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Review in translational cardiovascular medicine

From bench to bedside: The critical need for standardized senescence detection☆

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ABSTRACT

Cellular senescence, identified as a state of permanent cell cycle arrest, has become central to understanding aging and disease. Initially seen as a cellular aging mechanism, it is now recognized for its roles in development, tissu repair and tumour suppression. However, the accumulation of senescent cells with age contributes to chronic diseases such as diabetes, atherosclerosis and neurodegeneration. Recent efforts have focused on "senotherapeutics", including senolytics, which aim to eliminate senescent cells to mitigate age-related decline. Despite significant advances, senescence research faces critical challenges because of inconsistent detection methods. Common markers, such as p16INK4a and senescence-associated β-galactosidase, vary across tissues and contexts, complicating cross-study comparisons and clinical applications. A standardized multifaceted approach to senescence detection is essential, and should incorporate complementary methods, clear thresholds for senescence classification and considerations for cell type-specific variations. Such standardization would enhance reproducibility, streamline research and facilitate clinical translation, advancing therapeutic applications in aging and disease management.

1. Abbreviations

DNA	deoxyribonucleic acid
SA-β-gal	senescence-associated β-galactosidase
SASP	senescence-associated secretory phenotype
SCCNV	senescent cancer cell-derived nanovesicle

2. Background

Cellular senescence, a state of permanent cell cycle arrest first described by Hayflick and Moorhead in 1961, has emerged as a critical factor in several diseases [1]; their seminal work, demonstrating the limited replicative capacity of human fibroblasts in culture, laid the foundation for a research domain that has since burgeoned into a multifaceted discipline with far-reaching implications for human health and longevity [2]. Over the ensuing decades, our understanding of senescence has progressed from a simple model of cellular aging to a complex biological process implicated in development, tissue homeostasis, wound healing and various pathological conditions [3]. This phenomenon plays diverse roles in biological processes, including embryonic development, tumour suppression, wound healing and tissue repair. Senescent cells accumulate with age, and contribute to various chronic conditions such as diabetes, atherosclerosis and neurodegenerative diseases [4]. The interdisciplinary nature of senescence research has both accelerated progress and complicated efforts to establish standardized detection methods. Senescence intersects with diverse fields, including cancer biology, regenerative medicine and aging research, each bringing its own perspectives and methodologies. This cross-pollination of ideas has enriched our understanding but has also led to a proliferation of context-specific markers and detection techniques. The resulting heterogeneity in approaches has made cross-study comparisons challenging and has hindered the translation of basic research findings into clinical applications [5].

The senescent phenotype is complex, characterized by morphological changes, altered gene expression and secretion of bioactive factors known as the senescence-associated secretory phenotype (SASP). Although senescence serves important physiological functions, the accumulation of senescent cells beyond a certain threshold can activate age-related pathological changes [6]. Recent

☆ X post (Tweet): Cellular senescence plays a crucial role in ageing and disease. As senescent cells accumulate, they contribute to chronic conditions. A standardized approach to senescence detection is vital for advancing research and therapeutic applications in age-related diseases.

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research has focused on developing "senotherapeutics", i.e. interventions targeting senescent cells to ameliorate age-related decline and extend health span [7]. However, the field faces a significant challenge – the lack of standardized detection methods. Current approaches employ various markers and techniques, including senescence-associated β-galactosidase (SA-β-gal) activity, p16INK4a expression and persistent deoxyribonucleic acid (DNA) damage foci [8,9]. Yet, no single marker definitively identifies all senescent cells across different tissues and contexts. The translational potential of senescence research has garnered significant attention, particularly in the realm of senotherapeutics. The development of senolytics, drugs that selectively eliminate senescent cells, has shown promise in preclinical models for treating various age-related conditions [9,10]. However, the clinical translation of these findings hinges on our ability to reliably detect and quantify senescent cells in human tissues [9]. Standardized detection methods would not only accelerate the development and validation of senotherapeutics, but would also enable more accurate patient stratification and treatment monitoring in clinical trials. As the field rapidly expands, there is an urgent need to establish standardized procedures for senescence detection. Developing a unified framework incorporating multiple complementary approaches would enhance the reliability and comparability of senescence-related research, accelerating scientific progress and paving the way for more robust clinical applications [11].

This review aims to address the critical need for standardized senescence detection methods, exploring current challenges, proposing key components for a standardized approach and discussing the potential implications for high-mortality diseases and future research directions.

3. Standardized senescence detection: a catalyst for stem cell biology and regenerative medicine

The development of uniform methods for detecting senescence has significant implications for stem cell biology and regenerative medicine. These fields rely on accurate identification and characterization of senescent cells to maximize the therapeutic potential of stem cells. Reliable senescence detection is essential for evaluating stem cell quality and functionality, particularly as stem cells age or undergoes multiple passages, which can diminish their regenerative capabilities [12]. Standardized methods could optimize stem cell culture conditions, rejuvenate senescent stem cells and improve tissue engineering by enhancing quality control and developing senescence-resistant biomaterials [13]. For regenerative therapies, reducing the burden of senescent cells is essential for ensuring safety and effectiveness. Consistent detection methods could improve donor cell screening, facilitate in vivo monitoring of transplanted cells and optimize senolytic pretreatments to enhance graft survival – critical factors for the success of stem cell clinical trials [14]. Furthermore, refining disease models using induced pluripotent stem cells relies on accurate senescence detection to create physiologically relevant models and to understand the role of cellular aging in disease progression, particularly in cardiovascular diseases, cancer and neurodegenerative disorders [15]. Overall, integrating standardized senescence detection into stem cell research and regenerative medicine is a critical step toward advancing these fields and improving their translation to clinical practice [11].

4. Variability in senescence detection: a case for standardization

The field of cellular senescence research has been hampered by inconsistencies in detection methods, even when studying identical cell lines. This variability underscores the need for a standardized approach to defining and identifying senescent cells. Several studies have demonstrated this issue, highlighting how different methodologies can lead to divergent conclusions about the senescent state of cells.

A study by Tuttle et al. (2021) [16], investigated senescence markers in human tissue samples from individuals with age-related diseases. The researchers observed that while p16INK4a was commonly used as a primary marker for senescence, its expression levels varied significantly across different tissues and disease states. This finding underscores the challenges of relying on a single marker for senescence detection in diverse biological contexts. In a review, Kudlova et al. (2022) [8], explored the heterogeneity of senescence markers across various contexts. The authors highlighted that even widely recognized markers, such as SA-β-gal, can exhibit variable activity levels in senescent cells, influenced by factors such as the inducing stimulus and cell type. This variability poses difficulties in establishing universal thresholds for detecting senescence.

Another example comes from studies on human diploid fibroblasts; Coppé et al. (2008) [17] and Rodier et al. (2009) [18] both investigated senescence in human fetal lung (IMR-90) fibroblasts, but reported different SASP profiles. Coppé et al. identified a broader range of secreted factors, including several matrix metalloproteinases, whereas Rodier et al. focused on a more limited set of inflammatory cytokines. These differences probably stem from variations in cell culture conditions, senescence induction methods and the sensitivity of the detection assays used. The discrepancies extend beyond in vitro studies; in vivo senescence detection has also shown considerable variability. Baker et al. (2016) [19] and Chang et al. (2015) [20] both examined senescence in mouse tissues using p16INK4a as a marker. However, they reported different percentages of senescent cells in similar tissues, probably as a result of differences in detection methods and the criteria used to define senescence positivity.

These examples illustrate how the lack of standardized protocols can lead to inconsistent results, even when studying the same cell types or tissues. Such variability complicates the interpretation of senescence-related data and hinders progress in understanding the role of senescent cells in aging and disease. The observed inconsistencies emphasize the need for a more defined approach to senescence detection. Standardized protocols would improve the reproducibility of results across different laboratories, and also enhance the ability of the scientific community to compare findings from various studies. By highlighting these discrepancies, we underscore the importance of this commentary in advocating for a concerted effort to define the minimum criteria for cellular senescence.

5. Current state of senescence detection

Cellular senescence detection is a complex challenge in aging research, characterized by a diverse array of methods, each with unique strengths and limitations. These methods can be categorized into morphological, molecular and functional assays, often used in combination to provide a comprehensive assessment of the senescent state [21]. Morphological analysis is one of the earliest approaches, focusing on characteristic changes in cell and nuclear shape and size [21]. Senescent cells typically exhibit enlarged flattened morphologies and increased granularity. However, these changes can be subtle, and vary across cell types, making this method subjective and prone to misinterpretation [22]. The most widely used molecular marker is SA-β-gal activity, detectable through histochemical staining. Although this assay is relatively simple, it lacks specificity; some non-senescent cells

can also express SA-β-gal under certain conditions, such as cell culture stress and quiescence, and specific tissue environments can influence cellular behaviour [1]. For example, in the developing avian retina, newly differentiated ganglion cells exhibit high SA-β-gal activity at pH 6.0, a level typically associated with senescence [23]. Similarly, non-proliferating or terminally differentiated neurons can display increased lysosomal activity, which can mimic the SA-β-gal signal [24]. Macrophages, too, often test positive for SA-β-gal without being senescent [25]. As the assay is performed at pH 6.0 to detect senescence (compared with pH 4.5 for general SA-β-gal activity), overlapping results can occur. These complexities underscore the importance of using multiple markers and careful analysis in senescence research [26,27]. Additionally, staining intensity can vary based on cell culture conditions and fixation methods, leading to inconsistent results [25]. Other molecular markers include cyclin-dependent kinase inhibitors p16INK4a and p21CIP1, which are often upregulated in senescent cells. These can be detected through immunohistochemistry. However, their expression is not exclusive to senescence, and can fluctuate based on cell type and the specific senescence inducer [22,28,29]. Functional assays, such as proliferation tests and cell cycle analysis, provide valuable insights, but can be time-consuming, and may not distinguish between senescence and other forms of growth arrest [1,5]. Telomere length analysis measures the progressive shortening of telomeres associated with replicative senescence. Although informative, this method does not capture all forms of senescence, such as stress-induced premature senescence, oncogene-induced senescence, developmental senescence, therapy-induced senescence, epigenetically induced senescence and mitochondrial dysfunction-induced senescence, which requires specialized equipment and expertise [22,30–32]. Different laboratories often employ distinct combinations of assays, complicating cross-study comparisons. The threshold for considering a senescent cell can vary widely, and the interpretation of results is often context dependent, which impacts research reproducibility [6].

To this end, it is essential to establish a standardized multifaceted approach to senescence detection that is context dependent. The approach should consider incorporating multiple complementary assays, define clear thresholds for senescence classification and account for cell type-specific variations. By doing this, we can enhance the reproducibility of senescence research and accelerate its translation into clinical applications.

6. Key components to consider when defining senescence

Establishing a standardized procedure for senescence detection requires careful consideration of essential markers, diverse assays and rigorous controls. We propose considering a tiered approach combining morphological, molecular and functional assays to provide a comprehensive and reliable assessment of the senescent state across various experimental contexts. To address the challenge of detecting true senescence, we suggest minimum criteria, including at least one marker of growth arrest, one SASP marker and a functional assay demonstrating stable cell cycle exit.

Key assays and markers for senescence detection include SA-β-gal, which remains a cornerstone, but should not be used in isolation. The cell cycle inhibitors p16INK4a and p21CIP1 provide crucial information about growth arrest, and DNA damage markers, such as γ-H2AX foci or 53BP1 staining, indicate persistent DNA damage response. SASP components, including inflammatory cytokines (interleukin-6, interleukin-8), chemokines (C-X-C motif ligand [CXCL] and C-C motif ligand [CCL]), growth factors (vascular endothelial growth factor [VEGF], epidermal growth factor [EGF] and transforming growth factor beta [TGF-β]) and matrix-remodelling enzymes (matrix metalloproteinases 1 [MMP-1], 3 [MMP-3] and 9 [MMP-9]) offer insights into the paracrine effects of senescent cells. Telomere length assessment remains relevant, especially in the context of replicative senescence. By combining multiple markers and assays, we increase the likelihood of accurately identifying truly senescent cells. This approach aims to provide a comprehensive, reliable and adaptable framework for senescence detection, recognizing that while no single marker is definitive, the convergence of multiple indicators provides strong evidence of senescence.

A comprehensive approach to senescence detection integrates morphological analysis, protein profiling, secretory protein assessment and functional assays, supported by rigorous controls and standards. High-content imaging systems provide quantitative morphological data, and protein profiling combines SA-β-gal staining with immunofluorescence for key senescence markers. Gene expression analysis targets senescence-associated genes, including SASP factors, offering a broader perspective on the senescent phenotype. SASP profiling through a multiplex enzyme-linked immunosorbent assay (ELISA) or mass spectrometry-based proteomics characterizes the secretory phenotype. Functional assays, such as 5-ethynyl-2′-deoxyuridine (EdU) incorporation, and flow cytometry-based cell cycle analysis confirm growth arrest. The use of appropriate controls, including tissue-matched positive and negative controls, and calibration standards ensure better comparability across laboratories. Time-course analyses are crucial for distinguishing between transient responses and true senescence. This multifaceted approach, incorporating insights from various studies [5,19,33–35], provides a robust and adaptable framework for senescence detection across various experimental contexts, recognizing the variability of senescent markers across different tissues and cell types.

Recent advances in senescence research have marked significant progress, yet challenges persist in standardizing detection methodologies and establishing definitive thresholds for identifying senescent cells. Despite employing identical techniques, researchers often encounter inconsistent results, primarily as a result of biological heterogeneity, technical variability and divergent interpretations [36,37]. Comprehensive mapping efforts, such as those undertaken by the National Institutes of Health (NIH) SenNet Consortium [38] – which aims to generate four-dimensional atlases of senescent cells throughout the human lifespan – are essential for addressing these complexities and furthering our knowledge [37]. Continually refining these guidelines based on new discoveries is encouraged.

7. Implications and future directions

The standardization of senescence detection methods holds profound implications for our understanding and treatment of high-mortality diseases, while also opening up exciting new avenues for research and clinical applications. As we look to the future, the potential transformative impact of this standardization on the field of senescence research becomes increasingly apparent.

7.1. Impact of senescence on high-mortality diseases

7.1.1. Cardiovascular diseases

Standardized senescence detection could revolutionize our approach to cardiovascular diseases, which are the leading cause of death globally. By enabling precise quantification of senescent cells in arterial walls, we can better track atherosclerosis progression and assess plaque vulnerability. This could lead to more accurate risk stratification and personalized treatment strategies. For instance, patients with a high burden of senescent cells in their

vasculature might benefit from more aggressive lipid-lowering therapies or emerging senolytic treatments.

7.1.1.1. Advancing senomorphics: clinical trials and cardiovascular applications. Senolytic therapies are gaining momentum, with more than 30 clinical trials, including rigorous phase 2, randomized, double-blind, placebo-controlled studies, either underway or in the planning stages [39]. Early investigations have focused on repurposing existing drugs or utilizing natural compounds with established safety records. For instance, dasatinib and quercetin were evaluated in two pilot studies, demonstrating promising initial outcomes [40,41].

The clinical application of senolytics for cardiovascular diseases has been slow. However, several studies are now poised to address this. One such effort, the Q-CABG trial, is examining the potential of quercetin monotherapy to improve outcomes in patients undergoing coronary artery bypass grafting [42]. Additional research is being planned to explore the use of senolytic therapies for diverse conditions, including heart failure and atrial fibrillation, and for improving the durability of arteriovenous dialysis fistulas, signalling a growing interest in applying these therapies to cardiovascular health [43].

In the context of heart failure, reliably detecting senescent cells is essential for deepening our understanding of disease mechanisms. This capability could inform the development of precise therapies designed to maintain cardiac function by addressing the senescent cell burden directly [43–47].

7.1.2. Cancer

7.1.2.1. Exploring senescent cancer cells as a basis for cancer vaccines. Therapy-induced senescence is a common outcome of anticancer treatments, often contributing to challenges such as drug resistance, immunosuppression or tumour recurrence, but also offering a unique opportunity to enhance immune responses against cancer [48]. Recent findings by Liu et al. (2023) [49] highlight how senescent cancer cells could be repurposed into cancer vaccines by stimulating antitumour immunity. Their study revealed that dendritic cells, when exposed to senescent cancer cells in a co-culture system, became activated. In animal models, the injection of either senescent cancer cells or dendritic cells preconditioned with these senescent cells led to tumour protection, notable tumour regression and improved responses to immunotherapy.

An alternative method involves creating vaccines from senescent cancer cell-derived nanovesicles (SCCNVs) [50]. These nanovesicles can be produced by inducing senescence in cancer cells from individual patients under controlled conditions outside the body. After a purification process, the SCCNVs can be readministered to patients, leveraging their natural immunostimulatory properties. Importantly, cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) – key components of the SASP – serve as built-in adjuvants, enhancing immune activity without the need for external agents [50]. The SCCNVs also carry a diverse array of antigens capable of triggering robust immune responses, including CD8+ T-cell activation. Studies using SCCNVs derived from B16F10 melanoma cells treated with doxorubicin showed significant reductions in tumour progression and metastasis in mice. However, further investigation is needed to evaluate potential risks, such as immune reactions triggered by non-specific antigens on SCCNVs [50].

Further supporting the concept, Marin et al. (2023) [51] demonstrated that immune activation is not limited to senescent cancer cells; normal senescent cells can also provoke strong immune responses. Their work identified a set of approximately 70 peptides associated with the senescent phenotype that make these cells immunogenic and capable of activating CD8+ T cells. Extending their analysis to senescent cancer cells, they showed that these cells outperformed immunogenic cell death in eliciting protective immune responses against tumour relapse [51]. This suggests that senescent cell-based vaccination, whether using cancerous or non-cancerous cells, holds significant promise as a strategy for preventing tumour growth and recurrence.

In oncology, the implications of standardized senescence detection are particularly necessary. A universally accepted "senescence index" could serve as a powerful biomarker for early cancer detection and prognosis. Furthermore, consistent detection methods will facilitate the evaluation of senescence-inducing therapies – a promising approach in cancer treatment. By accurately quantifying therapy-induced senescence in tumours, we can better assess treatment efficacy and predict long-term outcomes. This could be particularly valuable in the context of combination therapies, where senescence induction might synergize with other treatment modalities [4].

7.1.3. Neurodegenerative diseases

In the realm of neurodegenerative diseases, standardized senescence detection could provide new insights into disease progression and potential therapeutic targets. For instance, in Alzheimer's disease, senescent astrocytes, microglia, endothelial cells and neurons have been identified in patients' brains and animal models [52,53]. Precise quantification of these senescent populations may serve as a biomarker for disease progression and therapeutic response, particularly in relation to the accumulation of senescent cells alongside amyloid-β plaques and tau tangles [54].

Senolytic therapies have progressed from preclinical research to clinical testing, with several trials currently underway [55,56]. A phase I feasibility study evaluated the safety of oral quercetin and dasatinib, confirming their tolerability, and showing that dasatinib effectively crosses the blood-brain barrier [55]. Although no significant improvements were observed in cognitive performance or neuroimaging outcomes, cerebrospinal fluid analysis indicated promising trends, including reduced concentrations of senescence-associated cytokines and chemokines and an increase in amyloid-β concentrations. These preliminary clinical findings support the potential of quercetin and dasatinib as a safe and feasible approach for treating Alzheimer's disease [55].

In Parkinson's disease, the presence of senescent astrocytes and dopaminergic neurons in the substantia nigra has been documented [57], and standardized detection techniques could clarify the role of cellular senescence in α-synuclein aggregation, potentially identifying novel therapeutic targets.

In multiple sclerosis, senescent oligodendrocyte progenitor cells have been implicated in disease progression [58]. Accurate quantification of these senescent cells could enhance monitoring of disease activity and the effectiveness of remyelination therapies.

In neurodegenerative disorders, consistent senescence detection could enhance disease staging and prognosis and evaluation of senolytic therapies; it may also uncover disease-specific senescence profiles, enabling more targeted treatments. Developing robust multimarker strategies for senescence detection is a crucial goal to advance the understanding and clinical treatment of these conditions.

8. Challenges and limitations

Standardizing senescence detection methods poses a range of complex challenges and limitations. First, biological heterogeneity in senescent cells, driven by differences in cell types, tissue environments and the stimuli inducing senescence, makes it difficult to create a universal detection method. Standardized

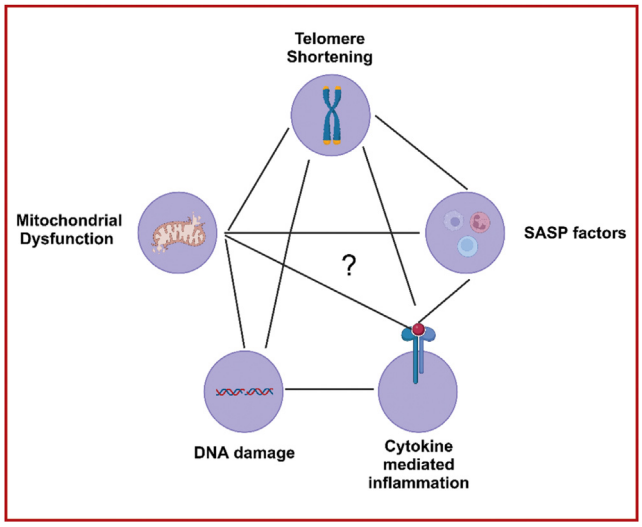


Fig. 1. Understanding the different uptake markers of senescence activation. DNA: deoxyribonucleic acid; SASP: senescence-associated secretory phenotype.

approaches must be adaptable enough to address this variability, while ensuring consistent results across various experimental settings. Technical variability across laboratories, including differences in expertise, equipment and resources, can also lead to inconsistent outcomes.

Ensuring reliable implementation of consistent senescence detection methods across diverse environments is essential, but challenging. The growing understanding of senescence, with the discovery of new markers and mechanisms, requires detection methods to be flexible and adaptable. Bridging the gap between in vitro and in vivo applications remains a significant hurdle, as does addressing the high cost and complexity of advanced technologies, such as high-throughput sequencing and imaging, which may limit accessibility for smaller laboratories.

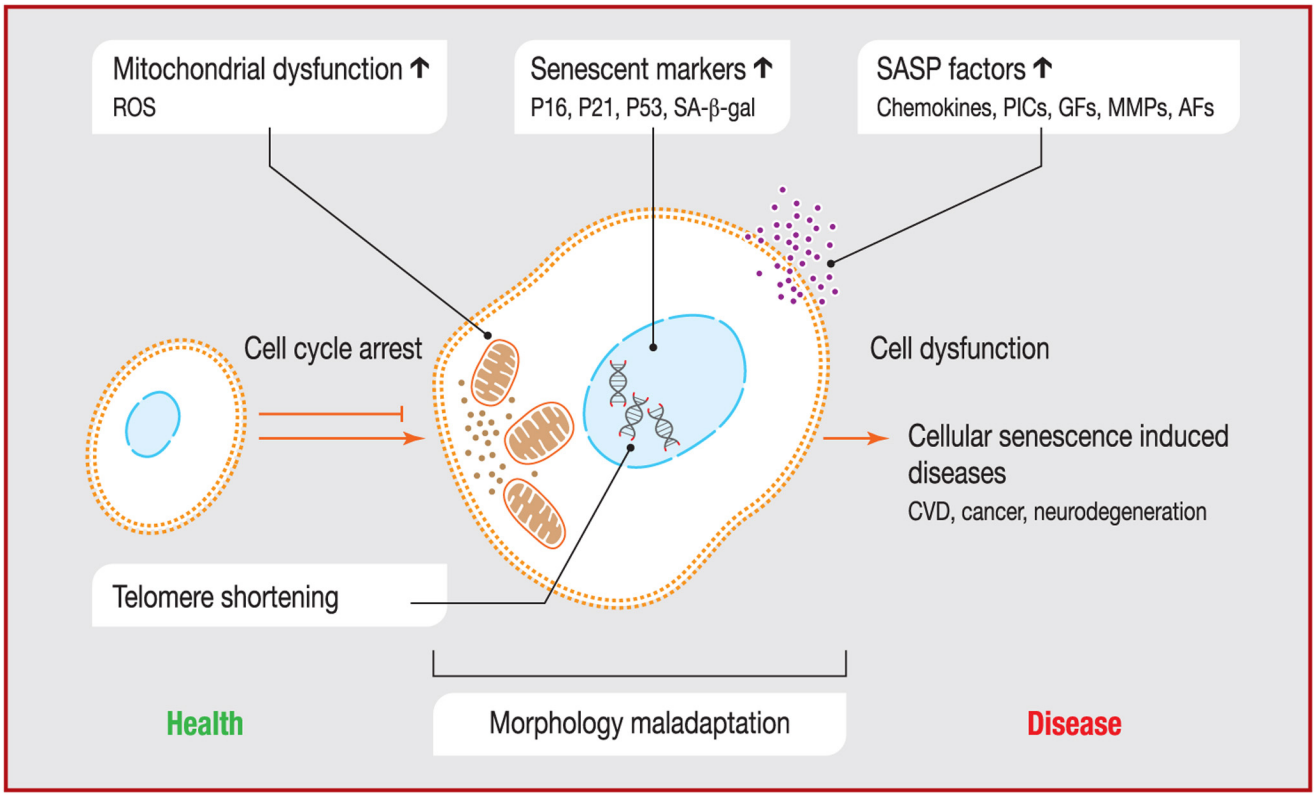
Senescence is dynamic, with cellular traits changing over time, making it difficult to capture these shifts, especially in vivo. Distinguishing senescence from similar states, such as quiescence or terminal differentiation, as well as accounting for tissue-specific variations, adds further complexity.

As senolytic therapies advance, updated protocols will be needed to detect residual or altered senescent cells in treated tissues. Implementing these methods in clinical settings will face ethical and regulatory challenges, particularly when using novel biomarkers subject to varying international rules. Additionally, managing the vast data generated by standardized approaches will require a robust infrastructure for sharing and analysis. Overcoming these obstacles will necessitate a coordinated effort from the research community (Fig. 1).

9. Conclusions

Creating consistent methods for detecting senescence is a major step forward in biomedical research, with wide-ranging impacts on many fields and diseases. By reducing variability in current techniques, we are opening the door to a new era in senescence research that could transform how we understand cellular aging and its role in disease.

Senotherapies represent an innovative strategy aimed at addressing the fundamental biological mechanisms of aging, offering the potential to prevent and treat a wide range of age-related conditions while promoting a longer health span. By targeting the root causes of aging rather than managing individual diseases or disorders, these therapies may also reduce the need for multiple medications, thereby mitigating the risks associated with polypharmacy, such as adverse effects and drug interactions. Senolytics are particularly effective, even with intermittent use, thanks to their short elimination half-lives. Although more research is needed, they have shown promise by reducing the risk of cumulative toxic effects and improving safety in the clinical arena (Central Illustration).



Central Illustration. Cellular senescence, defined as a permanent irreversible halt in the cell cycle, plays a crucial role in cellular dysfunction and disease. Senescence is observed with an increase in mitochondrial dysfunction that in turn increases reactive oxygen species (ROS) production, there is an increase in cell and nuclear senescent markers including P16, P21, P53 and senescence-associated β-galactosidase (SA- β-gal). Senescent cells secrete senescent associated secretory protein (SASP) factors including proinflammatory cytokines (PICs), growth factors (GFs), matrix metalloproteinases (MMPs) and angiogenic factors (AFs). The nucleus and cell morphology significantly undergoes maladaptation in senescent cell that appear larger and more flattened. This leads to cellular senescence induced disease including cardiovascular disease (CVD), cancer and neurodegeneration.

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