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Senescence and senotherapeutics: a new field in cancer therapy

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ABSTRACT

Cellular senescence is a stress response mechanism ensuring homeostasis. Its temporal activation during embryonic development or normal adult life is linked with beneficial properties. In contrast, persistent (chronic) senescence seems to exert detrimental effects fostering aging and age-related disorders, such as cancer. Due to the lack of a reliable marker able to detect senescence *in vivo*, its precise impact in age-related diseases is to a large extent still undetermined. A novel reagent termed GL13 (SenTraGor™) that we developed, allowing senescence recognition in any type of biological material, emerges as a powerful tool to study the phenomenon of senescence *in vivo*. Exploiting the advantages of this novel methodological approach, scientists will be able to detect and connect senescence with aggressive behavior in human malignancies, such as tolerance to chemotherapy in classical Hodgkin Lymphoma and Langerhans Cell Histiocytosis. The latter depicts the importance of developing the new and rapidly expanding field of senotherapeutic agents targeting and driving to cell death senescent cells. We discuss in detail the current progress of this exciting area of senotherapeutics and suggest its future perspectives and applications.

Contents

Abbreviations: ARF, alternate reading frame; cHL, classical Hodgkin lymphoma; LCH, Langerhans Cell Histiocytosis; DDR, DNA damage response; DNA-SCARS, DNA segments with chromatin alterations reinforcing senescence; HRS, Hodgkin and Reed-Sternberg; IKK, IkB kinase; IkB, Inhibitor of kappa B; LAD, Lamin-B1 associated chromatin domains; mTOR, mammalian target of rapamycin; Nrf2, nuclear factor E2-related factor 2; OIS, oncogene induced senescence; ROS, reactive oxygen species; SA-β-gal, senescence-associated β- galactosidase; SAHF, senescence associated heterochromatin foci; SASP, Senescence Associated Secretory Phenotype; SIRT, sirtuin; TIS, therapy induced senescence; UPR, unfolded protein response.

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1. Introduction

All organisms, from single cells to multi-cellular ones, activate a variety of complex biochemical pathways to ensure their homeostasis when intrinsic or extrinsic stressogenic insults occur (Gorgoulis et al., 2005; Bartkova et al., 2005; Bartkova et al., 2006; Halazonetis, Gorgoulis, & Bartek, 2008; Pateras et al., 2015; Petrakis et al., 2016; Gorgoulis, Pefani, Pateras, & Trougakos, 2018). Under physiological conditions these mechanisms manage to counterbalance detrimental forces that jeopardize proper cellular function, while in a non-physiological context this balance is disrupted leading to accumulation of cellular defects (Gorgoulis et al., 2018; Halazonetis et al., 2008; Pateras et al., 2015). As a consequence, cellular malfunctioning is established, promoting ageing and disease.

Cellular senescence is such a stress response mechanism, that similarly to apoptosis, aims to preserve cellular/tissue homeostasis. It was first described by Hayflick and Moorhead (1961) and termed replicative senescence, since this form was triggered by telomere attrition. Currently other forms of senescence have been defined that are collectively known as stress induced premature senescence. They can be induced independently of telomere length shortening by a variety of stress signals (Burton & Krizhanovsky, 2014; Georgakopoulou et al., 2016; Gorgoulis & Halazonetis, 2010; Munoz-Espin & Serrano, 2014). Oncogene induced senescence (OIS) is a well-established representative of stress induced premature senescence (Bartkova et al., 2006; Halazonetis et al., 2008; Gorgoulis & Halazonetis, 2010; Petrakis et al., 2016; Gorgoulis et al., 2018). Regardless of the initiating stimulus, cells that undergo senescence survive exhibiting a variety of phenotypical and molecular features (Fig. 1). Some of these are increased size with abnormal shape, cell division blockage, tolerance against apoptosis, metabolic dysfunction and a specialized secretory activity termed Senescence Associated Secretory Phenotype (SASP) (Campisi, 2013; Childs et al., 2017; Ewald, Desotelle, Wilding, & Jarrard, 2010). Additional characteristics include nuclear $p16^{INK4A/ARF}$ and $p21^{WAF1/Cip1}$ expression, occasionally DNA damage and senescence associated heterochromatin foci (SAHF), and increased lysosomal senescence-associated β-galactosidase (SA-β-gal) activity. Recently, lipofuscin accumulation was also established as a hallmark of senescent cells (Evangelou et al., 2017; Georgakopoulou et al., 2013).

A large body of experimental evidence accumulated over the last decades has demonstrated that senescent cells can have beneficial as well as harmful outcomes. Their transient occurrence in embryonic (developmental senescence) and adult life (acute senescence) due to their clearance by the immune system ensures proper tissue/organ development and homeostasis by withdrawal of stressed and/or damaged cells (Fig. 2) (He & Sharpless, 2017; Munoz-Espin & Serrano, 2014). On the contrary, senescent cell persistence (chronic senescence) and accumulation in tissues with age, driven by the imbalance in senescent cell induction/elimination (simultaneous accumulating stress/damage and weakening of the immune system), seems to be related with aging and the development and progression of age-related diseases (Fig. 2) (Burton & Krizhanovsky, 2014; He & Sharpless, 2017; Munoz-Espin & Serrano, 2014). In cancer, a severe age-related disease, senescence acts in a bimodal manner. While in early stages it has a tumor-suppressive role, in latter ones it drives cancer evolution via SASP (Bartkova et al., 2006; Burton & Krizhanovsky, 2014; Coppe, Desprez, Krtolica, & Campisi, 2010; Gorgoulis & Halazonetis, 2010; Halazonetis et al., 2008; Liontos et al., 2007; Petrakis et al., 2016; Rodier & Campisi, 2011).

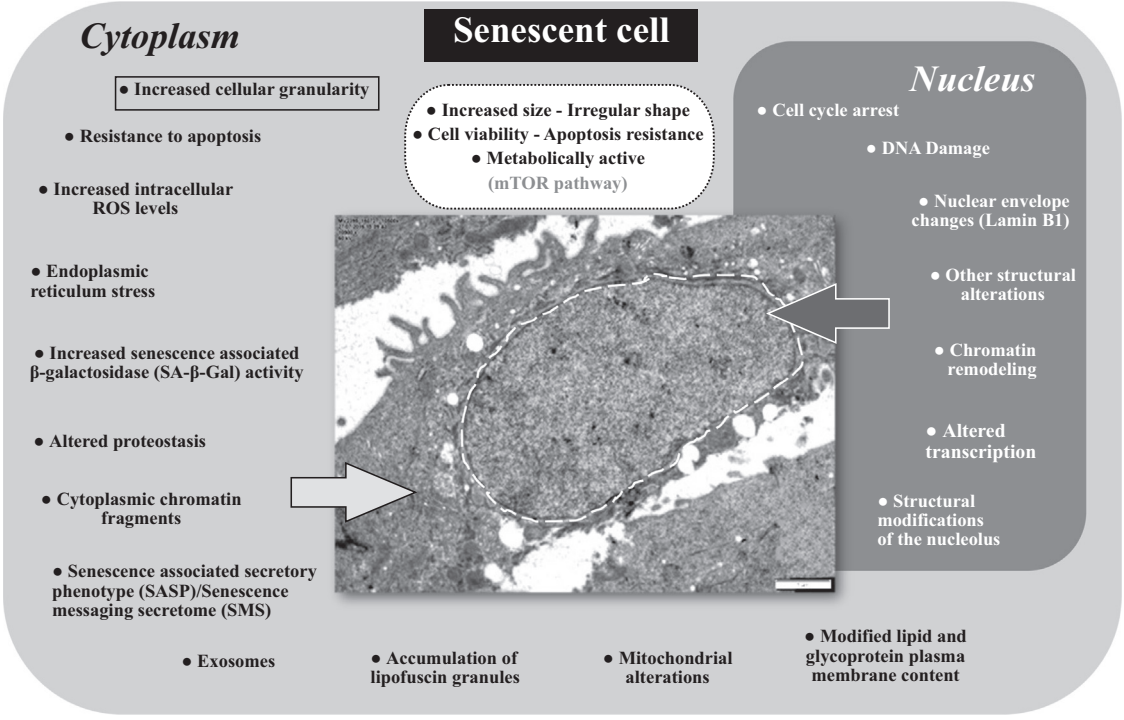

Fig. 1. Image depicting the key characteristics of senescent cells per cellular compartment (see text for details).

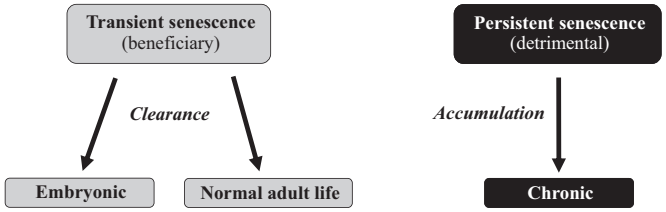

Fig. 2. Types of cellular senescence during life time and their effects (see text for details).

Notably, cancer cells adopt a senescent phenotype following antitumor interventions. This therapy induced senescence (TIS) has been associated with tumor relapse, metastasis and adverse prognosis, as it confers stemness to neoplastic cells (Milanovic et al., 2018). In addition, although senescence has been considered an irreversible cell-cycle arrest program for many years, new findings suggest that senescent cells under specific conditions can escape from this condition and re-enter the cell cycle, acquiring aggressive features (Galanos et al., 2016; Galanos et al., 2018; Komseli et al., 2018). This impact of the senescence state in modulating cancer cell plasticity reveals an important aspect of its "dark" potential (Fig. 3).

As evident so far, detection of senescence is essential. A major conundrum in the field of senescence was until recently its detection in *in vivo* settings, due to the lack of a reliable and applicable in clinical material, marker. Currently available methods are either insufficient or exhibit inherent inabilities for *in vivo* recognition of senescent cells (Munoz-Espin & Serrano, 2014). We bypassed this problem by generating a novel chemical compound (GL13; SenTraGor™) that detects specifically with high sensitivity senescent cells in any type of biological material, thus opening new horizons in understanding the role of senescence in aging and age-related disorders (Evangelou et al., 2017). Exploiting this new methodology in clinical settings and in order to shed light to this exciting discovery, some examples are presented here for the first time, detecting senescence in a primary human malignancy, namely classical Hodgkin Lymphoma (cHL). Notably, increased number of senescent cells in cHL confers a poorer clinical outcome, in terms of resistance to first and second line treatment (Figs. 4–5, Suppl Table 1–3). By the same means, we identified senescent cells also in the context of primary human Langerhans Cell Histiocytosis (LCH), a neoplastic lesion with high prevalence of the BRAF V600E oncogene activating mutation (Fig. 6). The latter is associated with fatal consequences in a significant cluster of disseminated cases (Badalian-Very, 2014).

Such findings denote the emerging role of senescence in age-related disorders and aging that has been the springboard for the quest of senescence-oriented therapeutic strategies, aiming its detrimental effects. To date, two main anti-senescent drug categories have been introduced into basic and clinical research, with each one including several classes of compounds: "senocidals" (including senolytics and senoptotics) and senomorphics (Kirkland, Tchkonia, Zhu, Niedernhofer, & Robbins, 2017; Niedernhofer & Robbins, 2018; Schmitt, 2017; Soto-Gamez & Demaria, 2017). The former term represents pharmaceutical agents that specifically eliminate senescent cells, either by apoptotic (senoptosis) or nonapoptotic (senolysis) means. The latter consists of drugs that suppress markers of senescence or the secretory phenotype of senescent cells, without inducing cellular death (Childs et al., 2017; Kirkland et al., 2017; Soto-Gamez & Demaria, 2017; Zhu et al., 2015). Moreover, pro-senescent strategies, meaning the application of systemic senescence-

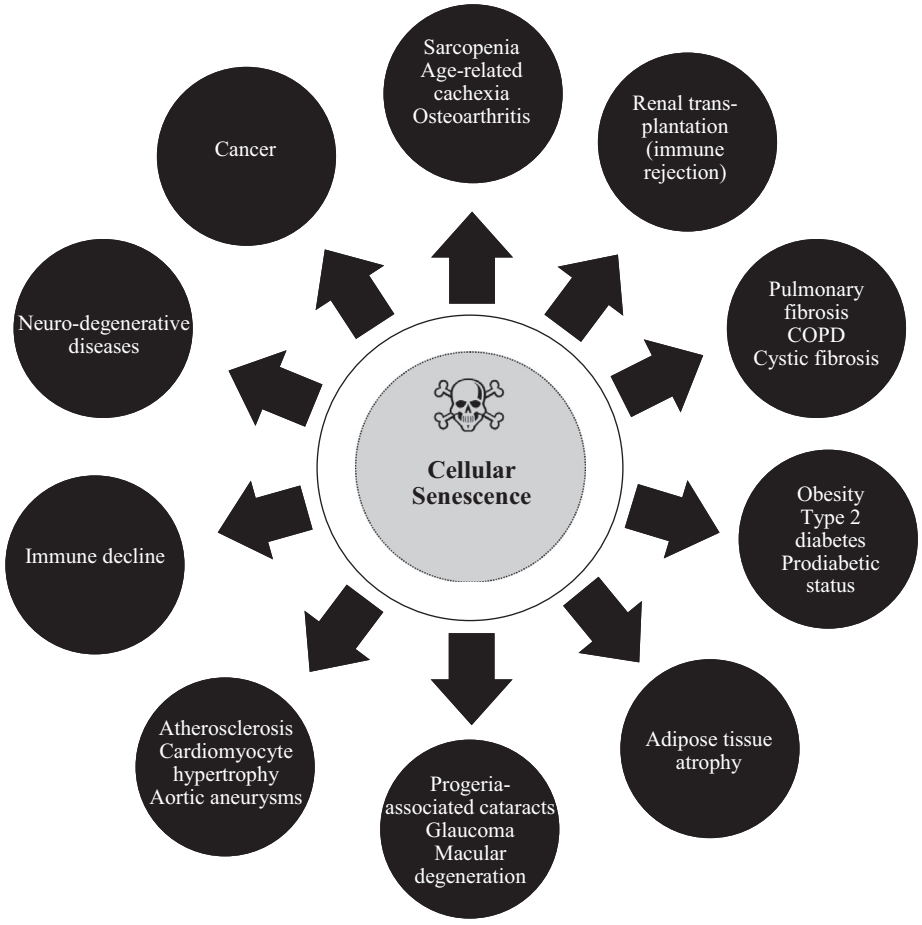

Fig. 3. The "dark side" of cellular senescence (for details see text). (COPD: chronic obstructive pulmonary disease).

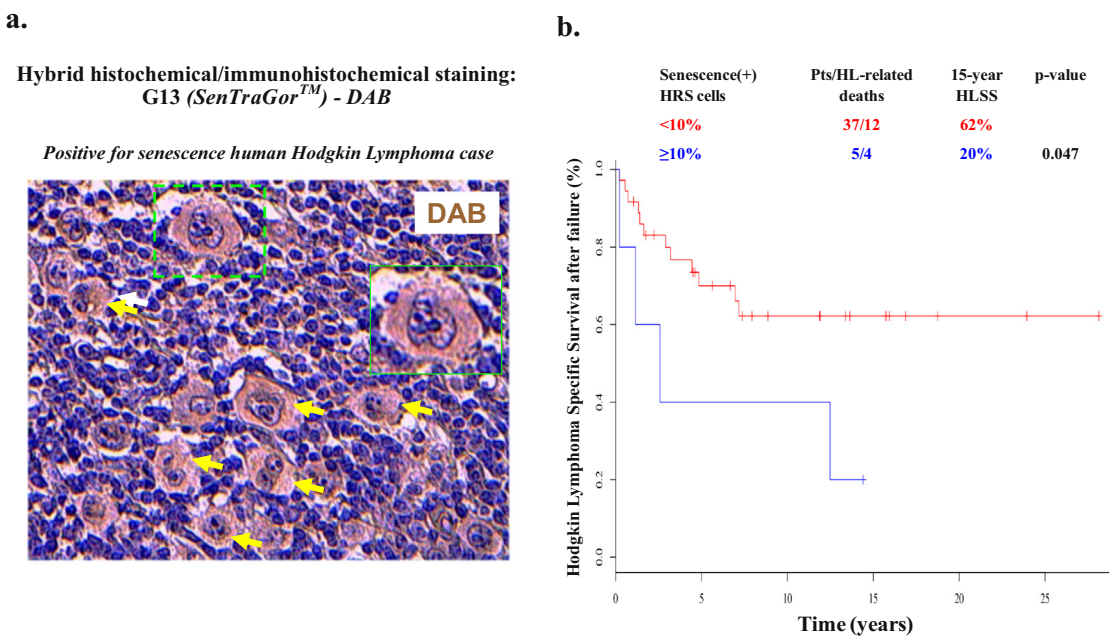

Fig. 4. Senescence in primary human cHLs. a. Representative result from applying a hybrid Histo (GL13)-Immunohistochemical (anti-biotin) method. Arrows indicate positive GL13 HRS cells. Diaminobenzidine (DAB) chromogenic reaction, hematoxylin counterstain. Magnifications: x400, inset: x630. b. Kaplan-Meier curves showing the differences in post-relapse HLSS, using 10% as cutoff.

promoting stress, such as ionizing radiation or DNA-damaging chemotherapy, or the restoration of defective stress-response pathways, have also been developed (Lujambio, 2016; Watanabe, Kawamoto, Ohtani, & Hara, 2017). Preliminary results suggest that the rapidly evolving repertoire of senotherapeutics could have the potential to score a significant impact on the fight against cancer, introducing a new era in cancer therapeutics (Kirkland et al., 2017; Niedernhofer & Robbins, 2018; Schmitt, 2017; Soto-Gamez & Demaria, 2017). However, given the complex nature of both cellular senescence and cancer, as well as the various pros and cons of each senotherapeutic modality, many issues remain to be resolved.

In this review, we provide an updated, comprehensive insight on cellular senescence and on current and future senotherapeutic strategies, which is a highly upcoming field of research with clinical and pharmaceutical orientation. For this purpose, in the first parts, we provide critical aspects of this biological phenomenon which are essential for its understanding and unravel possible therapeutic targets. Next, we highlight issues related to its recognition, focusing on a new detection assay and on the clinical relevance. Regarding the latter, we present novel *in vivo* aspects on the importance of cellular senescence in human malignancies. In the last part, we confer an updated overview of the spectrum of pharmacological interventions that target senescent cells, as well as associated limitations and challenges.

2. Cellular and molecular features of senescence

Senescent cells can exhibit a plethora of morphological and molecular characteristics that are non specific alone and not globally acquired (Fig. 1) (Munoz-Espin & Serrano, 2014; Salama, Sadaie, Hoare, & Narita, 2014). Until recently a combination of them was required to be evident in a cell to imply acquisition of the senescent state. These cellular (viability, large size/irregular shape, growth arrest, resistance to apoptosis) and subcellular ($p21^{WAF1/Cip1}$, $p16^{INK4A}$, SA-β-Gal activity, lipofuscin accumulation and SASP) traits, reflect orchestrated molecular responses mediated by a wide spectrum of genetic and epigenetic events.

In this context, for many years ARF, the Alternative Reading Frame product of the *INK4A/ARF* locus, was considered the sole response mechanism against activated oncogenes triggering either cell cycle arrest/senescence or apoptosis. However, since genomic instability is a hallmark of cancer (Gorgoulis et al., 2018; Negrini, Gorgoulis, & Halazonetis, 2010) we hypothesized that the DNA Damage Response pathway (DDR), that ensures genomic integrity (Gorgoulis et al., 2018; Halazonetis et al., 2008; Negrini et al., 2010), could also act as a complementary partner in this response. We demonstrated that DDR and ARF activation occurs in time-related manner during carcinogenesis and depending on the oncogenic load and cellular milieu they set in motion either the senescent or the apoptotic antitumor barrier (Evangelou et al., 2013; Velimezi et al., 2013). Specifically, DDR is activated from the earliest stages of cancer due to a lower oncogenic threshold, whereas ARF induction occurs later, as oncogenic stimuli increase (Evangelou et al., 2013; Gorgoulis et al., 2005; Velimezi et al., 2013). Moreover, ARF acts as a second defense line in the case that components of the DDR machinery, like serine/threonine kinase ATM, are disabled (Evangelou et al., 2013; Velimezi et al., 2013). As a result p53/$p21^{WAF1/Cip1}$ activation occurs inducing the antitumor barriers (senescence or apoptosis) preventing transformation of incipient cancer cells (Fig. 7) (Evangelou et al., 2013; Velimezi et al., 2013). Activation of the p53/$p21^{WAF1/Cip1}$ axis promotes a steady increase in reactive oxygen species (ROS) generation that further fuels DNA damage (Munoz-Espin & Serrano, 2014). This positive feedback loop seems to be both necessary and sufficient to maintain cell cycle arrest during the establishment of senescence (Passos et al., 2010). Progression to cancer requires evasion of the antitumor barriers and occurs when critical DDR and ARF pathway components, like p53, are impaired (Galanos et al., 2016; Gorgoulis et al., 2018; Halazonetis et al., 2008; Petrakis et al., 2016; Petrakis, Vougas, & Gorgoulis, 2012; Sideridou et al., 2011).

The p53/$p21^{WAF1/Cip1}$ axis and the products of the *INK4A/ARF* locus are key players in senescence induction when cells respond also to various, other than oncogenes, stress insults. It has been suggested that while the p53/$p21^{WAF1}$ axis acts to initiate the senescence response, $p16^{INK4A}$ functions for the maintenance of this state (Childs, Baker, Kirkland, Campisi, & van Deursen, 2014). There are certain molecular patterns followed depending on the type of senescence induced (Fig. 2). For example, embryonic senescence strictly relies on $p21^{WAF1/Cip1}$, TGF-b/SMAD and PI3K/FOXO pathways, but is independent of

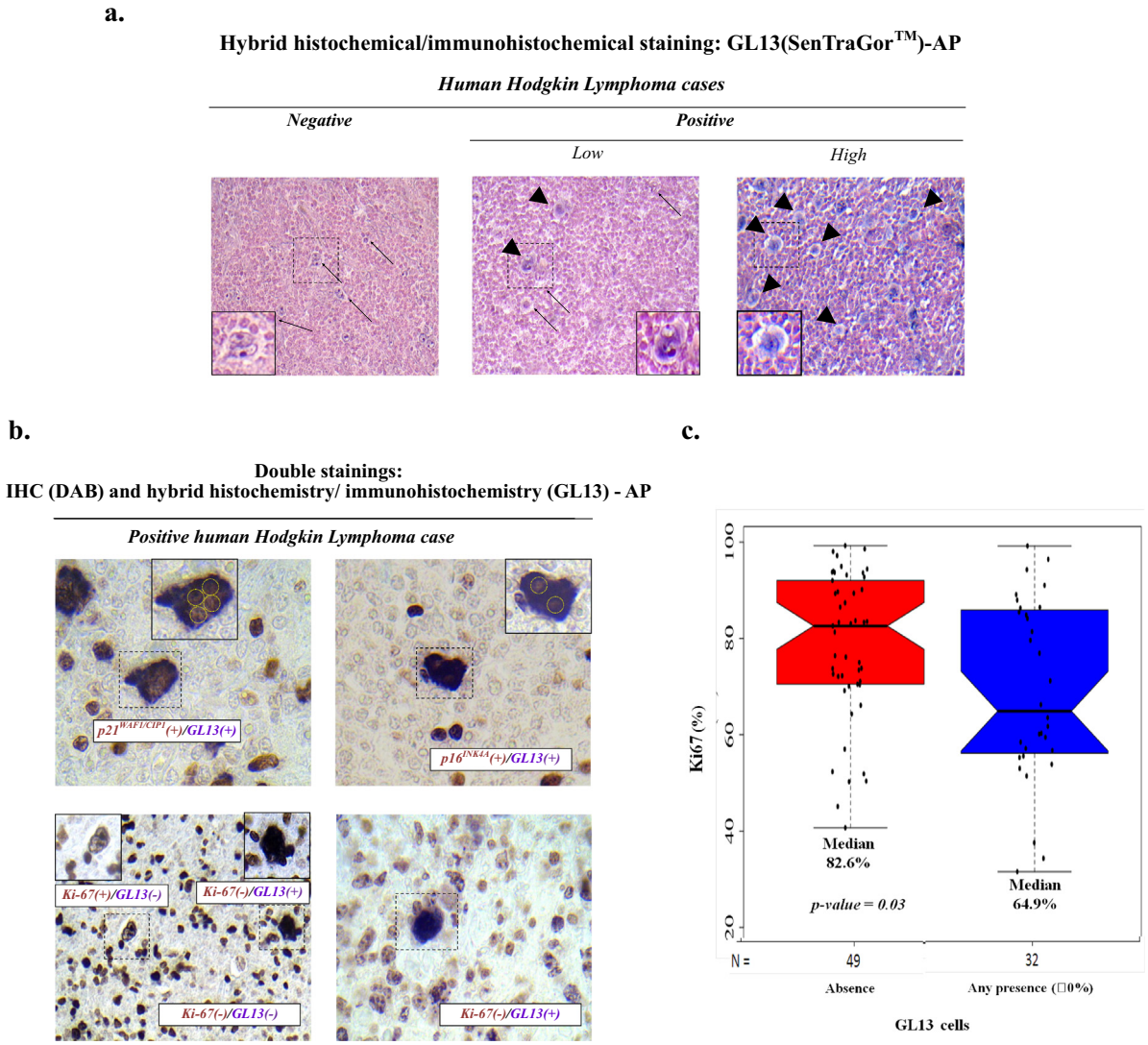

Fig. 5. a. Hybrid Histo (GL13)-Immunohistochemical (anti-biotin) method in primary human cHLs. Representative results of the hybrid Histo GL13-Immunohistochemical (anti-biotin) method. Left panel: negative cHL case. Middle and right panel: positive cHLs cases with low and high cytoplasmic GL13 staining (dark blue). Arrows indicate negative GL13 HRS cells. b. Double immunohistochemical/hybrid Histo-Immunohistochemical stainings in primary human cHLs. Double staining results showing senescent HRS cells with concurrent cytoplasmic GL13 positivity (dark blue) and nuclear reaction (dashed yellow circles) for $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ (upper panels). Mutual exclusive staining between GL13 and Ki-67 in HRS cells (lower panels). Black arrowhead denotes a double negative HRS cell. [a and b: Alkaline Phosphatase (AP) chromogenic reaction: dark blue cytoplasmic product, Diaminobenzidine (DAB) reaction: nuclear brown signal, a: Counterstain: Nuclear fast red]. Magnifications: x400, insets: x63. For left Ki-67/GL13 double staining image: magnification: x200, insets: x40. c. Boxplot indicating the percentage of Ki67(+) HRS cells in senescence(-) and senescence(+) cases.

DNA damage, p53, or other cell-cycle inhibitors (Munoz-Espin et al., 2013; Storer et al., 2013). Senescence can also be induced upon DNA damage via the Rb-$p16^{INK4A}$ route or in response to oxidative stress via $p38^{MAPK}$ mediated $p16^{INK4A}$ up-regulation (Iwasa, Han, & Ishikawa, 2003). $p38^{MAPK}$ belongs to the stress-activated protein kinase family that responds to a variety of stresses, including oxidative stress (Kyriakis & Avruch, 2012). ARF, as previously shown, is also a sensor of oxidative stress signals (Liontos, Pateras, Evangelou, & Gorgoulis, 2012). Moreover, the $p16^{INK4a}$/Rb-pathway is implicated in a ROS-depended positive feedback loop, which reinforces the irreversible cell cycle arrest in senescent cells (Takahashi et al., 2006).

In addition to cell division arrest, resistance to apoptosis is also a key feature of the senescent phenotype, thereby favoring clearance by the immune system (Fig. 1). Members of the BCL-2 family and other factors such ephrins, PI3K, FOXO4 and HSP90 are implicated in this process. Notably, senescence and apoptosis share common signaling cascades such as the p53 pathway. Which outcome is eventually selected by the cell, depends on the so called "hallmarks of stress" (Childs et al., 2014; Georgakopoulou et al., 2016; Gorgoulis et al., 2018). In this context, we have suggested that stromal cells enter preferentially senescence upon stress to preserve tissue homeostasis, while epithelial cells that exhibit an increased capacity of renewal, and respond by inducing apoptosis (Georgakopoulou et al., 2016; Liakou et al., 2016).

Senescent cells are also characterized by the acquisition of a distinctive pro-inflammatory, proteolytic secretome, termed SASP or Senescence Messaging Secretome (SMS) (Fig. 1) (Coppe et al., 2010; Evan & d'Adda di Fagagna, 2009; Kuilman & Peeper, 2009). SASP consists of a complex mixture of secreted cytokines, chemokines, growth factors, and proteases. This secretory function exerts an immunomodulatory role by augmenting and promoting senescence in an autocrine and paracrine manner. SASP induction relies on the activation of NF-κB and mTOR (mammalian target of rapamycin) and on $p38^{MAPK}$ signaling (He & Sharpless, 2017; Herranz et al., 2015; Laberge et al., 2015). Additionally, ectodomain shedding as well as secretion via small extracellular vesicles have been both recognized as key phenomena implicated in the release or secretion, respectively, of some SASP factors, thus

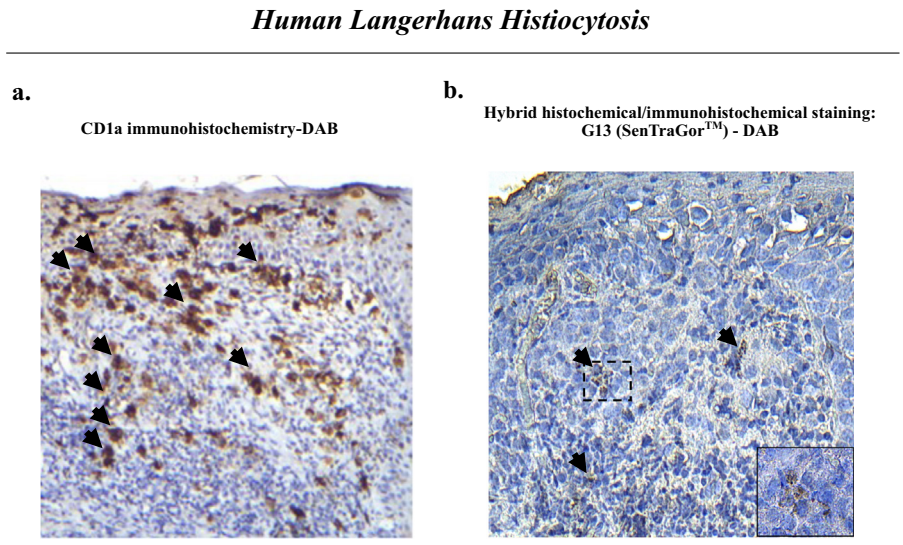

Fig. 6. Senescence detection in human Langerhans Cell Histiocytosis (LCH). Serial sections of a representative LCH case stained for CD1a (a) and GL13-Sentragor™ (b). Arrows indicate positive cells. Diaminobenzidine (DAB) chromogenic reaction, hematoxylin counterstain. Magnification: ×200, Inset: ×630.

mediating a more remote effect (Hernandez-Segura, Nehme, & Demaria, 2018; Lehmann et al., 2008; Stow & Murray, 2013; Takasugi et al., 2017). In this regard, extracellular vesicles have been implicated in cancer cell proliferation, inflammation, as well as telomere regulation (Takasugi, 2018).

Senescent cells are characterized by increased metabolic activity, despite cell cycle arrest. Under certain circumstances (replicative senescence) a transition from oxidative phosphorylation to glycolysis is evident (Weichhart, 2018). In general, activation of anabolic pathways and down-regulation of catabolic processes has been linked with aging. The opposite pattern that ensures energy sufficiency under calorie restriction and removal of damaged organelles, has been related with lifespan extension in a various organisms (Schreiber, O'Leary, & Kennedy, 2016). The mTOR network is a crucial mediator of this balance, as it integrates stimuli from nutrients, growth factors, energy status and stress (Schreiber et al., 2016). mTOR is active from the onset of cellular senescence and drives the biosynthesis of molecules vital for cell integrity such as proteins, lipids and nucleic acids. At the same time it decreases the autophagic activity to levels that ensure cell survival but further accelerate the progression of senescence by diminishing the clearance of damaged biomolecules and organelles (Weichhart, 2018). In contrast, calorie restriction which is related to longevity results in mTOR pathway inhibition, increased autophagy and senescence delay (Weichhart,

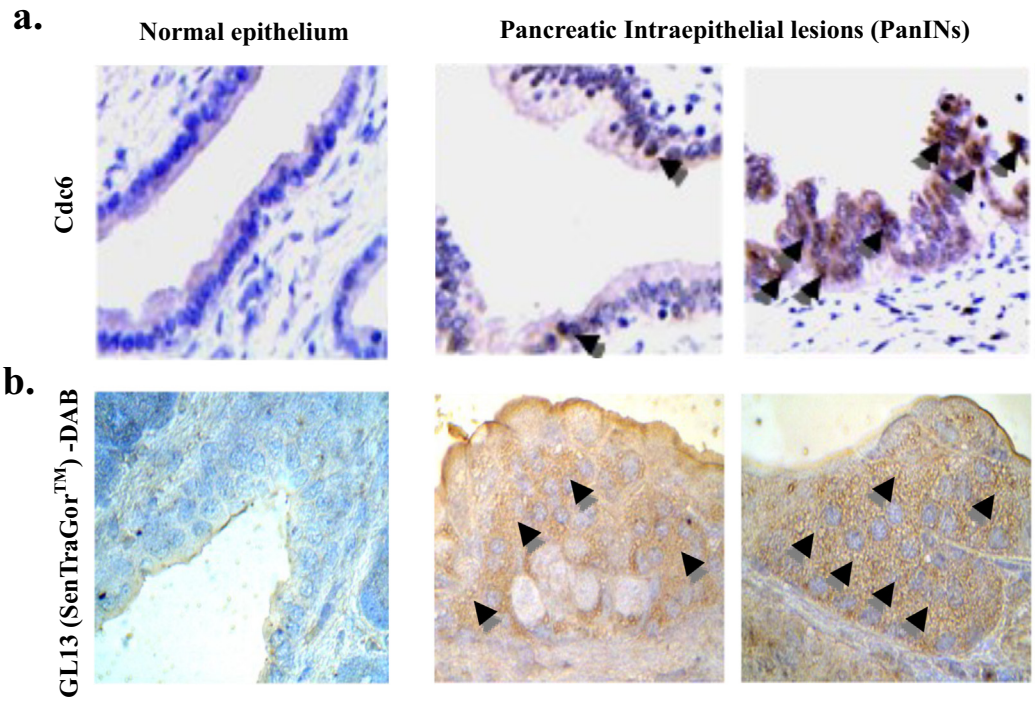

Fig. 7. Oncogenic Cdc6 driven senescence, as an antitumor barrier, in pancreatic carcinogenesis. a. Aberrant Cdc6 expression occurs from the earliest stages of pancreatic carcinogenesis. b. Application of the hybrid Histo (GL13)-Immunohistochemical (anti-biotin) method reveals senescence in low grade Pancreatic Intraepitelial lesions (PanINs). Arrowheads indicate positive Cdc6 and GL13 cells. Diaminobenzidine (DAB) reaction: cytoplasmic brown signal, Counterstain=Hematoxylin. Magnifications: ×200 (a), ×400 (b).

2018). This seemingly inverse interplay between autophagy and senescence is however more complex and controversial and further studies are required.

Senescent cells, as already mentioned, rely on base line autophagic activity to counterbalance metabolic stress and stay alive (Weichhart, 2018). To this end, lysosome function seems also essential. Cellular senescence is usually characterized by increased lysosomal β-galactosidase (commonly termed "SA-β-gal") activity, which is attributed to active autophagy along with high lysosomal content (Fig. 1) (Young et al., 2009; Dimri et al., 1995; Kurz, Decary, Hong, & Erusalimsky, 2000; Lee et al., 2006). The accumulation of aged lysosomes along with increased lysosome biogenesis seems to drive the enhanced lysosomal content that characterizes senescent cells (Hernandez-Segura et al., 2018). In addition, as we have demonstrated aggregation of lipofuscin- the waste material of metabolism- is a key property of senescent cells (Fig. 1) (Georgakopoulou et al., 2013). This material consists of heavily oxidized proteins that undergo various biochemical modifications and form cross links and finally a scaffold that is non-degradable by proteolysis (Höhn & Grune, 2013; König et al., 2017). Oxidized lipids, carbohydrates and metals are added during this process. Lipofuscin is a very resilient material since it was extracted and detected (Rizou et al., 2018) in bone remains in the proximity of a fossil cranium [Apidima 1 (LAO1/S1)], of an estimated age of 160.000 years (Fig. 8). Impaired proteostasis in senescent cells is a frequent trait and occurs at various levels. mTOR activation drives increased protein biosynthesis while autophagy down-regulation leads to accumulation of misfolded/unfolded proteins (Schreiber et al., 2016). The latter is boosted by inhibition of the 26S subunit of the proteasome due to lipofuscin accumulation, which is an active material and produces ROS (Gorgoulis et al., 2018; Höhn & Grune, 2013; König et al., 2017). Endoplasmic reticulum stress has also been implicated in excess of unfolded proteins in senescent cells, triggering a reaction termed unfolded protein response (UPR) (Fig. 1) (Pluquet, Pourtier, & Abbadie, 2015). Upon failure of UPR to counteract prolonged or unresolved endoplasmic reticulum stress cell death occurs (Pluquet et al., 2015). Of great interest, mitochondrial function and life cycle during senescence are also under regulation by the mTOR/autophagy (mitophagy) interplay. In fact, mitochondria seem to exert a critical role in the onset of the senescent phenotype (Correia-Melo et al., 2016). mTOR activation increases mitochondrial function and biogenesis while senescent cells accumulate dysfunctional mitochondria, due to perturbed turnover of mitochondria (mitophagy). This mitochondrial dysfunction (Senescence-Associated Mitochondrial Dysfunction) is characterized by decreased oxidative phosphorylation and increased endogenous ROS formation (Fig. 1) (Correia-Melo & Passos, 2015; Lawless et al., 2012; Passos et al., 2007; Passos et al., 2010).

Extensive epigenetic modifications form a specialized landscape that characterizes the senescence phenotype (Moudry et al., 2016). Gain of active histone modifications or loss of repressive histone marks in the promoter of genes related to aging and senescence has been reported (Shah et al., 2013). More specifically, H3K4me3 has been found enriched at up-regulated senescence associated genes (eg SASP, antiproliferation and stress response genes) and is characteristically depleted at down-regulated genes. In contrast, H3K27me3 demonstrates the opposite pattern; it is lost at up-regulated "senescence" genes and enriched in suppressed ones that are related to proliferation. These alterations result in the formation of areas enriched with H3K4me3 and H3K27me3 ("mesas") and areas depleted in H3K27me3 ("canyons"), across the senescence genome. Mesas have been shown to localize at lamin B1-associated chromatin domains (LADs) while canyons are mainly located between LADs (Shah et al., 2013). Apart from LADs and the organization of the inner side of the nuclear membrane, lamins are also implicated in nuclear shaping and DNA replication (Gruenbaum & Foisner, 2015). These molecules have recently gained an increased attention since discontinuous nuclear lamina, due to loss of lamin B1 (Shah et al., 2013), has been proposed as new promising marker of senescence (Fig. 1) (Munoz-Espin & Serrano, 2014; Salama et al., 2014).

Epigenetic regulation during senescence seems also to be mediated by a class of enzymes termed sirtuins (SIRTs) that exhibit additional metabolic and DNA repair functions (Tasselli et al., 2016). These factors possess histone deacetylase or mono-ribosyltransferase activity and are related to longevity in a species-specific manner (Ghosh & Zhou, 2015). Their down-regulation has been associated with aging and senescence (Ghosh & Zhou, 2015; Giblin & Lombard, 2016). SIRTs mediate deacetylation of lysine residues of histone tails increasing thus chromatin condensation (Giblin & Lombard, 2016). This in turn negatively affects accessibility of transcription factors and RNA polymerase II to chromatin regions and alters gene transcription (Giblin & Lombard, 2016). Other chromatin modifications, such as DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) or SAHFs are often observed in certain genetic backgrounds and are therefore neither sensitive nor specific. SAHFs are observed in the frame of RAS induced senescence (Di Micco et al., 2011), while their absence is evident in cells undergoing Cdc6 driven OIS (Komseli et al., 2018). SAHFs formation is related to down-regulation ("switch off") of genes implicated in cell proliferation

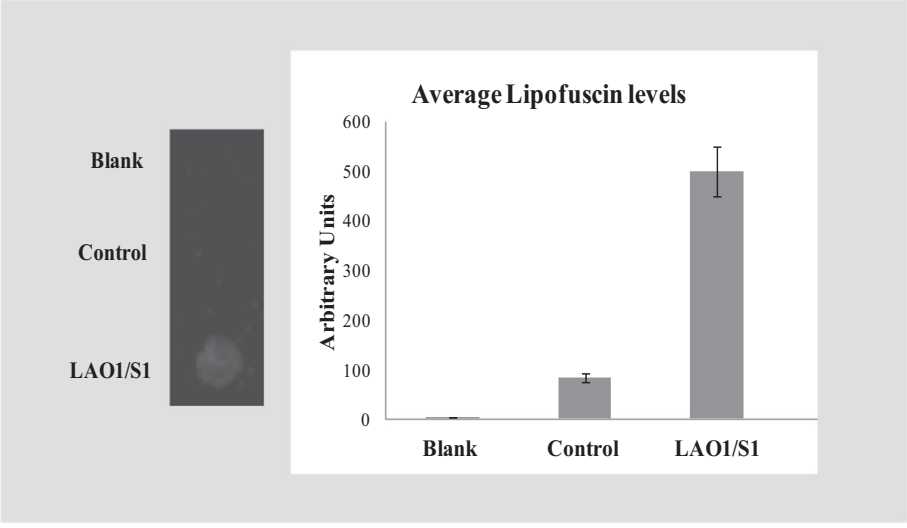

Fig. 8. Quantitative estimation of lipofuscin amounts extracted from bone remains in the proximity of a fossil cranium of an estimated age of 160.000 years [Apidima 1 (LAO1/S1)] using a novel, SentraGor™ based, methodology.

(Munoz-Espin & Serrano, 2014; Salama et al., 2014). Non-coding RNAs have also been implicated in the epigenetic regulation of the senescent phenotype (Komseli et al., 2018).

Interestingly, chromatin fragments extruded from the nucleus into the cytoplasm are targeted by the lysosomal/autophagy machinery (Ivanov et al., 2013). The functional role of cytoplasmic chromatin is generally unclear, however recent evidence suggest that it may serve as a cytoplasmic "danger signal" to alarm the immune system (Dou et al., 2017). It has been shown that the cytoplasmic chromatin- cyclic GMP-AMP synthase (cGAS) - Stimulator of interferon genes (STING) pathway is involved in expression of pro-inflammatory genes in cancer cells that promote senescence (Glück et al., 2017; Yang, Wang, Rena, Chen, & Chen, 2017).

Cellular senescence in culture is typically accompanied by specific morphological alterations (Fig. 1), which rely on cytoskeleton rearrangements (Cormenier et al., 2018; Druelle et al., 2016). Of note, these traits are less prominent *in vivo*. Rearrangement of the cytoskeleton, specifically of the vimentin filaments, through the ATF6a- branch of the UPR pathway, seems to contribute to the senescence-associated shape alterations (Cormenier et al., 2018; Druelle et al., 2016). Additionally, not only cell size and shape modifications, but also plasma membrane composition changes occur during senescence (with the more prominent being the upregulation of caveolin-1), thus affecting inter-cellular communication and intra-cellular signaling pathways (Althubiti et al., 2014; Hernandez-Segura et al., 2018; Ohno-Iwashita, Shimada, Hayashi, & Inomata, 2010).

3. Senescence detecting methods

The lack of a reliable marker to detect senescent cells, particularly *in vivo*, has been until recently a critical limiting factor in the "senescent field". While *in vitro*, their morphological features (large, flat, vacuolated and multinucleated) can help in their recognition, these characteristics are quite impossible to spot *in vivo* (Munoz-Espin & Serrano, 2014). Since the discovery of senescence a number of markers have been used for their identification (Table 1). However, most of them are also implicated in non-senescent cellular programs, while the specificity of the applied methods is questionable (Hernandez-Segura et al., 2018).

The assay traditionally used for detecting cellular senescence, the SA-β-gal activity, identifies as already mentioned the increased activity of lysosomal β-D-galactosidase in senescent cells in conditions of suboptimal pH (pH: 6.0) (Dimri et al., 1995; Georgakopoulou et al., 2013; Munoz-Espin & Serrano, 2014). Its foremost limitation is the requirement of fresh tissue to retain enzymatic activity, rendering it unapplicable in archival (Formalin Fixed Paraffin Embedded) material, which represents the vast majority of tissues stored in Pathology and Research laboratories (Debacq-Chainiaux, Erusalimsky, Campisi, & Toussaint, 2009; Rodier & Campisi, 2011). Further drawbacks of the SA-β-gal assay include false positive staining under certain cell culture conditions, such as serum starvation and confluence, as well as false negative results in cells that fully undergo senescence, but may not exhibit SA-β-gal activity (Munoz-Espin & Serrano, 2014; Salama et al., 2014; Severino, Allen, Balin, Balin, & Cristofalo, 2000). Several modifications that use other chromogenic or fluorescent (one or two-photon fluorescence excitation) probes for the visualization of SA-β-gal activity (Lee et al., 2014; Lozano-Torres et al., 2017; Zhang et al., 2017) exhibit similar disadvantages and their specificity in recognizing senescent cells should be always confirmed with other senescence biomarkers (immunohistochemical expression of $p21^{WAF/CIP1}$, $p16^{INK4a}$ etc, or GL13 positivity as described below).

To circumvent this obstacle, we recently generated a novel reagent (GL13) and developed a hybrid histochemical/immunohistochemical method that facilitates, with high specificity and sensitivity, *in situ* identification of senescent cells in any type of biological material including archival tissues (Evangelou et al., 2017). GL13 (commercially available as SenTraGor™) is a biotinylated Sudan Black-B based chemical derivative that strongly binds to lipofuscin, a well established property of senescent cells (Evangelou & Gorgoulis, 2017; Georgakopoulou et al., 2013). The method provides the major advantage of co-visualization with other markers; thus potentially unrevealing new players involved in imposing senescence (Evangelou et al., 2017; Komseli et al., 2018).

Table 1
List of markers/methods for senescent cell detection.

Marker/Method	References
Large and flat morphology (Various microscopical and staining approaches)	(Hayflick & Moorhead, 1961; Hernandez-Segura et al., 2018)
Lack of cell proliferation markers: absence of Ki-67, BrdU/EdU-incorporation, no colony formation	(Hayflick & Moorhead, 1961; Hernandez-Segura et al., 2018)
Lack of response to growth signals	(Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013)
Resistance to apoptosis: BCL family members (Bcl-2, Bcl-w, or Bcl-xL)	(Hernandez-Segura et al., 2018)
p53	(Lopez-Otin et al., 2013)
ARF	(Lopez-Otin et al., 2013)
CDKIs (p16INK4a, p15INK4B, p21WAF/CIP1, p27KIP1)	(Lopez-Otin et al., 2013)
DDR markers (ATM, 53BP1, γH2AX, MBS1, CHK2)	(d'Adda di Fagagna, 2008).
Lamin B1 reduction	(Shimi et al., 2011)
DEC1 (BHLHE40)	(Collado et al., 2005)
DCR2 (TNFRSF10D)	(Collado et al., 2005)
PML nuclear bodies	(Lopez-Otin et al., 2013)
SAHF (senescence-associated heterochromatic foci)/markers (HP1-γ, H3K9me3)	(Hernandez-Segura et al., 2018; Narita et al., 2003)
HMGA proteins	(O'Sullivan & Karlseder, 2012)
TIF (telomere dysfunction-induced foci)	(Takai, Smogorzewska, & de Lange, 2003)
TAF (telomere-associated foci)	(Takai et al., 2003)
DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence)	(Rodier & Campisi, 2011)
Cell surface proteins (DEP1, B2MG and DPP4)	(Althubiti et al., 2014; Kim et al., 2017)
SA-β-gal (senescence-associated β-galactosidase activity)	(Dimri et al., 1995)
Modifications: chromogenic or fluorescent (one or two-photon fluorescence excitation) probes for the visualization of SA-β-gal activity	Modified: (Debacq-Chainiaux et al., 2009; Lee et al., 2014; Lozano-Torres et al., 2017; Zhang et al., 2017)
Lipofuscin accumulation - Hybrid histochemical/immunohistochemical method	(Evangelou et al., 2017; Evangelou & Gorgoulis, 2017; Georgakopoulou et al., 2013)
SASP factors, ligands and receptors (IL-1a, IL-6,and IL-8, CCL2 and metalloproteinases)	(Hernandez-Segura et al., 2018; Kuilman & Peeper, 2009)
Quantitative identification based on a combination of SA-β-gal and molecular marker staining with flow cytometry and high content image analysis	(Biran et al., 2017)
Senescence chips for ultrahigh-throughput isolation and removal of senescent cells	(Chen, Mao, Snijders, & Wang, 2018)

4. Clinical relevance - role of senescence in cancer

Our knowledge regarding the role of cellular senescence in various age-related diseases stems mainly from *in vitro* studies, due to the lack of available *in vivo* detection systems, as mentioned earlier. The *in vitro* settings were conducted in isolated cells derived from experimental models and clinical panels. The main conclusion drawn by these reports is that senescence exerts a bimodal behavior, beneficial or detrimental. Favorable effects have been related to the attenuation of liver fibrosis in cirrhotic models, the reduction of skin scarring and oral fibrosis, the mitigation of renal fibrosis upon urethral obstruction

or ischemic kidney injury, the limitation of cardiac fibrosis after myocardial infarction, the restriction of atherosclerotic plaque formation, and pulmonary hypertension (Munoz-Espin & Serrano, 2014). Conversely, adverse effects of senescence have been associated with other clinical entities described in Table 2.

In cancer the dual behavior of senescence is also evident. In early stages it operates as a tumor barrier, in response to activated oncogenic insults (Gorgoulis et al., 2018; Halazonetis et al., 2008; Komseli et al., 2018). Apart from cell proliferation blockage, senescent cells secrete SASP factors that contribute to tumor suppression in a paracrine manner by recruiting cells of the immune system (Gorgoulis & Halazonetis, 2010; Kang et al., 2011; Pateras et al., 2015). If senescent cells are not cleared timely, they can facilitate in the development of an immunosuppressive and protumorigenic microenvironment via SASP, promoting cancer progression (Gonzalez-Meljem et al., 2017; Gonzalez-Meljem, Apps, Fraser, & Martinez-Barbera, 2018; Jackson et al., 2012; Krtolica, Parrinello, Lockett, Desprez, & Campisi, 2001; Kuilman, Michaloglou, Mooi, & Peeper, 2010). In addition, SASP in treated cancers seems to be implicated in tumor recurrence and adverse prognosis. In this context, therapy induced senescence has also been linked to cancer cell stemness (Senescent Associated Stemness, SAS) that exerts its deleterious, highly aggressive growth potential upon escape from cell-cycle inhibition, driving tumor relapses (Milanovic et al., 2018).

In view of the above mentioned "dark side" of senescence, identifying cancer types that harbor such cells is of outmost importance. Exploiting the advantages of GL13 we identified for the first time *in vivo* senescent cells in two distinct malignant entities, namely Hodgkin lymphoma and LCH (Table 3). The cHL is one of the most frequent lymphomas in the Western world, with an estimated incidence of 8500 new cases and 1050 deaths in 2018 in the US. It is a highly curable disease, however, 20-30% of patients eventually relapse and half of them ultimately die from disease-related causes (Broeckelman, Angelopoulou, & Vassilakopoulos, 2016; Vassilakopoulos & Johnson, 2016). cHL is characterized by rare tumor cells, the Hodgkin and Reed-Sternberg (HRS) cells that evolve in an abundant reactive microenvironment. Interestingly, an increased number of senescent HRS was detected in a subset of examined primary cHL cases (Figs. 4–5, Suppl Tables 1–3). Moreover, high percentage of senescent HRS cells (>10% or >15%) was related to unresponsiveness not only to first-line but also to salvage chemotherapy, as evident from decrease in Hodgkin-lymphoma-specific-survival (HLSS) after failure (Figs. 4–5, Suppl Tables 1–3). Two putative scenarios could explain this association. First, senescent HRS cells in primary lesions, via SASP may foster the tumor microenvironment leading to tolerance to currently available chemotherapeutic strategies (Gordon & Nelson, 2012; Schosserer, Grillari, & Breitenbach, 2017). Such a process has also been reported for TIS and could be a potential mechanism of relapses after the initial

Table 2
Highlighting cellular senescence as a potential contributor to the pathogenesis of numerous age-related diseases (cancer is not included herein). Nevertheless, the characterization of cellular senescence as the causal factor of these pathologies is far from being well-documented since several important questions remain to be addressed. Indeed, in some cases the overall effect of senescence may be beneficial instead of detrimental (See Munoz-Espin & Serrano, 2014).

Disease	Senescence phenotype and pathological markers	Refs
Kidney disease	Renal senescent cell numbers are increased in response to injury in various experimental animal models and human renal diseases, (including IgA nephropathy, diabetic nephropathy, glomerular disease), and is associated with detrimental effects contributing to histopathological and functional deterioration. Senescence decreases renal transplantation success.	(Braun et al., 2012; Sturmlechner, Durik, Sieben, Baker, & van Deursen, 2017; Valentijn, Falke, Nguyen, & Goldschmeding, 2018)
Heart failure	Endothelial cell senescence promotes the typical hemodynamic and structural changes of heart failure with preserved ejection fraction in an aging mouse model.	(Gevaert, Lemmens, Vrints, & Van Craenenbroeck, 2017; Gevaert et al., 2017)
Atherosclerosis	Senescent intimal foam cells accumulate in atherosclerotic lesions and act as the key drivers of atheroma formation, whereas leukocyte senescence is involved in the progression of atherosclerotic plaque.	(Childs et al., 2016)
Diseases of the aorta	Endothelial cells and vascular smooth muscle cells from patients with abdominal aortic aneurysm exert phenotypic features similar to those observed in senescent cells. Senescence of endothelial cells and vascular smooth muscle cells in the aorta increase vascular stiffness.	(Cafueri et al., 2012; Durik et al., 2012)
Metabolic syndrome	Vascular endothelial cell senescence induces systemic metabolic dysfunction. Endothelial cell senescence suppresses skeletal muscle metabolism, leading to systemic glucose intolerance.	(Lumeng & Saltiel, 2011; Pillon, Bilan, Fink, & Klip, 2013; Yokoyama et al., 2014)
Type 2 diabetes	Senescent cells is associated with the pathogenesis of type 2 diabetes pathogenesis through direct impact on pancreatic β-cell function, SASP-mediated tissue damage, and involvement in adipose tissue dysfunction. From the opposite point of view, features of the pathology, such as high circulating glucose, altered lipid metabolism, and growth hormone axis perturbations, can promote senescent cell formation.	(Palmer et al., 2015)
Osteoarthritis	Senescent cells have been shown to accumulate in cartilage and synovium in a model of surgically induced osteoarthritis. Senescence load has been shown to correlate with disease severity in patients with knee osteoarthritis. Senescent chondrocytes are able to affect the surrounding microenvironment, determine a senescent status of cartilage precursor cells, and promote osteoarthritis when transplanted in healthy joints.	(Gao et al., 2016; Jeon et al., 2017; Xu et al., 2017)
Liver disorders	The risk of developing non-alcoholic fatty liver disease is predicted by the presence of senescent hepatocytes. Hepatocyte senescence correlates with severity of non-alcoholic fatty liver disease. Cellular senescence drives hepatic steatosis by inducing mitochondrial dysfunction, resulting in reduced fat metabolism.	(Ogrodnik et al., 2017; Pellicoro, Ramachandran, Iredale, & Fallowfield, 2014)
Diseases of the eye	Senescence has been involved in the pathogenesis of cataracts, macular degeneration and glaucoma.	(Caprioli, 2013; Kozhevnikova, Telegina, Devyatkin, & Kolosova, 2018; Wang et al., 2018)
Pulmonary fibrosis	Fibrotic lung disease is mediated, in part, by senescent cells.	(Schafer et al., 2017)
Age-related cachexia	Senescent cells prevent adipocyte differentiation and contribute to an age-dependent loss of adaptive thermogenic capacity and metabolic dysfunction.	(Berry et al., 2017; Xu, Palmer, et al., 2015)
Neurodegenerative diseases	Astrocytes, the major cell division-competent cell type in the brain, undergo senescence in vivo in humans. Senescent astrocytes are more prominent in both neurodegenerative diseases (Parkinson disease, Alzheimer disease) and aging.	(Chinta et al., 2015)

Table 3
Clinical entities in which senescence has been recognized by applying the SenTraGor methodology.

Entity		Cellular type	Referencess
Normal	Thymic involution	Senescent Thymic Epithelial cells	Barbouti et al., 2018
Benign	Nevi	Senescent Nevous Cells	Evangelou et al., 2017
Preneoplastic	Pancreatic Intraepithelial Lesions (PanINs)	Senescent Epithelial Cells	Fig. 7
Malignant	Classical Hodgkin Lymphoma (cHL)	Senescent Hodgkin & Reed-Sternberg cells	Fig. 4, Fig. 5
	Langerhans cell Histiocytosis	Senescent Neolastic Histiocytes	Fig. 6
Post Therapeutic	Irradiated Laryngeal carcinomas	Senescent Stromal Cells	Evangelou et al., 2017
	Breast cancers following chemotherapy	Senescent Stromal Cells	Evangelou et al., 2017

cycle of chemotherapy (Ewald et al., 2010). Secondly, senescent HRS cells can eventually escape from senescence and re-enter the cell cycle, having acquired an aggressive features and resistance against chemotherapy. We described such a phenomenon recently in two distinct settings. We showed that oncogene-driven genomic instability, taking place during the OIS phase, shapes the genetic landscape favoring the emergence of a subpopulation of malignant cells that "escape" from senescence harboring more deleterious characteristics (Galanos et al., 2016; Galanos et al., 2018; Komseli et al., 2018).

It appears that under certain circumstances OIS skews cancer cell plasticity towards the direction of more aggressive features, as is also the case of senescence associated stemness that follows therapeutic interventions (Milanovic et al., 2018). Regardless of the underlying mechanism, the potential aggressive role of senescent HRS cells in cHL and the use of seno-strategies should be taken, from now on, into consideration when new therapeutic approaches are designed to deal with unresponsive relapsed cases (Chang et al., 2016). Similarly, senescence could play a role in the unresponsiveness to BRAF-V600E inhibitors administrated to treat LCH, a dendritic cell neoplasm with a strong inflammatory component (Abla & Weitzman, 2015). Indeed, we observed in a few primary cutaneous LCH lesions the presence of GL13(+) neoplastic cells (Fig. 6, Table 3). This novel finding needs further examination to the direction of developing new therapeutic interventions that will combine inhibitors of the BRAF pathway complemented by senolytic agents, for patients with refractory or multiply-relapsed LCH (Abla & Weitzman, 2015).

5. Pharmacological interventions that target senescence

The discovery of senotherapeutic drugs represents a developing and highly promising field of current research for new therapies. As extensively discussed in the previous sections, strategies that aim in reducing the burden of senescent cells of an organism are extremely likely to contribute in many favorable ways to protection against a wide array of serious pathological states and age-related abnormalities. Of equal interest are alternative approaches that do not aim directly at survival of senescent cells but at SASP involving signaling modules and pro-inflammatory factors that mediate many of the undesirable effects of aging and act as senescence promoters. This distinction gives rise to the classification of senotherapeutic molecules into senolytics, i.e. compounds that preferably induce death of senescent cells in a selective manner and senomorphics, i.e. molecules that can inhibit SASP and suppress senescence indirectly.

As this field of research is relatively young, knowledge on putative molecular targets or mechanisms of action concerning small molecules with senotherapeutic properties is currently limited. However, the existing studies have already provided convincing evidence that both strategies of either eliminating aged cells or suppressing senescence through SASP inhibition are equally feasible. As such, these pioneering research efforts have established senotherapeutics as a particularly vivid and exceedingly attractive field of contemporary drug discovery. At this point, it is interesting to note that so far in the discovery of small molecules with senotherapeutic properties, several cutting-edge concepts and technologies related to drug discovery such as the target-based approach and reverse pharmacology, sophisticated chemical biology techniques, drug repurposing and bioinformatics have been successfully implemented alongside with more traditional methodologies such as production of transgenic animals, cell-based assays and *in vivo* disease models. The chemical structures of compounds that are currently known to show senotherapeutic properties are summarized in Fig. 9.

An elegant example of target validation and subsequent identification of compounds with senolytic properties has been reported in a series of rationally designed studies led by the research group of J. Kirkland in Mayo Clinic, USA. A hypothesis was stated that survival of senescent cells is heavily dependent on specific genes implicated in pro-survival and anti-apoptotic pathways and that these signaling modules could be targeted to facilitate clearance of senescent cells in a selective fashion over normal cells. By implementing the advanced *ATTAC* genetic methodology, the researchers have created transgenic animals (*INK-ATTAC* mice) where the senescence biomarker $p16^{INK4a}$ promoter was coupled to a 'suicide' gene triggering caspase-dependent apoptosis upon dimerization of the protein it encodes (Baker et al., 2011). Implementation of this technology permitted the selective elimination of cells that are $p16^{INK4a}$-positive by administration of the small molecule AP20187 which acts as a dimerization inducer of the *ATTAC*-encoded protein. Treating the transgenic animals with AP20187 led to increased lifespan, providing strong evidence that removal of aged cells can indeed have a favorable impact on living organisms. The findings of this study were fully confirmed by a later report showing that clearance of $p16^{INK4A}$-positive cells not only could extend lifespan but, most importantly, enabled a side-effect free protection in the organism of transgenic mice from age-related deterioration and delay of tumorigenesis (Baker et al., 2016). Having shown that inhibiting a key pro-survival pathway succeeded in eliminating senescent cells *in vivo*, the researchers moved forward and tried to probe for the validity of an array of potential drug targets, as these were suggested by genetic data and studies of differential gene expression between senescent and normal cells (Zhu et al., 2015). By following a genetic interference approach, siRNA was used for the sequential knockdown of each of 39 previously identified candidate genes. The effects of RNA interference in cell proliferation were combined with a bioinformatics analysis for determining possible interactions among the candidate genes. The network analysis guided selection of the most promising target genes that affected viability of senescent cells in a differential manner with respect to non-senescent cells. The products of these genes were subsequently targeted *in vitro* by a collection of small molecules with well-known modulating activities and the first compounds with senolytic properties identified by this rational approach were dasatinib and quercetin.

Dasatinib (Sprycel®) is an ATP-competitive kinase inhibitor currently in clinical use (Das et al., 2006). This widely used therapeutic molecule targets several protein tyrosine kinases including BCR/Abl and c-Kit but also members of the Scr family such as SRC, LYN, FYN and LCK (Han, Schuringa, Mulder, & Vellenga, 2010; Keating, 2017). Interestingly, dasatinib has been described as an inhibitor of the Eph receptors (Li et al., 2010). Ephrins are the natural ligands of Eph receptors and two ephrins (EFNB1 and EFNB3) were gene products successfully identified by the aforementioned combined transcriptomic

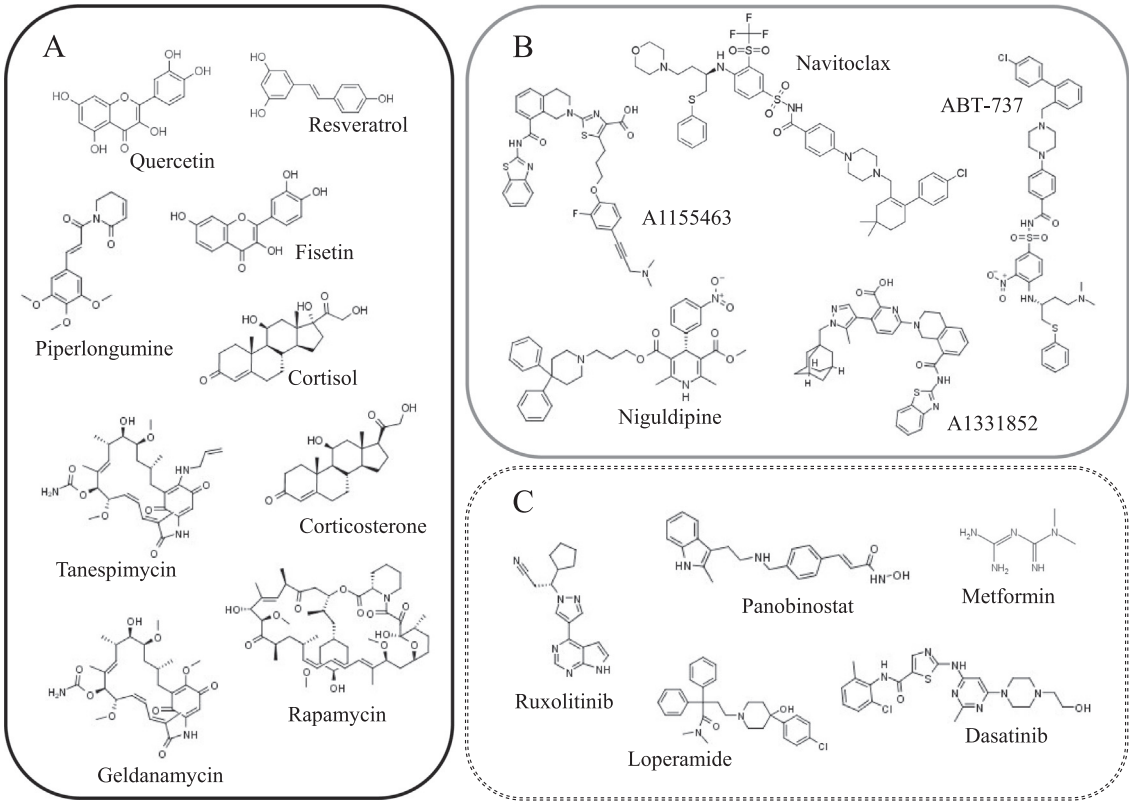

Fig. 9. Structures of the most studied compounds with an established senotherapeutic potential. While classifying the molecules between senolytics and senomorphics was avoided due to the limited knowledge that is currently available regarding the exact underlying mechanisms and their possible overlaps, compounds were partitioned in naturally occurring or endogenous compounds and derivatives (inlet A), investigational chemical tools (inlet B) and clinically used drugs (inlet C).

and informatics analysis as members of a critical pro-survival pathway in senescent cells. Administration of dasatinib reduced viability of senescent cells *in vitro* and the effects were reproduced *in vivo* where alleviation of aging phenotypes was additionally observed in treated animals. The second newly discovered senolytic compound by the same researchers was quercetin, a widespread natural product from the class of flavonoids (Ay et al., 2016). Quercetin is a polyphenol with potent antioxidant activity (Jaffe & Mani, 2014), abundant in natural sources related to nutrition. Like most flavonoids (Middleton Jr, Kandaswami, & Theoharides, 2000), quercetin is characterized by a multitude of biological activities (Wang et al., 2016) including those of cancer prevention (Schnekenburger & Diederich, 2015; Shankar, Antony, & Anto, 2015) and kinase inhibition (Hubbard et al., 2003; Hubbard, Wolffram, Lovegrove, & Gibbins, 2004). Most interestingly, quercetin has been identified as a modulator of several signaling pathways involved in proliferation such as NF-kB, PI3K/Akt, mTOR and estrogen receptor signaling (Feitelson et al., 2015; Maggiolini et al., 2004; Russo et al., 2014; Williams, Spencer, & Rice-Evans, 2004). In this first screen for senolytic molecules, the transcriptomic analysis indicated inhibition of kinase PI3Kδ as a potential mechanism of the observed senolytic action for quercetin (Zhu et al., 2015). The flavonoid showed a similar overall activity against senescent cells both *in vitro* and *in vivo* as dasatinib. Of interest is a follow-up study providing sound evidence regarding the drastic effects of the combined administration of dasatinib and quercetin in mitigating physical dysfunction and increasing survival of mice that were either normally aged or transplanted with senescent cells (Xu et al., 2018). However, the efficiency of the two molecules varied considerably between different cell types. Results showing cell type dependence of senolytic effects are quite abundant in studies dealing with senotherapeutics and to our view such findings further emphasize the need for a more complete and profound understanding of the mechanisms through which these compounds exert their effects. For example, it might be interesting to investigate whether the activity against senescent cells might be responsible, at least in part, for the excellent clinical efficacy of dasatinib against several types of cancer (McCormack & Keam, 2011). Or, given that in many cases the observed phenotype is a collective effect originating from interactions with many different signaling pathways and molecular targets, it could be worth investigating whether PI3Kδ is indeed the main target for flavonoids like quercetin in senescent cells. The activity of quercetin toward the β and γ isoforms of PI3K is reported to be comparable with that of δ (Navarro-Núñez et al., 2009) and this might indicate that inhibition of other kinases or targets from different protein families may also account for the observed senolytic effects of the flavonol. In addition to the overall complexity of the studied systems, the reported activity of quercetin toward senescence underlines the paramount importance of selecting the most appropriate model system for deriving valid and consistent results. For example, a separate group of researchers evaluated quercetin and hyperoside, a simple 3-O-glycoside of quercetin, in a different experimental setting based on a cell line that was the adult analogue of the one used in the previous study of Zhu et al. (2015) and they failed to reproduce the senolytic effect of the flavonol (Hwang, Tran, Rebuffatti, Li, & Knowlton, 2018). It should be stressed that in many studies reporting senotherapeutic molecules, the authors admit that ruling out off-target effects was not trivial and that designing such experiments could be a very challenging endeavor (Zhu et al., 2015). To this direction, the additive effects initially reported for dasatinib and quercetin and later for other senolytics as well, probably indicate that modules and networks that are cell-type dependent contribute to the beneficial phenotypes of senotherapeutics by poorly understood modes.

Right after dasatinib and quercetin, the next senolytic compound to emerge was navitoclax (or ABT-263), a small molecule developed by Abbott as an inhibitor of the BCL-2 family of proteins regulating apoptosis (Tse et al., 2008). The compound is a disruptor of the interaction

between BCL-2 proteins and their endogenous inhibitory regulators, namely BH3 domain-containing proteins, and in this sense navitoclax is regarded as a protein-protein interaction inhibitor (PPI). However, its low selectivity which resulted in severe toxicity has hindered further clinical evaluation of the compound as a therapeutic drug (Garland, Benezra, & Chaudhary, 2013). The capacity of navitoclax to selectively eliminate senescent cells was reported simultaneously by two groups (Chang et al., 2016; Zhu et al., 2016). In the first study a small molecule collection including bioactive compounds and drugs was subjected to a phenotypic screening, while in the second a target-based approach was utilized and the BCL-2 family was selected on the basis of the above mentioned transcriptomic analysis indicating that this particular pro-survival pathway was vital for senescent cells. The same researchers showed that the main targets of navitoclax related with the senolytic response were not BCL-2 itself but BCL-W and BCL-XL and this was soon confirmed by an independent study (Yosef et al., 2016). Since its establishment as a senolytic, navitoclax has been extensively used as a chemical tool to study the mechanisms of aging in a series of model systems (Chang et al., 2016; Demaria et al., 2017; Kim et al., 2017; Pan et al., 2017). This finding prompted a second round of evaluations with molecules that are selective BCL-XL inhibitors and three additional senolytics were identified, compounds A1331852 and A1155463 (Zhu et al., 2017) and ABT-737 (Yosef et al., 2016). The senolytic potential of the first two compounds was validated *in vitro* whereas the activity of ABT-737 was measured *in vivo* using two independent senescence models. Notably, ABT-737 shares a functional similarity with navitoclax as both molecules are PPI inhibitors that mimic the natural BCL-2 inhibitory motif BH3. The compound has been evaluated for its apoptotic properties in specific cancer cells and it has demonstrated a particularly interesting activity profile (Del Gaizo Moore et al., 2007; Konopleva et al., 2006; van Delft et al., 2006).

Along with A1331852 and A1155463 an additional member of the flavonoid family with senolytic properties was discovered, namely fisetin (Zhu et al., 2017). In this case the authors came to the conclusion that the mode of action of fisetin is similar to that of quercetin and this is quite reasonable since the two flavonols differ by a single hydroxyl group at position 5 of the chromone scaffold. As a flavonoid, fisetin is a potent antioxidant (Naeimi & Alizadeh, 2017) but in a similar fashion to quercetin, a number of additional biological activities and molecular targets have been reported for it. More specifically, the flavonoid has been shown to interact among others with topoisomerases and cyclin-dependent kinases, signaling proteins that belong to the NF-kB, PPAR (peroxisome proliferator-activated receptor), PARP1 (poly (ADP-ribose) polymerase 1) and PI3K/Akt/mTOR pathways and epigenetic modules (Adhami, Syed, Khan, & Mukhtar, 2012; Deeba, Vaqar, Mohammad Imran, & Hasan, 2013; Esselen & Barth, 2014; Gupta et al., 2014; Kim, Lee, & Lee, 2014; Lu, Chang, Baratte, Meijer, & Schulze-Gahmen, 2005; Mathers, Strathdee, & Relton, 2010; Webb & Ebeler, 2004). In addition, fisetin has been attributed with considerable cancer chemopreventive properties (Lall, Adhami, & Mukhtar, 2016), anti-inflammatory activity (Khan, Syed, Ahmad, & Mukhtar, 2013) and delay of age-related implications in the central nervous system (Maher, 2015). In light of this multitude of biological activities, it may be reasonably hypothesized that several of the health promoting properties assigned to fisetin, quercetin and possibly to other members of the flavonoid family in the future are related to a good extent with their activities against senescence and SASP. To this direction, more detailed studies are needed to validate additional molecular targets of flavonoids and facilitate a more complete evaluation of the roles that those multifaceted natural products play in preserving a state of good health and delaying aging.

Another natural compound with senolytic activity is piperlongumine (Wang et al., 2016). Piperlongumine is an alkaloid found in trees of the genus *Piper* and it was identified as a senolytic by using an identical experimental setting that led to the discovery of navitoclax. The natural product has been studied in the past as a potent inducer of apoptosis in cancer cells and in this case, the mechanism of action was the formation of ROS (Raj et al., 2011). With respect to senescence, piperlongumine along with a number of structurally related analogues were evaluated as chemical inducers of apoptosis in aged cells. Although the set of analogues was insufficient large for sustaining systematic structure-activity relationships observations, it though provided indications that the senolytic action was independent from ROS, yet without offering adequate data for any alternate hypothesis to be stated. Nonetheless, a recent report presented evidence that piperlongumine can induce apoptosis in cancer cells through modulation of the NF-kB pathway, providing indications of common modalities between the potential mechanisms of action for flavonoids and alkaloids such as piperlongumine (Zheng et al., 2016).

A totally different class of drug candidates for which senolytic properties have been discovered are inhibitors of heat shock protein HSP90. The heat-shock proteins and particularly HSP90 are molecular chaperones widely studied as drug targets (Celine et al., 2007; David & Neal, 2006). As a continuation of the rational approach initially adopted by the researchers who identified the first senolytics, a screening platform based on a SA-β-gal assay was established and subsequently utilized for evaluating compounds on a medium-throughput fashion (Fuhrmann-Stroissnigg et al., 2017). A collection of small molecule autophagy regulators was screened and at least 15 compounds that span 11 different target classes were identified for their activity as senescence modulators. Among them, the most promising in terms of activity and specificity against normal cells were the HSP90 inhibitors geldanamycin and tanespimycin (17-AAG). Geldanamycin is a natural antitumor antibiotic isolated from bacteria of the *Streptomyces* genus and 17-AAG is one of its semisynthetic analogues previously developed in an effort to limit toxicity of the natural lead (Dimopoulos, Mitsiades, Anderson, & Richardson, 2011; Ochel, Eichhorn, & Gademann, 2001; Singh, Genilloud, & Peláez, 2010). Tanespimycin has undergone clinical trials as an anticancer drug by Bristol-Myers Squibb, but they were concluded unsuccessfully (Sharp, Jones, & Workman, 2014). Both are macrocyclic lactams that act as HSP90 inhibitors by binding its N-terminal ATP pocket (Roe et al., 1999). An interesting aspect of their evaluation as senolytics is that in this study, evidence is provided showing that the senolytic activity of HSP90 inhibitors is not as much cell type-dependent as the effects observed for other compounds targeting aged cells. Regarding the potential mechanism of senolytic action for HSP90 inhibitors, the researchers suggest that it is likely related to down-regulation of the PI3K/Akt signaling pathway. In this respect, HSP90 inhibitors act by a mechanism closely related to senolytics such as quercetin which are thought to interfere with the pathway of PI3K.

An inspiring aspect of senolysis as a potential therapeutic mechanism is underlined in one of the most recent studies reporting such a drug (Samaraweera, Adomako, Rodriguez-Gabin, & McDaid, 2017). In this study, the senolytic potential of the clinically used non-selective histone deacetylase inhibitor panobinostat (Farydak®) was demonstrated and the synergistic therapeutic effect observed in a co-treatment with taxol was shown to be mediated by the senolytic potential of panobinostat. Most importantly, post-treatment with the histone deacetylase inhibitor was determined to be a more efficacious therapeutic approach than repeat dosing of treatment with standard drugs such as taxol or cisplatin. Based on these findings, the authors suggest that panobinostat can be used in a repurposing concept as a drug selectively targeting persistent senescent cells which arise following taxol or cisplatin therapy.

As already mentioned, senomorphics are a different class of molecules that can interfere with senescence in an indirect way by affecting the SASP. These molecules have no effect on proliferation of senescent cells but they are implicated in the expression of factors that regulate specific senescence biomarkers. Interestingly, flavonoids structurally related with quercetin and fisetin such as apigenin or kaempferol have been shown to act as senotherapeutics through SASP inhibition (Lim, Park, & Kim, 2015). In this study, a number of natural as well as

synthetic flavonoids were examined and although the compounds could not influence the progress of bleomycin-induced senescence as measured by specific markers, they nonetheless demonstrated notable inhibition of certain components of SASP such as in IL (interleukin)-6, CXCL1(chemokine C-X-C motif ligand 1) and GM-CSF (granulocyte-macrophage colony-stimulating factor) *in vivo*. A number of structure-activity relationships were determined concerning the substitution of flavonoid scaffold and SASP inhibitory activity, while the effect on senescence phenotype was partly attributed to interference of the compounds with the NF-kB pathway and more specifically the NF-kB p65 subunit and the NF-kB inhibitor IkB (inhibitor of kappa B). Of note, in this study quercetin was found moderately active but not to the extent of apigenin and kaempferol.

Rapamycin (Rapamune®) is a selective inhibitor of the mTOR kinase, a signaling molecule with a variety of key functions involved with cell proliferation and survival (Ballou & Lin, 2008). Rapamycin is a natural macrolide acting as an allosteric ligand for mTOR and it is mainly used in clinical practice as an immunosuppressant (Li, Kim, & Blenis, 2014). It is isolated from *Streptomyces* strains and it comprises a prototype inhibitor for this particularly promising drug target. Concerning aging, rapamycin has been attributed with considerable lifespan extension properties in model systems (Arriola Apelo & Lamming, 2016) while it has been described as a potent suppressor of replicative senescence in rodent embryonic cells (Pospelova et al., 2012). To probe for the effects of mTOR inhibition on senescence, in a recent study researchers have used cell-based model systems and *in vivo* experiments to show that rapamycin activity on senescence is correlated with suppression of SASP and this result is mediated by an Nrf2 (nuclear factor E2-related factor 2)-independent mechanism (Wang et al., 2017). In this aspect, it is interesting to note that Nrf2 is regarded a crucial pro-longevity signaling pathway (Sykiotis & Bohmann, 2008). Yet in the same study, the direct effects of the mTOR inhibitor against senescence as determined by senescence-related biomarkers such as $p21^{WAF/CIP1}$ were found to be Nrf2-dependent, thus revealing for one more time the complexity of mechanisms underlying maintenance and propagation of senescence. The activity of rapamycin against SASP has been also shown to correlate with downregulation of IL-6 but most importantly with inhibition of IL-1α translation (Laberge et al., 2015). A notable conclusion confirmed by both studies of Wang et al. and Laberge et al. where rapamycin was used is that the studied mTOR inhibitor has the capacity to inhibit senescence but it cannot invert the condition.

Inhibiting the JAK/STAT pathway is an alternative route than may be proven beneficial for senescence. By using JAK inhibitors such as ruxolitinib (Jakafi®), researchers demonstrated that a beneficial effect on SASP could be obtained by targeting JAK kinases (Xu et al., 2015). While treatment with JAK inhibitors could not reduce senescence, it though resulted to a considerable decrease of systemic inflammation in aged animals and contributed into improving age-related dysfunctions and alleviating frailty. Interestingly, by taking advantage of the availability of inhibitors that are selective against different kinases of the JAK group, the researchers specifically identified JAK1/2 as the key pathway members that mediated SASP alleviation. In a continuation of this study, the same group showed that JAK inhibition could exert its protective effect against senescence by limiting excretion of activin A, a signaling protein facilitating SASP that increases with aging (Xu et al., 2015). In light of the pronounced contribution of kinases such as JAK, IkB and mTOR in senescence, efforts to identify modules of unknown contribution to senolytic response within the kinome can be of great value in accelerating both drug repurposing and medicinal chemistry studies. In one such case (Ferrand et al., 2015) a range of kinases with pro-senescence properties was identified and notably, most of the enzymes were shown to exert their functions through activation of an NF-kB-dependent transcriptional cascade.

In the recent past, several studies have correlated the antidiabetic drug metformin (Glucophage®) with beneficial effects on aging and life span extension (Barzilai, Crandall, Kritchevsky, & Espeland, 2016). This prompted the investigation of its possible effect on senescence. By implementing a drug repurposing approach, metformin was indeed identified as a compound with strong capacity to alleviate SASP (Moiseeva et al., 2013). By interrogating the underlying mechanism, the researchers found that suppression of SASP was mainly mediated through inhibition of phosphorylation of the two catalytic subunits of IKK (IkB kinase), namely subunits α and β. As IkB is an upstream activator of NK-kB, these results provide evidence that the activity of metformin against the senolytic phenotype is independent of AMPK (AMP-activated protein kinase) activation, the principal molecular target responsible for the clinically relevant, antidiabetic action of the drug (Rena, Hardie, & Pearson, 2017). However, inhibiting AMPK is anticipated to affect mTOR in a negative way and this might also contribute to the beneficial result of metformin in aging. The notion that metformin can indeed be considered as a senolytic drug was greatly supported by a recent study demonstrating its efficacy in protecting against senescence using an *in vivo* setting based on the intervertebral disc degeneration model (Chen et al., 2016). As a result of such compelling evidence that a drug proven successful and already in clinical use can have a beneficial effect on aging-related diseases, a study aiming at the approval of an additional therapeutic indication for metformin was launched in consultation with FDA (Food and Drug Administration) (Barzilai, 2017). The study is called TAME (Targeting Aging with MEtformin) and is an inspiring example of how drug repurposing may contribute to the development of new therapeutic approaches against cancer based on drugs that are considered mild and generally safe (Sleigh & Barton, 2010; Druzhyna et al., 2016; Patel & Patel, 2018; Datta, Kim, Mcgee, et al., 2018).

Targeting SASP has been also attempted through another class of approved drugs, namely glucocorticoids. In a study utilizing a screening assay based on human fibroblasts, a collection of clinically used drugs was evaluated and a number of compounds were identified as potent suppressors of the senescence phenotype (Laberge et al., 2012). The most active molecules were found to be corticosterone and cortisol. Nonetheless, in contrast to this finding a study focusing on dexamethasone, a highly relevant clinically used glucocorticoid, resulted in the conclusion that the drug is not a senomorphic but an inducer of senescence through SIRT1 inhibition and $p53/p21^{WAF/CIP1}$ activation (Poulsen et al., 2014). The authors extend this outcome to suggest that this adverse function of dexamethasone might account for several of its detrimental side effects. In either case, it is reasonable to think that, unlike metformin, an attempt to establish additional therapeutic indications for drugs of the class of steroids would be excessively risky. The same might be true for small molecules that act as covalent inhibitors of specific targets related to senescence or SASP propagation. For example, while it could be intriguing to speculate that nitrofuran derivatives which inhibit the stimulator-of-interferon genes (STING) protein could possibly have a beneficial impact to SASP by downregulating inflammatory cytokine production, the fact that these molecules act as irreversible inhibitors by attacking a specific transmembrane cysteine residue renders them a highly challenging option in terms of balancing benefit and risk (Haag et al., 2018).

Another interesting research effort deals with the evaluation of mRNA splicing factors as potential targets for reversing the senescent phenotype (Latorre et al., 2017). In this study, the researchers set forth with the hypothesis that altering mRNA regulator processing through small molecules could have an impact on the senescence phenotype. They used fibroblast cells and they selected the natural product resveratrol as a lead compound on the basis of its well-known effects on lifespan extension (Hubbard & Sinclair, 2014; Sinclair & Guarente, 2014). They synthesized a small collection of derivatives carrying a polar substitution on the 4′ position of resveratrol with the aim to examine the relation between its already known activity toward SIRT1 (Borra, Smith, & Denu, 2005) and its possible senolytic effects. Results provided evidence that resveratrol and its synthetic

analogues were active in restoring splicing factor expression. This effect was coupled to improved telomere maintenance but most importantly, it was found to be independent of SIRT1 activation. The beneficial result of resveratrol on splicing factors was accompanied by inhibition of several undesirable aspects of the senescence phenotype.

Resveratrol is a polyphenol of the stilbene class and is abundant in foods such as wine and fruits. Like flavonoids, resveratrol has demonstrated an impressively wide variety of biological activities (Bhat, Kosmeder, & Pezzuto, 2001; King, Bomser, & Min, 2006; Yang, Wang, Zhu, Zhang, & Yan, 2015). The main health benefits attributed to this natural compound concern cancer chemoprevention, protection against cardiovascular diseases, anti-inflammatory activity and corrective effects on metabolism (Pollack & Crandall, 2013; Sarkar, Li, Wang, & Kong, 2009). Resveratrol has been widely studied in the clinic and although more research is still needed to fully elucidate its complex bioactivity profile, existing data from clinical trials shows that it can interfere with a number of critical pathways such as those of NF-kB, IGF-1R/Akt/Wnt and PI3K (Berman, Motechin, Wiesenfeld, & Holz, 2017; Kundu, Shin, Kim, & Surh, 2006; Parekh, Motiwale, Naik, & Rao, 2011; Vanamala, Reddivari, Radhakrishnan, & Tarver, 2010). Regarding NK-kB, it should be noted that resveratrol is a known inhibitor of IkB (Holmes-McNary & Baldwin, 2000), a kinase previously encountered as a possible target of metformin. As already mentioned, one of the main targets of resveratrol is SIRT1, a member of the class III histone deacetylases (Chung et al., 2010) which hold a seemingly important yet still controversial role in the regulation of lifespan (Dang, 2014). Indeed, in line with the potential molecular targets of resveratrol, many of its reported activities are related with beneficial effects on aging or aging-related issues such as increased life and health span, resiliency against age related stress factors and ultimately longevity (Huffman, Schafer, & LeBrasseur, 2016). Whether a senolytic, a senomorphic or an indirect senescence modulator, resveratrol has been moreover characterized as a compound that can attenuate development of SASP in human MRC5 fibroblasts by reducing the release of pro-inflammatory cytokines without affecting senescence (Pitozzi et al., 2013) whereas in another experimental setting based on murine embryonic fibroblasts, resveratrol showed no statistically significant effect on senescent cells at all (Fuhrmann-Stroissnigg et al., 2017). In this study however, other clinically used or investigational drugs such as loperamide and niguldipine, respectively, have shown senomorphic properties while there is a mention on unpublished data of a similar activity determined for dopamine and serotonin antagonists.

However, as clearly stated in the previous sections, cellular senescence is a condition characterized by a number of known beneficial functions as well. The role of these functionalities in senolysis-based therapeutic interventions is still controversial and remains to be further clarified. For example, a class of compounds that can increase endogenous p53 levels have been examined as senescence inducers in model systems where such a condition was deemed as therapeutically significant. Nutlins are potent stabilizers of p53, an effect achieved through inhibition between the tumor suppressor and its principal negative regulator, namely MDM2 (Vassilev et al., 2004). Nutlin-3a is a known senescence inducer and as such it has been used with success in an *in vivo* model of pulmonary hypertension to prevent or partially invert the disease. The beneficial result was achieved by the p53 activation-mediated induction of senescence on pulmonary-artery smooth muscle cells, the increased expression of $p21^{WAF/CIP1}$ and the subsequent cell cycle arrest that accompanied the compound administration (Mouraret et al., 2013). To summarize, the existing data collectively and clearly suggest that a delicate balance exists between the various constructive and detrimental effects of senescence in living organisms. This aspect is of critical importance for the unbiased evaluation and safe implementation of any therapeutic intervention that may arise in the future based on small drug-like molecules. To this direction, the need for a deeper understanding on the therapeutic limits posed by the inherent complexity of aging mechanisms themselves is of utmost significance.

6. Conclusion and future perspectives

In this review, we describe the latest advances in the discovery of drug-like molecules that demonstrate senescence-modulating properties and we present a critical overview of their experimental evaluation as promising agents against age-related pathologies, with a particular emphasis on mechanisms related to cancer. A thorough review of the currently available knowledge on such compounds and their corresponding modes of action concludes toward two principal but contrasting notions. In terms of the underlying biology and future therapeutic opportunities, the field is truly exciting and particularly promising and we support this notion by reporting primary data relating to the implication of senescence in survival after failure of initial treatment in Hodgkin lymphoma. However, when the advancement in the field of senotherapeutics is examined from a point of view focusing on discovery and validation of new drug targets and development of original bioactive molecules, current progress seems awkwardly poor. Indeed, almost all currently known senotherapeutics are either drugs, clinically used or investigational, or widely studied natural products. The same is true for the limited set of signaling pathways and individual modules currently identified as the potential targets of these small molecules. It is thus reasonable to suggest that at this specific time-point, the landscape of senescence-based therapeutics is rather underexplored and as a result, research efforts still need to be intense, systematic and, most of all, multi-disciplinary. Wide-scale omics studies along with chemical biology approaches and development of chemical probes may provide invaluable hints and starting points for identification and validation of new targets. Subsequently, systematic iterative medicinal chemistry efforts entailing computational exploration of chemical space, synthesis of original scaffolds and implementation of high-throughput biochemical or biophysical screening assays may afford cell-active molecules for sustaining advanced biological evaluations. Finally, sophisticated *in vivo* experiments and systems pharmacology may advance our knowledge on aging to the point of supporting the clinical evaluation of new first-in-class medications having as primary indication senescence therapeutics.

With respect to future research directions, two aspects are considered of key importance for enabling rapid progress in the field. The first is to improve our capacity to precisely detect senescence *in vivo*, as demonstrated by the revolutionary method that we generated. The second is the development and establishment of models describing the factors that govern the fate of stressed/damaged cells, including tumor ones, toward either senescence or cell death. Both are anticipated to greatly advance our current understanding of the mechanistic complexity underlying senescence and also to facilitate rationalization of the whole process related to senotherapeutic drug discovery. More specifically utilizing SenTraGor™ could sustain development of experimental systems and prospectively in clinical settings for evaluating senotheraputic candidate compounds in a high-throughput fashion (Rizou et al., 2018). Moreover, the role of cellular functionalities with fundamental homeostatic significance, such as DDR and damage repair of biomolecules in general (proteins, lipids), needs to be specifically explored under the viewpoint of senescence. It is possible that selective targeting of senescent cells after, for example, primary radiation or chemotherapy treatment may provide a valuable weapon for deleting cancer recurrence or metastasis. Within this context, development of drugs with the ability to securely drive senescent cells to cell death would be of enormous importance not only for optimization of cancer treatment, but also for the alleviation of the aging phenotype in a variety of morbidities or co-morbidities after chemotherapy treatment (cardiovascular, neurodegenerative, immune ageing etc.). For such therapeutic approaches to be efficient and safe, specificity in targeting senescent cells is deemed as a factor of paramount significance. To this direction

various traditional or innovative medicinal chemistry strategies can be devised. A facile drug delivery system based on encapsulation of known senolytics with galacto-oligosaccharides has already been developed for this purpose and successfully used for selectively targeting senescent cells with cytotoxic drugs (Muñoz-Espín et al., 2018). A different idea would be the development of bifunctional prodrugs comprised by a senolytic pharmacophore linked to a scaffold facilitating localization in senescent cells at a selective fashion. Notably, SenTraGor™ may fulfill this scope. Under such an approach, the local and controlled release of active senolytic compounds could be fine-tuned. This would be expected to minimize unwanted toxicity and systematic side effects, thus achieving a pronouncedly safe therapeutic result. To conclude, this is the beginning of a brand new domain of biomedical research and to this end, intense and well-orchestrated efforts at multiple levels, from medicinal chemistry and chemical biology to cellular biology and systems pharmacology will provide the foundation for revealing the true potential of senescence-targeting therapies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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