma, OIS in mouse hepatocytes, culture-induced senescence in mouse hepatic stellate cells) obtained in Amor's study (Amor *et al.*, 2020), revealed eight common upregulated genes, which after filtration of the highly expressed in non-senescent cells and in vital tissues led to the identification of a senescence-specific cell surface marker called urokinase-type plasminogen activator receptor (uPAR) (encoded by *PLAUR* gene) (Amor *et al.*, 2020). This membrane protein is a promoter of extracellular matrix degradation during fibrinolysis, wound healing along with invasion, motility and survival of neoplasic cells (Amor *et al.*, 2020).

Further, a previous study had already reported the *in vitro* effectiveness of a CAR T cells targeting uPAR therapy for ovarian cancer using the natural ligand part of the uPAR in alternative to single-chain variable fragment (Wang *et al.*, 2019). Cells expressing uPAR displayed similar histological features as cells with high p16 and IL-6 expression along with high SA-β-gal activity, also, uPAR positive-cells were found to be abnormally present in pancreatic intraepithelial neoplasia lesions from patients suffering from pancreatic cancer and human atherosclerotic plaques from carotid endarterectomy specimens. uPAR can also be converted into a soluble formed (suPAR) (generated by cleaving a uPAR portion upon ligand binding) which is a component of the SASP (Coppe *et al.*, 2008) and a serum biomarker for diabetes and kidney disease (Hayek *et al.*, 2015). Amor and colleagues further confirmed uPAR upregulation *in vitro* and *in vivo* senescent models including replication-induced senescent human primary melanocytes and therapy-induced senescence in mouse KP lung cancer cells and therefore used uPAR as a target for CAR-T cell therapy. The designed uPAR-targeting CAR T-cell therapy was shown to complement MEK and CDK4/6 inhibitors anti-tumor treatment of mice suffering from lung adenocarcinoma prolonging their survival, and to minimize diet induced liver fibrosis in mice. However, some specimens exhibited transient hypothermia along with weight loss and increased levels of inflammatory cytokines such as GM-CSF, G-CSF , IFN-γ and IL-6, characteristic of the CAR T-cell-associated cytokine release syndrome, a condition that has been reported as a main side effect in a wide range of previous CAR-T cells-based trials along with the recently described immune effector cell-associated neurotoxicity syndrome (Borrega et al., 2019). Overall, this study is an indicator of the potential displayed by this uPAR-specific CAR T-cell therapy in the treatment of senescent-associated diseases. However, future clinical studies should pay attention to possible related toxicities since the development of several side effects was already described (Borrega *et al.*, 2019; Rivera *et al.*, 2020)**.**

Galacto-oligosaccharide-coated nanoparticles for safe delivery of diagnostic or therapeutic agents (gal-encapsulation)

As mentioned earlier, senescent cells are characterized by abnormal increased activity of lysosomal SA-β-gal along with increased endosomal traffic (Kurz *et al.*, 2000; Munoz-Espin and Serrano, 2014; Sharpless and Sherr, 2015; Udono *et al.*, 2015). Despite not being a totally specific cellular senescence biomarker, SA-β-gal is enriched in damaged or pathological affected tissues (Sharpless and Sherr, 2015). An article by Muñoz-Espín and colleagues describes a versatile senescent cell-specific drug-delivery technique they named gal-encapsulation, that takes advantage of the increased SA-β-gal activity seen in senescent cells (Munoz-Espin *et al.*, 2018). This approach consists in the encapsulation of the chosen compound in spherical particles of ± 100 nm diameter of porous silica coated with galacto-oligosaccharides that prevents the diffusion of the content out of the silica matrix (Agostini *et al.*, 2012). These particles can enter cells by endocytosis travel to the lysosomes. In senescent cells, highly active SA-β-gal can promote the dissolution of the spherical particles' galacto-oligosaccharide coat releasing their inside content into the intracellular space, while in non-senescent cells the particles stay intact and eventually are released by exocytosis. This should prevent drug delivery to non-senescent cells highly contributing to the reduction of drug-related toxicity. This study confirmed the senescent specific delivery capacity of this mechanism on *in vitro* (chemotherapeutic (Palbociclib) induced senescent human cells) and *in vivo* (palbociclib treated in tumor xenografts and in lungs damage using bleomycin). Gal-encapsulated doxorubicin (chemotherapeutic with senescent and non-senescent cell killing) was able to significantly improve lung elasticity and reduce fibrosis while non-encapsulated doxorubicin failed to restore lung function (Munoz-Espin *et al.*, 2018). Curiously, Gal-encapsulated rhodamine (fluorophore) administrated to bleomycin treated mice, besides being found in lung cells, was also found in lung macrophages which, as already mentioned, despite not being senescent cells display similar abnormal increased levels of SA-β-gal activity (Hall *et al.*, 2016, 2017; Munoz-Espin *et al.*, 2018). Despite being a further indicator of gal-encapsulation's specificity to high activity SA-β-gal cells, this also stands out as a liability in this method for not being entirely specific to senescent cells. As such, off-target effects caused by affinity towards activated macrophages might vary according to the encapsulated compound or might not even

be clinically significant, more *in vivo* (and posteriorly clinical) experimentation should be employed in order to assess this. Both Gal-encapsulated doxorubicin and Gal-encapsulated navitoclax (reviewed above) in combination with palbociclib were able to promote xenograft tumor regression. Without palbociclib (senescence-inducing factor on tumors) administration, this effect was abrogated suggesting specificity for senescent tumor cells. Further, gal-encapsulation reduced significantly navitoclax and doxorubicin known toxic effects such as thrombocytopenia and cardiotoxicity suggesting that gal-encapsulation might effectively prevent drug exposure to non-senescent cells. Finally, this mechanism has also been shown to serve has a cellular detection mechanism as it can be loaded with fluorophores such as rhodamine which allows for visualized by *in vivo* imaging (Munoz-Espin *et al.*, 2018).

Galactose-modified prodrugs (duocarmycin derivatives)

Galactose-modification is described throughout literature as a pharmacokinetic enhancing system that promotes effective and specific drug delivery. One great example is a technique referred to as antibody-directed enzyme prodrug therapy (ADEPT) in which target-cell specific antibodies are conjugated with an enzyme that is able to convert a prodrug into a cytotoxic drug (Sharma and Bagshawe, 2017). The natural antibiotic duocarmycin is a DNA alkylator agent that inhibits DNA synthesis and can lead to cell death, its glycosidic derivatives are often used as prodrugs in ADEPT (Tietze *et al.*, 2010). Considering SA-β-gal abnormal high activity during senescence, in a recent study, Guerrero and colleagues tested galactose-modified duocarmycin derivatives prodrugs mainly JHB75B (referred to as prodrug A) and seco-duocarmycin analog dimer (JHB71A) for senolytic activity (Guerrero *et al.*, 2020). This would not require the usual simultaneous treatment with antibody-enzyme (SA-β-gal) used in ADEPT, due to SA-β-gal increased activity being a highly specific cellular senescence trait which theoretically would promote the conversion of these galactose-modified duocarmycin derivatives prodrugs into cytotoxic drugs specifically by senescent cells. They verified that these drugs were able to eliminate multiple types of senescent cells *in vitro* including in OIS cells (lung fibroblast (IMR90 ER:RAS), human mammary epithelial cells (HMEC ER:RAS)),

IMR90 senescent cells induced by doxorubicin or etoposide treatment, serial passage or irradiation. Duocarmycin prodrugs also alleviated senescent cell burden in the lung of irradiated senescent mouse model and were able to clear pro-tumorigenic senescent clusters in *Hesx1*$^{Cre/+}$*; Ctnnb1*$^{lox(ex3)/+}$ adamantinomatous craniopharyngioma (pituitary pediatric tumor) mouse model, with no effect on other cell types in the pituitary gland (Guerrero *et al.*, 2020). These prodrugs also seemed to prevent the accumulation of bystander senescent cells after whole-body irradiation treatment of mice. These results although very promising are still in early stages of development and therefore further studies are much needed for assessing efficiency and viability of this senolytic strategy before moving on to the clinic (Guerrero *et al.*, 2020).

VI. Conclusion and Future perspectives

Discovery of new senotherapeutic strategies and optimization of the existing ones will be dependent upon the endeavor to find more solid and specific cellular senescence biomarkers which would benefit from more research elucidating more details about cellular senescence mechanisms and better describe the role that cellular senescence plays in disease. The heterogeneity of the senescence phenotype found throughout tissues has remained an obstacle for the deployment of current senotherapeutic strategies as different senescent cells express different senescence markers, adopt different SASP profiles and use different SCAP. Therefore, for producing a senolytic effect on multiple tissues, finding optimal combinations between multiple senotherapeutics that inhibit different SCAP might be a solution in the future (van Deursen, 2014; Zhu *et al.*, 2015). Another problem that must be tackled in the future is the toxicity associated with current senotherapeutic therapies. Investment must be made in finding new ways to get around or compensate for the acute cellular senescence processes that might be affected by senescent cell elimination. This would require gathering more knowledge on how cellular senescence is important to the organism and the development or improvement of existing drug delivery mechanisms, such as gal-encapsulation developed by Muñoz-Espín and colleagues (described above) (Munoz-Espin *et al.*, 2018) in order to increase senescence specificity and decrease off-target effects or even non-pharmacological approaches such as the

CAR-T cell-based senolytic immunotherapeutic strategy developed by Amor and colleagues (described above) (Amor *et al.*, 2020). It would also be important to assess possible differences between cellular senescence in humans and model organisms. The prevention of the secretion of pro-inflammatory SASP mediators has rendered senomorphic therapy a potential treatment option for inflammaging associated diseases(Sun *et al.*, 2018). However, senolytic therapy has revealed to be a more specific (given SASP mediators non-specificity for senescent cells) and feasible strategy as senolytic acute administration intermittently has been shown to alleviate senescent cell burden, therefore minimizing the effects of the associated toxicity and making future treatments more tolerable (Kirkland and Tchkonia, 2017; Kirkland *et al.*, 2017). Overall, despite the Senotherapy field still being in its early days, the current work has laid many foundations for future success in treating aging-related conditions which we expect to have tremendous impact in the extension of the average human health span.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lucas, V., Aveleira, C., and Cavadas, C.

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Conflict-of-interest statement

No author has an actual or perceived conflict of interest with the contents of this article.

Tables

Table 1- Summary of the Cellular Senescence Biomarkers currently used. 1. Examples of the presence of the Biomarker in different cells/cell types upon Senescence **(Biomarker Expression)**; 2. Biomarker quality (Features); 3. Detection methodologies (Detection)

Biomarker	**Biomarker Expression**	**Features**	**Detection**
Beta-galactosidase (SA-β-gal) *(Dimri et al., 1996) (Kurz et al., 2000) (Hall et al., 2016, 2017) (Castro et al., 2003) (Sousa-Victor et al., 2014)*	Present in the majority of senescent cells	High activity at pH 6.0 Present in non-senescent activated macrophages; Not present in senescent geriatric satellite cells (not 100% specific)	Measurement using a commercial kit (β-Galactosidase Staining Kit)
$p16^{INK4a}$ *(Rayess et al., 2012)((Baker et al., 2016; Shimizu and Minamino, 2019)*	Present in the majority of senescent cells	Exhibits low expression in healthy cells; phosphorylated in senescent epithelial cells; present in non-senescent activated macrophages (not 100% specific)	Can be detected through RT-qPCR, western blot, single-cell RNA-seq, and p16(LUC);
Senescence-Associated Secretory Phenotype (SASP) *(Hari and Acosta, 2017) (Becher et al., 2016; Wang et al., 2017; Furue et al., 2020) (Lu et al., 2006; Green, 2008; YZ Xiao et al., 2020).*	Some SASP factors are ubiquitous to most senescent cells	Feature of various non-senescent cells (Not specific)	ELISA assay, Western blot, immunostaining & High Content Microscopy Analysis
Tumor suppressor genes p53/p21 & DDR markers (e.g. ATM, ATR, 53BP1, γH2AX…etc) *(Wang et al., 2015; YZ Xiao et al., 2020)(Turinetto and Giachino, 2015)*	Present in most cells in an irreversible cell cycle arrest state	Not specific since can be upregulated during other processes apoptosis and transient cell cycle arrest	RT-qPCR, western blot, single-cell RNA-seq
Telomere length			Measured through Southern

(Kimura et al., 2010) (Lin et al., 2019) (Bradford et al., 2009; YZ Xiao et al., 2020)	Cells under replicative senescence	Not senescence specific; Used as a confirmation marker;	Blot or Flow cytometry & Flow-FISH
Proliferation markers Ki-67;PCNA *(Nagai et al., 2014; El Hasasna et al., 2015) (Cuylen et al., 2016; YZ Xiao et al., 2020)*	Highly expressed in Proliferating Cells	Complementary biomarkers, (Combined with high-density DNA damage foci (γH2AX)) absence indicates cell proliferation arrest	Immunohistochemistry
Clusterin *(Foster et al., 2019) (Zhou et al., 2015) (Matos et al., 2012; Li et al., 2013)*	e.g. Senescent Glioblastoma multiforme cells or WI-38 fibroblasts, upregulated in many metastatic cancers	Low-grade specificity	RT-qPCR or Western Blotting
Senescence-associated heterochromatin foci (SAHF) *(Narita et al., 2003; Zhang and Adams, 2007).*	Cells undergoing irreversible cell cycle arrest e.g. primary human embryonic fibroblasts cell lines IMR90 and WI38 cells	SAHF formation and senescence are not always coupled. It may not be a suitable biomarker for single detection of senescence, should be combined with other biomarkers such as SA-β-gal	SAHF punctate can be observed using DAPI cell staining
Lamin B1 & Lamin B receptor (LBR) *(Dreesen et al., 2013) (Freund et al., 2012)*	e.g. HeLa, normal human diploid fibroblast TIG-7 cells, Senescent dermal fibroblasts, and keratinocytes	Lamin B1 and LBR potential reliable cellular senescence biomarkers (Specificity and sensitivity still in question.	RT-qPCR, Western Blotting, immunohistochemistry

Table 2 - Summary of all reviewed senotherapeutic compounds and strategies: Classified according to type, target elements, and type of existing evidence that support its potential for senotherapeutic use (Existence of Evidence (+) Absence of Evidence (Ø)).

Compound(s)/ Stategy	**Type**	**Target**	***in vitro***	***in vivo***	***Clinical***
Metformin *(Fang et al., 2018) (Chen et al., 2016) Karnewar et al., 2018)*	Senomorphic	AMPK, NF-κB, NRF2	+	+	**Ø**
Rapamycin *(Volonte et al., 2013; Wang et al., 2017;)(Laberge et al., 2015; Weichhart, 2018) (Herranz et al., 2015)*	Senomorphic	mTORC1	+	+	**Ø**
Roxolitinib *Verstovsek et al., 2012) Xu et al., 2015a) (Xu, Tchkonia, et al., 2015) (Griveau et al., 2020)*	Senomorphic	JAK1/2	+	+	**Ø**
ABT-737 *Yosef et al., 2016) (Anantram and Degani, 2019) (Oltersdorf et al., 2005)*	Senolytic	Bcl-2, Bcl-w & Bcl-xL	+	+	**Ø**
Navitoclax (ABT-263) *(Anantram and Degani, 2019) (Zhu et al., 2016) (Chang et al., 2016) (Yang et al., 2020) (Childs et al., 2016)*	Senolytic	Bcl-2, Bcl-w & Bcl-xL	+	+	**Ø**
A1331852 *(Y Zhu et al., 2017)*	Senolytic	BCL-XL	+	**Ø**	**Ø**
A1155463 *(Y Zhu et al., 2017)*	Senolytic	BCL-XL	+	**Ø**	**Ø**
Dasatinib *(Demaria et al., 2017; Nath et al., 2018; Xu et al., 2018; PS Zhang et al., 2019) Zhu et al., 2015) (Hickson et al.,2019) (Martyanov et al., 2019) Justice et al., 2019)*	Senolytic	Ephrins	+	+	+
Quercetin *((Demaria et al., 2017; Nath et al., 2018; Xu et al., 2018; PS Zhang et al., 2019) Zhu*	Senolytic	BCL-2 protein family, HIF-1α, PI3-kinase or p21	+	+	+

et al., 2015) (Kim et al., 2019) (Hickson et al., 2019) (Martyanov et al., 2019)(***Justice et al., 2019)***					
Fisetin *(Syed et al., 2013; Zhu et al., 2020 (Yousefzadeh et al., 2018) (Y Zhu et al., 2017)*	Senolytic	PI3K/AKT pathway	+	+	+
Piperlongumine *(Wang et al., 2016)*	Senolytic	PI3K/Akt mTOR	+	**Ø**	**Ø**
Geldanamycin *Fuhrmann-Stroissnigg et al., 2017)*	Senolytic	Hsp90 PI3K-Akt pathway	+	**Ø**	**Ø**
17-AAG (tanespimycin) *Fuhrmann-Stroissnigg et al., 2017)*	Senolytic	Hsp90 PI3K-Akt pathway	+	**Ø**	**Ø**
17-DMAG (alvespimycin) *Fuhrmann-Stroissnigg et al., 2017)*	Senolytic	Hsp90 PI3K-Akt pathway	+	+	**Ø**
FOXO4-DRI Peptide *(Bourgeois and Madl, 2018) (Baar et al., 2017)*	Senolytic	FOXO4	+	+	**Ø**
Epigallocatechin gallate *(Kumar et al., 2019) (Udroiu et al., 2019) (Shin et al., 2016)*	Senolytic	Nrf2, mTOR, PI3K/Akt, AMPK, Cox-2, ROS, iNOS and NF-κB, p53, Bcl-2	+	**Ø**	**Ø**
uPAR-CAR T-cells *(Amor et al., 2020)*	Senolytic	uPAR	+	+	**Ø**
Gal encapsulation *(Munoz-Espin et al., 2018)*	Senolytic drug delivery system	Cells with high SA-β-gal Activity	+	+	**Ø**
Galactose-modified prodrugs (duocarmycin derivatives) *(Guerrero et al., 2020)*	Senolytic	SA-β-gal	+	+	**Ø**
Cardiac glycosides *(Guerrero et al., 2019; Triana-Martinez et al., 2019) (L'Hôte et al., 2021).*	Senolytic	Na^+/K^+ ATPase pump, Bcl2 proteins (mainly NOXA), JNK, GSK3-ß, p38	+	+	**Ø**
BET family protein degrader (BETd)(ARV825) *(Wakita* et al., *2020)*	Senolytic	non-homologous end-joining (NHEJ) repair BRD4	+	+	**Ø**

Gingerenone A ***(Moaddel etal., 2022)***	Senolytic Senomorphic	Caspase-3 (predicted)	+	**Ø**	**Ø**

Figure Legends

Figure 1- Various mechanisms of Cellular Senescence and respective inducing stimuli. Cellular senescence can be divided into replicative senescence and non-replicative senescence. Replicative senescence (1) Is induced by the cell division-related gradual telomere erosion which leaves the chromosome ends vulnerable to stressing agents promoting the development of various senescence-related traits (Turner *et al.*, 2019). Non-replicative senescence is triggered by various stressing elements that can emerge during aging and pathology. DNA Damage (2) which results in DDR activation can lead to cell cycle arrest through ATM or ATR kinases activation that signal p53 stabilization and subsequent p21 increased expression; this leads to Cyclin-dependent kinase 2 (CDK2) inhibition which allows Rb tumor suppressor protein (Rb) to remain attached and therefore inhibiting E2F (G1 to S phase transition inducing transcription factor); DNA damage can also promote Rb mediated E2F inhibition through $p16^{INK4a}$ upregulation which inhibits CDK4/6 (Rb detachment promoter) Paracrine-induced senescence (3) mediated by: paracrine SASP components secreted by nearby senescent cells which trigger specific signaling such as IL-6 that upon binding to its receptor two GP130 molecules complex lead JAK-STAT signaling pathway activation (Jones, Scheller and Rose-John 2011); Mitochondrial Dysfunction-Associated Senescence (MIDAS)): mitochondrial dysfunction originates a distinct cellular senescence type (MIDAS); Dysfunctional mitochondria display a low NAD^+/NADH ratio which promotes growth arrest through p53; p53 activation modulates SASP by downregulating the IL1-dependent inflammatory arm through an NF-kB-independent mechanism and promote the expression of other factors such as CCL27, TNFα and IL10 (Gallage and Gil, 2016; Wiley *et al.*, 2016). Oncogene-induced Senescence (OIS) 5) works as an intrinsic suppressive mechanism through impaired tumor cell proliferation (Di Micco *et al.*, 2006; Kuilman *et al.*, 2010; Rufini *et al.*, 2013); both oncogene activation and tumor suppressor repression can lead to DNA damage which can originate irreversible growth arrest through p53/p21 and $p16^{INK4a}$ pathways activation (Calcinotto *et al.*, 2019). Epigenetically-induced Senescence (6) consists in the formation of senescence-associated heterochromatin foci (SAHFs) (discussed in 1.2.) specialized domains of facultative heterochromatin such as

H3k9me2/3 that result from the spatial repositioning of preexisting repressive marks to promoter sites of proliferation-inducing genes such as E2F target genes such as cyclin A (Pluquet *et al.*, 2015). Oxidative stress-induced senescence (7): generation of reactive oxygen species (ROS) for example through endoplasmic reticulum (ER) stress can result in DNA and mtDNA damage which can trigger DDR and MIDAS respectively. Chemotherapy-induced Senescence (8): the absence of senescence associated tumor suppressors such as p53 and Rb, in tumor cells upon being submitted to chemotherapeutic agents (Ewald *et al.*, 2010; Amaya-Montoya *et al.*, 2020); Senescent-related morphological features) Senescent cells display morphological alterations including enlarged and flat form along cytoskeleton abnormalities (Cho *et al.*, 2004). This seems to be mediated by the increased expression of the integral membrane protein caveolin-1 in senescent cells which was shown to regulate focal adhesion kinase activity and actin stress fiber formation through activation of RhoA, Rac1, and Cdc42 resulting in membrane ruffles/lamellipodia and filopodia formation (Cho *et al.*, 2004). (Created with BioRender.com).

Figure 2- ER stress response mechanisms. During cellular senescence, there is an accumulation of dysfunctional protein accumulation that can lead to ER stress. In response, cells activate the unfolded protein response (UPR) through the phosphorylation of 3 key proteins: I) Serine/threonine-protein kinase/endoribonuclease IRE1 (IRE1α) that upon activation leads to unconventional splicing of X-box binding protein 1 (XBP1) mRNA into XBP1s that migrates to the nucleus where it functions as a transcription factor increasing the expression of genes involved in protein quality control (QC) and several ER Stress response mechanisms II) Cyclic AMP-dependent transcription factor ATF-6 alpha (ATF6α) when activated, leaves the ER and is cleaved by two Golgi apparatus membrane proteases sphingosine-1-phosphate 1 and 2 (S1P and S2P). As a result, a 50-kDa ATF6α domain is released and travels through the cytosol to the nucleus where it promotes the expression of genes involved in the protein (QC) such as Binding immunoglobulin protein (BiP) and even XBP1. III) PKR-like ER kinase (PERK) when activated promotes downregulation of protein synthesis through the phosphorylation of the translation

initiation factor eIF2α and the upregulation of genes involved in Antioxidant (A.O.) and Amino acid (Aa) response through induction of ATF4 and BiP mRNA translation. (Created with BioRender.com).

Figure 3- Cellular senescence and physiological functions. 1 Wound healing: senescent fibroblast and endothelial cells secrete PDGF-AA inducing myofibroblasts differentiation; senescent hepatic stellate cells prevent excessive fibrosis, the proliferation of damaged cells, and signal the immune system to remove senescent cells. 2. cellular senescence plays a role in the formation of some embryonic structures. 3. Oncogene-induced senescence (OIS), suppresses tumor cell proliferation and development. 4. Immune cell recruitment and activation by senescent cells promote dysfunctional cell clearance. (Created with BioRender.com).

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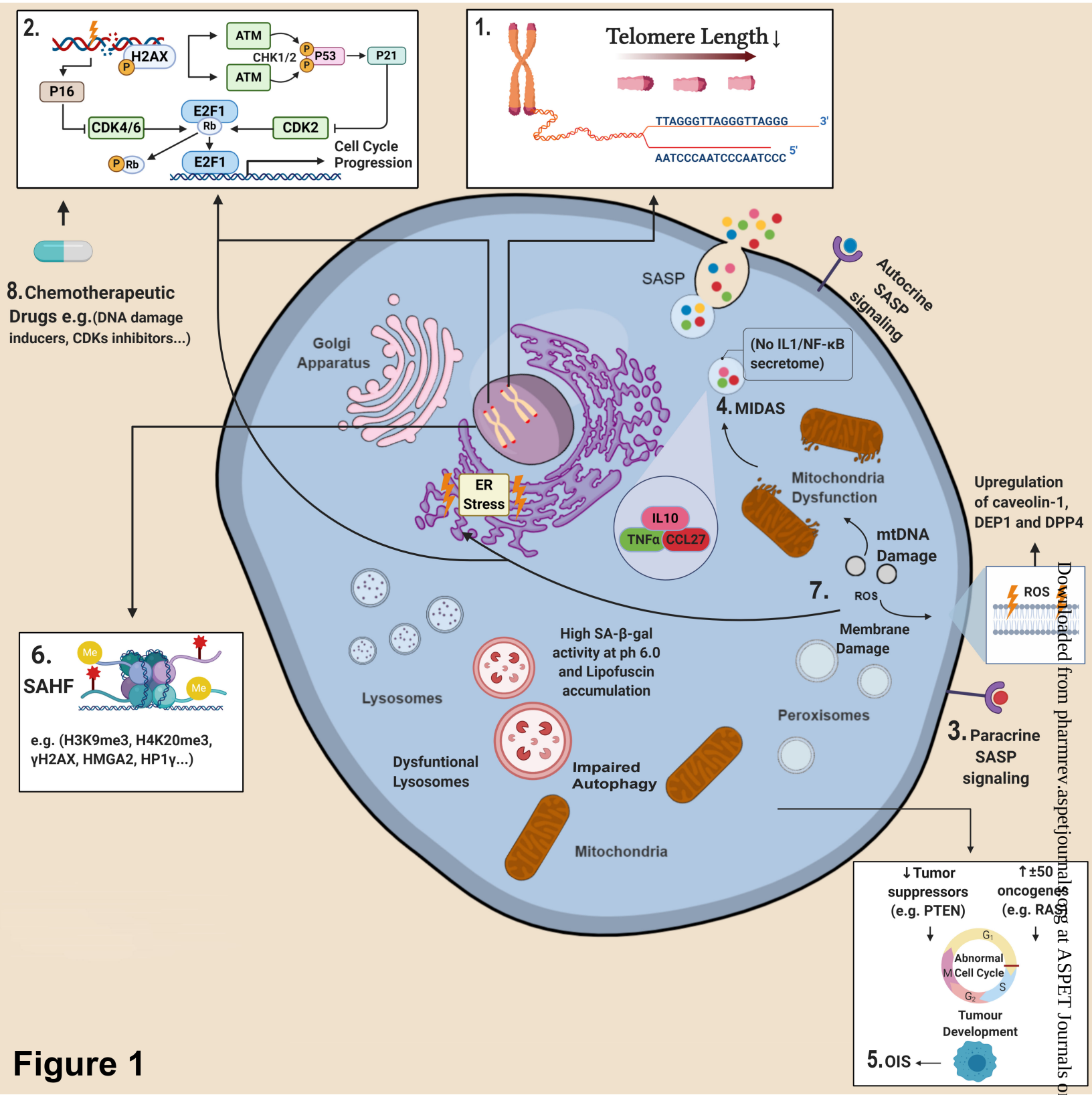

Figure 1

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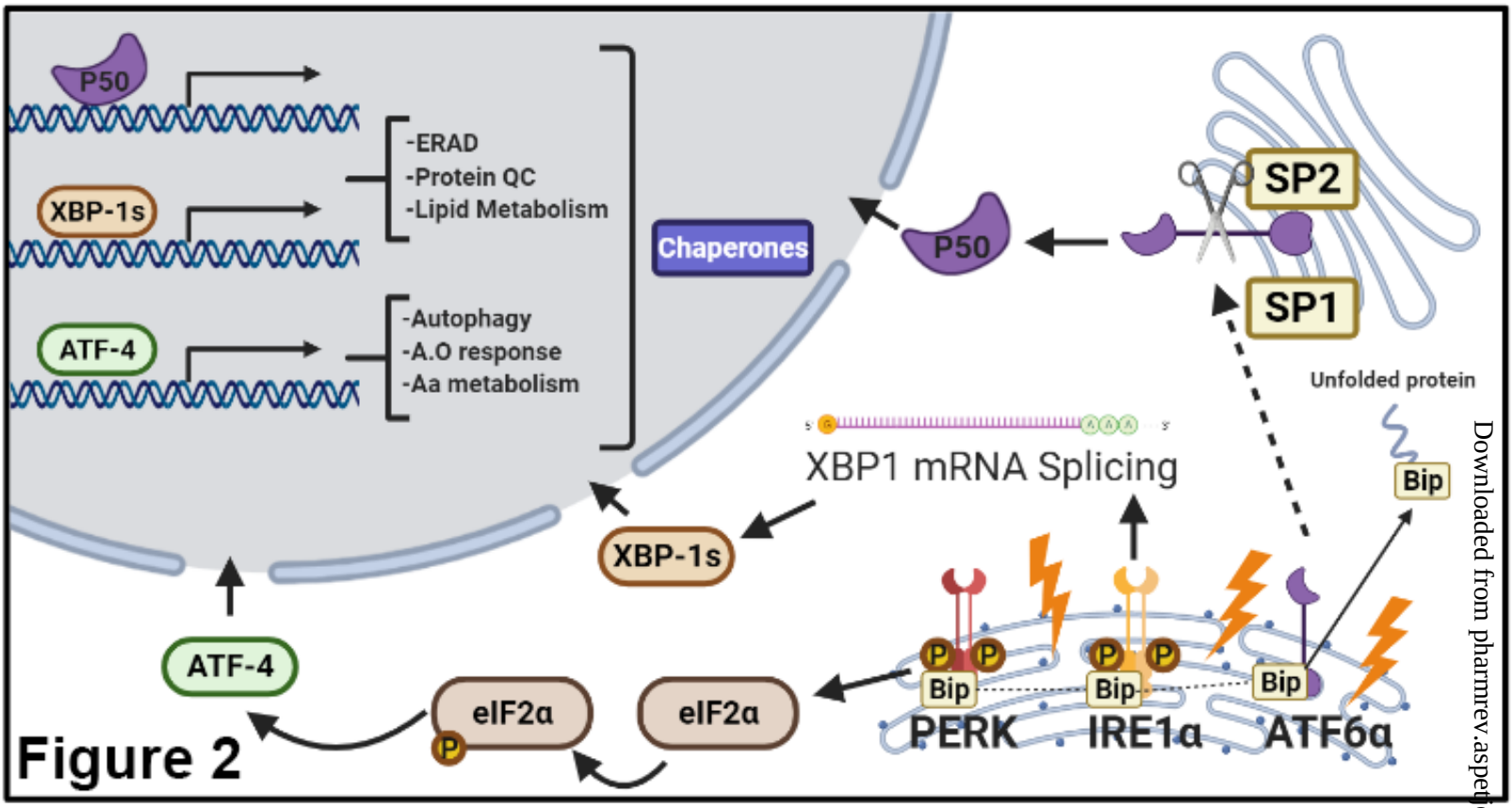

Figure 2

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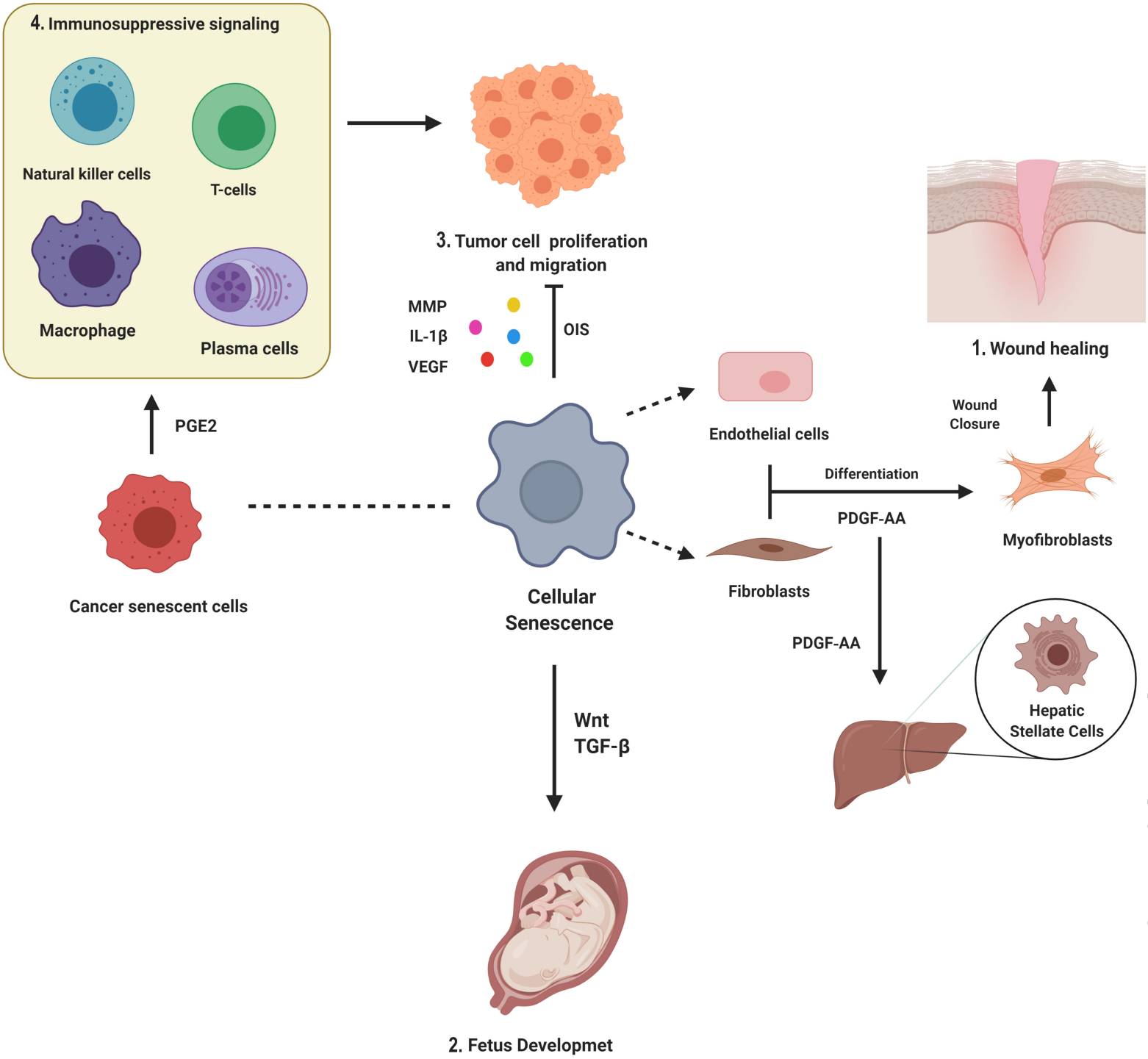

Figure 3