Cellular senescence: defining a path forward

Vassilis Gorgoulis[1]*, Peter D. Adams[2,], Andrea Alimonti[3], Dorothy C. Bennett[4], Oliver Bischof[5], Cleo Bishop[6], Judith Campisi[7], Manuel Collado[8], Konstantinos Evangelou[9], Gerardo Ferbeyre[10], Jesús Gil[11], Eiji Hara[12], Valery Krizhanovsky[13], Diana Jurk[14], Andrea B. Maier[15], Masashi Narita[16], Laura Niedernhofer[17], João F. Passos[14], Paul D. Robbins[17], Clemens A. Schmitt[18], John Sedivy[19], Konstantinos Vougas[20], Thomas von Zglinicki[21], Daohong Zhou[22], Manuel Serrano[23]*, Marco Demaria[24]*

[1]Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece; Faculty Institute for Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK; Biomedical Research Foundation, Academy of Athens, Athens, Greece; Center for New Biotechnologies and Precision Medicine, Medical School, National and Kapodistrian University of Athens, Athens, Greece

[2]Institute of Cancer Sciences, University of Glasgow, Glasgow G61 1BD, UK; CRUK Beatson Institute, Glasgow G61 1BD, UK; Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA

[3]Institute of Oncology Research (IOR), Oncology Institute of Southern Switzerland, Bellinzona, Switzerland; Università della Svizzera Italiana, Faculty of Biomedical Sciences, Lugano, Switzerland; Department of Medicine, University of Padova, Padova, Italy; Veneto Institute of Molecular Medicine, Padova, Italy;

[4]Molecular and Clinical Sciences Research Institute, St. George's, University of London, London SW17 0RE, UK

[5]Laboratory of Nuclear Organization and Oncogenesis, Department of Cell Biology and Infection, INSERM U.993, Institute Pasteur, Paris, France

[6]Centre for Cell Biology and Cutaneous Research, Blizard Institute, Barts & The London School of Medicine and Dentistry, Queen Mary University of London, 4 Newark St, London, E1 2AT

[7]Buck Institute for Research on Aging, Novato CA, USA

[8]Health Research Institute of Santiago de Compostela (IDIS), Clinical University Hospital (CHUS), Santiago de Compostela, Spain

[9]Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece;

[10]Faculty of Medicine, Department of Biochemistry, Université de Montréal and CRCHUM, Montreal, Quebec, Canada

[11]MRC London Institute of Medical Sciences (LMS), Du Cane Road, London, UK; Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London, UK

[12]Department of Molecular Microbiology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
[13]Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel
[14]Robert and Arlene Kogod Center on Aging, Mayo Clinic, Rochester, Minnesota.
[15]Department of Human Movement Sciences, Faculty of Behavioural and Movement Sciences, Amsterdam Movement Sciences, Vrije Universiteit, Amsterdam, The Netherlands; Department of Medicine and Aged Care, The Royal Melbourne Hospital, The University of Melbourne, Melbourne, Victoria, Australia.
[16]Cancer Research UK Cambridge Institute, Li Ka Shing Centre, University of Cambridge, Cambridge CB2 0RE, United Kingdom
[17]Institute on the Biology of Aging and Metabolism, University of Minnesota
[18]Charité - University Medical Center, Department of Hematology, Oncology and Tumor Immunology, Virchow Campus, and Molekulares Krebsforschungszentrum, Berlin, Germany; Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany; Kepler University Hospital, Department of Hematology and Oncology, Johannes Kepler University, Linz, Austria
[19]Department of Molecular Biology, Cell Biology and Biochemistry, and Center for the Biology of Aging, Brown University, Providence RI, USA
[20]Biomedical Research Foundation, Academy of Athens, Athens, Greece
[21]Newcastle University Institute for Ageing, Institute for Cell and Molecular Biology, Campus for Ageing and Vitality, Newcastle University, Newcastle upon Tyne NE4 5PL, UK
[22]Department of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville FL, USA
[23]Institute for Research in Biomedicine (IRB Barcelona), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain; Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain.
[24]University of Groningen (RUG), European Research Institute for the Biology of Aging (ERIBA), University Medical Center Groningen (UMCG)

***correspondence to:** *Vassilis Gorgoulis: vgorg@med.uoa.gr; Manuel Serrano: manuel.serrano@irbbarcelona.org; Marco Demaria: m.demaria@umcg.nl*

ABSTRACT

Cellular senescence is a cell state implicated in various physiological processes and a wide spectrum of age-related diseases. Recently, interest in therapeutically targeting senescence to improve healthy aging and age-related disease (senotherapeutics) has been growing rapidly. Thus, the accurate detection of senescent cells, especially *in vivo*, is essential. Here, we present a consensus from the International Cell Senescence Association (ICSA), defining and discussing key cellular and molecular features of senescence and offering recommendations on how to use them as biomarkers. We also present a resource tool to facilitate the identification of genes linked with senescence (SeneQuest, available at http://Senequest.net). Lastly, we propose an algorithm to accurately assess and quantify senescence, both in cultured cells and *in vivo*.

MAIN TEXT

Cellular senescence: walking a line between life and death

Cell states link both physiological and stress signals to tissue homeostasis and organismal health. In both cases, the outcomes vary and are determined by the signal characteristics (*type*, *magnitude* and *duration*), spatiotemporal parameters (*where* and *when*) and cellular capacity to respond (Gorgoulis et al., 2018). In the case of potentially damaging stress, damage can be reversed and cells restored structural and functional integrity. Alternatively, damage can be irreversible and cells activate death mechanisms mainly to restrict the impact on tissue degeneration. Between these extremes, cells can acquire other states, often associated with survival, but also with permanent structural and functional changes. An example is the non-proliferative but viable state, distinct from G0 quiescence and terminal differentiation, termed cellular senescence (Rodier and Campisi, 2011). Formally described in 1961 by Hayflick and colleagues, cellular *senescenc*e derived from the latin word *"senex"* meaning "old" (Hayflick and Moorhead, 1961)**,** was originally observed in normal diploid cells that ceased to proliferate after a finite number of divisions (*Hayflick limit*), later attributed to telomere shortening **(**see section "*Cell cycle withdrawal"*).

Cellular senescence has since been identified as a response to numerous stressors, including exposure to genotoxic agents, nutrient deprivation, hypoxia, mitochondrial dysfunction and oncogene activation **(Table 1: Senescence inducers)**. Over the last decade, improved experimental tools and the development of reporter/ablation mouse models have significantly advanced our knowledge about causes and phenotypic consequences of senescent cells. However, specific markers and a consensus definition of what constitutes senescent cells are lacking. Further, although a link to organismal aging is clear, aging and senescence are not

synonymous (Rodier and Campisi, 2011)**.** Indeed, cells can undergo senescence, regardless of organismal age, due to myriad signals, including those independent of telomere shortening. Consequently, senescent cells are detected at any life stage, from embryogenesis, where they contribute to tissue development, to adulthood, where they prevent the propagation of damaged cells and contribute to tissue repair and tumor suppression. Thus, cellular senescence might be an example of evolutionary antagonistic pleiotropy or an abortive cellular program with detrimental effects. Here, we clarify the nature of cellular senescence by: **i)** presenting key features of senescent cells; **ii)** providing a comprehensive definition of senescence, **iii)** suggesting means to identify senescent cells; and **iv)** delineating the role of senescent cells in physiological and pathological processes, that altogether/overall pave the way for developing new therapeutic strategies. paving the way for new therapeutic strategies.

Definition and characteristics of cellular senescence

Cellular senescence is a cell state triggered by stressful insults and certain physiological processes, characterized by a prolonged and generally irreversible- cell-cycle arrest with secretory features, macromolecular damage and altered metabolism (**Figure 1**. These features can be inter-dependent (Figure 1) but for clarity are described here separately.

Cell cycle arrest

One common feature of senescent cells is an essentially irreversible cell cycle arrest which can be an alarm-response instigated by deleterious stimuli or aberrant proliferation. This cell cycle withdrawal differs from quiescence and terminal differentiation (He and Sharpless, 2017). Quiescence is a temporary arrest state, with proliferation re-instated by appropriate stimuli; terminal differentiation is the acquisition of specific cellular functions, accompanied by

a durable cell cycle arrest mediated by pathways distinct from those of cellular senescence (**Figure 2**). In turn, senescent cells acquire a new phenotype, which can lead to an abortive differentiation program. Although the senescence cell cycle arrest is generally irreversible, cell cycle re-entry can occur under certain circumstances, particularly in tumor cells (Galanos et al., 2016; Milanovic et al., 2018; Patel et al., 2016; Saleh et al., 2019) (**Figure 2**).

In mammalian cells, the retinoblastoma (RB) family and p53 protein are important for establishing senescent cell cycle arrest (Rodier and Campisi, 2011). RB1 and its family members p107 (RBL1) and p130 (RBL2) are phosphorylated by specific CDKs (CDK4, CDK6, CDK2). This phosphorylation reduces the ability of RB family members to repress E2F-family transcription factor activity, which is required for cell cycle progression (Sharpless and Sherr, 2015). In senescent cells, however, the CDK2 inhibitor $p21^{WAF1/Cip1}$ (CDKN1A) and CDK4/6 inhibitor $p16^{INK4A}$ (CDKN2A) accumulate. This accumulation results in persistent activation of RB-family proteins, inhibition of E2F transactivation and consequent cell cycle arrest, which, in time, cannot be reversed by subsequent inactivation of RB-family proteins or p53 (Beausejour et al., 2003). This persistence is enforced by heterochromatinization of E2F target genes (Salama et al., 2014), the effects of cytokines secreted by senescent cells (Rodier and Campisi, 2011), and/or enduring ROS production (Takahashi et al., 2006). Notably, in senescent murine cells, ARF, an alternate reading frame protein of the $p16^{INK4a}$ gene locus that activates p53, also has an important role in regulating cell cycle arrest (Sharpless and Sherr, 2015).

Additional features of the senescent cell-cycle arrest include ribosome biogenesis defects and derepression of retrotransposons (De Cecco et al., 2019; Lessard et al., 2018). However, currently no specific marker of the senescent cell-cycle arrest has been identified (Hernandez-Segura et al., 2017). For example, RB and p53 activation also occurs in other forms of cell-cycle

arrest (Rodier and Campisi, 2011). Even $p16^{INK4A}$, which is considered more specific to senescence, is expressed in certain non-senescent cells (Sharpless and Sherr, 2015), and is not expressed by all senescent cells (Hernandez-Segura et al., 2017). Thus, detecting a senescence-associated cell cycle arrest requires quantification of multiple factors/features.

Secretion

Senescent cells secrete a plethora of factors, including pro-inflammatory cytokines and chemokines, growth modulators, angiogenic factors and matrix metalloproteinases (MMPs), collectively termed the Senescent Associated Secretory Phenotype (SASP or Senescence Messaging Secretome (SMS) (**Figure 1**, **Table 2**) (Coppe et al., 2010; Kuilman and Peeper, 2009). The SASP constitutes a hallmark of senescent cells and mediates many of their patho-physiological effects. For example, the SASP reinforces and spreads senescence in autocrine and paracrine fashions (Acosta et al., 2013; Coppe et al., 2010; Kuilman and Peeper, 2009), and activates immune responses that eliminate senescent cells (Krizhanovsky et al., 2008a; Munoz-Espin and Serrano, 2014). SASP factors mediate developmental senescence (Munoz-Espin et al., 2013; Storer et al., 2013), wound healing (Demaria et al., 2014) and tissue plasticity (Mosteiro et al., 2016), and contribute to persistent chronic inflammation (known as inflammaging) (Franceschi and Campisi, 2014). Thus, the SASP can explain some of the deleterious, pro-aging effects of senescent cells. Further, the SASP can recruit immature immune-suppressive myeloid cells to prostate and liver tumors (Di Mitri et al., 2014; Eggert et al., 2016) and stimulate tumorigenesis by driving angiogenesis and metastasis (Coppe et al., 2010).

While the senescent cell cycle arrest is regulated by the p53 and $p16^{INK4A}$/Rb tumor suppressor pathways, the SASP is controlled by enhancer remodeling and activation of transcription factors such as NF-κB, C/EBPβ and GATA4 (Ito et al., 2017; Kang et al., 2015;

Kuilman and Peeper, 2009; Salama et al., 2014), and mTOR (mammalian target of rapamycin) and p38MAPK signaling pathways (Freund et al., 2011; Ito et al., 2017; Kuilman and Peeper, 2009). Upstream signals triggering SASP activation are multiple, and differ depending on the senescence inducer, but include DNA damage, cytoplasmic chromatin fragments (CCFs) that trigger a type1 interferon response, and damage-associated molecular patterns (DAMPs) that activate the inflammasome (Acosta et al., 2013; Davalos et al., 2013; Li and Chen, 2018).

The SASP composition and strength varies substantially, depending on the duration of senescence, origin of the pro-senescence stimulus and cell type (Childs et al., 2015). Further, single cell RNA-Seq reveals considerable cell-to-cell variability of SASP expression (Wiley et al., 2017b). For example, transition from an early TGF-β-dependent to a pro-inflammatory secretome is governed by fluctuation of Notch1 activity (Ito et al., 2017). Moreover, an interferon type I response occurs as a later event, and is driven in part by derepression of LINE-1 retrotransposable elements (De Cecco et al., 2019). Senescent cells also communicate with their microenvironment through juxtacrine NOTCH/JAG1 signalling (Ito et al., 2017), release of ROS (Kuilman et al., 2010), cytoplasmic bridges (**Suppl. Video 1**) (Biran et al., 2015) and extracellular vesicles, such as exosomes (Takasugi et al., 2017). Overall, defining the senescent secretome in each biological context will help identify senescence-based molecular signatures.

Macromolecular damage

DNA damage

The first molecular feature associated with senescence was telomere shortening, a result of the DNA end-replication problem, during serial passages (Shay and Wright, 2019). Telomeres are repetitive DNA structures, found in terminal loops at chromosomal ends, and stabilized by the Shelterin protein complex. This organization renders telomeres unrecognizable by the DDR

and DSB repair pathways (de Lange, 2018; Shay and Wright, 2019). Telomerase, the enzyme that maintains telomere length, is not expressed by most normal somatic (non-stem) cells, but is expressed by most cancer cells that have overcome senescence. Moreover, telomerase activity reconstitution in normal cells leads to telomere elongation, extending their replicative life-span in culture (Bodnar et al., 1998; Shay and Wright, 2019).

Telomere shortening during proliferation culminates in telomeric DNA loop destabilization and telomere uncapping, generating Telomere dysfunction-Induced Foci (TIFs) that activate the DDR, eventually causing cell-cycle arrest. This response can also be elicited by inhibiting or altering genes involved in telomere maintenance (d'Adda di Fagagna, 2008). Another form of DNA damage, termed Telomere-Associated Foci (TAFs), can exist at telomeres due to oxidative DNA damage at telomeric G-reach repeats, irrespective of telomere length or Shelterin loss (de Lange, 2018; Shay and Wright, 2019).

Although half the persistent DNA damage foci in senescent cells localize to telomeres, other stressful subcytotoxic insults can trigger senescence by inducing irreparable DNA damage (Figure 1). Numerous genotoxic agents, including radiation (ionizing and UV), pharmacological agents (e.g., certain chemotherapeutics), and oxidative stress trigger senescence. Moreover, activated oncogenes can induce senescence (known as OIS) as a tumor suppressive response, restricting the uncontrolled proliferation of potentially oncogenic cells. OIS is often mediated by the tumor suppressors $p16^{INK4A}$ and ARF, both encoded by the C*DKN2A* locus, imposing a cell-cycle arrest (Kuilman et al., 2010; Serrano et al., 1997). But the DDR also plays a major role in triggering OIS (Gorgoulis and Halazonetis, 2010; Gorgoulis et al., 2018; Halazonetis et al., 2008). In this case, the damage signal originates at collapsed replication forks as a result of oncogene-driven hyperproliferation. Recently, it was shown that the DDR and ARF pathways

can act in concert during OIS with the former requiring a lower oncogenic load than the latter (Gorgoulis et al., 2018).

Senescent cells harbor persistent nuclear DNA damage foci termed DNA-SCARS (DNA-segments with chromatin alterations reinforcing senescence). DNA-SCARS are distinct from transient damage foci; unlike transient foci, they specifically associate with promyelocytic leukemian (PML) nuclear bodies, lack the DNA repair proteins RPA and RAD51 and ssDNA and contain activated forms of the DDR mediators CHK2 and p53 (Rodier et al., 2011). DNA-SCARS are dynamic structures, with the potential to regulate multiple aspects of the senescent cells, including the growth arrest and SASP (Rodier et al., 2011). However, as not all senescence-inducing stimuli generate a persistent DNA damage response, DNA-SCARS are not a global feature of the senescent cells. CCFs are another type of DNA damage in senescent cells (Ivanov et al., 2013). These cytoplasmic chromatin fragments activate a proinflammatory response, mediated by the cGAS–cGAMP–STING pathway (Ivanov et al., 2013; Li and Chen, 2018), that can serve as another non-inclusive senescence-associated marker.

Protein damage

Proteotoxicity is a hallmark of aging and cellular senescence (Kaushik and Cuervo, 2015). Hence, damaged proteins help identify senescent cells (**Figure 1**). A prominent source of protein damage is ROS, which oxidize both methionine and cysteine residues and alter protein folding and function (Hohn et al., 2017). Many protein tyrosine phosphatases (PTPs) contain cysteine residues in their active sites that can be inactivated by oxidation. This inactivation can trigger senescence by hyperactivating ERK signaling, similar to the effect of activated oncogenes (Deschenes-Simard et al., 2013). High phospho-ERK levels were detected in pre-neoplastic lesions, rich in senescent cells, such as melanocytic nevi and benign prostatic hyperplasia (BPH)

(Deschenes-Simard et al., 2013) and are a characteristic of therapy-induced senescence (Haugstetter et al., 2010). The PTP oxidation pattern (the oxPTPome) can be revealed by a monoclonal antibody that recognizes oxidized cysteine (Karisch et al., 2011).

ROS, in the presence of metals, can carbonylate proline, threonine, lysine and arginine residues. Protein carbonylation exposes hydrophobic surfaces, leading to unfolding and aggregation, and protein carbonyl residues can be specifically detected using antibodies (Nystrom, 2005). Moreover, carbonyl residues can react with amino groups to form Schiff-bases, contributing to protein aggregation. Subsequent cross-linking with sugars and lipids forms insoluble aggregates, termed lipofuscin from the Greek "lipo" meaning fat and "fuscus" meaning dark. Lipofuscin can be visualized in lysosomes by light microscopy or a histochemical method using a biotinylated Sudan Black-B analogue (GL13) (Evangelou et al., 2017). The latter is emerging as another indicator of senescent cells in culture and *in vivo* (Evangelou et al., 2017; Gorgoulis et al., 2018; Myrianthopoulos et al., 2019*).* It should be noted that damage accumulation continues, even when cell division ceases, and can continue for months or even years.

Most protein oxidative damage is not reversible, and degradation by the ubiquitin proteasome system (UPS) or autophagy often eliminates these proteins. As the UPS (Deschenes-Simard et al., 2013) and autophagy are active in senescent cells, they could prove to be useful in chacterizing the senescent state (Ogrodnik et al., 2019a). Similarly, PML bodies act as sensors of reactive oxygen species and oxidative damage (Niwa-Kawakita et al., 2017) and can also be non-exclusive biomarkers of cellular senescence (Vernier et al., 2011).

Lipid damage

Lipids are essential for cell membrane integrity, energy production and signal transduction. Some age-related diseases are characterized by altered lipid metabolism, resulting in lipid profile changes (Ademowo et al., 2017). Although senescent cells are marked by changes in lipid metabolism, it is unclear how this contributes to the senescent phenotype (Figure 1).

Mitochondrial dysfunction during senescence can result in ROS-driven lipid damage, lipid deposits (Correia-Melo et al., 2016; Ogrodnik et al., 2017) and lipofucin accumulation (Gorgoulis et al., 2018). Apart from oxidation, lipid-derived aldehyde modifications [e.g., 4-hydroxy-2-nonenal (4-HNE)] have been reported in senescent cells (Ademowo et al., 2017; Jurk et al., 2012).

Lipid accumulation in senescent cells can be visualized using various commercial dyes and assays (Ogrodnik et al., 2017) or immunostaining for lipid associated proteins such as Perilipin 2 (Ogrodnik et al., 2017). Importantly, genetic or pharmacological clearance of senescent cells in obese and aging mice reduced lipid deposits in liver (Ogrodnik et al., 2017) and brain (Ogrodnik et al., 2019b).

Despite the association with lipid accumulation, our knowledge about specific lipid metabolite composition in senescent cells is sparse. Fatty acids, their precursors and phospholipid catabolites, such as eicosapentaenoate (EPA), malonate, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-HOCA) and 1-stearoylglycerophosphoinositol increase in senescent fibroblasts, whereas linoleate, dihomo-linoleate and 10-heptadecenoate decline (James et al., 2015). Moreover, free cholesterol rises, accompanied by reduced phospholipids and cholesteryl esters derived from acetate, while fatty acid synthase and stearoyl-CoA desaturase-1 declines (Maeda et al., 2009). Several methods are available to detect lipid changes in tissues and cells, but their

use as a senescence biomarker remains limited due to high variability of the senescence-associated lipid profile. For example, lipid metabolites vary significantly between oncogene-induced senescence and replicative senescence (Quijano et al., 2012).

Deregulated metabolic profile

Mitochondria

Senescent cells exhibit several changes in mitochondrial function, dynamics and morphology. Mitochondria in senescent cells are less functional, showing decreased membrane potential, increased proton leak, reduced fusion and fission rates, increased mass and abundance of tricarboxylic acid (TCA) cycle metabolites (Kaplon et al., 2013; Passos et al., 2010). While mitochondria are more abundant, it appears their ability to produce ATP is compromised (Birch and Passos, 2017; Korolchuk et al., 2017). In contrast, senescent cells often produce more ROS, which can cause protein and lipid damage, as discussed in previous sections (see '*protein damage*' and '*lipid damage*'), but also telomere shortening and DDR activation (Passos et al., 2007). Targeting aspects of mitochondrial biology, such as the electron transport chain (ETC), complex I assembly, mitochondrial fission rates and biogenesis, mitochondrial sirtuins and/or disruption of the TCA cycle can trigger senescence (Correia-Melo et al., 2016; Jiang et al., 2013; Kaplon et al., 2013; Miwa et al., 2014; Moiseeva et al., 2009; Park et al., 2010; Wiley et al., 2016). Altered AMP:ATP and ADP:ATP ratios during senescence contribute to cell-cycle withdrawal by activating AMPK (AMP-activated protein kinase), a main sensor of energy deprivation (Birch and Passos, 2017).

Mitochondrial dysfunction during senescence is also implicated in SASP regulation. Mitophagy (mitochondrial clearance) in senescent cells appears to suppress the SASP (Correia- Melo et al., 2016). Genetic or pharmacological inhibition of the ETC can induce

senescence even though cells lack expression of key pro-inflammatory SASP factors, such as IL-6 and IL-8 (Wiley et al., 2016). NAD^+/NADH ratios are reduced in senescent cells (Wiley et al., 2016), which could alter the activity of poly-ADP ribose polymerase (PARP) and sirtuins, both involved in activation of the SASP-regulator NF-kB (Birch and Passos, 2017).

While substantial data support a role for mitochondria in senescence in culture, less is known *in vivo*. Mouse models of mitochondrial dysfunction and enhanced oxidative stress show increased senescence (Wiley et al., 2016), but a detailed characterization of mitochondrial function in senescent cells *in vivo* is lacking. Because mitochondrial dysfunction characterizes other cellular processes (Eisner et al., 2018), it is not a consistent biomarker of senescence. Finally, it is not clear whether senescent cells contribute to declining mitochondrial function during aging and age-related diseases (Srivastava, 2017).

Lysosomes

Secretion requires simultaneous activation of anabolic and catabolic processes (see "Secretion") (Salama et al., 2014). Increased catabolism provides energy and raw materials, and is favored by the lysosome, the end-degradation compartment of phagocytosis, endocytosis and autophagy (Settembre and Ballabio, 2014)**.** Lysosome biogenesis is transcriptionally-driven, and depends on the cellular energetic or degradative needs (Settembre and Ballabio, 2014). Intriguingly, when amino acid levels in the lysosomal lumen are high, mTOR1 is recruited and activated and *vice versa* (Settembre and Ballabio, 2014)**.** Additionally, lysosomes interact with mitochondria to preserve mitochondrial homeostasis (see *"Mitochondria"*) (Park et al., 2018).

Lysosomes in senescent cells increase in number and size, evident by the cytoplasmic granularity seen microscopically (Robbins et al., 1970); **Figure 1**, **Suppl Video 1,** for non-senescent cells see **Suppl Video 2**). The increased lysosomal number might reflect an attempt to

balance the gradual accumulation of dysfunctional lysosomes by producing more new lysosomes. Thus, the balance between anabolism and catabolism, vital for secretion, is extended. This balance is maintained during OIS through the TOR-autophagy spatial-coupling-compartment (TASCC), which coordinates the production of SASP factors (Salama et al., 2014).

The elevated lysosomal content does not necessarily reflect increased activity, as the degradation stage of autophagy also declines (Park et al., 2018). Thus, the lysosome-mitochondrial axis degrades, leading to decreased mitochondrial turnover that increases ROS production. Subsequently, ROS targets cellular structures, including lysosomes, forming a vicious feedback loop that induces more damage (Park et al., 2018). The increased lysosomal mass has been linked to SA-β-gal activity (Hernandez-Segura et al., 2018), a senescence biomarker. However, although the SA-β-gal is prominent in senescent cells (Dimri et al., 1995; Hernandez-Segura et al., 2018), it is neither required nor a determinant of the senescent phenotype (Hernandez-Segura et al., 2018). From a therapeutic viewpoint, the enlarged lysosomal compartment offers an increased capacity to trap drugs that can be protonated, such as the selective CDK4/6 inhibitors palbociclib, ribociclib and abemaciclib. This capacity reduces their effective concentration in the cytosol and nucleus, but counteracted by the slow release of the drugs from the lysosomes, thereby increasing drug exposure time (Llanos et al., 2019). Another senescence trait, related to lysosomal malfunction, is the intra-lysosomal accumulation of lipofuscin aggresomes (see "*Protein damage*" and "*Lipid damage*", reviewed in (Gorgoulis et al., 2018). Interestingly, lipofuscin was reported to stimulate expression of the anti-apoptotic factor Bcl-2, conferring resistance to apoptosis, another characteristic of senescent cells (McHugh and Gil, 2018). Lysosomes in senescent cells also participate in chromatin processing (CCFs) (see *"DNA damage"* and "*Secretion"*) (Ivanov et al., 2013).

Senescence-associated (epi)-genetic and gene expression changes

The features listed above are associated with changes in gene expression, determined by transcriptional regulation of coding and non-coding RNAs, which can be exploited for senescence detection (**Figure 1**). Here, we discuss such major alterations, and describe a novel database that can aid the identification of genes associated with senescence, termed SeneQuest (http://Senequest.net) [see **Supplementary Information and Suppl. Table 1]**.

Chromatin landscape

Epigenetic modifications occur during senescence, but are mostly context-dependent (Cheng et al., 2017). For example, replicative senescence has been correlated with global loss of DNA methylation at CpG sites (Cheng et al., 2017). In addition to the global loss of DNA methylation, cellular senescence is entails focal increases in DNA methylation at certain CpG islands (Cruickshanks et al., 2013). Interestingly, this DNA methylation profile somewhat resembles the cancer- and aging-associated methylome patterns (Cruickshanks et al., 2013; Xie et al., 2018). Cells undergoing OIS fail to show such alterations in DNA methylation (Xie et al., 2018), reinforcing the diverse nature of epigenetic alterations during senescence.

Senescent cells also exhibit a global increase in chromatin accessibility, but the genome-wide profile varies depending on the stimulus (De Cecco et al., 2013). Individual histone modifications and variants (Cheng et al., 2017; Hernandez-Segura et al., 2018; Rai et al., 2014) demonstrate alterations during senescence. For instance, H4K16ac is often enriched at active promoters in senescent, but not proliferating, cells (Rai et al., 2014). Its accumulation correlates closely with histone variant H3.3, which is deposited into chromatin in a DNA replication-independent manner by the HIRA/UBN1/CABIN1 and ASF1a chaperones (Rai et al., 2014).

Notably, N-terminus proteolytic cleavage of H3.3 correlates with gene repression in a different subset of genes during senescence (Ivanov et al., 2013). Global loss of linker histone H1 is another senescence feature (Funayama et al., 2006). Certain histone modifications are vital, such as elevated H4K20me3 and H3K9me3, which contribute to the proliferation arrest (Cheng et al., 2017; Di Micco et al., 2011; Salama et al., 2014), whereas elevated H3K27ac at gene enhancers promotes a SASP (Hernandez-Segura et al., 2018).

Senescence is also associated with chromatin morphological changes. Senescence-associated heterochromatin foci (SAHF), visualized as DAPI-dense foci, are enriched in Heterochromatin Protein (HP) 1. SAHFs derive from chromatin factors, including RB, histone variant macroH2A, high mobility group A proteins, the HIRA/UBN1/CABIN1 and ASF1a chaperones, and increased nuclear pore density (Boumendil et al., 2019; Salama et al., 2014). SAHFs were initially hypothesized to contribute to gene regulation (Salama et al., 2014). However, SAHFs were since shown to comprise largely late-replicating gene poor heterochromatic regions, even in proliferating cells, suggesting a small role in senescence-associated gene expression (Salama et al., 2014). Senescence is also correlated with global loss of linker histone H1 (Funayama et al., 2006). Notably, SAHFs seem to be cell type- and stimulus-dependent, as they are not seen in all senescent cells (Di Micco et al., 2011; Kennedy et al., 2010; Sharpless and Sherr, 2015), rendering them useful for senescence identification, while the functional significance remains to be elucidated.

Another chromatin feature, termed senescence-associated distension of satellites (SADS), corresponds to de-compaction of (peri-)centric constitutive heterochromatin (Cruickshanks et al., 2013; De Cecco et al., 2013; Swanson et al., 2013). SADS precede SAHF formation and might be widely linked to senescence (Swanson et al., 2013). Retrotransposable elements are another

type of constitutive heterochromatin related to senescence. The normally-repressed LINE-1 (L1) retrotransposons are activated, stimulating the cGAS-STING pathway that elicits a type I interferon response (see "*Secretion*") (De Cecco et al., 2013). Hence, in addition to triggering genomic instability, these elements fuel the SASP (Criscione et al., 2016).

Downregulation of lamin B1, a major component of the nuclear lamina, is another key feature of senescence (Dou et al., 2015; Freund et al., 2012; Shah et al., 2013; Shimi et al., 2011). Lamin B1 loss correlates with epigenetic profiles (Salama et al., 2014), as well as senescence-associated chromatin structures (SAHF and SADS) (Salama et al., 2014; Swanson et al., 2013). Its reduction occurs predominantly at H3K9me3-rich regions, a process that appears to liberate H3K9me3 from the nuclear lamina promoting spatial rearrangement of H3K9me3-heterochromatin to form SAHF (Salama et al., 2014). Hi-C analysis (genome-wide mapping of chromatin contacts) in OIS revealed a reduction in local connectivity at regions enriched for H3K9me3 and lamin B1, perturbing these long-range interactions (Chandra et al., 2015). Replicative senescence, on the other hand, showed loss of long-range and gain of short-range interactions within chromosomes (Criscione et al., 2016), implying that the nature of senescence-associated high-order chromatin interactions is stimulus and context-dependent (Zirkel et al., 2018). Furthermore, lamin B1 loss and reduced nuclear integrity is suggested to fuel the SASP by contributing to CCF formation (Dou et al., 2015; Ivanov et al., 2013), thereby stimulating the cGAS-STING pathway and interferon response (see "*Secretion*") (Li and Chen, 2018). Autophagy-mediated CCF formation (Dou et al., 2015) together with reduced histone synthesis (O'Sullivan et al., 2010) might also lead to a global loss of core histones during senescence, affecting the chromatin landscape (Chan and Narita, 2019; Ivanov et al., 2013).

Transcriptional signatures

Several genes linked to the cell cycle arrest and SASP are frequently interrogated in combination with other biomarkers to validate the senescence phenotype or type of senescence (**Figure 1**). For example, increased expression of the cyclin-dependent kinase inhibitors CDKN1A ($p21^{WAF1/Cip1}$), CDKN2A ($p16^{INK4A}$) and CDK2B ($p15^{INK4B}$) and a subset of SASP genes, along with decreased expression of cyclins CCNA2 and CCNE2 and LMNB1 should be determined. In addition, the transcriptome of putative senescent cells should be established, which can then be compared with the increasing number of existing senescence transcriptomes (Hernandez-Segura et al., 2018).

Whole-transcriptome studies have been instrumental in defining major signaling pathways involved in establishing senescence phenotypes, and in some cases predicting drug targets (Zhu et al., 2015). A set of 13 genes was differentially regulated in several cell types undergoing distinct forms of senescence, including oncogene-, replicative- and DNA damage-induced senescence (Hernandez-Segura et al., 2017). More recently, a similar study, which considered only fibroblasts and endothelial cells, also attempted to define senescence-associated transcriptome signatures (Casella et al., 2019). Due to the current paucity of transcriptome data sets, and the availability of more single-cell studies that allow evaluation of intra-population variability (Wiley et al., 2017a; Zirkel et al., 2018), these gene signatures will likely change in coming years. But ultimately a senescence gene expression signature will prove valuable for identifying senescence under many conditions in culture and *in vivo*.

miRNAs and non-coding RNAs

Non-coding RNAs, particularly microRNAs (miRNAs), can influence the senescence program, alone or in concert. Functional studies revealed several miRNAs that directly or indirectly modulate the abundance of key senescence effectors, including p53, $p21^{WAF1/Cip1}$ and

SIRT1 (Suh, 2018). miR-504 targets the p53 3'UTR, reducing p53 abundance and activity (Hu et al., 2010). Also, Gld2-mediated stabilization of miR-122 enables its binding to the CBEP 3'UTR, resulting in decreased p53 mRNA polyadenylation and translation (Burns et al., 2011). Conversely, miR-605 targets MDM2, triggering p53-mediated senescence (Xiao et al., 2011), and multiple miRNAs downregulate $p21^{WAF1/Cip1}$, including 28 miRNAs that block OIS (Borgdorff et al., 2010). Likewise, miR-24 suppresses $p16^{INK4a}$ in cells (Lal et al., 2008) and disease models, including osteoarthritis (Philipot et al., 2014). Intricate miRNA feedback loops can modulate senescence programs. For example, a p53/miRNA/CCNA2 pathway drives senescence independently of the p53/$p21^{WAF1/Cip1}$ axis (Xu et al., 2019). Similarly, p53-dependent upregulation of miR-34a/b/c downregulates cell proliferation and survival factors (Hermeking, 2010). Non-coding RNAs also regulate the SASP (Panda et al., 2017). MiR-146a/b, for example, increases weeks after senescence induction and dampens a proinflammatory arm of the SASP (Bhaumik et al., 2009). miRNAs also downregulate repressors of senescence, including Polycomb Group (PcG) members CBX7, EED, EZH2 and SUZ12 (miR-26b, 181a, 210 and 424), leading to $p16^{INK4a}$ derepression and senescence initiation (Overhoff et al., 2014). Finally, the role of miRNAs in senescence extends beyond their classical functions. For example, Argonaute 2 (AGO2) binds let-7f in the nucleus, forming a complex with RB1 (pRB), resulting in repressive chromatin at CDC2 and CDCA8 promoters (Benhamed et al., 2012). Silencing these E2F target genes is required for senescence initiation.

Long non-coding RNAs (lncRNAs) (> 200 nt) can bind RNA, DNA or proteins to regulate senescence. For example, ANRIL, a 30-40kb antisense transcript encoded by theCDKN2A locus, binds CBX7 to repress INK4b/ARF/INK4a expression (Kim et al., 2017). Likewise, the lncRNA PANDA recruits PcG complexes, suppressing senescence-promoting genes (Kim et al.,

476 2017), whereas silencing of GUARDIN, a p53-responsive lncRNA, causes senescence or
477 apoptosis (Hu et al., 2018). By contrast, following OIS induced by RAF, the lncRNA VAD
478 preserves senescence by decreasing repressive H2A.Z deposition at INK promoters (Kim et al.,
479 2017). Also, lncRNA UCA1 disrupts association of the RNA binding protein hnRNP A1 with
480 $p16^{INK4A}$, but not $p14^{ARF}$, transcripts (Kim et al., 2017). In addition, non-coding RNA profiling,
481 with a focus on miRNAs, provides a senescence signature (Suh, 2018). Intriguingly, the miRNA
482 content of small extracellular vesicles released by senescent cells varies, evolving over time
483 (Terlecki-Zaniewicz et al., 2018).

484 *Immune-regulation and anti-apoptotic proteins*

485 The search for senescent protein markers started in OIS. In addition to identifying known
486 cell cycle regulators, these studies identified DCR2 as a common marker of senescence (Collado
487 et al., 2005), later shown to characterize other types of senescence. DCR2 is a decoy death
488 receptor that protects senescent cells from immunity-mediated apoptosis, thus blocking immune
489 surveillance of senescent cells (Sagiv et al., 2013). Similarly, the natural killer (NK) cell
490 activating receptor (NKG2D) ligands MICA and ULBP2 increase upon replicative, OIS and
491 DNA damage-induced senescence (Krizhanovsky et al., 2008b; Sagiv et al., 2016). Cell surface
492 markers are of special interest because they should allow quantification, isolation and single cell
493 transcriptional analysis of senescent cells extracted from tissues. However, DCR2 and NKG2D
494 ligands are not conserved among species, making mouse/human comparisons not possible.
495 Recently, two additional upregulated cell surface markers, Notch1 in OIS and DPP4 in
496 replicative and OIS, were identified (Hoare et al., 2016). Both proteins have roles in regulating
497 the SASP. Furthermore, an oxidized form of membrane-bound vimentin was identified as a
498 senescence marker, which could be used to target these cells by the adaptive immune system

(Frescas et al., 2017). Finally, senescent cells are resistant to apoptosis, which can be mediated by increased expression of anti-apoptotic BCL-2 family members (Yosef et al., 2016).

In vivo models to study cellular senescence

Senescence reporter mice

Several transgenic mice were developed to estimate *p16*^{Ink4a} expression *in vivo* or *ex vivo* using luciferase or fluorescent protein reporters. Measuring luciferase activity longitudinally revealed an increase in *p16*^{INK4A} expression as mice age, as well as an age-dependent increase in inter-animal variability, whereas isolation of fluorescent p16+ cells allowed phenotyping (Liu et al., 2019; Ohtani et al., 2010). This approach allows the endogenous *p16*^{INK4A} promoter to drive signals, but causes p16 hemizygosity. Another mouse (p16-3MR) used a luciferase (rLUC), monomeric Red Fluorescent Protein (mRFP) and Herpes simplex Virus-Thymidine Kinase (HSV-TK) fusion protein driven by the *p16*^{INK4A} promoter present on a bacterial artificial chromosome, integrated into the mouse genome (Demaria et al., 2014). This approach allows detection and killing of senescent cells, and does not perturb the endogenous CDKN2A locus. Finally, INK-ATTAC mice express a FKBP-Caspase 8 fusion-protein and eGFP reporter to kill and detect p16^{+} cells, driven from a 1.6 kB fragment of the *p16*^{INK4A} promoter (Baker et al., 2011; Folgueras et al., 2018). Despite differences between these mice, they have been valuable in showing that senescent cells contribute to a wide range of age-related pathologies (Calcinotto et al., 2019). Mice expressing luciferase and eGFP from *p21*$^{WAF1/Cip1}$ promoter are also available (Ohtani et al., 2007).

Murine models of accelerated senescence and aging

Several progeric mouse models have been developed to mimic human progeric syndromes, including DNA repair and genome integrity deficiencies (Folgueras et al., 2018). Progeroid mice with accelerated senescence and shortened lifespans are also useful for assessing the role of cellular senescence in aging and testing senotherapeutics. For example, the demonstration that ablation of *p16*^{INK4A} expressing cells slowed age-related declines in progeroid *BubR1*$^{H/H}$ mice provided the first evidence that senescent cells are causal for certain aging phenotypes (Baker et al., 2011; Folgueras et al., 2018). BUBR1 is important for the mitotic spindle assembly checkpoint (Guo et al., 2012). *BubR1*$^{H/H}$ mice, which express 10% of the normal level of BUBR1, have increased aneuploidy, several progeroid features and increased expression of senescence markers in several organs (Folgueras AR et al., 2018). Selective removal of *p16*$^{INK4A+}$ cells from *BubR1*$^{H/H}$-INK-ATTAC mice delays kyphosis, cataracts and muscle atrophy, but not cardiac arrhythmias and arterial wall stiffening, nor does it extend lifespan (Baker et al., 2011; Folgueras et al., 2018).

Similarly, *Ercc1*$^{-/\Delta}$ progeroid mice, harboring a DNA repair defect, prematurely develop multiple morbidities associated with age, driven in part by accelerated accumulation of senescent cells in numerous tissues (Folgueras AR et al., 2018). *Ercc1*$^{-/\Delta}$ mice (Folgueras AR et al., 2018) express 5% of the normal level of the endonuclease ERCC1-XPF, important for nucleotide excision, interstrand crosslink and double-strand break repair. These mice develop numerous age-related histopathologic lesions in virtually every tissue (Folgueras AR et al., 2018), and accumulate oxidative DNA damage faster than wild-type mice (Wang et al., 2012). Treatment of *Ercc1*$^{-/\Delta}$ mice with senolytic drugs reduces senescence markers and extends health span (Fuhrmann-Stroissnigg et al., 2017; Yousefzadeh et al., 2018; Zhu et al., 2015). Cross-breeding

543 of these models with the *p16*^{INK4A} reporter transgenes permits monitoring senescent cell burden
544 longitudinally in live animals (Robinson et al., 2018; Yousefzadeh et al., 2018).

545 Hutchinson-Gilford Progeria Syndrome (HGPS) is a segmental or tissue-specific
546 progeria, caused by mutations that compromise lamin A processing (Cau et al., 2014). Mice
547 with altered or deleted LMNA develop HGPS-like phenotypes. They also accumulate senescent
548 cells, as determined by SA-ß-gal staining and mRNA levels of senescence markers, in skeletal
549 muscle and heart, consistent with sites of age-related pathology and disease (Folgueras AR et al.,
550 2018). Similarly, in a mouse model of HGPS that recapitulates the pathogenic LMN splicing
551 mutation, *Lmna*$^{G609G/G609G}$ mice, senescence in the liver and kidney was observed (Osorio et al.,
552 2011). However, senescent cells have not yet been shown to be causative for HGPS pathology.

553 A mouse model of trichothiodystrophy (TTD) (Andressoo et al., 2006), caused by a
554 specific mutation in the *Xpd* gene, also indicated a role for senescent cells in premature aging.
555 Here the role of senescence in driving aging in the *Xpd*$^{TTD/TTD}$ was clearly documented by the fact
556 that treatment with a D-retro inverso (DRI)-isoform peptide of FOXO4 able to disrupt FOXO4
557 interaction with p53. Treatment with the FOXO4-DRI peptide reduced lethargy in *Xpd*$^{TTD/TTD}$
558 mice and improved fur density, running wheel activity, and physical responses to stimuli (Baar et
559 al., 2017).

560 Loss of Cu/Zn-superoxide dismutase (*Sod1*) in mice accelerates aging (Zhang et al.,
561 2017). *Sod1*$^{-/-}$ mice show increased oxidative DNA damage, senescence (*p16*^{INK4A}, *p21*$^{WAF1/Cip1}$),
562 SASP factors (*Il1β, Il6*), SA-βgal^{+} cells and age-associated pathology in kidneys (Zhang et al.,
563 2017). To date, senescence has not been demonstrated to drive pathology in *Sod1*$^{-/-}$ mice.

564 Deletion of the nfkb1 subunit of the transcription factor NF-κB induces premature ageing
565 in mice. These mice have been shown to experience chronic, progressive low-grade

inflammation which contributes to a wide spectrum of ageing phenotypes and early mortality (however, in contrast to some of the widely used progeria mouse models these mice have a maximum lifespan of approximately 20 months). Furthermore, these mice show increased incidence of senescent cells in multiple tissues (Jurk et al., 2014).

Finally, the selective inbreeding of AKR/J mice resulted in numerous senescence-accelerated mouse (SAMP) strains including SAMP1-3 and SAMP6-11 (Takeda et al., 1997). Although these mice have increased senescence and thus can be used for testing senotherapeutics, it remains unclear which mutant genes drive senescence in these strains.

Identification of cellular senescence *in vivo*

A simplified algorithm for detecting senescent cells in situ

In vivo, senescent cells reside in complex tissues. Their impact on tissue function can be local or global due to the SASP (Xu et al., 2018). To understand how senescence affects tissue function, tissue remodeling and aging, we need tools to identify senescent cells in tissues.

Single cell analyses can be performed on most tissues. Common techniques include immunostaining, in-situ hybridization and multicolour (imaging) flow cytometry. Even higher numbers of markers can be assessed by mass cytometry (Cytometry by Time-Of-Flight, CYTOF) (Abdelaal et al., 2019). Although promising, limitations include loss of information about spatial associations and variable efficiency of isolation of different cell types, including senescent vs non-senescent cells. Therefore, microscopic imaging remains a preferred method for *in situ* senescence detection.

As mentioned, there is currently no single marker with absolute specificity for senescent cells. Marker specificity varies, depending on cell type, tissue, organismal developmental stage,

589 species and other factors. However, some markers have more global/universal value/validity
590 while others are related to specific senescence types. Therefore, we advise a multi-marker
591 approach, encompassing/combining broader and more specific markers for more robust detection
592 of senescent cells *in situ* (**Figure 3)**.

593 *Challenges to detect senescent cells in humans*

594 The role of senescence in human disease is clear from cellular studies, while *in vivo*
595 evidence is only now catching up (Childs et al., 2015; He and Sharpless, 2017; Munoz-Espin and
596 Serrano, 2014). OIS, initially described in culture, was the first type of senescence validated in
597 humans (Serrano et al., 1997). OIS or senescence induced by loss of a tumor suppressor was
598 verified *in vivo* in human preneoplastic lesions (Collado et al., 2005; Gorgoulis and Halazonetis,
599 2010; Kuilman and Peeper, 2009) and primary or treated neoplasias (Haugstetter et al., 2010).
600 Later reports on the diverse activities of the senescence secretome (see "Secretion") led to the
601 recognition of its pro-tumorigenic properties, establishing what is now accepted as the dual role
602 of senescence in carcinogenesis (Lee and Schmitt, 2019). Evidence linking senescence to other
603 common age-associated human diseases has recently emerged. These diseases include
604 neurodegenerative disorders, glaucoma, cataract, atherosclerosis/cardiovascular disease,
605 diabetes, osteoarthritis, pulmonary, and renal and liver fibrosis (Childs et al., 2015; He and
606 Sharpless, 2017; Munoz-Espin and Serrano, 2014) (**Suppl Table 2**).

607 In most studies, senescence is assessed in *ex vivo* cultures or fresh samples by SA-β-gal
608 staining or indirect markers in formalin-fixed tissues (Haugstetter et al., 2010; He and Sharpless,
609 2017; Kuilman and Peeper, 2009; Munoz-Espin and Serrano, 2014; Serrano et al., 1997). Since
610 SA-β-gal is not suitable for fixed tissues, analyzing senescence in human samples is challenging.
611 The recently developed assay and reagent Sudan Black-B (SBB) interacts with lipofuscin,

another hallmark of senescent cells (Georgakopoulou et al., 2013). Lipofuscin is preserved in fixed material (Georgakopoulou et al., 2013) and is resilient, as it was isolated from a 210,000 year old human fossil (Harvati et al., 2019; Myrianthopoulos et al., 2019). The test reagent is amenable to immunohistochemistry (Evangelou et al., 2017), and identified senescent Hodgkin and Reed-Sternberg (HRS) cells in Hodgkin lymphomas (cHL), where they predicted poor prognosis (Myrianthopoulos et al., 2019). These cells are giant in size, have a large and occasionally multilobular nucleus (indication of an abortive cell cycle), have increased secretory activities, are embedded within an inflammatory milieu, and show a histological pattern strongly reflecting features of the senescence phenotype (Kuppers et al., 2012) (**Figure 1)**. Another method for identifying and quantifying senescent cells *in vivo* is SA-β-gal staining combined with ImageStream X analysis (Biran et al., 2017).

Despite promising results that each marker provides, no marker is completely senescence-specific (Sharpless and Sherr, 2015) (Sharpless and Sherr, 2015). We recommend combining cytoplasmic (e.g., SA-β-gal, lipofuscin), nuclear (e.g., $p16^{INK4A}$, $p21^{WAF1/Cip1}$, Ki67) and context/cell type-specific markers (Childs et al., 2015) (**Figure 3**).

Conclusions, open questions and perspectives

From the first description of cellular senescence by Hayflick and colleagues almost 60 years ago, significant progress has been made in understanding the characteristics and functions of senescent cells. A limitation, particularly for studying biospecimens, remains the absence of specific markers. To overcome this obstacle, we propose a multi-marker approach **(Figure 3).** This strategy could also be used to evaluate the efficacy of senolysis, an emerging therapeutic

634 approach that recently entered clinical trials for treatment of various age-related pathologies
635 (Myrianthopoulos et al., 2019).

636 Conceptually, senescence can be considered a non-linear, multivariable [F(x,y)=z]
637 function where the dependent variable (outcome) z depends on the independent variables x
638 (stimulus) and y (environment). The non-linear processing is dictated by dynamic genetic and
639 epigenetic processes that can lead to reprogramming cycles until a steady-state is achieved. At
640 first glance, the outcomes appear to be cell cycle withdrawal and secretion of bioactive
641 molecules. However, recent evidence suggest that the cell cycle arrest is not always a necessary
642 outcome, as post-mitotic cells, already unable to proliferate, can assume senescence-like
643 features, and under certain conditions senescent cells can re-enter the cell cycle. The SASP
644 appears a common senescence-associated feature, but it is highly heterogeneous. Thus, to
645 understand the pleiotropic phenotypes of senescent cells, a shift from traditional reductionism to
646 more systematic, multi-parametric approaches is needed. The development of sophisticated high
647 throughput methods and machine learning tools that can handle multi–omics data will help
648 achieve this goal (Vougas et al., 2019). Although "old and new" have pros and cons, we can
649 combine the best to achieve a "de profundis" analysis of senescent phenotypes. This approach
650 will likely unveil more specific senescence-associated signatures to address important
651 unanswered questions: What causes and regulates the SASP? How do genetic and epigenetic
652 determinants interact with triggering stimuli and cellular microenvironments? Which genomic
653 repair systems act in different senescence scenarios? What causes cells to evade the growth
654 arrest, and what phenotypes do 'escaped' senescent cells acquire? Answers to these and other
655 questions will help develop specific panels of markers for each senescence subtype (step 3 in the

workflow) and guide the evolving field of senotherapy (van Deursen, 2019), achieving the best outcome within the spirit of precision medicine.

CONFLICT OF INTEREST

The authors declare conflicts of interest related to this work.

ACKNOWLEDGMENTS

We would like to thank Nikolaos Kastrinakis, Panagiotis VS Vasileiou, Gkikas Magiorkinis, Eleni Fitsiou and Michela Borghesan for their valuable support to this work. We apologize in advance that for reason of space we have omitted the citations of relevant papers and reviews.

FIGURE LEGENDS

Figure 1. The hallmarks of the senescence phenotype. Senescent cells exhibit four inter-dependent (shown by the dashed thin outer cycle and bidirectional arrows) hallmarks: 1) cell cycle withdrawal, 2) macromolecular damage, 3) Secretory Phenotype (SASP) and 4) deregulated metabolism, as depicted in the outer circle (see text). The inner cycle includes distinct morphological and functional features that reflect the proposed hallmarks. Several of these traits are strongly evident in the malignant entity, the classical Hodgkin Lymphoma (see section 5). Multilobular nuclei commonly present in (senescent) HRS cells, as a result of S/M phase dissociation, are linked to cell cycle withdrawal ($p21^{WAF1/Cip1}$ immunopositivity-left

679 image) while the inflammatory milieu is associated with SASP. Lipofouscin accumulation
680 assessed with GL13 staining (brown cytoplasmic staining-right image) reflects macromolecular
681 damage conferring to increased granularity (left centered image). The latter is also linked to
682 deregulated metabolism. Altered/increased gene expression (right centered image) that is also
683 accompanied by increased transcriptional "noise" also confers to macromolecular damage
684 (Ogrodnik et al., 2019).

685 **Figure 2. Cell cycle withdrawal in senescent, quiescent and terminally differentiated cells.**
686 Depicted are differences in cell cycle arrest reversibility, activated signals **(see text),** secretory
687 functions and macromolecular damage that allow discrimination between these cellular states.
688 Macromolecular damage is a common feature of senescence. Secretion is another common
689 feature of senescence and is sometimes (context-dependently) found in the differentiated state.
690 Cell cycle arrest is generally considered irreversible during senescence and terminal
691 differentiation, although cell cycle re-entry can occur under certain conditions. Green color:
692 active/present, red color: inactive/absent. Double-headed arrows: solid=established connection,
693 hatched: uncertain.

694 **Figure 3. A multi-marker, three-step workflow for detecting senescent cells.** The first step
695 of the proposed workflow includes assessing senescence-associated beta-galactosidasde (SA-β-
696 gal) activity and/or lipofuscin accumulation (SBB or GL13 staining). Secondly, co-staining with
697 other markers frequently observed in ($p16^{INK4A}$, $p21^{WAF1/Cip1}$) or absent from (proliferation
698 markers, Lamin B1) senescent cells. In the third step, identification of factors anticipated to be
699 altered in specific senescence contexts should be identified. This multi-marker workflow can
700 lead to the recognition of senescent cells with the highest accuracy.

701

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