 doi:10.20944/preprints202207.0160.v2

NIH SenNet Consortium: Mapping senescent cells in the human body to understand health and disease

SenNet Consortium

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 doi:10.20944/preprints202207.0160.v2

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 doi:10.20944/preprints202207.0160.v2

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 doi:10.20944/preprints202207.0160.v2

Abstract

Cells respond to a myriad of stressors by senescing, acquiring stable growth arrest, morphologic and metabolic changes, and a senescence-associated-secretory-phenotype (SASP). The heterogeneity of senescent cells (SnCs) and their SASP is vast, yet poorly characterized. SnCs have diverse roles in health and disease and are therapeutically targetable, making characterization of SnCs and harmonization of their nomenclature a priority. The Cellular Senescence Network (SenNet), a NIH Common Fund initiative, will leverage emerging single cell and spatial-omics to identify and map SnCs in numerous organs across the lifespan of humans and mice. A common coordinate framework will integrate the data, using validated, standardized methods, creating public 4-dimensional SnC atlases. Key SenNet deliverables include development of innovative tools/technologies to detect SnCs, biomarker discovery, common annotations to describe SnCs and extensive public data sets. The goal is to comprehensively understand and map SnCs for diagnostic and therapeutic purposes to improve human health.

Keywords
Cellular Senescence Network, Normal Aging, Senescence, Senescence-associated secretory phenotype, SenNet

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Introduction

Senescence is a cell state triggered by numerous types of cell - intrinsic and –extrinsic stress, including mitotic, oxidative, genotoxic, mechanical, or nutrient stress, and organelle dysfunction[1]. Senescence is driven by p53/p21^{CIP1} and p16^{INK4a}/Rb tumor suppressor pathways and possibly other signaling events yet to be identified[1-3]. The senescence response is amplified by several mediators, including ATM, IKK/NF-κB, JAK/STAT, GATA-4, and mTOR. Senescent cells (SnCs) acquire diverse characteristics such as increased cell size and protein content, altered organelle function, evidence of chronic nuclear genotoxic stress, a robust secretome, and resistance to apoptosis[1]. One constant characteristic of SnCs is a stable cell cycle arrest, illustrating the main goal of senescence – to prevent a damaged cell from replicating itself, potentially leading to mutations and the risk of cancer.

Multiple lines of evidence suggest that SnCs drive aging itself, and many diverse age-related diseases in preclinical models[1,4-10]. Reports suggest that interventions targeting SnCs can impact more than one disease of old age[11]. However, at least certain types of SnCs also play important roles in normal physiology, e.g., development and wound healing[12,13]. SnCs have a senescence-associated secretory phenotype (SASP) comprised of diverse molecules, including proinflammatory cytokines, chemokines, growth factors, proteases, receptors, extracellular vesicles, and specific extracellular matrix proteins[14-16]. The SASP can drive the loss of tissue homeostasis and secondary senescence (pathological role). The SASP is also thought to be critical for attracting immune cells that mediate tissue regeneration and clearance of SnCs (normal physiology)[17]. Given the heterogeneity and evolving definitions of SnCs and SASP, there is a need to expand efforts to characterize, detect and integrate SnCs, with deeper ontology-based understanding and standardization of nomenclatures, as well as detection methodologies.

In 2011, it was firmly established that genetic clearance of SnCs delays onset of multiple age-related pathologic conditions affecting numerous tissues in transgenic mice[18]. In 2016 it was established that genetic clearance of SnCs in mice delays all-cause mortality, extending median, but not maximum lifespan[19], thus implicating SnCs in contributing to all diseases that kill mice, including cancer, chronic kidney disease, and cardiomyopathy[19]. These genetic studies incentivized the development of senotherapeutics – drugs that selectively target SnCs, either killing them (senolytics) or suppressing markers of senescence including the SASP (senomorphics). The first senolytics were described in 2015[20]. Since then, dozens of senotherapeutics have been described, including natural products[21,22], repurposed drugs[6,23], peptides[24], proteolysis-targeted chimeras[25], and CAR-T cells[26].

Senolytics have proven efficacious in pre-clinical models of frailty, cardiovascular disease, kidney disease, atherosclerosis, diabetes, osteoarthritis, osteoporosis, hepatic and pulmonary fibrosis, steatosis, obesity, depression, mortality due to acute ß-coronavirus infection, and Alzheimer's disease[27,28]. Currently, there are numerous ongoing clinical trials testing senolytics in a variety of age-related diseases including frailty, idiopathic pulmonary fibrosis, Alzheimer's disease, chronic kidney disease, osteoporosis, and COVID-19 in older adults. Preliminary data from these studies indicate that at least the senolytic cocktail, dasatinib plus quercetin, appears to be safe in humans, reducing SnC burden[29,30]. Moreover, in mice, a short course of senolytics, administered intermittently, is sufficient to improve multiple measures of physical fitness even

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when administered late in life[31], highlighting the potential impact of such strategies on human health and healthcare costs.

Nevertheless, despite this promise, there is a lack of information about the identity and heterogeneity of SnCs in human tissues and their unique characteristics. Little is known about where and when SnCs arise in humans across lifespan and health status, or the extent of SnC heterogeneity *in vivo,* and release of senescence factors/SASP into blood circulation. Such knowledge could guide therapeutic and organ-specific targeting of SnCs, improving senotherapeutic approaches to treating multi-morbidities associated with aging. Clearly there is a significant need to develop tools to map and identify the types of human SnCs with spatial and temporal resolution over the human lifespan.

Establishment of SenNet: The characterization of human SnCs to date has largely been conducted *in vitro*. SnC characteristics very much depend on cell-type, inducing stimulus, temporal dynamics, and physiological context. Such phenotypic and temporal heterogeneity make it challenging for the scientific community to identify and agree on biomarkers that are common to most SnCs. As a result, no single laboratory, grant, or approach will be adequate to comprehensively define cellular senescence. Yet, a detailed characterization of SnCs and the unique SASP associated with them is urgently needed if we are to harness knowledge about cellular senescence for the benefit of human health. The number of tissues, diseases and conditions affected by the accumulation of SnCs during aging and other physiological processes supports the need for a community-wide scientific effort to tackle this challenge. The Common Fund occupies a unique and exciting space at NIH and is specifically designed to address large challenges and opportunities that are of high priority for the entire NIH (all 27 institutes and centers) and the biomedical community broadly.

In 2021, the NIH Common Fund launched the Senescence Network (SenNet) program with the goal of identifying and functionally characterizing SnCs in healthy human tissues across the human lifespan. The geographic spread of the tissue mapping centers (TMCs) / Technology Development and Application awards (TDAs) are shown in **Fig.1**. This vision of creating a comprehensive atlas of SnCs was catalyzed by several NIH-sponsored workshops engaging experts in senescence working across numerous disciplines who indicated a need to develop novel tools and technologies to identify SnCs *in vivo* and to harmonize data across laboratories to accurately characterize the heterogeneity of SnCs at single cell resolution. Model systems and perturbations to validate the characteristics of SnCs identified in tissues were also identified as needs[32]. While distinct from human models, murine models offer extraordinary value by enabling genetic and pharmacologic manipulation of SnCs and longitudinal assessments to determine how/when cells acquire senescence features, how those features change over time and what the physiologic roles may be in vivo. SenNet will incorporate mechanisms for establishing a murine atlas of SnCs. The SenNet is designed to have a single Consortium Organization and Data Coordinating Center (CODCC) to harmonize and integrate SnC characterization efforts from numerous sites to create an atlas of SnCs across tissues and organisms that captures information about the evolution of senescence in space and time (Four dimensional (4D) atlases). These efforts will be of immeasurable value to the broader biomedical community, while addressing incredible challenges that will require technological advances for the following reasons.

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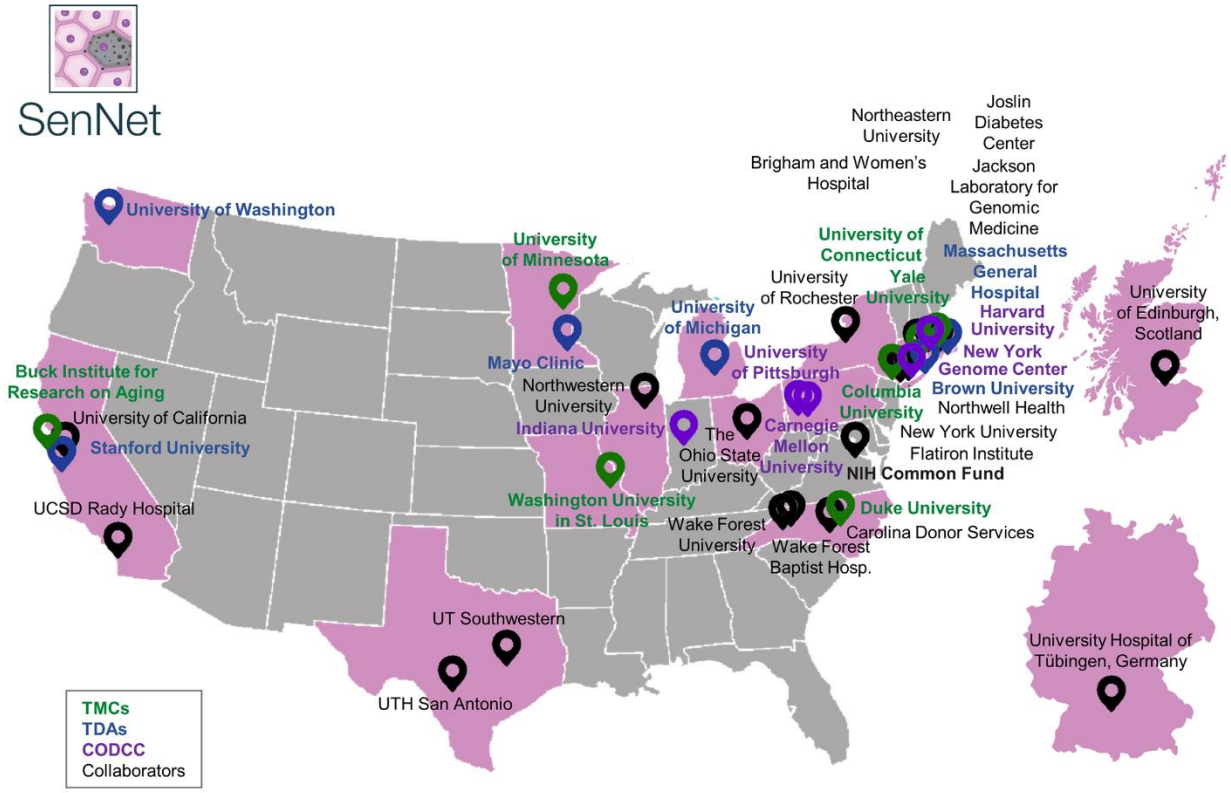

Figure 1. Geographic distribution of 2021 SenNet Awards focused on mapping SnCs in human tissues. TMC = Tissue Mapping Center (RFA-RM-21-008 U54); TDA = Technology Development and Application (RFA-RM-21-009 UG3/UH3), CODCC = Consortium Organization and Data Coordinating Center (RFA-RM-21-010 U24). Bold font identifies institutes of contact PIs.

Characterization of SnCs: The complexity of the senescence response entails kinetic alterations in virtually all aspects of a cell's biology, from epigenetic remodeling of chromatin[33] to changes in the quantity and function of organelles[34]. Current biomarkers used to identify SnCs include increased expression of the cell cycle regulators *p16*^{INK4a} [35] and *p21*^{CIP1} [36], increased lysosomal senescence-associated β-galactosidase activity (SA-βgal)[37], decreased Lamin B1[38], secretion of HMGB1[39], and several markers of genotoxic stress. These include SADFs (senescence-associated DNA damage foci characterized by co-localization of γH2AX and 53BP1), TAFs/TIFs (telomere-associated or telomere dysfunction-induced foci characterized by γH2AX foci at telomeres)[40], SAHFs (senescence-associated heterochromatic foci characterized by co-localization of dense DAPI staining and modified histones) and SADS (senescence-associated distensions of satellite DNA characterized by CENP-B foci at centromeres). In addition, activation of LINE-1 retrotransposable elements[41,42], and cytoplasmic chromatin fragments[43], as well as mitochondrial DNA[44], have also been described as stress markers. None of these molecular endpoints are specific to SnCs, which suggest the need to measure multiple endpoints to more precisely identify senescence[1,45]. Most published studies to date rely on bulk tissue analysis or, if at single cell resolution, implement a singular approach (e.g., transcriptomics, CyTOF), neither of which is adequate to identify SnCs lineages with the precision necessary to move the field forward – particularly in the therapeutic realm. Numerous other molecules have been attributed to SnCs or their SASP. However, in the absence of cross-validation with established (yet non-specific) SnC biomarkers at the level of single cell resolution and validation

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with appropriate perturbations provoking or targeting SnCs, these molecules are only *potential* biomarkers of SnCs. Hence, there is a significant need for a creative, comprehensive, and most importantly united approach (in terms of cross-validation to establish rigor and reproducibility) to characterize SnCs at single cell resolution in tissues and *in vivo*.

SnC Atlas: Other challenges to creating a 4D SnC atlas include: 1) SnCs are rare *in vivo*; 2) spatial-omics is currently a nascent technology validated only for mapping of well-characterized cell types, implying an additional burden of validation for ill-characterized cell types such as SnCs; 3) for any single SnC biomarker, it is not yet established whether changes in mRNA, protein or the epigenome (or some combination) best reflect a senescent state; 4) implementing a biomarker panel that includes a combination of proteins, nucleic acids, morphology markers, and measure of enzymatic activity endpoints limit the ability to co-localize the biomarkers at the single cell resolution; 5) SnCs in different tissues will likely express common as well as tissue-specific patterns of senescence regulators, effectors, and other features; and 6) a lack of tools to confidently discriminate pathological vs. physiological SnCs. In complex tissues, comprising of numerous cell types, both the physiological and pathological roles of SnCs may be occurring in close proximity (e.g., chronic tissue damage foci with adjacent areas of tissue regeneration). Ideally, to optimize senotherapeutics and to minimize side effects of this new class of drugs, one would like to distinguish between SnCs involved in these two processes and to do so using a biomarker measured in an easily accessed tissue or biofluid. This will require tissue mapping advances as well as biomarker discovery in human biofluids.

SenNet Deliverables: The expected deliverables from SenNet include a SnC atlas at single cell resolution for human and murine tissues, novel tools, technologies, and data sets that can be readily accessed, searched, and visualized to enable the broad community to query these sets to better define SnCs. A clear and comprehensive definition of SnCs in various tissues that will yield information about molecular targets unique or enriched in SnCs that could form the basis of selective senotherapeutics to advance the treatment of age-related multi-morbidities as well as diseases. Biomarkers will ideally be validated within and across tissues, ultimately enabling predictive modeling, optimizing SnC targeting and ensuring the safety and efficacy of senotherapeutics in both health maintenance and disease. Finally, it is expected that the SenNet program will interface with other existing cell mapping programs such as Human Bimolecular Atlas Program (HuBMAP), Human Cell Atlas (HCA), and the Kidney Precision Medicine Project (KPMP). Data integration will allow greater identification of translatable intersections as well as deploy common knowledge, data platforms, pipelines, tools, and technologies in an integrated manner to map SnCs.

To achieve the goals of SenNet, a substantial part of the consortium effort is dedicated to establishing multiple TMCs. Each TMC is charged with creating 4D atlases of at least two human or five murine tissues at the single cell level via emerging spatial mapping techniques including multiplexing of imaging, proteomics, and transcriptomics in tissue sections. Collectively, the TMCs are currently covering 18 primary human tissues (**Fig. 2**). Complementing this atlas construction effort, SenNet also has a significant technology development component. These efforts are designed to explore novel techniques and/or to improve existing methods to detect and characterize the heterogeneity of SnCs at single cell resolution. Examples include deep

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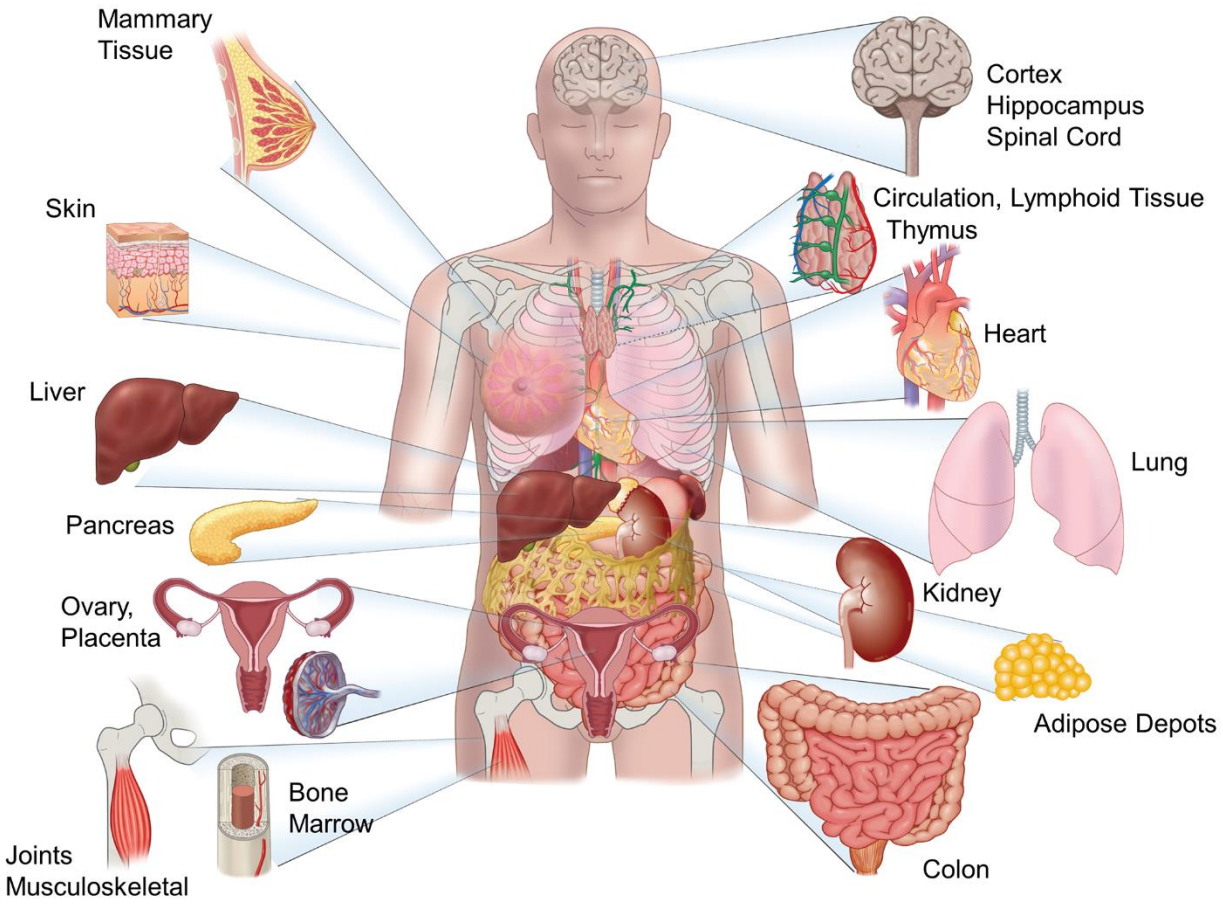

Figure 2. Human tissues in which SnCs will be mapped and characterized by SenNet Consortium to produce 4D atlases of senescence across the lifespan of humans.

phenotyping of the 4D nucleome of SnCs, high throughput quantitation of TAFs, and *in vivo* detection of SnCs via PET imaging. Through SenNet, these new technologies will be applied broadly and collaboratively across multiple tissues that will be characterized in SenNet TMCs **(Fig. 2)**.

SnCs are important for normal physiology, including embryonic development, initiation, parturition, tissue regeneration, wound healing, as well as contribute to numerous disease processes. However, whether SnCs are beneficial or deleterious, as thought to occur with aging, is unclear. There is no doubt that a deeper, temporal understanding of SnCs will help research communities to develop therapies that promote the beneficial effects of SnCs while suppressing or removing the deleterious effects. While the current goal of SenNet is mapping of SnCs in "normal/healthy" human and murine tissues to generate reference atlases of SnCs, we anticipate that future efforts will be directed towards leveraging these data sets to study the role of SnCs in various age-related pathologies and diseases.

Anticipated Results

The SenNet Consortium is initially focused on advancing our knowledge of the identity and definition of SnCs in healthy human tissue across the age-span. Like tumorigenesis, senescence

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is likely not a single state, entity or phenotype. Rather, the molecular identity of any given SnC is ultimately determined by a combination of its cell lineage, surrounding cells and environment, the endogenous or exogeneous stressors driving senescence, and the purpose of that cell entering senescence (e.g., preventing oncogene-mediated proliferation or promoting wound healing). Collectively, these variables are likely to create a unique molecular fingerprint. SenNet aims to address the challenges in defining the heterogeneity of SnCs and their impact on their tissue microenvironment at a single cell resolution, which will require the implementation of an array of cutting-edge enabling technologies to comprehensively identify, profile, and spatially map SnCs across tissues in humans and mice. These technologies are broadly categorized into two groups – bulk/single cell omics and spatial mapping. An overview of the technologies is shown in **Fig. 3**.

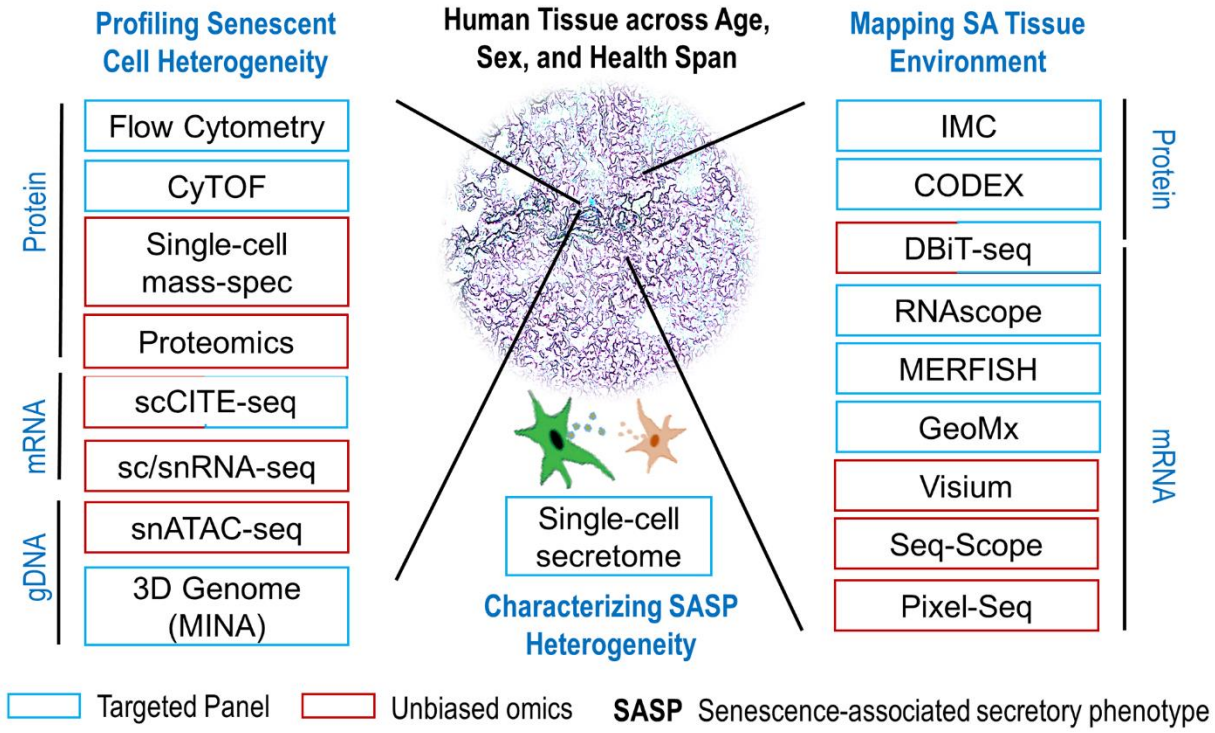

Figure 3. Overview of technologies that will be implemented and developed by SenNet Consortium scientists to detect, characterize, and spatially map the location of SnCs. CyTOF = Cytometry by Time-of-Flight; scCITE-Seq = Cellular Indexing of Transcriptomes and Epitopes by Sequencing; sc/snRNA-seq = single cell or single nuclear RNA sequencing; snATAC-seq = single nuclear Assay for Transposase-Accessible Chromatin using sequencing; MiNA = Multiplexed Imaging of Nucleome Architectures; IMC = Imaging Mass Cytometry; CODEX = CO-Detection by inDEXing immunofluorescence; DBiT-seq = deterministic barcoding in tissue for spatial omics sequencing for co-mapping mRNAs and proteins; RNAScope = RNA in situ hybridization visualization of single molecules; MERFISH = multiplexed error-robust fluorescence in situ hybridization; GeoMx = NanoString GeoMx Digital Spatial Profiling; Visium = Visium 10x Genomics molecular profiling; Seq-Scope = a spatial barcoding technology with spatial resolution comparable to optical microscopy; Pixel-Seq = Polony-indexed library sequencing.

Technologies used by SenNet:

Transcriptomic Approaches: To achieve single cell resolution and overcome the scarcity of SnCs, high-throughput single cell and single-nucleus transcriptomic techniques have become mainstay tools for surveying tens of thousands of cells to identify transcriptional signatures in rare cell populations as low as 0.5%, enabling discovery of potential new SnC biomarkers[46,47].

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Perturbations that drive or eliminate SnCs will be critical for validating SnC identity in a context-dependent way, which will also help optimize detection methods. Incorporation of antibody-based targeted proteomics, such as CITE-seq[48], allows for the simultaneous measurement of hundreds of protein markers and whole transcriptome to link cell-type information to transcriptional profiling to uncover cell type-specific senescence biomarkers. Aligning SenNet with HuBMAP's Organ Mapping Antibody Panels (OMPAs)[49], Anatomical Structures, Cell Types, plus Biomarkers (ASCT+B) tables[50], and Affinity Reagent groups will leverage incredible experience in SOPs for validating antibodies and the products of that for discerning cell types.

Proteomic Approaches: SenNet investigators are further developing novel single cell mass spectrometry methods[51], for unbiased discovery of proteomic signatures of SnCs. These methods can be scaled to hundreds of thousands of single cells[49]. These methods can be extended to analyzing protein modifications and covariations, which can reflect regulatory processes in SnCs[52]. SenNet investigators will also examine the epigenetic regulation that may drive transcriptional alterations observed in SnCs. Single cell profiling of chromatin accessibility using ATAC (assay for transposase-accessible chromatin) or chromatin modification using ChIP-seq or CUT&Tag, in combination with single cell/single nucleus RNAseq, will allow for simultaneous profiling of the same cells/nuclei for transcriptome and epigenetic changes to define functional senescence signatures at the single cell level. A hallmark of SnCs is the SASP, which will eventually require a combinatorial approach using proteomics, metabolomics and lipidomics analyses, especially when characterizing SASP-associated extracellular vesicles. The SenNet program will employ emerging technologies to measure large panels of secreted proteins from single cells[53], enabling the characterization of SASP in live SnCs. Caveats to characterizing SnCs include the fact that upregulation of cell cycle regulators in SnCs is modest at best and often below detection by single cell methods. SnCs also tend to be morphologically large and fragile, making them potentially incompatible with single cell dissociation methods. Finally, *in vivo* SASPs may be quite distinct from *in vitro*, which is where mouse, and other animal, models can be informative.

Imaging approaches: High resolution molecular and cellular imaging will be also critical for the study of SnCs in the tissue context and the construction of a common coordinate system and organ-specific two-dimensional (2D) and three dimensional (3D) atlases, which is the main goal of the SenNet TMCs. Static universal tissue agnostic senescence markers may not exist. Instead, there are likely dynamic senescence signatures (gene expression patterns anchored by frequent, but unobligated presence of some of the "known" markers and others yet to be identified), that may differ with cell type, age, and environmental stimuli. As such, it is important to use multiplex imaging to study the coexistence of cellular, molecular (RNA, protein, epigenetic, etc.), or even SASP factors as potential senescence biomarkers. SenNet will use a range of multiplexed imaging tools including Imaging Mass Cytometry, Co-Detection by Indexing (CODEX), Single-Molecule Fluorescence *In Situ* Hybridization (smFISH), Multiplexed Error-Robust Fluorescence *In Situ* Hybridization (MERFISH), Spatial Molecular Imaging, Positron Emission Tomography (PET) Imaging, RNAscope, Multiplex Immunofluorescence, Super Resolution Microscopy, Iterative Indirect Immunofluorescence Imaging (4i), Mitochondrial Network Analysis (MiNA) and Lightsheet Microscopy An example of indexing of co-detection

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imaging methods is CODEX[54], which relies on DNA-conjugated antibodies and the cyclic addition and removal of complementary fluorescently labeled DNA probes to simultaneously visualize, at subcellular resolution, up to 60 markers on tissue sections.

In parallel, SenNet investigators are developing novel technologies to image select epigenetic signatures in tissues by visualizing 3D nuclear architecture and open chromatin regions[55]. Although feasible to computationally construct 3D atlases using series of 2D images with multiplexed staining, native 3D imaging, such as light sheet microscopy[56], require much larger sampling volumes are required to build atlases. Another limitation of light sheet microscopy is the small number of detectable markers (3-5) available for each run, which would be insufficient for accurate SnC detection. Current efforts within and outside of SenNet are pushing for greater multiplexity in 3D imaging using barcoded antibodies that will ultimately expand the application of 3D imaging in atlas building for rare, heterogeneous cells such as SnCs. These technologies measure a finite panel of RNA or protein targets, therefore integration with single cell multi-omics data will further advance our capacity to computationally incorporate genome-wide information using spatial molecular or cellular tissue maps.

Spatial mapping approaches: The latest advent in Next-Generation Sequencing (NGS)-based spatial omics technologies is poised to bridge the gap to realize both genome scale and cellular resolution in mapping SnCs in tissue. GeoMx allows for profiling thousands of genes in specific regions of interest and Visium 10X Genomics can map whole transcriptome pixel-by-pixel in a tissue section with a spot size of 55 µm. Higher resolution Visium HD, NanoString CosMx and novel technologies such as Seq-Scope[57] and Pixel-Seq developed within SenNet will further enable the mapping of SnCs with single cell or even subcellular resolution. Investigators in SenNet also developed the first spatial multi-omics technology to co-profile whole transcriptomes and hundreds of proteins via deterministic barcoding in tissue followed by NGS (DBiT-seq) with 10 µm pixel size[58]. This was further expanded to spatial epigenome mapping to measure genome-wide chromatin accessibility (spatial-ATAC-seq) or chromatin modification (spatial-CUT&Tag)[59] to link epigenetic regulation to transcriptional or proteomic markers. Integration with high-resolution imaging makes spatialomics the crucial linchpin in connecting mechanistic underpinnings and molecular signatures with morphological features and spatial distribution, critical to construct a map of SnCs in the native tissue environment.

Validation: SenNet investigators will use a variety of *in vitro* approaches to validate characterization of SnCs, including differentiation of hiPSCs organoids[60] and precision cut tissue slices (PCTS) senolytic agents[61]. Organoids contain structural cells, such as epithelial (stem) cells, fibroblasts, and endothelial cells, which can further be supplemented with immune cell populations. As such, organoids are useful tools to determine the autocrine and paracrine mechanisms of SnCs in a multi-cellular 3D assay system. PCTS, which are 300-500 µm tissue slices, have the advantage of allowing multicellular analyses of cells in their natural 3D environment with high spatiotemporal resolution. Organoids and PCTS will be subjected to the single cell high-content technologies described above as well as to 2D and 3D imaging techniques. Using these approaches, two major outcomes are anticipated: 1) the identification of cell- and driver-specific SnC signatures that will increase the precision and sensitivity of SnC detection *in vivo* by providing a basis for imputing perturbation-specific senescence-associated

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gene and protein lists with the atlases generated from whole tissues; and 2) information about the selectivity and potential utility of senolytic agents *ex vivo* as well as across SnC types, perturbations and cell states. In this way, novel senolytic and senomorphic agents can be integrated into a validation pipeline.

Discussion

In recent years, advances in single cell technologies have enabled tissue mapping efforts of human tissues at unprecedented resolution, and the NIH Common Fund has rapidly mobilized such technologies for the advancement of human health. The NIH Common Fund is managed by the Office of Strategic Coordination within the Division of Program Coordination, Planning, and Strategic Coordination Office of the NIH Director. Common Fund programs must address emerging scientific opportunities and pressing challenges in biomedical research that are transformative, catalytic, synergistic, cross-cutting, and unique. Examples of these initiatives include the Human Biomolecular Atlas Program[62], and Somatic Cell Genomic Editing[63], 4DNucelome[64] and GTex[65]. This vision of creating a comprehensive blueprint for senescence was catalyzed by community input that indicated the need for development of novel tools and technologies to identify, trace, and track these heterogeneous populations within tissues. What also emerged from internal and external advisory workshop is the recognition that to achieve these goals new model systems and perturbation agents are necessary to validate *in vitro* and *in vivo* observational studies.

Single cell technologies for imaging and deep-phenotyping SnCs have significant clinical and translational potential with diagnostic, monitoring, and predictive applications. Complementary, multi-modality characterization of SnCs will not only deepen our understanding of senescence biology in health but also reveals the clinical significance of SnCs in cancer, fibrosis, metabolic disorders, and diverse degenerative processes that are fundamental to disease. SnC identification, at single cell resolution, organ and whole-body, would launch impactful conceptual, technical, and clinically applicable advancements that prevent, diagnose and/or treat diverse, disease conditions.

A key impetus for aspiring to create SnC 4D tissue atlases is the advent of relatively new classes of drugs and biologics termed senolytics and senomorphics, as defined earlier in this report. Until we can distinguish between beneficial vs detrimental senescence, and understand what SnC sub-types exists, the senescence field is in danger of developing interventions that can be deleterious or carcinogenic. Cutting-edge, emerging multi-omics and imaging tools employed by SenNet scientists will make critical contributions to achieving the high resolution, multi-dimensional molecular Common Coordinate Framework (CCF) that is used in the Human Reference Atlas (HRA). Specifically, SenNet will add cellular atlases of SnCs from most major human tissue types across the lifespan and health span, serving as a valuable resource and a stepping stone to a new era of cellular senescence and aging research.

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Common Coordinate Framework (CCF) and Data visualization: The CCF consists of ontologies and reference object libraries, computer software (e.g., user interfaces), and training materials that support the efficient mapping, registration, and exploration of clinically, semantically, and spatially indexed human tissue data. SenNet will extend the HuBMAP CCF that consists of (1) a CCF Specimen Ontology, which provides CCF-relevant demographic and clinical metadata about the specimen and donor (the "who"); (2) a CCF Biological Structure Ontology, which describes "what" part of the body a tissue sample came from; and (3) a CCF Spatial Ontology, which indicates "where" the tissue is located in a 3D reference system. In addition, the CCF defines a "registration process" that makes it possible to annotate data and map it to the 3D reference system, as well as an "exploration process," which facilitates query, analysis, and visual examination of registered tissue data and prediction of properties, e.g., what cell types are commonly located in a specific anatomical structure or what antibodies should be used to identify a desired set of protein biomarkers (**Fig. 4)**. The CCF also provides three-dimensional representations of anatomy that are linked to anatomical structures, cell types, plus biomarker (ASCT+B) tables that provide detailed cell type level information for each organ of interest.[50] Note that the CCF is semantically explicit (i.e., terminology for anatomical structures, cell types, and biomarkers link to existing ontologies, namely Uberon/FMA, CL, and HGNC) as well as spatially explicit (e.g., 3D reference organs are used for registration and exploration). In February 2022, there were ASCT+B tables for 25 organs and there were 50 associated 3D reference object sets (1-4 per organ, e.g., one uterus but 4 kidneys to capture left/right and male/female versions), which represent the size, shape, position, and spatial orientation of major anatomical structures in an organ-specific manner. The ASCT+B tables and associated spatial reference objects represent the human body in a simplified manner as a partonomy where each cell is part of an anatomical structure that are part of larger anatomical structures and ultimately make up the entire body.

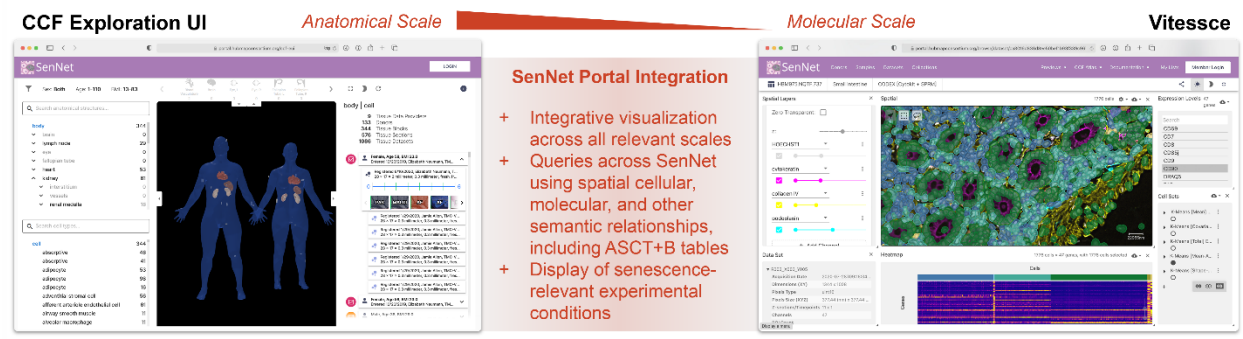

Figure 4. Integration of Common Coordinate Framework (CCF) Exploration UI and Vitessce to enable seamless navigation across scales and queries against SenNet data. The CCF Exploration UI enables registered tissue blocks from the Registration User Interface to be explored spatially (via body browser in the left screenshot, center) and using ontology terms (via hierarchy in the left screenshot, on left) at anatomic scale. A click on a tissue dataset (left screenshot, right) leads to Vitessce (right screenshot) which supports the exploration of cellular and molecular scale distributions. In summary, EUI provides clinical and spatial context and ontology crosslinks, while Vitessce supports details on-demand at the molecular scale.

The SenNet CCF Atlas and SenNet CODCC Data Portal will serve as the "hub" for a hub-and-spoke model of data coordination-integration, which will facilitate future systematic, standardized indexing of clinical, spatial and semantic metadata allowing harmonization amongst

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complementary meta data sets, such as HuBMAP. Armed with data sets from "normal" reference maps of senescence, along with requisite tools and technologies, one could envision studying senescence in relevant disease models and physiological systems along with focused studies of senolytics / senomorphics and other pharmacologic and biologic agents. Future extensions of the CCF will require closer integration of Electronic Health Record (EHR) data for characterization and integration into the current medical workflow. Building upon current efforts, future work would require integrating specimen ontology with clinical informatics and EHR-based clinical data for longitudinal data collections to characterize not just the state of patient when the sample was drawn, but the evolution of the person over their entire lifetime. This would naturally capture health characteristics, phenotypes and diseases in evolution, functioning as both markers for comorbidities as well as reference points, or even predictors, for health and disease trajectory. Furthermore, this may serve as an integration point for environmental factors or cumulative drug exposures a person may face over their lifetime within a health system. Such examples may then be used for interpretation of an individual's "health" atlas using artificial intelligence platforms.

Future Perspectives: While the current vision of SenNet is to identify and functionally characterize the heterogeneity of senescent cells across multiple human tissues in a lifespan at single cell resolution, future directions could expand to apply the knowledge to better understand the role of SnCs in various disease states. In addition, how senolytics, senomorphs and other pharmacological agents can be utilized for preventative or therapeutic purposes could also be developed. Importantly, we envision utilizing SenNet's emerging senescence biomarkers studies to monitor health, disease progression and response-to-therapeutics. Novel technologies will be developed and applied to characterize SnCs in human tissues, while new model systems can probe, perturb and validate senescence in various physiological contexts. SenNet will undoubtedly pave the way for exciting, limitless possibilities in the geroscience.

Methods

TMC and TDA

Each individual often multi-site TMC or TDA center will apply their own specific technological measurements (detailed **in Fig. 3**) on the various human or murine organs. Collaborations within SenNet TMCs and TDAs are established throughout this work, including exchange of tissues between the centers. TMCs and TDAs are the foundation of the SenNet mapping initiatives and the data generated will coalesce into the CODCC.

SenNet Biomarker Working Group

Currently, there are three main phenotypes that characterize SnCs, with the caveat that they are context dependent. In general, 1) SnCs enter an essentially permanent arrest of proliferation; 2) become *relatively* resistant to cell death, and 3) develop a SASP. Ideally, an endpoint associated with each of the three main phenotypes should be measured to determine if a cell is senescent. Relying on a single endpoint is fraught with error. For example, high SA-β-Gal activity is detected in cultured confluent fibroblasts[66,67] and certain activated macrophages[68,69], whereas p16 and p21 can be induced in a reversible manner in certain physiological contexts[68-72]. At present, there is no single, stand-alone marker to identify a SnC. Even more unclear are the markers indicate a cell is "destined" to senescence. As more cell types and physiological contexts are studied, a

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senescence-*specific* marker may never emerge. Consequently, several groups are compiling lists of cell traits, mRNAs, proteins, lipids and metabolites that may be used to identify, and ultimately define, SnCs. These efforts are generally scientifically sound and welcome as more cell types and tissues are being interrogated for the presence of SnCs. However, the field should be open to the possibility that this effort may ultimately fail due to heterogeneity in senescence responses. To address this, the SenNet consortium has formed a dedicated working groups, whose overall goal is to curate a database of senescence-associated biomarkers. The short-term goal of this working group is to generate a short list of senescence-associated biomarkers currently used by members of the SenNet consortium. The information collected will include cell type information, in what combinations they occur, the associated reagents and their compatibility with experimental methods and tissues. We expect this list will evolve over time, with some markers being removed due to lack of specificity/sensitivity, and others being added as our understanding of the senescent phenotype improves. Ultimately, this effort will generate a compendium of senescence biomarkers at the tissue and cell-type level, which will be integrated with the ASCT+B tables in collaboration with the HuBMAP CCF and ASCT+B working group.

Clinical-Medical Imaging (**Fig. 5)**

The ability to detect SnCs non-invasively and longitudinally in people would substantially improve our ability to monitor the effects of injury, inflammation, development of carcinogenesis, autoimmunity and potential responsiveness to specific drugs or biologics, ultimately identifying those who may benefit from senotherapies. However, to date the development of imaging biomarkers for *in vivo* detection of SnCs has received surprisingly little attention. Initial reports describe fluorescent biomarkers for the detection of SnCs with two-photon microscopy. Vats *et al.* used quantitative liver intravital microscopy to visualize SnCs in young and aged mouse liver using AF488-anti-p21^{CIP1} antibody[73]. In addition, radioactive probes have been used for real-time *in vivo* tracking of SnCs with positron emission tomography (PET), integrated PET/computed tomography (PET/CT), microCT for mouse models, and integrated PET/MRI: For example, Koslowsky *et al.* used ^{18}F-labelled antisense oligonucleotide probes to monitor expression of the p21 in human colon carcinoma cells using PET imaging[74]. Krueger *et al.* described the detection of SnCs in colorectal tumors in mice with the radiotracer [^{18}F] FPyGal (2-[Fluorine-18]Fluoro-3-pyridinyl-β-D-galactopyranoside) and integrated PET/CT and PET/MRI[75]. Qiu *et al.* developed a novel near-infrared fluorescent probe to detect SA-β-Gal activity in KSL0401 cells using fluorescence imaging[76]. Furthermore, recent advances in radioluminescence imaging improved analyses of single cell pharmacokinetics by incorporating two scintillators instead of one, which increases microscope sensitivity and thereby reduces image acquisition times. Kim *et al.* performed radioluminescence imaging using a low-light microscope on MDA-MB-231 cells labeled with radioactive glucose analogue [^{18}F] FDG, as a radiotracer[77].

Another marker, albeit non-specific, for SnCs is reduced cell saturation density at confluence and this could be measured using laser scanning cytometry, which is a microscope-based quantitative-image analyzer offering dual advantages of flow cytometry and image analysis as demonstrated by Zhao *et al*. in A549 cells induced to undergo senescence[78]. Oja. *et al.* performed

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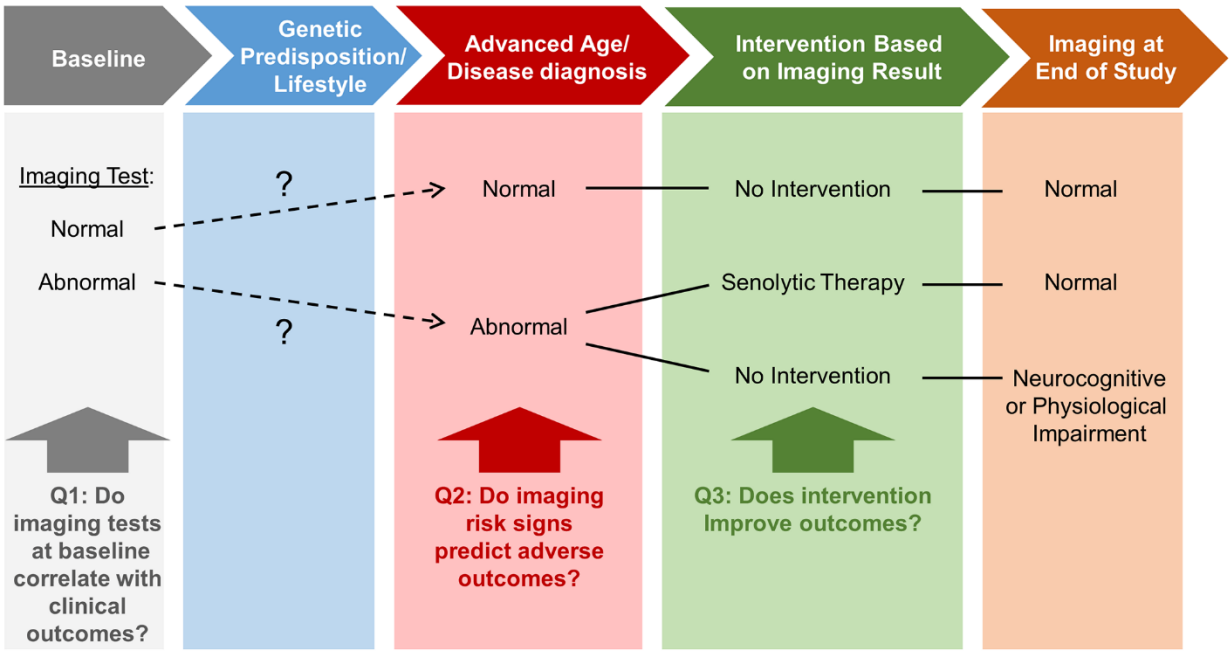

Figure 5. Flowchart of Novel Imaging Biomarkers for Senescence Imaging: 1) A baseline imaging test may or may not show senescent cells in specific organ systems, at this stage, prospective research can investigate correlation between quantitative senescence imaging biomarkers and clinical outcomes; 2) the presence or absence of senescent cells may be dependent on genetic predisposition or lifestyle factors, 3) people with specific risk factors, such as advanced age or a disease diagnosis, might demonstrate a normal or abnormal imaging test. At this stage, prospective research can investigate correlations between quantitative senescence imaging biomarkers and clinical outcomes; 4) as a result of senescence imaging tests senotherapies may be used. Prospective research can investigate if senescence imaging biomarkers can be used to stratify patients to personalize senotherapies; 5) ultimately, novel senescence imaging tests might predict clinical outcomes.

an automated image analysis using Cell Omics Morphology Explorer software on images of mesenchymal stromal cells acquired by using high-content screening microscope to give information about nine different parameters indicating cell size and shape[79]. Lee *et al.* utilized time-lapse imaging to illustrate how senescent tumor cells build 3D clusters, using a highly malignant cell line MDA-MB-231[80]. Confocal 3D immunostained images were acquired to look at the size growth of SnCs; the time-lapse images showed their emergence from their initial seeding, to migration and then to 3D clusters. Advances in radio imaging, fluorescence-based imaging approaches and imaging techniques to assess cell morphology and will enable detection and tracking of SnCs *in vivo* longitudinally.

Microphysiologic Platforms

Cellular senescence is often interrogated *in vitro*, which is static relative to *in vivo* conditions. Micro-physiologic platforms have overcome the deficiencies of traditional cell culture techniques and revolutionized the ability to culture tissues *ex vivo* in a manner that recapitulates the dynamic flow of nutrient, gas and waste exchange and tissue interactions that more closely recapitulate the *in vivo* micro-environment. Within SenNet, projects will evaluate SnCs using platforms that enable dynamic flow conditions to better mimic *in vivo* cell biology and thereby generate more accurate markers to identify SnCs *in vivo*. These micro-physiologic platforms will enable the study of cellular senescence in diverse modeling platforms and inform drug-testing in a more complex, physiologic biomimetic system. Moreover, they will allow evaluation of cell interactions, both

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senescent and non-senescent, to better understand bystander and/or paracrine effects driven by SASP.

Deep Learning Method to Assess Cellular Senescence

A deep neural network classifier, trained on nuclear morphology, can predict senescence using DAPI- or H&E-stained images[81]. This provides an automated and efficient method to characterize SnCs in tissues with spatial detail. U-NET, a convolutional neural network, relies on image segmentation to identify nuclei, which are then normalized to filter out background, standardize size, and mask internal structures. A collection of nuclei can then be assessed for senescence using several predictor models, trained on multiple forms of senescence, such as replicative, ischemia-reperfusion (IR), and drug-induced senescence. Applied to primary human fibroblast cell cultures, the predictor model showed remarkably high accuracy in detecting SnCs, which can now be applied to histological tissue sections. The top performing model, applicable to images of DAPI-stained nuclei in culture, has an f1 score of 94%, accuracy of 95%, and AUC of 0.99. The generalized model, based on feature reduction, had lower performance which was improved using a deep ensemble. Furthermore, the deep ensemble can filter ambiguous predictions by raising the classification threshold to restrict to higher confidence predictions.

With images from several individuals, the predictor showed an age-dependent increase in SnCs in mice and human tissue samples. We can also apply the predictor to samples with disease to determine how disease affects the predicted rate of senescence, relative to age-matched healthy individuals. The predictor generates granular senescence scores, which can be used to compare groups of individuals with different characteristics. For instance, groups of individuals with different gene expression profiles can be evaluated to uncover correlations with senescence. These novel image analytic methods can be focused on key image regions to estimate senescence by tissue (eventually cell) type and help determine how different regions contribute to overall senescence or disease. In one application, images were classified by tissue type using a deep learning model for image segmentation, indicating regions of adipose, connective, and epithelial tissue. Analyzing predicted senescence suggests that senescence develops differently in each region.

The deep learning predictor can produce SnC scores per nucleus, the location of which can be tracked for spatial mapping. The spatial distribution of predicted SnCs in tissues will enable discovery of how senescence propagates locally. The high precision senescence score also enables more detailed and quantitative analysis, such as an association to other factors of the individual (e.g., biological, physiological, environmental, clinical) or tissue (histological characteristics, gene expression levels, or any other quantitative factor). While this approach has great potential, the development of deep learning methods to accurately identify SnCs in tissues based on morphology is still in its infancy and will require an in-depth characterization of the heterogeneity of SnCs in different tissues, which is one of the goals of SenNet.

Bioinformatics approach to catalog SnC biomarkers

Given that there is no widely agreed-upon SnC biomarker list, we leveraged existing search engines such as Uniprot, a protein database. Senescence markers were identified using the

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query, “senescence” AND “reviewed: yes” AND organism: "Homo sapiens (Human) [9606]"). Data collection was focused on key words: "senescence", "human specific", and "peer-reviewed". After collecting information on several senescence markers, further details were assembled, including protein function, subcellular location, and tissue specificity. Each individual marker was tagged with relevant PubMed papers documenting this information. The data were downloaded into excel sheets and further organized. The markers were categorized into secreted and non-secreted proteins using “keyword search” as well as sorting through individual markers. Then, the markers were further categorized into protein function and subcellular localization. Once these proteins are validated and integrated with data from the Biomarker and ASCT+B working groups, standardized, validated categories of SnC biomarkers can start to be formulated.

Data Integration and Harmonization

Given the multi-organ and multi-modal data generation envisioned as part of the SenNet program, a structured, cross-team data management, organization, and analysis plan is essential to the success of SenNet. The SenNet CODCC will manage data curation, integration, analysis, atlas creation, and dissemination through the SenNet Data Portal (**Fig. 6**). These data harmonization and integration efforts will be coordinated with Common Fund Data Ecosystem (CFDE) to align the SenNet data for integration with data sets from other Common Fund programs. Uniformly processed molecular and cellular data will be integrated with the CCF and will be the basis for the construction of an atlas of SnCs. To facilitate the development of uniform data processing and quality control pipelines within CODCC, and re-use by other data consumers, CODCC will mandate data submission using common data formats that are aligned with CCF reference atlas construction. Examples are the use of Azimuth for cell type annotation or OMAP-validated antibody panels. Uniform processing pipelines will implement state-of-the-art algorithms for the analysis of imaging, sequencing, and multi-omics data, which will generate standardized data sets that are spatially registered, segmented and annotated using CCF ASCT+B terminology and

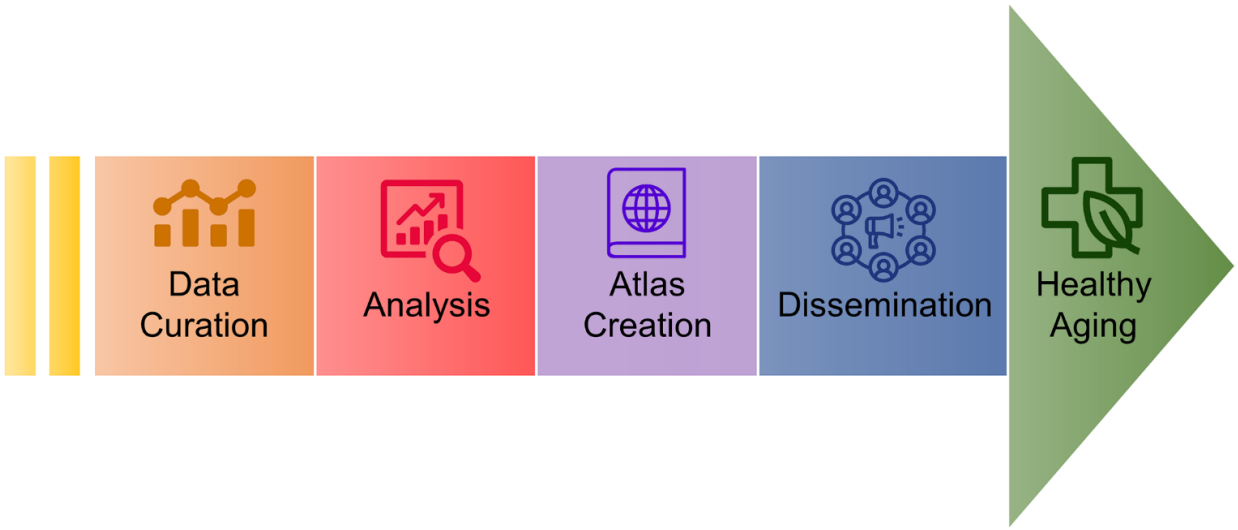

Figure 6. The SenNet Consortium and CODCC workflow. Data generated by the TMCs and TDAs are input into the CODCC along with associated metadata. The data sets are organized and de-identified (curation) then analyzed and integrated. The goal is to create an atlas and public database of curated data that can be searched, analyzed, and visualized as 3D images of organs using unified annotations. Identifying, characterizing, localizing SnCs in human tissues is predicted to promote multiple opportunities for improving human health.

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hence linked to existing ontologies. Integrated and harmonized data sets will be made available through the data portal, along with the raw data.

The SenNet Data Portal will also integrate the CCF registration and exploration user interfaces (known as RUI and EUI, respectively) and the Vitessce framework in support of exploratory visualization of existing data across levels—from the whole body to single organs to molecular and cellular level data sets and vice versa (**Fig. 4, 7)**. Clinical data will also be standardized and shared in an extension of the CODCC / CCF efforts and will be the basis for standardized implementation and association with EHR clinical data in the future.

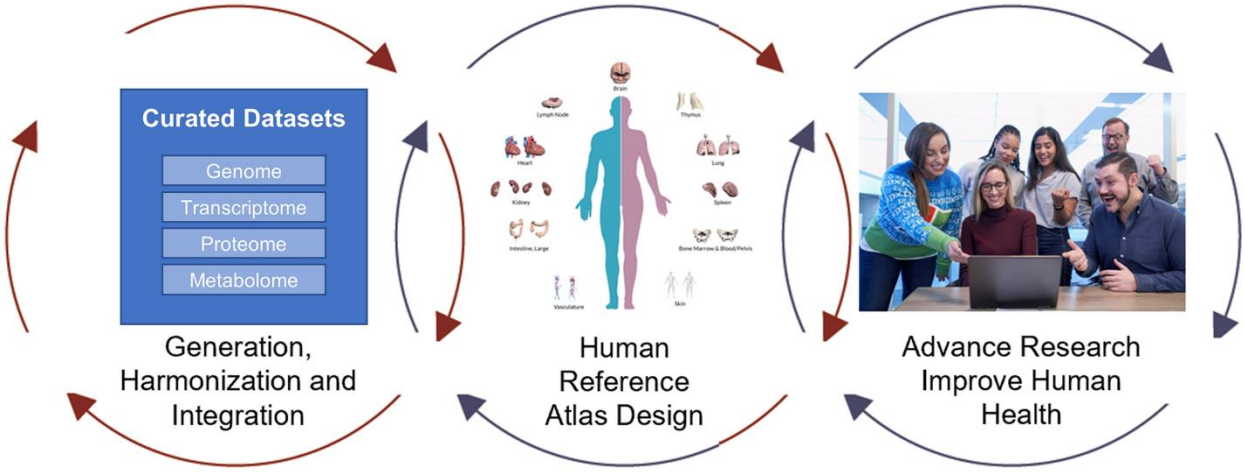

Figure 7. Summary of SenNet Consortium Goals. High quality experimental data is needed to create a human reference atlas. The evolving reference atlas supports data standardization and federation, making it possible to integrate data from different specimen, laboratories, and assay types. The atlas characterizes the healthy human—from the whole body down to the single cell level; it can be compared across ages and diseases to understand differences, advance research, and improve human health. Use case scenarios for different stakeholders (researchers, practitioners, students) guide atlas construction and usage but also experimental data acquisition and analysis.

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Acknowledgements

We would like to thank Valerie Bekker, SCENT Program Manager for administrative and formatting assistance. We would also like to thank Dennis Mathias, Science Illustrator, for his assistance with figures. This research is supported by the NIH Common Fund, through the Office of Strategic Coordination/Office of the NIH Director under awards: 1U54AG075932-01, 1UG3CA268112-01, 1U54AG075934-01, 1UG3CA268117-01, 1U54AG076043-01, 1U54AG075931-01, 1UG3CA268096-01, 1U54AG075941-01, 1UG3CA268091-01, 1U54AG075936-01, 1UG3CA268105-01, 1UG3CA268202-01, 1U54AG076041-01, 1UG3CA268103-01, 1U54AG076040-01.