Senescence-induced inflammation: an important player and key therapeutic target in atherosclerosis

Stevan D. Stojanović [1,2]**, Jan Fiedler** [1]**, Johann Bauersachs**[2]**, Thomas Thum** [1]***,
and Daniel G. Sedding** [3]*

[1]Institute of Molecular and Translational Therapeutic Strategies (IMTTS), Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany; [2]Department of Cardiology and Angiology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany; and [3]Department of Internal Medicine III, Cardiology, Angiology and Intensive Care Medicine, Martin-Luther-University Halle (Saale), Ernst-Grube-Strasse 40, 06120 Halle (Saale), Germany

Inflammation is a hallmark and potent driver of pathological vascular remodelling in atherosclerosis. However, current anti-inflammatory therapeutic strategies have shown mixed results. As an alternative perspective on the conundrum of chronic inflammation emerging evidence points towards a small subset of senescent cells as a critical player and central node driving atherosclerosis. Senescent cells belonging to various cell types are a dominant and chronic source of a large array of pro-inflammatory cytokines and various additional plaque destabilizing factors, being involved with various aspects of atherosclerosis pathogenesis. Antagonizing these key agitators of local chronic inflammation and plaque instability may provide a causative and multi-purpose therapeutic strategy to treat atherosclerosis. Anti-senescence treatment options with translational potential are currently in development. However, several questions and challenges remain to be addressed before these novel treatment approaches may enter the clinical setting.

Keywords   Atherosclerosis • Inflammation • Ageing • Cardiovascular disease • Vascular disease

Introduction

Inflammation is a critical driver of atherosclerosis and an independent risk factor for myocardial infarction and cardiovascular death.[1] The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) showed that targeting inflammation can provide significant, clinically relevant cardiovascular benefit.[2] In another attempt to target inflammation, the Cardiovascular Inflammation Reduction Trial (CIRT)[3] yielded negative, but highly educative results. These trials highlighted the potential, but also the limitations of current anti-inflammatory approaches, including immune suppression and the associated risk of sepsis.[4] Furthermore, targeting individual cytokines of the pro-inflammatory cascade may not fully address the effects of various other pro-inflammatory elements.[5] Strategies to target upstream effectors in the inflammatory signalling cascade may provide a solution for these issues.[5]
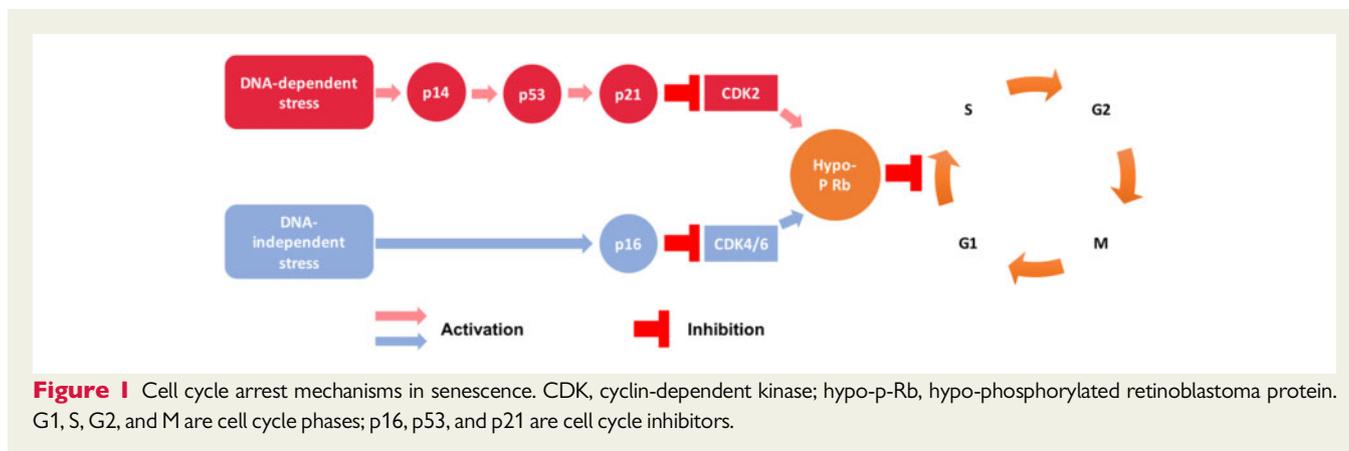
A strong suspect for the central upstream source of inflammatory factors is ageing, being the strongest independent risk factor for cardiovascular disease (CVD).[6] On the cellular level, ageing is driven by the accumulation of senescent cells.[7] Cellular senescence is defined as an irreversible cell cycle arrest (mechanism outlined in *Figure 1*)[8] and is accompanied by a pro-inflammatory phenotype. The phenotype is referred to as the senescence-associated secretory phenotype (SASP), producing pro-inflammatory cytokines [interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18, CCL-2, tumour necrosis factor α (TNF-α)], metal-loproteinases (MMP-1, -2, -3, -7, -8, -9, -10, -12, -13, and -14), and other factors.[9,10] Emerging experimental evidence indicates that, in contrast to an acute inflammatory response, the SASP is a protracted and chronic source of inflammatory and other plaque-destabilizing factors,[9,10] gradually contributing to atherosclerosis pathogenesis.

Intriguingly, cellular senescence and atherosclerosis share multiple causative stimuli[1,11,12]: oxidized and electronegative low-density

* Corresponding author. Tel: +49 511 532 5272, Fax: +49 511 532 5274, Email: Thum.Thomas@mh-hannover.de; Tel: +49 345 557-2601, Fax: +49 345 5572072, Email: Daniel.Sedding@uk-halle.de

Figure 1 Cell cycle arrest mechanisms in senescence. CDK, cyclin-dependent kinase; hypo-p-Rb, hypo-phosphorylated retinoblastoma protein. G1, S, G2, and M are cell cycle phases; p16, p53, and p21 are cell cycle inhibitors.

lipoprotein (LDL),[13,14] inflammation,[15] reactive oxygen species,[8] cigarette smoke,[16] hypertension,[17] hyperglycaemia,[18] cytomegalovirus (CMV) infection,[19] telomere attrition,[20] mitochondrial failure,[21] dysfunctional autophagy,[22] and genomic damage.[8] Senescent cells are more than simple bystanders, as multiple molecules involved in the SASP act as promoters of atherosclerosis and are biomarkers of CVD risk.[23] Furthermore, effects of experimental overexpression of many molecules drives both atherosclerosis and senescence. On the other hand, antagonizing cellular senescence and ageing processes has anti-atherosclerotic effects. These overlapping features of senescence and atherosclerosis are shown in *Table 1*. For a better understanding of the cell-specific roles of these molecules refer to *Figure 2*, which explains expression patterns on a cellular level of senescence and their roles in atherogenesis.

These observations suggest that senescent cells are an important source of local, chronic, low-grade inflammation and plaque destabilization, and thus, a promising upstream therapeutic target. Targeting of senescent cells and ageing processes therefore holds unexplored potential to ameliorate atherosclerosis as a causal approach to decrease inflammation and provide plaque stabilization, possibly without overt immunosuppression.

In this review, we present the current experimental knowledge close ties of senescence with many aspects of atherosclerosis pathophysiology. Further on, we present clinical evidence connecting cellular senescence and atherosclerosis. We elaborate on how targeting senescence can be utilized to develop novel therapeutic strategies for atherosclerosis. Finally, we discuss the challenges and unmet needs that remain to be addressed before anti-senescence therapies can be introduced in the clinical setting.

Evidence on the cellular level of the contribution of senescence to atherosclerosis

Cellular senescence evolves with ageing and in sites of disease[7] in response to sub-lethal exposure to various stimuli,[81] which are outlined in the Introduction section. Senescent cells, within their physiological roles, facilitate tissue repair in response to acute damage,[82] but are promptly cleared by the innate immune system after injury resolution.[83] However, some senescent cells remain and gradually accumulate in tissues.[84] These persisting senescent cells have been recently tied to multiple chronic diseases other than atherosclerosis, including diabetes mellitus,[85] obesity,[85] Alzheimer's disease,[86] heart failure,[10,87] idiopathic pulmonary fibrosis,[88,89] chronic obstructive pulmonary disease,[90] chronic kidney disease,[91] cancer,[92] and osteoarthritis.[93] It is thus not surprising that senescent cell burden is a good predictor of animal lifespan.[94,95]

Cellular senescence is observed in early stages of atherosclerosis,[9] affecting different cell types in the arterial wall that have distinct roles in the pathogenesis of this disease: endothelial cells (ECs),[96–98] vascular smooth muscle cells (VSMCs),[45,99–101] macrophages, foam cells,[9,102] monocytes,[103] fibroblasts,[104–106] and T cells.[107,108]

Originating from these different cell types, senescent cells directly facilitate multiple key pathophysiological processes in atherosclerosis, which are displayed in *Figure 2* (necrotic core enlargement, extracellular matrix degeneration and cap thinning, erosion, calcification, intra-plaque angiogenesis).[109] *Take home figure* details the complex mechanisms leading to plaque destabilizing outcomes. *Take home figure* also exemplifies that some features of cellular senescence are shared by different senescent cell types, while some are cell population specific.

All senescent cells contribute to plaque inflammation. Consistent SASP elements in the majority of senescent cells are IL-1α, IL-1β, IL-6, IL-8, IL-18, and TNF-α,[15,105,110] all of which are clinically validated CVD risk factors.[1,5] These inflammatory cytokines promote senescence locally in a paracrine manner[30] and perhaps, at a systemic level.[111] Other features depend on the cell type.

Endothelial cell senescence directly compromised the endothelial barrier through disruption of cellular proliferation, permeability, and motility,[112–114] possibly contributing to endothelial erosion and intra-plaque haemorrhage. As seen in a retinopathy model, the overall effect of the SASP leads to pathological neo-angiogenesis.[115]

Vascular smooth muscle cell senescence was associated with necrotic core enlargement[80] and plaque calcification[62] in human atherosclerosis. Senescent VSMCs acquire an osteoblastic secretory phenotype and activate several osteogenic pathways [e.g. Runt-related transcription factor 2 (RUNX-2), bone morphogenetic protein 2 (BMP-2), alkaline phosphatase (ALP), osteopontin (OPN), and osteoprotegerin (OPG)], thus contributing to plaque calcification.[62] OPG, a soluble factor and key element of the SASP,[63] is a CVD risk factor.[64] Plaque destabilization through cap thinning is promoted by

Table 1 Clinical and experimental expression of molecules in atherosclerosis and senescence

Molecules	Effect on CVD risk	Over-expression effect on atherosclerosis	Over-expression effect on senescence	Down-regulation effect on atherosclerosis	Down-regulation effect on senescence	References
Ang II	↑	↑	↑	↓	↓	24–26
AMPK	?	↓	↓	↑	↑	27–29
CCL2	↑	↑	↑	↓	?	30–32
FOXO3	?	↑	↓	↑	↑	33–35
GATA4	?	↑	↑	↑	↓	36–38
HMGB1	↑	↑	↑	↓	?	39–41
HSP90	?	↑	↑	↓	↓	42–44
IL1α/β	↑	↑	↑	↓	↓	1,30,45
IL6	↑	↑	↑	↓	↓	1,30,45
IL18	↑	↑	↑	↓	↓	46–48
MMP9	↑	↑	↑	↓	↓	45,48–50
MMP12	↑	↑	↑	↓	?	51–53
mTOR	?	?	↑	↓	↓	27,54
NF-κB	↑	↑	↑	↑↓	↓	55,56
NLRP3	↑	↑	↑	↓	↓	57–60
NR3C2	↑	↑	↑	↓	↓	25,61
OPG	↑	↑	↑	↑	?	62–65
PI3K	?	↑	↑	↓	↓	27,54,66,67
p66Shc	↑	?	?	↓	↓	68–71
SIRT1	↓	↓	↓	↑	↑	27,72–74
STAT3	?	↑	↑	↓	↓	75,76
TRAF6	?	?	↑	↑	↓	77–79
TRF2	?	↓	↓	↑	↑	80

↑, promoting effect; ↓, inhibitory effect; ?, unknown/unclear evidence; Ang II, angiotensin II; AMPK, AMP-activated protein kinase; CVD, cardiovascular risk; FOXO, forkhead box class O; HMGB1, high-mobility group box 1; IL, interleukin; MMP, matrix metalloproteinase; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor kappa B; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; NR3C-2, mineralocorticoid receptor; PI3K, phosphoinositide 3-kinase; OPG, osteoprotegerin; SIRT, sirtuin; STAT3, signal transducer and activator of transcription 3; TRAF6, tumour necrosis factor receptor-associated factor 6; TRF2, telomeric repeat-binding factor 2.

various MMPs,[49] such as MMP-1, -2, -3, -7, -8, -9, -10, -12, -13, and -14,[45,49,51] which are secreted as part of the SASP of senescent VSMCs, monocytes, macrophages, and foam cells.

Furthermore, CD4+, CD28-null, CD45 re-expressing senescent T-cells (also known as CD4+ T-EMRA cells) were found to be highly active subpopulations, expressing increased amounts of TNF-α, INF-γ, CXCR-3 (C-X-C chemokine receptor), CCR-6, CCR-7 (C-C motif chemokine receptor 6 and 7), and the anti-apoptotic Bcl-2 family proteins.[19,116] In addition to the pro-inflammatory phenotype, T-EMRA cells showed atypical cytotoxic activity towards the plaque endothelium,[117] likely contributing to plaque erosion.
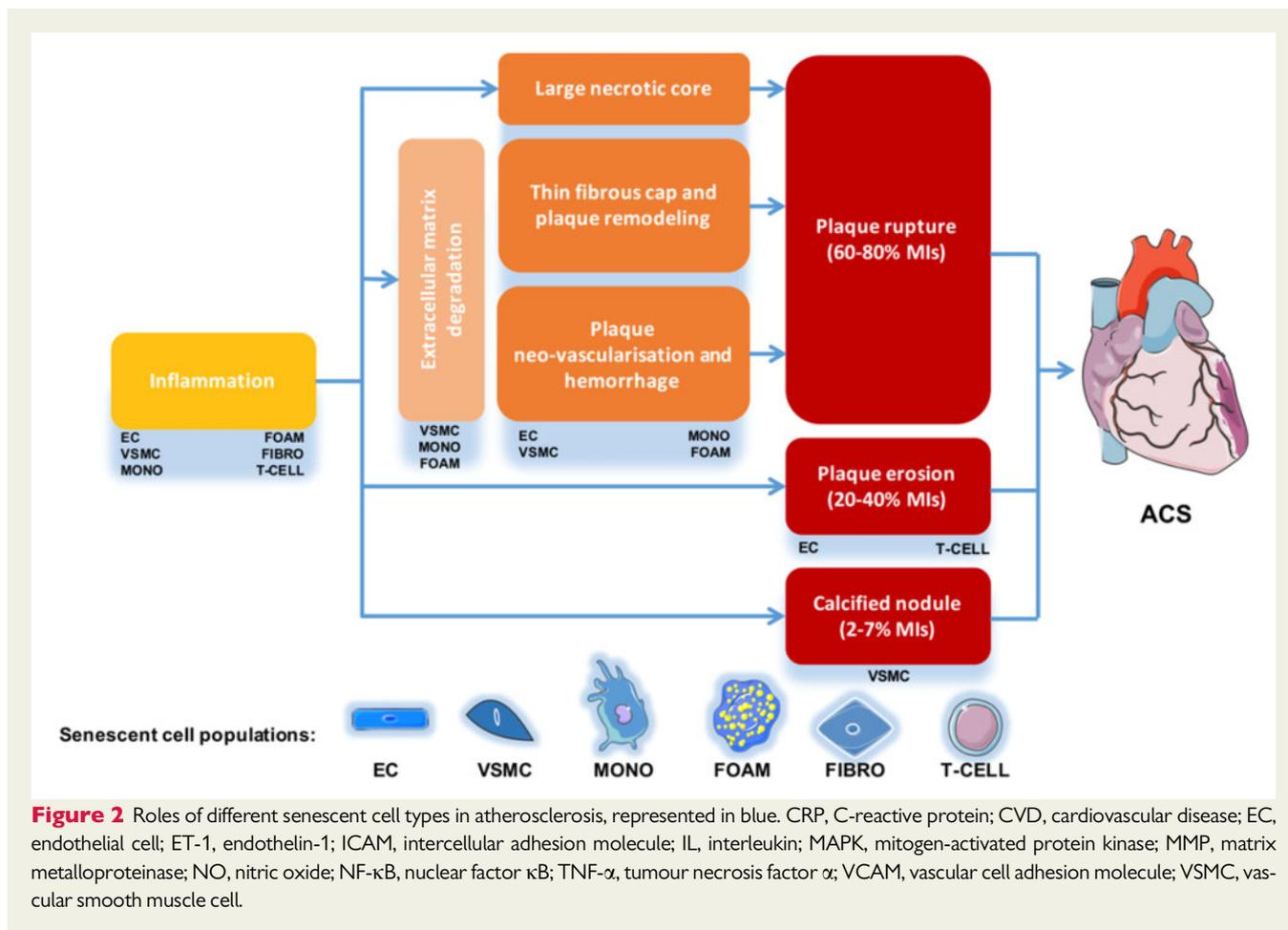
Together, these findings indicate that different types of senescent cells in the vasculature exhibit features that are considered highly unfavourable in the setting of atherosclerosis.

Clinical evidence linking ageing and senescence processes to atherosclerosis

Ageing is well-documented as a risk factor for CVD[118] and age is considered in various cardiovascular risk scoring systems (Framingham, Reynolds, PROCAM, ESC, and Diamond Forrester). In addition, ageing-associated morphological and haemodynamical arterial disturbances are known.[119,120] The majority of individuals older than 70 years of age (57.1% male and 68.7% female) have atherosclerotic lesions.[121] The trend progressively worsens, as 87.4% of male and 88.9% of female patients older than 85 were affected by subclinical or clinical forms of vascular disease.[122] However, ageing has a strong residual effect that cannot be simply explained by longer exposure to classical risk factors. Only 11.9% and 40.3% of the effect of age can be attributed to longer exposure to risk factors in men and women, respectively.[123]

Cellular senescence may account for a part of this void, acting a hub between various cardiovascular risk factors. As depicted in *Figure 3*, cellular senescence can be a consequence of two processes which may run in parallel and are intertwined. The first is replicative exhaustion resulting from chronological age or intense proliferation (telomere dependent)[20,124,125] and the latter is exposure to cardiovascular risk factors (stress-induced).[13–18] Therefore, cellular senescence burden is a phenomenon only partly correlated with age from birth; it is rather a consequence of a combined effect of chronological ageing and risk factor exposure. Being a shared consequence of the effect of all of these various factors, senescence is an important upstream effector that promotes atherogenesis.

Figure 2 Roles of different senescent cell types in atherosclerosis, represented in blue. CRP, C-reactive protein; CVD, cardiovascular disease; EC, endothelial cell; ET-1, endothelin-1; ICAM, intercellular adhesion molecule; IL, interleukin; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NO, nitric oxide; NF-κB, nuclear factor κB; TNF-α, tumour necrosis factor α; VCAM, vascular cell adhesion molecule; VSMC, vascular smooth muscle cell.

In clinical practice, the capability of cellular senescence to drive atherosclerosis independently of chronological ageing and classical risk factor exposure is seen in drastic examples of human progeria syndromes, such as Hutchinson–Gilford progeria (HGS), Werner syndrome, and other laminopathies. Patients with these syndromes are characterized by a strong accumulation of senescent cells early in life and suffer from strikingly increased CVD risk due to a large atherosclerotic plaque burden.[101,126,127] Patients with HGS suffer from myocardial infarction or stroke in the absence of classical risk factors at the mean age of 13 years.[128]

Furthermore, cancer therapies that induce cellular senescence, such as cisplatin, doxorubicin and radiation,[129] accelerate atherosclerosis.[130]

Additional clinical evidence of the involvement of cellular senescence in the atherosclerotic vessel wall in the general population comes from post-mortem histological analysis,[96,131–134] that showed that senescent EC and VSMC accumulate substantially more in atherosclerotic than in physiologically aged healthy arteries.[96,131–134] Expression of senescence marker p16 INK4a in the diseased human coronary arteries positively coincided with unstable plaques and correlated with intra-plaque TNFα levels.[135] Furthermore, coronary vessels from ischaemic heart disease patients showed significant endothelial senescent cell burden, while the mostly plaque-free internal mammary arteries from the same donors had no evidence of senescence.[96] In human carotid artery atherosclerosis, senescent VSMCs were associated with phenotypical features of plaque instability[80,134,136,137] and accounted for 18% of all plaque cells.[134] Senescent T-EMRA cells were also found inside unstable plaques.[117] Additional studies with detailed quantification of senescent cells, plaque features, and correlation to clinical data are necessary to strengthen the causal link between arterial wall cellular senescence and atherosclerosis.

As specific and safe imaging methods to evaluate senescent cell burden in the arteries of patients still do not exist, tracking senescent cell populations in the blood appears to be the best option to evaluate the contribution of cellular senescence to CVD risk.

Senescence of blood–borne immune cells has been tied to CVD risk. Bulk analysis of leucocyte populations showed that short telomeres predict atherosclerosis development and CVD.[124,125] The ageing pro-oxidative marker p66Shc[68] is overexpressed in leucocytes from patients with acute coronary syndromes, but not stable coronary artery disease.[69] As these results from bulk leucocytes may be affected by telomere length and expression profiles of short-lived myeloid cells, the stable lymphoid populations attracted significant attention, specifically senescent T-EMRA cells. Accumulation of T-EMRA was associated with increased acute mortality and re-occurrence of MI, particularly among diabetic patients.[19] Increased numbers of these cells were found in hypertension and rheumatoid arthritis, predicting worsened CV outcomes within these high-risk
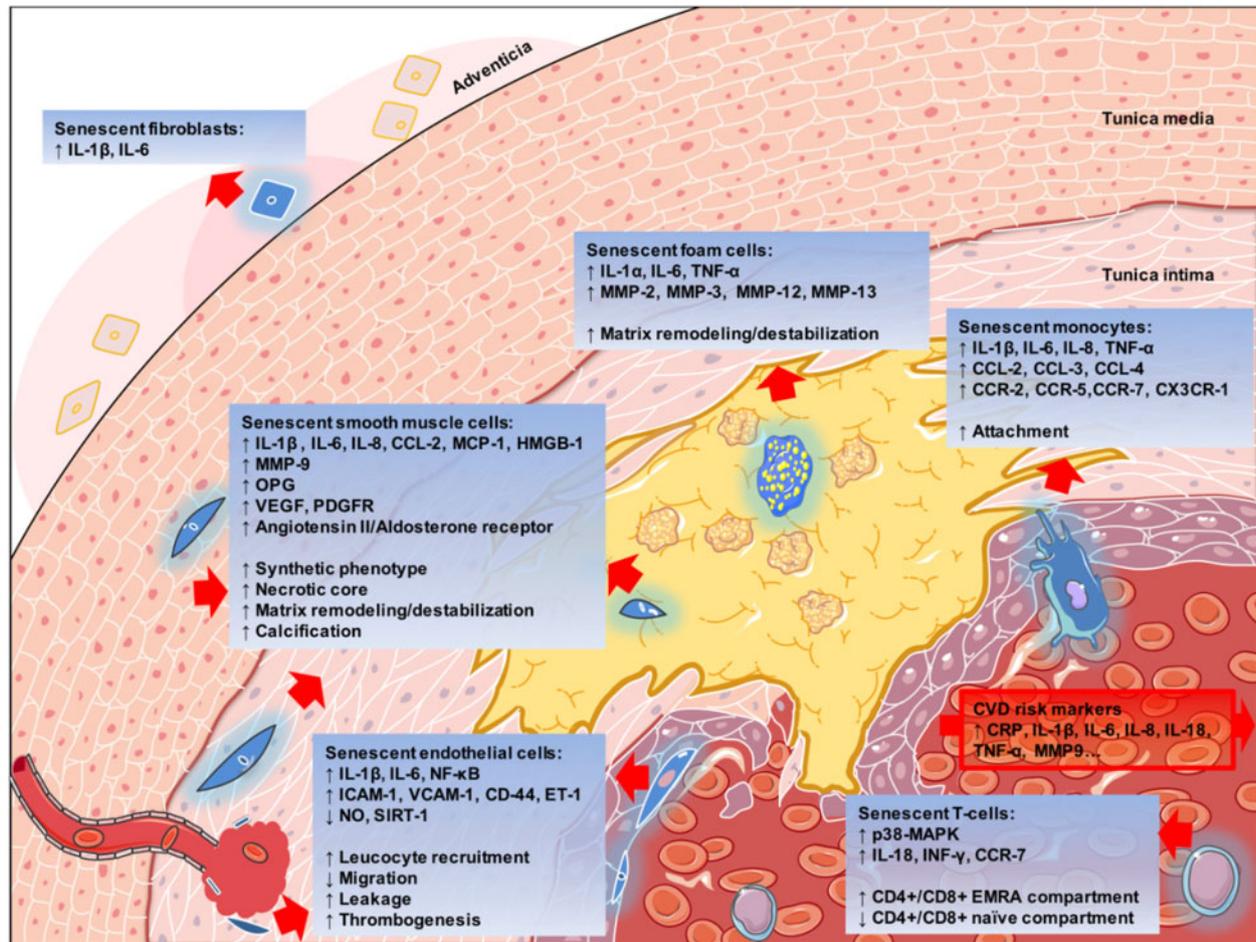
Take home figure Senescent cell contributions to the mechanisms of atherosclerotic plaque rupture.

conditions.[19] Both CD4+ and CD8+ T-EMRA cells are strong independent predictors of cardiovascular mortality in the elderly.[107] Cytomegalovirus infection activated senescence mostly in CD4+ EMRA cells,[19,107] providing an interesting connection between CMV infection, CD4+ EMRA senescence, and atherosclerosis.

In line herewith, the role of age-dependent accumulation of pro-inflammatory lymphocytes with somatic mutations (clonal haematopoiesis of indeterminate potential), mainly in *TET2* and *DNMT3a*, is emerging.[138] Senescence is associated wide perturbations in DNA methylation.[139] Given its critical functions in this process, it is possible that DMNT3a plays a role in the immuno-senescence programme. Silencing of *DMNT3a* reportedly activated senescence in cancer and skin experimental models.[140,141] However, it is unclear whether these mutations are involved in haematopoietic cellular senescence or SASP activation.

In summary, chronic senescent cells accumulate over time as a result of repeated tissue damage. Through the SASP, cellular senescence exerts many pro-atherogenic effects and it possibly is a key aetiologic driver of aberrant vascular remodelling, forming a perpetual loop that chronically amplifies the effects of risk factor exposure (*Figure 3*). Therefore, senescence appears to be a

therapeutic target worth exploring for the prevention or treatment of atherosclerosis.

Treatment options for targeting senescence

Multiple clinically approved treatment strategies for CVD have an anti-senescent effect upon closer analysis. Aldosterone and Angiotensin II drove VSMC senescence independently and synergistically, this being prevented by mineralocorticoid receptor and angiotensin II receptor blockers.[24,25] Pioglitazone ameliorated endothelial cell senescence by telomerase activation.[142] Statins were shown to delay endothelial and T-cell senescence, through p38-mediated SASP inhibition, telomerase and cell cycle regulation.[143] Metformin has anti-senescent and anti-atherosclerotic effects through SIRT-1 agonism.[27] Rivaroxaban attenuates VSMC senescence, by inhibiting the signalling cascade between Factor Xa and Insulin-like growth factor-binding protein 5.[137] The cardiovascular benefits of exercise in adulthood can be explained by a decrease of immuno-senescence.[144]
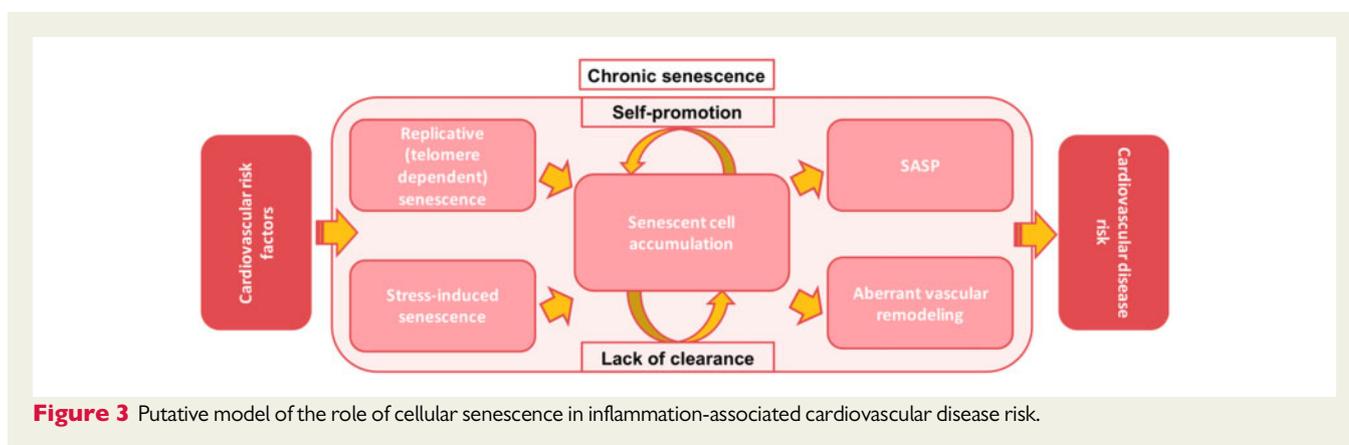
Figure 3 Putative model of the role of cellular senescence in inflammation-associated cardiovascular disease risk.

In a direct senescence-specific approach, several inflammatory and energy-sensing pathways and complex epigenetic modulators provide valid entry points to prevent, stabilize, or reverse atherosclerosis. Given the *status quo* of the clinical application of genome-based therapies in the CVD field, pharmacological approaches are discussed in detail below because of their immediate translational potential. *Figure 4* provides an overview of the known molecular therapeutic targets in senescence and divides them into six most promising therapeutic strategies: direct anti-inflammatory, anti-SASP, energy sensing, epigenetic modulation, senolytics, and reprogramming. These approaches and the complex relationships between various molecular targets are explained in further detail below.

# Targeting intracellular *signalling* molecules at the crossroads of inflammation and senescence

The idea of antagonizing pro-inflammatory molecules upstream of the IL-1-IL-6/TNFα-CRP cascade or other immune regulators[145] is viable, not only for a direct anti-inflammatory effect but also to ameliorate senescence. Inhibition of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 inflammasome (NLRP3) suppressed T-cell senescence[57] and atherosclerosis.[58] Even more upstream, inhibition of nuclear factor κB (NF-κB) abrogated p53-mediated senescence.[55] However, while inhibiting NF-κB in ECs was beneficial in atherosclerosis, in macrophages, this had a pro-atherogenic effect.[56] Immunosuppression and the inhibition of cellular debris traffic are strong reasons not to target these and other major inflammatory pathways.

A related concept is the inhibition of the SASP. Recent data suggest DNA leakage from the senescent nucleus into the cytoplasm and consequent activation of the GMP-AMP Synthase-Stimulator of Interferon Genes (cGAS-STING) anti-viral pathway as a mechanism of SASP onset.[146,147] This pathway is an attractive target to antagonize the SASP, but may come at the cost of lowering anti-viral defences. Alternatively, it is possible to modulate individual components of the SASP by inhibiting Janus kinase (JAK) 1/2[148] and

JAK2/signal transducer and activator of transcription 3 (STAT3).[149] TNF receptor-associated factor 6 (TRAF6) inhibition in macrophages is another anti-inflammatory strategy to counter atherosclerosis development.[77] The ATM-TRAF6-TAK1 axis is a driver of the SASP,[78] and an Serine-protein kinase ATM inhibitor was found to have an anti-senescence effect.[79] p38 Mitogen-activated protein kinase (MAPK) inhibition has been suggested as an anti-SASP strategy.[150] Independently of the senescence context, blockade of this pathway failed to improve cardiovascular outcomes 12 h to 2 weeks post-myocardial infarction.[151] However, anti-SASP strategies may not be effective in acute or subacute disease (see Future directions section).

Energy-sensing pathways, like those regulated by mammalian target of rapamycin (mTOR), sirtuin (SIRT)-1, Forkhead box class O 3 (FOXO3), and AMP-activated protein kinase (AMPK), have been shown to be largely responsible for the anti-ageing benefits of caloric restriction.[27] Inhibition of mTOR complex 1 by rapamycin prolonged the healthy lifespan of mice and exerted anti-senescence and anti-atherosclerotic effects.[152,153] However, mTOR inhibition can cause muscular atrophy.[154] The SIRT-1 agonist resveratrol ameliorated ageing pathology, in part by activating FOXO3.[33,155] Clinical application of resveratrol and related SIRT agonists is challenging due to stability and bioavailability issues.[156] Direct FOXO3 agonism is pharmacologically possible.[33] Importantly, more than 12.5-fold overexpression of FOXO3 has been shown to be harmful, possibly because of nicotinamide adenine dinucleotide (NAD) depletion[155]; thus, a careful dose-finding study is imperative. Recently, fine-tuning of FOXO3 levels has been achieved via a miRNA-132 antagonistic approach,[157] that is currently clinically tested (NCT04045405). NADP boosters (nicotinamide mononucleotide,[158] nicotinamide riboside,[159] P7C3,[160] apigenin[161]) can be used resolve the NAD depletion issue and are able to prevent senescence in ECs[158] and VSMCs.[159] The small scale Metformin in Longevity Study (MILES, NCT02432287) trial studied AMPK activation for anti-ageing purposes and the results are currently analysed. A larger scale study with a similar design is planned [Targeting Aging with Metformin (TAME) trial].

Epigenetic manipulation through long non-coding RNAs, miRNA modulators, and other oligonucleotides has yielded promising outcomes in experimental settings.[162–165] Some of these molecules are
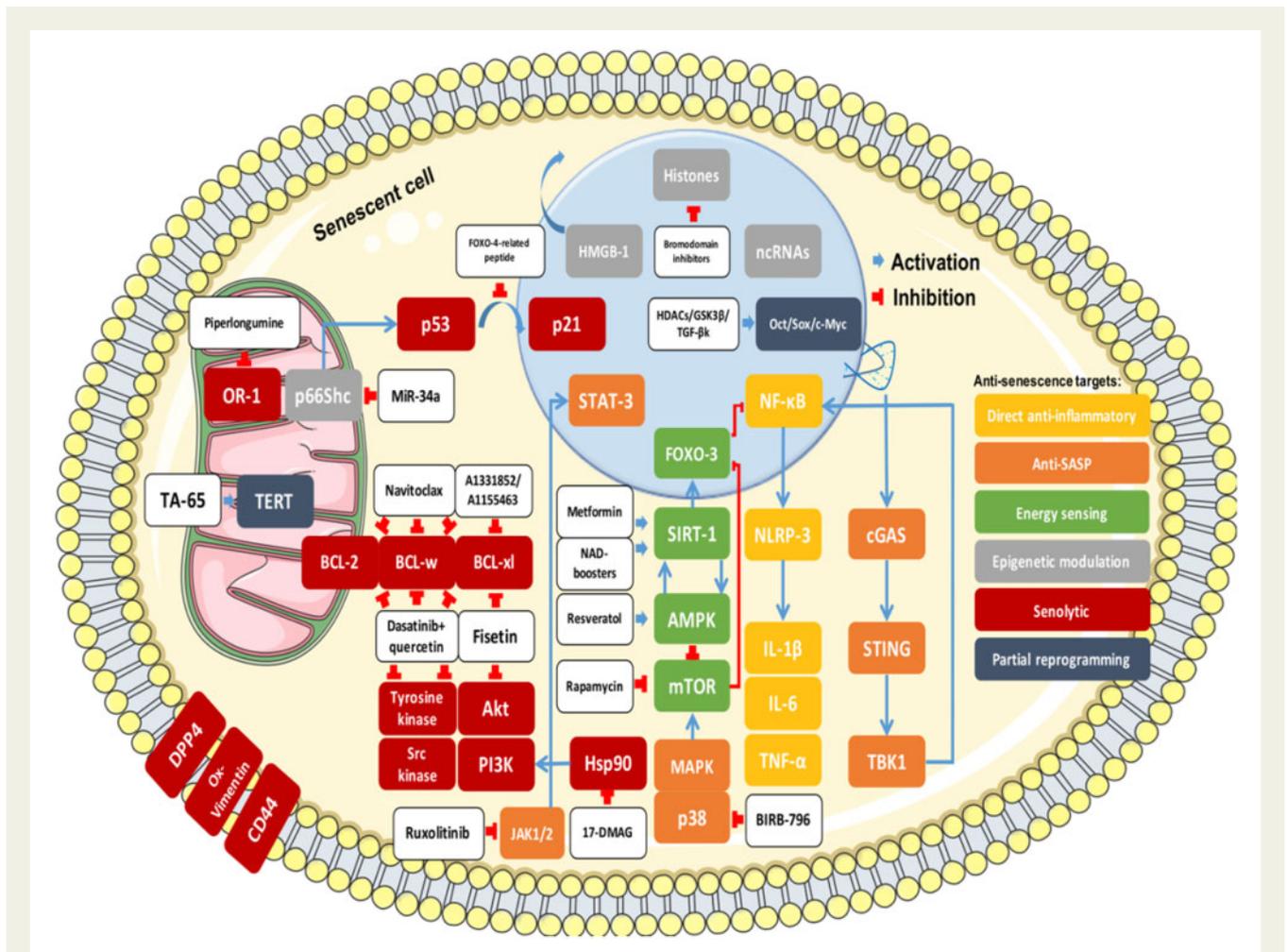
Figure 4 Anti-senescence strategies. Akt, protein kinase B; AMPK, AMP-activated protein kinase; cGAS, cyclic GMP-AMP synthase; DPP4, dipeptidyl peptidase 4; FOXO, Forkhead box class O; HMGB-1, high-mobility group box 1; IL, interleukin; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor kappa B; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; ox-vimentin, oxidized vimentin; PI3K, phosphoinositide 3-kinase; SASP, senescence-associated secretory phenotype; SIRT, sirtuin; STING, stimulator of interferon genes; TBK, serine/threonine-protein kinase TBK1; TNFα, tumour necrosis factor α.

currently clinically evaluated in CVD.[166] Epigenetic modification through histone modification via bromodomain inhibitors could suppress atherosclerosis,[167] senescence,[168] and the SASP,[169] indicating high potency for such interventions. The non-histone DNA-binding protein high mobility group protein B1 (HMGB1) is a major pro-inflammatory SASP factor, when externalized from the nucleus into the extracellular environment.[39] Soluble HMGB1 promoted atherosclerosis[40,41] and CVD risk.[170] It remains to be seen if HMGB1 nuclear retention ameliorates the epigenetic landscape of senescent cells and provides benefits in atherosclerosis. Another promising strategy is the epigenetic blockade of p66Shc, since pharmacological inhibitors are not currently available. p66Shc is a promoter of oxidative stress[70] and is highly expressed in senescent cells[68] and atherosclerosis.[171] Inhibiting p66Shc its key regulator MiR-34a is promising,[172] as p66Shc knock-out led to senescence prevention,[68] 30% animal lifespan extension,[173] and strong inhibition of atherogenesis.[70,71]

Targeting senescent cells as the root of inflammation: senolytic approaches or cellular reprogramming

Senolytic approaches

Safe removal of chronic senescent cells is an attractive approach to blocking ageing pathology and atherosclerosis. It is possible to specifically delete senescent cells through activation of apoptosis by compounds that selectively target senescent cells, called senolytics. INK-ATTACK transgenic mice contain an inducible suicide gene in the *CDKNA2* locus, which encodes p16 INK4a, a key molecule in senescent cells. This elegant transgenic model has been used to selectively eliminate senescent cells *in vivo*, leading to reversal of age-related characteristics without noticeable side effects.[94,95] The positive effect of senescent cell removal in atherosclerosis in LDL receptor-

knockout mice has been replicated in INK-ATTACK, p16-3MR, and INK-nitroreductase mouse models using the senolytic navitoclax (ABT-263).[9] Senescent cell elimination diminished plaque size, inflammation, and instability features.[9] These data provide a rational basis for the use of senolytics in the treatment of atherosclerosis.

Recently, multiple senolytic compounds have been developed and have been tested for the treatment of CVD (*Figure 4*).[42,174–185] Navitoclax, a Bcl-2 inhibitor, has beneficial effects in atherosclerosis,[9] and may be a good candidate for the targeting of T-EMRA cells, which overexpress Bcl-2 family proteins.[116] Combination therapy with dasatinib and quercetin (D+Q) removed senescent cells from the tunica media, but not the intima, improving vascular function parameters, but not arterial compliance and plaque burden.[33] Senolytic drugs were known to be more effective against particular senescent cell types *in vitro*, and less effective against others, for unclear reasons.[42,174–185] It is otherwise unknown how the cell type specificity of senolytics affects cells of the arterial wall, but it may explain why D+Q removed senescent cells only within the tunica media. Further studies are needed to corroborate the senolytic effect within plaque.

Another option for using senolytics may with the goal of improving outcomes of autologous transplantation therapies of bone marrow-derived angiogenic cells in ischaemic heart failure. In ischaemic heart failure patients, these cells are severely impaired[186,187] and display markers of senescence,[188,189] disabling their use for patient-derived cellular therapy. Senolytics may be used to remove senescent cells, either *in vivo* or *ex vivo*, leading to a rejuvenated population and more effective therapeutic outcomes.[186,189]

In the context of drug repurposing, some senolytic compounds are already clinically approved (dasatinib) or in clinical trials (navitoclax, HSP90 inhibitors) for indications in oncology. The results of the first human trial of dasatinib and quercetin in idiopathic pulmonary fibrosis (IPF) have been recently published.[88] This small scale single-arm study reported improvements of 6-min walk distance, 4 m gait speed, chair-stands, and Short Physical Performance Battery (SPPB) score, showing the promise of this therapy for IPF. An ongoing trial is evaluating dasatinib and quercetin for their senolytic ability in chronic kidney disease (NCT02848131).

Currently available senolytics drugs are limited by adverse effects. When used for cancer treatment, these drugs often exhibit adverse effects such as nausea, vomiting diarrhoea, and skin rashes; notably, Bcl-xl inhibitors induce severe thrombocytopenia.[190] To avoid these effects, localized delivery via percutaneous intervention may be required to target senescence in a CVD setting. This could be combined with senescent cell-specific delivery methods, such as the galactose-encapsulation of drugs that reduced thrombocytopenia after navitoclax application in an experimental setting.[191] Alternatively, the drugs could be applied periodically, with long off-drug periods.[175]

Cellular reprogramming

The induction or reprogramming of mesenchymal cells into a pluripotent state is possible via octamer-binding transcription factor (Oct)3/4, Sox2, c-Myc, and Krüppel-like factor (KLF4).[192] Cells can potentially be reverted to a non-senescent state, reversing their cellular biological clock. Short-term cyclic expression of Oct4, Sox2, KLF4, and c-Myc in LAKI 4F mice suppressed the ageing phenotype,

prevented VSMC degeneration, and reversed bradycardia without observed tumorigenesis.[193] These results suggest an intriguing possibility that low-dose short-term reprogramming may be beneficial for atherosclerosis. A drug cocktail of HDACs, GSK-3β, and TGF-β kinase enabled cell reprogramming *in vitro*, thus improving liver regeneration and function after acute injuries in mice.[194] The authors attributed this effect to the up-regulation of pluripotency genes. Extensive reprogramming may be tumourigenic, as it leads to up-regulation of strong oncogenes, such as c-Myc.[195] Extremely precise and reliable control of this anti-senescence strategy is an absolute must to avoid malignant transformation. Of note, senescent cells from elderly people could be reverted to a pluripotent state only by the additional over-expression of NANOG and LIN2 in addition to Oct3/4, Sox2, c-Myc, and KLF4.[196]

Another intriguing strategy is direct reprogramming or transdifferentiation, which enables a phenotypic change from one differentiated cell type into another without achieving pluripotency.[197] Hypothetically, senescent secretory VSMCs could thereby regain a contractile phenotype.

Future directions towards clinical application of anti-senescence strategies

Research regarding ageing mechanisms and cellular senescence is confronted with pre-clinical and clinical challenges that need to be addressed in order to pinpoint the role of cellular senescence in atherosclerosis and define optimal therapeutic strategies. Firstly, in pre-clinical research, the detection of senescent cells is hampered by the lack of specific markers. *In vitro*, the gold standard senescence-associated β-galactosidase staining and p16 INK4a expression detection can yield false-positive results in macrophages.[198] *In vivo*, the best currently available models for senescent cell detection are reporter-harbouring transgenic mice (p16-LUC, p16 tdTom)[199,200] and transgenic mouse strains used for selective senescent cell removal (INK-ATTAC, p16-3MR).[201]

Secondly, a better understanding of the role of cellular senescence in vascular disease development is needed. Studies in cell cycle inhibitor knock-out models have raised questions about the complex relationships between senescence, proliferation, and apoptosis in different phases of atherosclerosis. p16 INK4a, p14 ARF (p19 ARF in mice), p21, and p53 enable cell cycle arrest in senescence. However, polymorphisms that hinder their activity promote atherosclerosis in humans.[202] Knock-out of p16 INK4a, p19 ARF, p21, and p53 had a pro-atherosclerotic effect in mice.[203–205] On the other hand, cellular senescence promotes atherosclerosis in each developmental phase.[9,101] Selective removal of p16 INK4a-positive senescent cells prevented atherosclerosis, while stabilizing plaques and decreasing plaque inflammation.[9] This discrepancy can be explained by other critical roles of cell cycle inhibitors in cell physiology and disease development, all of which are afflicted by the abovementioned genetic polymorphisms and knock-outs in a nondiscriminatory way.

Thirdly, special care must be taken in selecting patients for possible anti-senescence clinical trials. Experimental evidence shows that

cellular senescence has roles in limiting myocardial[206] and liver[207] fibrosis, promoting skin healing,[82] as well as in embryonal development,[208] which have to be considered. However, these studies focused on the function of senescence in acute organ injury, not in chronic processes such as atherosclerosis. The aim of anti-senescent therapies would be to clear chronic senescent cells or suppress their noxious effects, while not permanently abrogating the senescence programme. Nevertheless, these findings may imply that initial anti-senescence/anti-SASP therapeutic trials need to be applied to patients with a clinically stable phase of vascular disease, with high residual CVD risk,[2] and not in a setting of acute injury. Another concern would be that inducing senescence in the short term initially slows cancer growth due to the cell cycle arrest induction,[81] which raises questions about oncogenesis as an adverse effect. However, chronic senescence promotes cancer.[92] These issues remain to be carefully addressed and analysed in future studies exploring anti-senescence strategies.

To track the effect of anti-senescence therapies in a clinical setting, it would be important to specifically detect total or localized senescent cell burden.[7] Using a matrix-biomarker approach of 48 SASP-associated cytokines and miRNAs[88] seemed not to be an effective strategy in evaluating the effects of senolytic therapy in IPF. The possible explanation is that immune cells can also produce these factors, making senescence-specific detection problematic. An alternative to the matrix approach may be the discovery of senescence-specific soluble antigens, such as oxidized vimentin.[209]

Anti-senescence approaches may be a crucial component of precision medicine therapy. The development and decreasing cost of – omics methods or imaging can lead to a clinically applicable biomarker set, which combines senescence-associated soluble biomarkers and cellular compartments to identify patients with a high senescence burden, and thus the best candidates for anti-senescence therapies. For example, patients with a high burden of Bcl-2 overexpressing senescent T-EMRA cells may be good candidates for therapy with a Bcl-2-targeting senolytic, navitoclax. On the other hand, if there is a dominance of endothelial senescence, SASP inhibitors may be more beneficial than senolytics to avoid endothelial erosion. Decision-making can be further tailored with individual genetic profiling. Speculatively, a patient with a down-regulating rs2802292 *FOXO3* T; T polymorphism[210] may benefit more from FOXO/SIRT-1 agonists and NAD boosters than from other forms of anti-senescence therapy. Ultimately, developing next-generation biomarkers and imaging methods seems essential to translate anti-senescence therapeutics to the clinic.

Conclusions

Current therapeutic strategies targeting known risk factors for CVD, such as hypertension, diabetes mellitus, and cholesterol levels lower CVD risk. However, the disease risk remains high and will increase in the ageing western population. Targeting specific upstream inflammatory processes involved in deleterious local plaque pathophysiology has emerged as a novel approach to limit atherosclerosis. Accumulation of senescent cells promotes inflammation and negatively affects plaque remodelling. Translating novel anti-senescence

strategies into the clinic has potential to causally and effectively prevent and treat atherosclerosis and/or CVD.

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