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Review Article

A Review of Cellular Senescence and Senolytic Drugs Use in Idiopathic Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a rare but deadly, and increasingly prevalent interstitial lung disease. Despite the recent approval for IPF treatment of the anti-fibrotic drug Pirfenidone and the tyrosine kinase inhibitor Nintedanib, the disease remains an inexorably progressive disease, associated with poor survival. In recent years, senescent cells appeared as attractive targets for the treatment of ageing-associated diseases with a chronic inflammatory component such as atherosclerosis, osteoarthritis, chronic obstructive pulmonary fibrosis and IPF. This growing interest is linked to two critical characteristics of senescent cells, i) Their resistance to apoptosis that might facilitate their accumulation in aged and diseased tissues, ii) and their ability to induce through the release of senescence-associated secretory phenotype mediators the secondary senescence of healthy neighbouring cells and a chronic low-grade inflammatory environment. In this article, we will review the literature on the role of cellular senescence in IPF, with an emphasis on histopathological findings, and the mechanisms involved in the induction and maintenance of alveolar epithelial cells and fibroblasts senescence. We will particularly look at the link between telomere functioning genes mutations and telomeres length in familial as well as the sporadic form of IPF. In recent years, the enthusiasm raised by the therapeutic potential of targeting senescent cells led to the development of senolytic drugs designed to eliminate them selectively. We will lastly review the mechanisms of action of the main senolytic drugs as well as the results obtained using them in experimental models of lung fibrosis and a few early clinical trials, including the first one involving IPF patients.

Keywords: *Idiopathic Pulmonary Fibrosis (IPF); Cellular Senescence; Senolytics; Senescence-Associated Secretory Phenotype (SASP), Lung Fibrosis; Fibroblasts; Alveolar Epithelial Cells; Cigarette Smoking*

Abbreviations

12-LOX: Arachidonate 12-Lipoxygenase; AEC: Alveolar Epithelial Cells; AECII: Alveolar Epithelial Cells Type Two; AM: Alveolar Macrophages; BAD: BCL2 Associated Agonist of Cell Death; BALF: Broncho-Alveolar Lavage Fluid; BAX: BCL2 Associated X; BCL-2: B-cell Lymphoma 2; BID: BH3 Interacting Domain Death Agonist; BIM: Bcl-2-Like Protein 11; CDK X: Cyclin Dependent Kinase X; CHK2: Checkpoint Kinase 2; CK2: Casein Kinase 2; COX-2: CycloOxygenase-2; CREB: cAMP Response Element-Binding Protein; CS: Cigarette Smoke/Smoking; CSE: Cigarette Smoking Extract; DCRX: Decoy Receptor X; DDR: DNA Damage Response; DNA-SCARS: DNA Segments with Chromatin Alterations Reinforcing Senescence; DNA: Deoxyribonucleic acid; DQC: Dasatinib/Quercetin Combination; DRX: Death Receptor X; EFNBX: Ephrin BX; FGF2: Fibroblast growth Factor 2; FOXO4: Forkhead Box Protein O4; GDF15: Growth; Differentiation Factor 15; HT-DF: healthy tissue-derived fibroblasts; ID-1: DNA-binding protein inhibitor ID-1; IGFBP5: Insulin Like Growth Factor Binding Protein 5; IL-x: Interleukin-X; ILD: Interstitial Lung Diseases; IPF: Idiopathic Pulmonary Fibrosis; IPF-DF: IPF-Derived Fibroblasts; MDM2: Mouse Double Minute 2; MEF: Mouse Embryonic Fibroblasts; MMPX: Matrix Metallo-Proteinase X; MOMP: Mitochondrial Outer Membrane Permeabilization;

mTOR: mammalian Target of Rapamycin; NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; OIS: Oncogene-Induced Senescence; PAI-X: Plasminogen Activator Inhibitor-X; PARK2: Parkinson Disease 2; PARN: Poly(A)-Specific Ribonuclease; PBL: Peripheral Blood Leukocytes; PDGF-AA: Platelet-Derived Growth Factor-AA; PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha; PGE-2: Prostaglandin E-2; PI3Kδ: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta Isoform; PINK1: PTEN-Induced Putative Kinase 1; PMAIP1: Phorbol-12-Myristate-13-Acetate-Induced Protein 1; POT1: Protection of Telomeres Protein 1; PTEN: Phosphatase and Tensin Homolog; PUMA: P53 Upregulated Modulator of Apoptosis; qPCR: Quantitative Polymerase Chain Reaction; RB: Retinoblastoma; RS: Replicative Senescence; RTEL1: Regulator of Telomere Elongation Helicase 1; SASP: Senescence-Associated Secretory Phenotype; SIPS: Stress-Induced Premature Senescence; SMA: Smooth Muscle Actin; TERC: Telomerase RNA Component; TERT: Telomerase Reverse Transcriptase; TGF-β1: Transforming Growth Factor-β1; TNF-α: Tumour Necrosis Factor-α; TRAIL: TNF-Related Apoptosis Inducing Ligand; TRFX: Telomeric Repeat-Binding Factor X; uPA: urokinase-type Plasminogen Activator; uPAR: Urokinase-Type Plasminogen Activator Receptor; XRCC1: X-Ray Repair Cross-Complementing Protein 1

Introducing Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF), is a rare and progressive interstitial lung disease (ILD) of unknown aetiology, whose affected patient's average life expectancy at diagnosis, range from 2 to 4 years in the absence of therapeutic intervention [1-3]. Although mostly a progressive disease, 5 to 15% of IPF patients a year presents episodes of rapid deteriorations of their pulmonary function outside any known cause. These episodes called acute exacerbations of IPF are fatal in approximately 50% of cases despite treatments with high doses of corticosteroids and antibiotics [4]. ILD, regroup lung diseases, characterised by interstitial inflammation, fibrosis, cellular proliferation or a combination of those in the absence of infection or cancer [3]. Some ILD such as chronic hypersensitivity pneumonitis, are caused by exposure to mould, and birds derived particles, or underlying autoimmune disease in the case of sarcoidosis, or even connective tissue diseases (i.e. connective tissue disease-related ILD). However, for other ILD known as idiopathic interstitial pneumonia, whom IPF is the most frequent one, the cause of the disease remains elusive [3].

IPF diagnosis

IPF diagnosis nowadays is mainly based on a combination of patients interrogation, high-resolution CT Scan, blood testing, and biopsy in atypical cases [3]. Most IPF patients present exertional dyspnoea due to reduced, forced vital capacity, total lung capacity, and carbon monoxide diffusing capacity [5]. Patients also suffer from chronic dry caught, fatigue and present manifestations of hypoxemia such as clubbing and acrocyanosis [3]. High-resolution CT Scan of IPF patients reveals a usual interstitial pneumonia pattern characterised by bilateral reticulation and honeycombing preferentially in the periphery of the lower lobes [6]. Since other ILD (e.g. Sarcoidosis) present a usual interstitial pneumonia pattern, they must be ruled out by the absence of their characteristic markers, or risk factors [3]. When biopsies have been required, the diagnosis of IPF is confirmed by the presence of 1) a patchy pattern of fibrosis mainly in the subpleural/paraseptal areas, 2) enlarged alveolar spaces filled with mucus and inflammatory cells lined by bronchiolar metaplastic epithelium called honeycombs, 3) and dome-shaped areas, formed by actively proliferating fibroblasts/myofibroblasts, embedded in a pale myxoid matrix, and covered by hyperplastic pneumocytes called fibroblastic foci [6,7].

Epidemiology of IPF

IPF incidence varies significantly among different geographical areas. Epidemiologic studies revealed incidence ranging between 3 to 9 cases per 100,000 individual per year in Europe and North America and less than 4 in South America and East Asia [8]. IPF incidence increases sharply with age as one study showed IPF prevalence among people over 65 years of age in the United-States to be 494 per 100,000 individual per year [3]. Moreover, comparisons with data gathered ten years earlier revealed a twofold increased in IPF prevalence on the period [3]. According to the 2015 United nation report on the world population ageing, the global population over 60 years of age was about 901 million in 2015 and is expected to grow by 56% in 2030 reaching 1.4 billion [9]. By 2050 the population over 60 years of age could be around 2.1 billion, that is to say, more than twice as much as in 2015 [9]. Thus, the burden of IPF should follow

the same trend, urging for a better understanding of its causes, mechanisms of progression and ultimately, management.

Risk factors associated with IPF

Although of unknown aetiology, recent epidemiological studies revealed distinct external/environmental and genetic risk factors for the development of IPF. For many IPF specialist, ageing is regarded as the most crucial associated risk factor [10]. However, healthy ageing being a physiological process, we will focus here on the pathologic risk factors that accelerate ageing and precipitate IPF development.

Among the external risk factors, early studies by Baumgartner., *et al*. identified smoking as a risk factor for IPF development as 72% of IPF patients had a history of ever smoking a cigarette against 62% in the control group. The study further showed a dose-dependent effect of cigarette smoking, as the risk of developing IPF was significantly higher in those smoking 21 - 40 pack a year [11]. Other studies have incriminated dust from various origins, including organic dust (e.g. livestock, farming), metal dust (e.g. steel), wood dust, mineral dust (e.g. stones, silica) often in a professional environment [12]. Besides, other health conditions such as gastroesophageal reflux, type 2 diabetes and some bacterial and viral infections (e.g. herpes, Epstein-bar and hepatitis C viruses) are frequently found in IPF patients [12,13].

On the genetic side, genome-wide association studies involving patients with sporadic as well as familial forms of IPF, revealed several genetic risk factors, including genes involved in lung epithelial cell functions (e.g. MUC5B, SFTPC, SFTPA2), telomere functioning (e.g. TERT, TERC, PARN, RTEL1) and innate immunity (e.g. TLR3, Tollip, IL-8) [14]. Altogether, these data suggest that IPF arise following a lifetime exposure of the lung to noxious agents in individuals whose genetics predispose to premature ageing, Endoplasmic Reticulum stress (ER stress), abnormal innate immune response or any combination of these [15].

Mechanisms of IPF development and therapeutic avenues

Clinicians and researchers initially perceived IPF as a fibrotic lung disease driven by chronic inflammation of unknown cause [16]. However, failure to improve, and even aggravation of the disease by the administration of immunosuppressive drugs (e.g. prednisolone, azathioprine), prompted the scientific community to revisit its view of IPF pathogeny [16]. Recent work by Newton., *et al*. suggest that the deleterious effects of immunosuppressive treatments (i.e. a higher rate of, death, lung transplantation, hospitalisation, FLC decline) mainly affects patients with short telomeres (10th percentile of normal <) as measured in their blood leucocytes [17]. It is however not clear whether immunosuppressants further decreases telomeres length or adversely affect these patient's health by other mechanisms. Moreover, several studies suggest that immunosuppressants treatments fail to inhibit alveolar macrophages activation and the recruitment of inflammatory cells to the lung of IPF patients [15].

The scientific community now regard aberrant epithelium/mesenchyme communications due to repetitive micro-injuries to the lung epithelium as the driving force of IPF progression [16]. Physio-pathological processes such as activation of the TGF-β pathway, epithelial/endothelial to mesenchymal transition and type II alveolar epithelial cells (AECII) ER stress, are believed to be critical drivers of IPF development in the current hypothesis [16,18]. In line with this hypothesis, since 2015 IPF treatment recommendation by the ATS/ERS/JRS/ALAT Clinical Practice Guideline now involved, the anti-fibrotic drug Pirfenidone and the tyrosine kinase inhibitor Nintedanib [19]. Although both molecules have shown an ability to slow down IPF progression and improve patient's survival, this comes at the cost of numerous side effects including, nausea, dyspepsia and a photosensitive rash with pirfenidone, and nausea and diarrhoea with Nintedanib [19].

In recent years, an accumulation of data has been published supporting a contribution of immune cells to IPF pathogeny notably through interactions with the mesenchymal compartment [15,20] and clinical trials targeting immune mediators such as IL-13 in IPF treatment are currently underway [19].

In addition to inflammation, cellular senescence has also attracted interest as a potential driver of IPF development as many studies have revealed, epithelial cells and fibroblasts senescence in IPF lungs [3,10]. Moreover, cellular senescence is one of the main consequences of ageing-associated telomeres shortening and is believed to be a mechanistic driver of many age-related diseases, including ILD [21,22]. In this review, we will analyse data on the mechanisms supporting cellular senescence in IPF, their potential contribution to the disease progression and the potential of senolytic drugs as therapeutic avenues for IPF treatment.

Cellular senescence, characteristics, markers and mechanisms

Cellular senescence defines the process by which cells irreversibly enter a state of growth arrest (i.e. permanent cell cycle arrest), become resistant to apoptosis, and secrete new mediators grouped under the terminology "senescence-associated secretory phenotype" (SASP) [23]. SASP mediators are mainly, cytokines (e.g. IL-1α, -6, -8, TNF-α), growth factors (e.g. PDGF-AA), and matrix metalloproteinase (e.g. MMP13) [24]. Other characteristics of senescent cells include cell enlargement, expression of cyclin-dependent kinase inhibitors, and the formation of senescence-associated heterochromatin foci [25].

Early *in vitro* studies by Hayflick., *et al.* in the 1960s, demonstrated that cells have limited replicative capacities and enter *in vitro* in a state of senescence after roughly 50 divisions [24]. These experiments defined replicative senescence (RS), a type of cellular senescence which is believed to occur *in vivo* and to be responsible for the accumulation of senescent cells during ageing as suggested by Karavassilis., *et al.* study of cultured endothelial and smooth muscle cells replicative potential evolution with donors age [26]. One of the main mechanisms supporting RS is the shortening of chromosomes telomeres after each cell division. Telomeres are formed by tandem repeats of the sequence "TTAGGG", which serve as a binding site for telomere-binding proteins [23]. Telomeres form a lariat-like structure called telomere-loop (t-loop) which are stabilized by several telomere binding proteins (e.g. TRF2, POT1), forming a so-called "shelterin" complex [23]. These shelterin complexes protect chromosomes from DNA damage, and their destabilization following telomere shortening results in increased DNA damage and a DNA damage response (DDR) that leads to cell senescence [23]. Cell senescence can also be induced by various stressors (e.g. Oxidative stress, Endoplasmic reticulum stress, Genotoxic agents) and is dubbed stress-induced premature senescence (SIPS) [23]. Although telomere size is not necessarily affected in SIPS, it often requires the triggering of a DDR [23]. However, some cellular senescence inducers such as oncogenes and tumour suppressors overexpression, chromatin perturbators can trigger pathways overlapping DDR pathways in the absence of DNA damage [27]. Although very close in term of their markers, a recent article by Sanokawa-Akakura., *et al*. showed that RS could be distinguished at least *in vitro* from SIPS, by reduced levels of 18S, 5.8S and 28S rRNA due to epigenetic silencing [28].

Cellular senescence was initially understood as a physiological response to DNA damage, aimed at preventing the proliferation of transformed cells and promote the recruitment of immune cells able to eliminate damaged cells (e.g. necrotic, apoptotic, cancerous), including senescent cells, thereby preventing the subsequent development of tumours [23,24]. However, later studies also highlighted physiological roles of senescent cells, in embryonic development, tissue regeneration and wound healing [24]. The observation during embryonic development of senescent cells devoid of any type of cell damage, and lacking classical senescent-associated markers, established a third category of senescence induction called programmed senescence that will not be further addressed in the context of this review [27,29].

Most of our knowledge on the mechanisms governing cellular senescence comes from *in vitro* studies using various cell lines (mainly fibroblastic) in which senescence was initiated by long-term cell culture (i.e. RS), or by exposure to radiations (i.e. X, Gamma, Ultraviolet), DNA damaging/chemotherapeutic agents, (e.g. Etoposide, Bleomycin, Doxorubicin), oxidants (e.g. Hydrogen peroxide aka H_2O_2), oncogenes overexpression (e.g. RAS, RAF), all of which defines SIPS [24,27,30]. In the following section, we will review the central mechanisms regulating senescence-associated, growth arrest, apoptosis resistance and secretory phenotype.

Mechanism of senescence-associated growth arrest

From a molecular standpoint, various pathways are activated and contribute to the phenotypic characteristics of senescent cells. One of the primary markers of senescent cells, the cyclin-dependent kinase inhibitor, $p16^{INK4a}$, was discovered by Serrano M., *et al*. in 1993 using a yeast two-hybrid interaction screen to find molecular partners of the Cyclin-Dependent Kinase 4 (CDK4), a critical player with CDK6 in the transition between the G0/G1 and the S phase of the cell cycle [31,32]. Further experiments showed that $p16^{INK4a}$ by inhibiting CDK4/6 was involved in RS of various cells including, human head and neck keratinocytes [33], human diploid fibroblasts [34] as wells as in oncogene-induced senescence (OIS) (i.e. RAS/RAF pathways activation) of human uroepithelial cells [35] and various human fibroblastic cell line [36-39].

It was later found that CDKN2A, the gene encoding $p16^{INK4a}$ also encoded with an alternative reading frame the p53 pathway regulator $p14^{ARF}$ (human homologue of $p19^{ARF}$) and was part of the 35kb INK4/ARF locus located on human chromosome 9p21. The INK4/ARF locus also comprised, the CDK inhibitor $p15^{INK4b}$ and the antisense non-coding RNA at INK4a/ARF Locus [40]. Similar to $p16^{INK4a}$, $p15^{INK4b}$ binds CDK4 and CDK6 preventing retinoblastoma (RB) phosphorylation and S phase entry, while $p14^{ARF}$ expression leads to the sequestration into the nucleolus of the p53 negative regulator MDM2 resulting in p53 activation, cell cycle arrest or apoptosis [40].

In normal cells, transcription of the INK4/ARF locus is silenced by repressive histone methylation marks (i.e. H3K27me3) due to the activity of polycomb repressive complexes (PRC) members such as BMI1, and CBX7 or 8 [40]. In addition to histone modifications, the epigenetic repression of $p16^{INK4a}$ might also include DNA methylation, as inhibition of the DNA methyltransferase DNMT1 expression by the HMG box-containing protein one during RS and OIS resulted in $p16^{INK4a}$ promoter hypomethylation and expression [41]. However, the mechanisms of $p16^{INK4a}$ inhibition are lost when cells are exposed to various stress. As shown, in human fibroblasts and mouse embryonic fibroblasts (MEFs), the exposition to UV radiation, hyperoxia or the overexpression of the oncogenes RAS/RAF, lead to the expression of the histone H3 demethylase JMJD3 and the subsequent expression of $p16^{INK4a}$ [42,43]. Following the removal of the repressive epigenetics marks, $p16^{INK4a}$ expression is promoted by transcription factors such as ETS-1 and 2 in response to OIS [44] or ageing as shown by *in vivo* mice experiments [45]. Additionally, the transcription factors sp1 [46] and PPAR-gamma [47] have been shown to promote $p16^{INK4a}$ expression in embryonic kidney/epithelial cells and human embryonic lung fibroblast RS, respectively. $p16^{INK4a}$, by inhibiting the AUF1-dependent decay of its mRNA, has also been shown to promote the expression of another critical senescence-associated CDKI named $p21^{WAF1}$ [48].

$p21^{WAF1}$ expression is mainly regulated by the genome safeguard p53 [24]. Following telomeres erosion, or DNA double-strand breaks, the activation of a DDR increases p53 activity by two mechanisms: 1) its phosphorylation by the kinases ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and RAD3-related protein (ATR) resulting in its stabilisation, 2) and $P19^{ARF}$ dependent inhibition of the ubiquitin ligase mouse double minute 2 (MDM2) which is involved in p53 degradation [24]. Once expressed in response to increased P53 transcriptional activity, $p21^{WAF1}$ inhibits CDK2, preventing its inhibitory phosphorylation of RB required for S phase entry resulting in cell cycle arrest [24,32].

The definitive growth arrest is secured by cooperation between $p16^{INK4a}$ and mitogenic signals, which favour the accumulation of intracellular ROS and subsequent protein kinase C delta (PKC-delta), activation. In a positive feedback loop, PKC-delta activation further increases ROS production and irreversibly block cytokinesis partly through the inhibition of the mitotic network exit kinase WARTS required for cytokinesis [49].

Mechanisms of senescence-associated apoptosis resistance

Apoptosis can be triggered by an extrinsic or an intrinsic pathway, both of which leads to the activation of the pro-apoptotic family of protein named caspases [50]. While the extrinsic pathway is triggered by immune cells or inflammatory mediators activating cytoplasmic death receptors such as the TNF-related apoptosis-inducing ligand receptor (TRAIL) and FAS, the intrinsic pathway is triggered by a wide

array of stimuli sensed intracellularly such as DNA damage, ER stress or Oxidative stress [50]. Whereas ligand binding at the surface of the extrinsic pathway death receptors, directly activates pro-caspases (e.g. pro-caspase-8 and -10), the diverse stimuli of the intrinsic pathway initially induce a process called mitochondrial outer membrane permeabilization (MOMP) [51]. The MOMP results in the release of mitochondrial cytochrome c into the cytoplasm, where it activates a protein complex named apoptosome responsible for caspases activation [51]. Due to its central role in apoptosis, MOMP is tightly regulated by members of the BCL-2 family of protein [52]. There are three subfamilies of BCL-2 proteins, whom two are pro-apoptotic, the BH3-only proteins (e.g. PUMA, BID, BIM) and the effector proteins (e.g. BAX, BAD), whereas one is anti-apoptotic, and includes members such as BCL-2, BCL-W, BCL-X_L [52]. The effector proteins activation at the mitochondrial membrane is responsible for the MOMP, and the members of the BH3-only protein support this activation in response to various stimuli [52]. Additionally, the anti-apoptotic BCL-2 family members can inhibit BH3-only proteins activity by direct binding, thereby preventing effectors activation, MOMP and caspases activation [50]. Although the intrinsic pathway mainly mediates MOMP, it can also be triggered by the extrinsic pathway through caspase-8 dependent activation of the BH3-only protein, BH3-interacting domain death agonist (BID) [50].

Senescent cells have been shown to resist apoptosis by mechanisms targeting both the intrinsic and extrinsic pathways [24]. Similar than what was observed in senescence-associated growth arrest, the level of activation and the pattern of post-translational modification of P53 play a critical role in directing cells fate toward apoptosis or senescence [24]. Thus, in senescent cells, a reduced level of p53 activation and a pro-senescent pattern of post-translational modification lead to a diminished increase in p53-induced apoptotic effectors such as PUMA, PMAIP1 and stronger expression of anti-apoptotic BCL-2 family members [24]. In a recent article Baar MP., *et al.* showed that senescent cells could resist apoptosis by nuclear accumulation of p53 following its interaction with FOXO4 [53]. By treating senescent cells with a peptide design to disrupt this interaction, the authors induced senescent cells apoptosis due to p53 cytoplasmic accumulation and activation of the intrinsic pathway [53]. Ryu., *et al*. further showed that BCL-2 expression in human diploid fibroblasts was regulated by phosphorylated cAMP response element-binding protein (CREB) [54]. Interestingly, they found that apoptosis inducers such as H_20_2, Staurosporine, Thapsigargin, downregulated p-CREB in young fibroblasts notably by inducing the protein phosphatase 2A, but failed to do so in senescent fibroblasts resulting in higher BLC-2 expression [54]. Similarly, Kim., *et al.* attributed senescent human diploid fibroblasts apoptosis resistance to a failed nuclear translocation of the BCL-2 repressors, and stress-induced map kinases ERK1/2 and p38 [55]. In fibroblasts undergoing RS, the epigenetic regulation of BCL-2 is marked by an increase of its association with active transcription marks such as histone acetylation (H4K16Ac), and decrease of repressive methylations (H4K20Me3), whereas that of the BCL-2 effector protein BAX evolved in the opposite direction [56]. Similarly, another study by Rochette., *et al.* showed that as cultured human fibroblasts age, UV stimulation tends to result in increased BCL-X_L expression as opposed to decreased expression in young cells [57].

On the extrinsic pathway side, it has been shown that senescence cells could overexpress decoy receptor 2 (DCR2), which prevent activation of the death receptor FAS by competitively binding its ligand expressed at the surface of cytotoxic immune cells [24].

Senescence-associated secretory phenotype

In addition to their growth arrest and resistance to apoptosis, senescent cells are characterised by the secretion of many mediators, including, cytokines, chemokines, growth factors, matrix-metal-proteinases [23]. Recent researches added to this list, haemostasis related factors [58] and small extracellular vesicles [59,60]. However, there is not a single senescent cells secretome, as the full spectrum of mediators released will depend upon the cell type and mechanisms of senescence induction [61,62]. To improve our knowledge of SASP mediators, Basisty., *et al*. recently published a proteomic atlas of the SASP induced by multiple senescence inducers in multiple cell types [63]. In this work, they identified potential circulating biomarkers of human senescence such as growth differentiation factor 15 (GDF15), STC1 and SERPINs that significantly correlates with age [63]. SASP mediators are responsible for most of the beneficial or deleterious effects attributed to senescent cells, such as immune surveillance through the recruitment of inflammatory cells, and cancer progression in late stages [24]. In this section, we will describe the main pathways involved in the development of a SASP, based mainly on *in vitro* studies carried out in human diploid fibroblastic cells.

A key driver of the SASP is the persistence of DNA damage at structures called "DNA segments with chromatin alterations reinforcing senescence" or DNA-SCARS [64], a phenomenon also observed in senescent cells of aged mammalian tissues [65]. Unlike transient DNA damage responses, persistent DNA damage responses linked to DNA-SCARS have been shown to support the secretion of IL-6 through DDR proteins such as ataxia telangiectasia mutated (ATM), Nibrin and CHK2 [66]. Using a model of OIS in human fibroblasts, Hoare., *et al*. observed a qualitative evolution of the SASP during senescence. Thus, they showed that early SASP is dominated by a TGF-β rich secretome driven by NOTCH1 activation, which also inhibits C/EBPβ activation and the secretion of pro-inflammatory mediators [67]. As cellular senescence progressed, inhibition of NOTCH1 facilitates the expression of C/EBP-dependent expression of pro-inflammatory mediators [67]. In contrast, a recent publication highlight the key role played by the IL-1 pathway in controlling the late SASP mainly composed of pro-inflammatory mediators regulated by the transcription factor NF-κB [68]. Orjaloa., *et al.* showed that IL-6 and IL-8 secretion by fibroblasts rendered senescent by DNA damaging agents, replicative exhaustion, oncogenic Ras or chromatin relaxation were dependent upon an autocrine stimulation by membrane-bound IL-1α [69]. Similarly, Acosta., *et al*. demonstrated using *in vitro* and *in vivo* models of OIS that the SASP was controlled by inflammasome mediated IL-1 signalling [70]. However, to adequately stimulate the SASP, membrane-bound IL-1α must be cleaved by caspase 5 [71]. Among the SASP mediators, the authors further identified TGF-family ligands, VEGF, CCL2 and CCL20 as key paracrine senescence inducers, involved in the bystander effect resulting in the senescence of healthy neighbouring cells known as secondary senescence [70]. SASP mediators was long regarded as the primary driver of secondary senescence; however, Teo., *et al*. using an OIS model in human fibroblasts showed that primary and secondary senescence are functionally distinct and that senescent cells NOTCH1 engagement with Notch ligand on non-senescent neighbouring cells is critical for the induction of secondary senescence [72]. Consistent with an inhibitory effect of NOTCH1 activation on the pro-inflammatory components of the SASP, secondary senescent cells present a blunted SASP and a higher expression of fibrillar collagens which are downregulated in primary senescence [72].

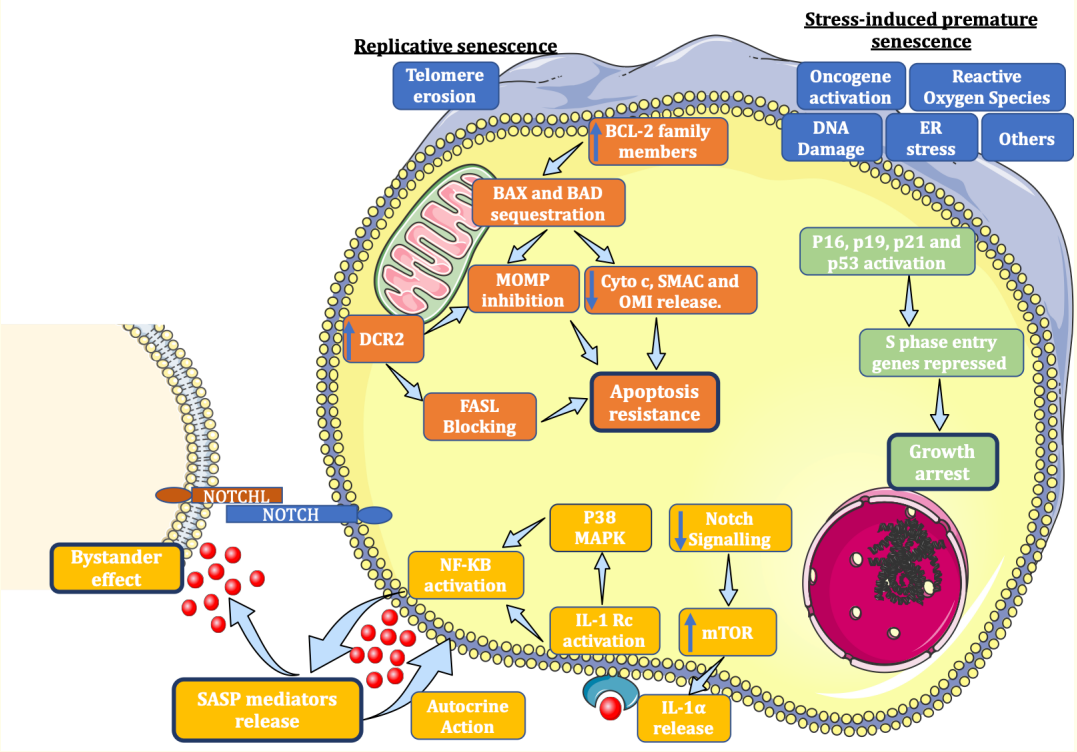

Figure 1: *Key molecular mechanisms supporting cellular senescence.*

Cellular senescence can result from telomere erosion following multiple cell divisions and define replicative senescence. Various stressors such as reactive oxygen species (ROS), genotoxic agents will define stress-induced premature senescence (SIPS). Cellular senescence present three main characteristics: 1) growth arrest, 2) apoptosis resistance and 3) a senescent-associated secretory phenotype (SASP). Following damages to DNA and other cellular molecules, the cell cycle inhibitors p16, p19, p21 and p53 are activated, resulting in S phase entry genes repression and growth arrest. Besides, cell senescence is also associated with increased BCL-2 family members expression (i.e. BCL-2, BCL-W, BCL-X_L), which bind to and sequester the proteins BAX and BCL-2-associated agonist of cell death (BAD). This, in turn, leads to mitochondrial outer membrane permeabilization (MOMP) inhibition as well as the inhibition of the release of pro-apoptotic cytochrome c, a second mitochondria-derived activator of caspase (SMAC); and OMI, resulting in intrinsic apoptosis resistance. On the other hand, senescent cells overexpress decoy receptor 2 (DCR2), which intercepts FAS ligand expressed on cytotoxic immune cells resulting in extrinsic apoptosis resistance. The long-term DDR is associated with a decrease in Notch signalling, leading to an increase mammalian target of rapamycin (mTOR) dependant IL-1α release. The autocrine activation of the IL-1 receptor results in the activation of the p38 MAPK and NF-κB pathways and the subsequent secretion of the senescent-associated secretory phenotype (SASP) mediators. These mediators act in an autocrine fashion to amplify the senescent phenotype and induce the secondary senescence of healthy neighbouring cells by a senescent cell bystander effect also involving NOTCH/NOTCH Ligand interaction.

In addition to IL-1, Kuilman., *et al.* identified in OIS of human fibroblast, IL-6 as a critical mediator for senescence entry, maintenance and amplification of the senescent inflammatory network through cooperation with the transcription factor C/EBPβ [73]. Consistent with a critical role of autocrine and paracrine stimulation by SASP mediators in senescence maintenance and propagation, Acosta., *et al.* demonstrated that OIS and RS of human fibroblasts was dependent upon the expression of the chemokine's receptor CXCR2 and its ligand following activation of the transcription factors NF-κB and C/EBPβ [74]. Apart from autocrine activation, senescence associated-mitochondrial dysfunction through the production of ROS is an essential driver of NF-κB activation and SASP acquisition [75]. Flanagan., *et al.* reported that a subset of the SASP mediators including the matricellular protein osteopontin, IL-6 and IL-8 was regulated by the combined action of C/EBPβ and the transcription factor c-Myb [76]. In addition to NF-κB, C/EBPβ, and c-Myb, recent studies highlighted the crucial role played by mTOR in IR and OIS of human fibroblasts. In one hand, mTOR promotes the translation of the membrane-bound IL1α mRNA, resulting in the subsequent expression of IL-6 and other SASP mediators mRNA [77]. In the other hand, mTOR activation prevents the degradation of numerous SASP mediator's mRNA by the RNA-binding protein ZFP36L1 in an MK2 (also known as MAPKAPK2) dependent manner [78]. Consistent, with a critical role of MK2 in SASP mediator's expression, its primary activator the p38MAPK is a novel DNA damage response-independent regulator of the SASP in models of IR and OIS of human fibroblasts [79]. Recent publications identified the p38MAPK and NF-κB activators TLR2 [80] and CD36 [81] as significant regulators of human fibroblasts SASP. The author identified a positive regulation loop in which TLR2 induces the expression of the acute-phase serum amyloid A1 and A2 (A-SAAs) which in turn act as damage-associated molecular patterns (DAMPs) to further activate TLR2 [80]. Finally, the authors report a role for the cytoplasmic chromatin sensors cGAS/STING in priming TLR2 and A-SAAs expression in this model [80]. This last result is consistent with previous data supporting a pivotal role for cytoplasmic chromatin sensing through the cGAS/STING pathway in SASP induction [29]. Activation of the cGAS/STING pathway in senescent cells seems to results from accumulation in these cells of cytoplasmic DNA following the downregulation of cytoplasmic DNases [82].

Finally, similar to what was described for senescence-related cell cycle arrest and apoptosis resistance, regulation of the SASP, also requires epigenetic regulation. For instance, IL-6 and IL-8 expression in RS or OIS fibroblasts required a proteasome-dependent degradation of the major histone H3K9 mono- and dimethyl transferases, G9a and GLP following their ubiquitination by the APC/CCdh1 ubiquitin ligase [83]. It was shown that activation of the APC/CCdh1 ubiquitin ligase, was downstream of the DDR effectors Cdc14B- and p21Waf1/Cip1 [83]. Similarly, the H3K27 methyltransferase EZH2 is repressed in senescent cells leading to a decrease in H3K27me3 marks and increased expression of the SASP mediators [84]. In addition to the inhibition of H3K9 methylation, the regulation of SASP

mediators such as IL-6 and -8 induced by IR of human fibroblasts require an increase in histones H3K9 and H4K16 acetylation, which was achieved by removing the histone deacetylase SIRT1 from their promoter [85]. Also, proteins involved in chromatin remodelling such as BRD4 and HMGB2 have been shown to regulate SASP mediator's expression in OIS and RS fibroblasts [86].

Cellular senescence and idiopathic pulmonary fibrosis

As stated earlier, IPF is a disease mainly affecting the elderly, a population in which markers of cellular senescence are common. In this section, we will explore the role of some IPF risk factors as cellular senescence drivers and review published articles focusing on the presence of senescent cells in the lungs of IPF patients as well as on their role in the disease progression.

Telomeres associated genes mutations, telomeres length and senescence in IPF

Genetic mutations in genes involved in telomere functioning are major risk factors in the familial form of IPF, and telomeres attrition is found in around 25% of the sporadic form of the disease [87]. In a study investigating the association between fibrotic lung diseases and mutation in telomeres functioning genes, Newton., *et al*. found telomerase reverse transcriptase (TERT) mutations to be the most frequent one (~ 65%), followed by poly(A)-specific ribonuclease (PARN) mutations (~ 17%), regulator of telomere elongation helicase 1 (RTEL1) mutations (~ 12%), and telomerase RNA component (TERC) mutations (~ 6%) [88]. About 50% had a diagnostic of IPF, followed by unclassifiable lung fibrosis (20%), chronic hypersensitivity (12%), pleuroparenchymal fibroelastosis (10%), interstitial pneumonia with autoimmune features (7%), idiopathic interstitial pneumonia (4%) and connective tissue disease-ILD (3%) [88]. Although patients with TERC mutation was diagnosed earlier than those with PARN mutations (51 ± 11 years versus 64 ± 8 years; $p = 0.03$) and had a higher incidence of haematological comorbidities, there was no significant association between a given mutation and a given ILD. Thus, in 80% of the families studied, similar mutations led to discordant diagnoses suggesting that the development of a specific ILD is due to a combination of genetic and non-genetic risk factors [88].

Telomeres length in IPF patients

Consistently, several studies showed that PBL (Peripheral Blood Leukocytes) from IPF patients presents shorter telomeres than those from age-matched controls [89-92] and that telomeres attrition adversely affect IPF patients survival [91,92]. According to a recent study on a Spanish cohort of sporadic IPF patients, telomeres shortening is especially predictive of poor survival in patients under 60 years of age with associated non-specific immunological or haematological abnormalities [93]. Interestingly, one study showed that in sporadic IPF patients without telomere functioning genes mutations, PBL telomeres length correlated with that of AECII [89]. This result was later confirmed by Snetselaar., *et al.* who showed using lung biopsies from IPF patients, that AEC telomeres attrition correlates with poorer survival [94].

Interestingly, the authors also demonstrated that in contrast with normal lung, AECII from fibrotic areas of IPF lungs have shorter telomeres than surrounding cells and that AECII from non-fibrotic areas have telomeres 56% longer than those of AECII from fibrotic areas [94]. As expected, patients with TERT mutation had even shorter AECII telomeres than those from sporadic IPF patients, whereas no difference could be observed in the telomere length of surrounding cells in these three groups [94]. This suggests that IPF pathogeny mainly exhaust AECII and hematopoietic cells regenerative potential. In line with this idea, Liu., *et al*. found that less than 4% of IPF-derived fibroblasts had telomeres attrition due to an increase in telomerase activity in these cells [95]. However, in this last study, the authors only found telomeres attrition in 6 % of PBL from IPF or chronic hypersensitivity pneumonitis patients [95]. The discrepancy of the results regarding telomeres attrition in PBL between this study and the previous ones cited is not well understood. A further study by McDonough., *et al.* showed that compare to control, IPF lungs had shorter telomeres and increased chromosomal damages [96]. The authors further showed that shorter telomeres were found mainly in areas of significant collagen deposition while chromosomal damages

as revealed by γ-H2A.X staining correlated with areas of profound structural remodelling and was often found in airway epithelial cells [96].

Telomere functioning genes mutations in experimental models of lung fibrosis

Despite these compelling correlations regarding the potential deleterious role of telomeres attrition in IPF pathogeny, studies evaluating the role of telomere functioning proteins in mouse models of lung fibrosis provided more contrasted results. Thus, by inducing lung fibrosis with single or repetitive intratracheal administration of bleomycin in TERT and TERC deficient mice, Degryse., *et al.* observed a reduction of telomere length in PBL and isolated AECII without any worsening of lung fibrosis [97]. In another study using the same model of bleomycin-induced lung fibrosis in TERT and TERC deficient mice Liu., *et al.* found a telomere length independent effect of TERT deficiency on bleomycin-induced lung fibrosis while TERC deficiency had no effect [95]. According to the authors, the discrepancy regarding the effect of TERT deficiency on bleomycin-induced lung fibrosis might be due to strains differences [95]. In sharp contrast with those two studies, Povedano., *et al.* developed two models of lung fibrosis driven by critically short telomeres (i.e. TERT-/- mice) or telomeres dysfunction without shortening (i.e. AECII specific deletion of Trf1) [98]. Interestingly, deletion of the telomere shelterin component Trf1 is associated with persistent DNA damage at telomeres ends rather than telomere shortening, and its deletion in AECII was sufficient to develop lung fibrosis [98]. Data showing stem cells failure and increased death rate in response to bleomycin in mice with a conditional deletion of Trf2 in AECII suggest a similar potential for lung fibrosis modelling [99].

On the other hand, global telomeres shortening in TERT deficient mice of the second and fourth generation did not spontaneously lead to lung fibrosis. To develop a lung fibrosis model driven by telomere shortening in these mice, the authors used the highest dose of bleomycin that does not induce fibrosis in wild-type mice, that is to say, 0.5 mg/kg. Thus, intratracheal administration of bleomycin to these mice at 0.5 mg/kg, (Vs 2.5 mg/kg in some protocols), led to the development of lung fibrosis [98]. These results suggest that a DDR in AECII is critical to the development of lung fibrosis and were confirmed by Naikawadi., *et al.* in a similar model of AECII conditional deletion of Trf1. Thus the authors observed two weeks after Trf 1 deletion an increased γ-H2A.X DNA damage foci, followed by progressive telomere attrition and senescence of AECII leading to lung fibrosis characterised by AECII hyperplasia, accumulation of α-SMA^{+} mesenchymal cells and collagen deposition at nine months [100].

Interestingly, mice with Trf1 deletion in collagen-1a2 expressing cells did not develop lung fibrosis but lung oedema, suggesting that mesenchymal cells senescence is not sufficient to develop lung fibrosis [100]. The repetitive instillation of bleomycin at 0.05 mg/kg in mice with a conditional deletion of Trf1 in collagen-1a2 expressing cells could give greater insight into the role of fibroblasts senescence in lung fibrosis development. Moreover, Povedano., *et al.* showed using a non-integrative AAV9 vector encoding TERT which preferentially targets regenerative AECII that, TERT expression protects mice from bleomycin-induced lung fibrosis by preventing AECII telomeres attrition, DNA damage and senescence [101]. Finally, Liu., *et al.* using a model of TERT deletion restricted to AECII, that these mice do not spontaneously develop lung fibrosis despite evidence of telomere shortening in AECII, but have an increased susceptibility to bleomycin-induced lung fibrosis [102]. Thus, the authors reported that TERT deletion in AECII led to their reduced proliferative potential and their senescence in bleomycin treated mice, supporting a role for AECII senescence in lung fibrosis development [102].

In conclusion, despite compelling pieces of evidence correlating telomere attrition to poor survival in IPF, experimental data are more contrasted. They suggest that telomeres-associated DNA damage rather than telomeres attrition in AECII is the critical determinant of experimental lung fibrosis. The bleomycin-induced lung fibrosis model has been criticised for poorly modelling IPF as it is based on a single acute inflammatory lesion in which fibrosis resolve in the long run [103]. The introduction of models with iterative bleomycin challenges attempted with some success to answer this criticism [104]. However, it is still difficult to model a long-term chronic disease like IPF in which epithelial cells and fibroblasts regenerative potential might be exhausted by decades of replications and exposures to noxious agents. In the future IPF models based on a combination of telomere functioning genes mutation and chronic exposure to IPF external risk factors such as CS, organic and metal dust should provide better insight into the mechanisms of IPF progression. Moreover, data from human hTERT mutation suggest that they affect telomerase activity, telomere length and cell growth by distinct mechanism

[105], suggesting that animal models based on telomere functioning genes deletion might fail to replicate the pathogeny induced by these mutations. Therefore, this is opening the door to personalised medicine and the use of organoid-based IPF models, particularly in the familiar form of the disease.

Cigarette smoking and epithelial cells senescence

In their 2001 International Consensus statement on IPF diagnosis and treatment, Castabel., *et al.* cited cigarette smoking (CS) as a potential risk factor for IPF [106]. The decision to list CS among IPF risk factor was based on previous epidemiological studies showing an increased prevalence of smokers or former smokers among IPF patients [107,108]. The toxicity of CS is due to the inhalation of particulate matter, as well as a multitude of chemicals among which the highly toxic reactive oxygen and nitrogen species [109]. These toxic particles and chemicals affect lung cells by promoting molecular damages (e.g. DNA, lipids) that leads to cellular dysfunction such as senescence and ultimately, death (e.g. apoptosis, necrosis). However, CS has primarily been associated with the development of emphysema, a condition characterised by alveolar wall destruction [110] and COPD, which is characterised by airway obstruction and emphysema [111]. Although CS is not sufficient to induce lung fibrosis or IPF, many studies suggest it can act in conjunction with other risk factors to promote the development of lung fibrosis. For instance, animal experiments showed that CS exposure potentiates lung fibrosis induced by bleomycin instillation [112,113].

Moreover, other studies demonstrated that prolonged exposure of the alveolar epithelial cell (AEC) line, A549 to CSE was able to increase their secretion of TGF-β1 [114]. The same way, in primary rat lung fibroblasts and a human pleural mesothelial cell line, CSE resulted in the activation of the TGF-β1 pathway [113]. The relevance of these results from animal models to human lung response was recently supported by Obeidat., *et al.* who have shown in a transcriptomic study of whole lung tissue, that 48% of the genes differentially expressed in a murine model of CS have also been reported as differentially regulated in the lung of human smokers [115]. Moreover, using RNA sequencing Voic., *et al.* recently compared the transcriptome of human bronchial epithelial cells undergoing RS or exposed to CSE. Out of the 1534 and 599 genes differentially expressed in response to RS and CSE respectively, 243 genes were common to both responses and related to the regulation of ROS, proteasome degradation, and NF-kB signalling [116].

Early studies on the impact of CS on lung bronchiolar and AEC showed that CS was able to induce oxidative stress, DNA damage and $p21^{WAF1/CIP1}$ expression, which was confirmed *in vivo* in a mouse model of CS exposure [117-119]. Moreover, Tsuji., *et al.* showed that AECII from smokers have a 50% decrease in telomere size and that smoking-induced emphysema was associated with a significant increase in $p16^{INK4A}$ and $p21^{WAF1/CIP1}$ positive AECII [120]. Results from an animal model of CS exposure later suggested that p21 could increase CS-induced DNA damage by binding to and inhibiting the DDR protein Poly (ADP-ribose) polymerase-1 (PARP-1) [121]. Wu., *et al.* showed that *in vitro*, CS induces airway epithelial cells senescence, as exemplified by increased β-galactosidase activity, impaired cell proliferation, increased expression of p21, HMGB1 and release of IL-6 and the TGF-β superfamily member, GDF15 [122]. Stimulation of the cultured airway epithelial cells with GDF15 triggered their senescence in an activin receptor-like kinase 1 receptor and Smad1 dependent pathway. Conversely, GDF15 inhibition significantly inhibited CS-induced senescence in these cells [122]. In addition to cigarette smoke, *in vitro* experiments and animal study suggest that silica exposure, another external risk factors for IPF development is genotoxic for small airway epithelial cells potentially by altering shelterin complex leading to DDR and lung fibrosis [123,124].

Cellular senescence in biological samples from IPF patients

One of the difficulties in assessing the presence of senescent cells in diseased human samples is the lack of specific markers. Currently, the visualization of senescent cells *in vivo* as well as *in vitro* relies on the detection of semi-selective markers, such as p16, p21, lipofuscin, β-galactosidase activity and the loss of nuclear High-Mobility Group Box 1 (HMGB1) or Lamin B1 [24].

One of the earliest studies evaluating senescence in IPF was carried out in 1996 by Kuwano., *et al.* which assessed p53 and p21

expression in conjunction with TUNEL staining in 14 lung specimens from IPF patients. This analysis demonstrated in contrast with a healthy lung, TUNEL, p53 and p21 signal in the hyperplastic bronchiolar and AEC of most diseased samples [125]. Further studies also reported increased p21 and β-galactosidase staining in the bronchial epithelial cells lining honeycombs and fibroblastic foci [126,127]. Along with p21 and β-galactosidase staining, the authors also observed an increase in SIRT6 expression. *In vitro* experiments showed that TGF-β induces human bronchial epithelial cells senescence, leading to IL-1β secretion and fibroblasts differentiation into myofibroblasts. Interestingly, SIRT6 overexpression in human bronchial epithelial cells was able to inhibit TGF-β induced senescence by promoting p21 proteasomal degradation [126]. Loman., *et al.* also reported $p16^{INK4A}$ expression in AECII overlying fibroblastic foci but not in the fibroblasts embedded in these fibroblastic foci [128], while Lehmann., *et al.* only reported p16 and p21 positive staining in AEC of IPF samples [129]. In contrast with these results, two other studies observed both p16-positive fibroblasts and epithelial cells in IPF samples [130,131]. In addition to $p16^{INK4A}$ expression, Schafer., *et al.* also observed in alveolar cells from IPF samples an increase in DNA damage foci (i.e. γH2A.X staining) sometimes at telomeres [130]. Moreover, increased DNA damage and shorter telomeres length in alveolar and airway epithelial cells of IPF samples have been shown to correlate with total collagen deposition [96]. Although, both IPF and chronic obstructive pulmonary fibrosis (COPD), have been associated with premature senescence [132], a recent comparative analysis by Okuda., *et al*. of p16, p21 and phosphorylated NF-κB immunostaining on formalin-fixed paraffin-embedded sections from IPF, COPD and CTD-ILD, revealed a higher expression of senescence and SASP markers in IPF samples [133]. As shown in table 1, there is a consensus regarding the expression of senescence markers (i.e. β-galactosidase; p16, p21) by bronchiolar and alveolar epithelial cells in IPF samples, particularly in the cells surrounding honeycombs and overlaying fibroblastic foci. For fibroblasts, on the other hand, only two studies out of the eleven studied reported an increase in p16 expression in fibroblastic foci, and one of them also reported higher p21 expression. However, this latter study only used 2 IPF samples, and the staining was of poor quality. Unlike for epithelial cells, these authors did not use fibroblastic

	IPF vs Healthy Epithelial cells		IPF vs Healthy (Myo)Fibroblasts			
Senescence markers	**Change**	**Epithelial markers/Senescent cells location**	**Change**	**Fibroblasts markers/Senescent cells location**	**N**	**Ref**
p21	↑	NSM/Hyperplastic bronchiolar and AEC.	NR	NSM	?	[125]
p21, β-Galactosidase	↑	NSM/+ cells around HCB and FF.	NR	NSM/β-Galactosidase +/p21- cells in FF	6	[126]
p21	↑	proSP-C for AECII.	→	NSM.	21	[127]
p16	↑	proSP-C; E-cadherin	→	α-SMA/ positive cells in FF	21	[128]
p16, p21	↑	proSP-C; KRT5/7	→	NSM	16	[129]
p16	↑	NSM/positive cells around HCB and FF	↑	NSM/ positive cells in FF	27	[130]
p16, p21*	↑	NSM/positive cells around HCB and FF.	↑	NSM/ positive cells in FF	2	[131]
p16, p21#	↑	NSM.	NR	p16 and $p21^{+}$ cells in FF/interstitium.	8	[133]
p16, p21, β-Galactosidase	↑	NSM/AECII overlying FF	NR	NSM/No positive cells visible	8 -12	[138]
P16, p21, β-Galactosidase	↑	SP-C /+ cells around HCB and fibrotic area	NR	NSM/Few + cells in the interstitium	12	[139]
p21, β-Galactosidase	↑	SP-C /+ hyperplastic AECII	NR	NSM/No positive cells visible	12	[140]

Table 1: *Summary of epithelial and fibroblast cells senescence findings in IPF by Immunohistochemistry.*

*Abbreviations: N: IPF Samples Number; NR: Not Reported; NSM: No Selective Marker Used; FF: Fibroblastic Foci; HCB: Honeycombs; KRT: Cytokeratin; Ref: References; ↑: Increase expression; →: No change in expression; *Very poor p21 staining quality; # Poor p16 staining quality, no display of p21 staining.*

markers to determine the identity of the senescent cells. The discrepancies regarding fibroblasts senescence in IPF lungs can be due to differences in tissue fixation, antigen retrieval procedure and antibodies used. Therefore, further studies using high quality monoclonal or recombinant antibodies directed against fibroblasts and senescence markers should be performed to conclude on the significance of fibroblasts senescence in IPF lung.

Signs of cellular senescence in IPF, however, are not restricted to the lung. Thus Cardenes., *et al.* observed increased senescence markers expression (i.e. p16, p21, β-galactosidase) and reduced proliferation potential in bone marrow-derived mesenchymal stem cells from IPF patients [134]. In contrast, Gao., *et al.* reported an increased expression of the senescence-associated long non-coding RNA TERRA in the PBL of IPF patients which was inversely correlated with the percentage of predicted forced vital capacity [135]. Besides, earlier this year, Barnes., *et al*. reported a decrease in circulating levels of the 'age-suppressing' hormone α-klotho in IPF patients compared to age-matched controls. Lastly, other authors have reported signs of immunosenescence in IPF patients. Thus Gilani., *et al.* observed in the blood of IPF patients a significant increase in the proportion of $CD28^{null}$ $CD4^{+}$ T lymphocytes compared to age-matched healthy patients. Moreover, CD28 downregulation was associated with a poor prognosis [136]. Interestingly, CD28 downregulation is regarded as a reliable marker of age-related decline in immunocompetence and correlates with a decrease in telomere length and human telomerase RNA component (hTERC) expression [137].

In the next section, we will review published data on mechanisms supporting cellular senescence in IPF lung fibroblasts and epithelial cells.

Epithelial cells senescence in IPF and experimental lung fibrosis

Repetitive micro-injuries to the bronchiolar and alveolar epithelium are believed to be the triggering events in IPF pathogeny. AECII senescence is believed to results from the long-term aggression of the lung epithelium and is a critical event in experimental lung fibrosis induced by radiation exposure [141]. Many *in vitro* studies have been undertaken to determine the mechanisms inducing epithelial cells senescence in IPF.

Role of the miR-34 family of miRNA in IPF lungs epithelial cells senescence

In ILD, the analysis of senescence markers (i.e. p16, p21, p53) expression by AECII, revealed their expressions were higher in IPF compared to other ILD [138], a phenotype that could be, regarding p53 expression, driven by the upregulation in these cells of the apoptosis-inducing ligand TRAIL and its cognate receptors DR4 and DR5 [127]. In particular β-galactosidase activity was found in 23.1% of AECII isolated from IPF samples compared to 1.2% in other ILD [138]. The authors also found in AECII from IPF patients a significant increase in the expression of several senescence-associated miRNAs (i.e. miR-34a, miR-34b, and miR-34c) and a decreased expression of their target mRNAs (E2F1, c-Myc, Cyclin E2) [138]. Interestingly, selective overexpression of miR-34a, miR-34b, and miR-34c in the AECII cell line A549, resulted in a significant increase in β-galactosidase activity [138]. Moreover, Cui., *et al.* showed that AEC miR-34a expression was increased in aged mice and further increased in fibrotic lungs [142]. Using the bleomycin-induced lung fibrosis model, in miR-34a deficient mice, they showed that miR-34a deficiency abrogated lung fibrosis, AEC senescence, apoptosis, and mitochondrial dysfunction by restoring in AEC the expression of 'anti-ageing' molecules such as Sirt1, E2F1 and Cyclin E2 [142]. Importantly bleomycin had previously been shown to induce AECII senescence *in vitro* [143]. Shetty., *et al.* further demonstrated in the bleomycin-induced lung fibrosis model the existence of positive feedback between miR-34a induction, p53 acetylation and PAI-1 expression. Indeed miR-34a inhibits the deacetylase Sirt1, involved in p53 deacetylation and expression, leading to the accumulation in IPF AEC of acetylated p53 and further miR34a expression [144]. Moreover, the authors found that bleomycin failed to induce miR-34a expression in the lungs of p53 or PAI-1 deficient mice, while miR-34a conditional knock-out in AEC prevented bleomycin-induced AEC injury, p53 acetylation, PAI-1 expression and lung fibrosis [144].

Role of the plasminogen activator inhibitor 1 (PAI-1) in IPF lungs epithelial cells senescence

A body of evidence suggests that PAI-1 could be involved in AEC senescence and lung fibrosis. Indeed, early studies revealed increased PAI-1 expression in the BALF of IPF patients [145]. Human and experimental data suggest that alveolar macrophages (AM) and epithelial cells are the primary source of BALF PAI-1 with macrophages being the leading source in healthy tissue while epithelial cells contribution increases in experimental fibrosis [146]. This idea is supported by immunohistochemistry and western blot analysis of IPF samples showing increased PAI-1 expression in AECII [144,147,148], paralleled by a decreased uPA expression in these same cells [148]. Using a combination of *in vivo* and *in vitro* models of PAI-1 inhibition in AECII, Jiang., *et al*. showed that PAI-1 induces AEC senescence through activation of the P53-p21-RB pathway in bleomycin-induced lung fibrosis [146]. Interestingly, *in vivo* and *in vitro* data, suggest that p53 binding to the 3' untranslated region of uPA, uPAR and PAI-1 mRNA, is required for bleomycin-induced inhibition of uPA and uPAR expression and stimulation of PAI-1 expression [149]. Further investigations revealed that PAI-1 induction in AECII is required for TGF-β1-induced senescence in a mechanism involving $p16^{INK4A}$ [150]. Moreover, the SASP mediators present in their conditioned medium promoted a profibrotic AM phenotype through the cytokines IL-4 and IL-13 [150]. A recent study, using a model of IR-induced lung fibrosis suggest that arachidonate 12-lipoxygenase (12-LOX) downstream products play a crucial role in AECII senescence, profibrotic cytokines secretion (i.e. IL-4, IL-13) and the generation of a profibrotic macrophages phenotype [151]. Whether there is in these cells, crosstalk between PAI-1 and 12-LOX in responses to senescence inducers is not known. In a mouse model of lung fibrosis induced by targeted injury of AECII, PAI-1, derived from injured AECII, was shown to favour the accumulation of $CD11b^{+}$ exudate macrophages that also produce PAI-1 in addition to type-1 collagen [152]. Moreover, global PAI-1 deficiency in this model inhibited fibrosis development [152]. The same way, in the accelerated ageing model of Klotho-deficient (kl/kl) mice, PAI-1 deficiency was associated with delayed senescence and impairment of organ structure and function resulting in increased life-span [153]. In addition to p53, data from IPF-derived fibroblasts suggest that PAI-1 expression in these cells is regulated by AKT activation [154]. In turn, experimental data showed that cigarette smoke-induced AKT activation in AECII is inhibited in p53 deficient mice and a lesser extent in PAI-1 deficient mice [155].

Role of PTEN loss and AKT activation in IPF lungs epithelial cells senescence

Recent pieces of evidence suggest a role for PTEN loss in IPF patients' AEC senescence. Indeed Tian., *et al.* observed by immunohistochemistry on formalin-fixed paraffin-embedded sections of IPF samples a loss of PTEN and an increased expression of $p21^{WAF1}$, $p16^{INK4A}$, activated NF-κβ and β-galactosidase activity in the alveolar epithelium [139]. These data are conflicting with a previous report by Xia., *et al*. showing a loss of caveolin and PTEN expression in IPF-DF but not in AEC [156]. Using bleomycin to induce A549 cells senescence, the authors further showed that PTEN loss in these cells exacerbates NF-κβ activation and senescence marker expression (i.e. p16, p21) induced by bleomycin [139]. Using immunohistochemistry staining and western-blot analysis of IPF samples, Qiu., *et al.* further showed that alveolar epithelial PTEN loss correlated with AKT activation (i.e. P-AKT473), a pattern reproduced in A459 cells *in vitro* by bleomycin treatment [140]. Interestingly, PTEN knockdown resulted in increased AKT activation independently of bleomycin treatment and was further increased in bleomycin-induced senescence, whereas its overexpression had the opposite effect [140]. Finally, pharmacological inhibition of AKT resulted in decreased bleomycin-induced senescence of A549 cells [140].

Role of mitochondrial dysfunction in IPF lungs epithelial cells senescence

In a 2015 article, Bueno., *et al.* showed that AECII from IPF lungs presented mitochondrial abnormalities (e.g. depolarisation, enlargement) associated with low expression of PTEN-induced putative kinase 1 (PINK1), a protein involved in mitophagy [157]. As PINK-1 is induced by PTEN [158] its downregulation in IPF lungs, AEC supports a decreased PTEN activity in these cells. Moreover, knock-out of PINK1 in AEC resulted in dysfunctional mitochondria and expression of pro-fibrotic mediators (i.e. TGF-β, FGF2) *in vitro*; while *in vivo*, its deletion aggravates lung fibrosis development induced by MHV68 infection in mice [157]. The authors showed that PINK1 expression by AEC was downregulated by the ER-Stress inducer Tunicamycin in a dose-dependent manner suggesting a causal link between ER stress, PINK1 repression and mitochondrial dysfunction in IPF [157]. Moreover, induction of ER-Stress by Tunicamycin treatment in the AECII cell line A549, resulted in an increased expression of senescence markers (i.e. p16, p19 and p21) [159]. Interestingly, many studies have highlighted the expression of ER stress markers by epithelial cells in IPF [160]. ER stress in AEC is believed to be driven by surfactant proteins mutations which prevent their proper folding, precipitating an unfolded protein response [160]. However, other IPF risk factors such as cigarette smoke, silica particulates or viral infections have been shown to induce ER stress in these cells [160]. The authors later identified the transcription factor ATF3 as an essential downstream mediator in ER stress-induced-PINK1 transcriptional repression [159]. Indeed, ATF3 expression in A549 cells resulted in reduced PINK1 expression increased production of mitochondrial

ROS as well as upregulation of ER Stress, fibrotic (i.e. Col1a1, Fibronectin, FGF2) and senescence (i.e. p21) markers [159]. Moreover, ATF3 was found to be overexpressed in IPF lungs AEC, and its conditional deletion in AECII protected mice from bleomycin-induced lung fibrosis [159]. Interestingly, ATF3 is also induced by other factors relevant to cellular senescence and IPF pathogeny, such as DNA damages and ROS. Moreover, ATF3 has been shown to promote cell stress-induced senescence, by regulating the transcription factors binding protein, id1 which control p16 expression [159]. In a third article, Bueno., *et al.* showed *in vitro,* that PINK1 deficiency in A549 cells led to mitochondrial DNA (mtDNA) oxidation and releases. The authors also reported increased mtDNA damage in IPF lungs, as well as an increased level of mtDNA in the plasma and BALF of IPF patients [161]. Finally, PINK1 deficiency and ER-stress was associated *in vivo* and *in vitro* with increased TLR9 expression. Interestingly, human precision lung slices stimulated with mtDNA, released a higher level of TGF-β compare to non-treated slices. This effect was further increased in response to mtDNA from old donors which was more oxidized. Finally, the authors showed that precision lung slices from TLR9 KO mice failed to release TGF-β in response to mtDNA from WT or PINK1 KO mice [161]. As too often in the scientific literature, the level of PINK1 expression is the subject of controversy, as another team, observed increased PINK1 protein level (i.e. WB, IHC) in IPF lung [162]. However, similar to what was found by Bueno., *et al.* in a different model, the authors showed that PINK-1 deletion exacerbated bleomycin-induced lung fibrosis due to a loss of protection against TGF-β-induced AECII cells death [162]. Moreover, another study showed that the anti-fibrotic and AEC protective effect of thyroid hormone treatment in the bleomycin-induced lung fibrosis model was lost in PINK1 KO mice suggesting it has a protective role in stress-induced AEC senescence and lung fibrosis.

In addition to PINK1, Parkinson Disease 2 (PARK2), another key player involved in mitophagy is inhibited in COPD lungs bronchial epithelial cells. Moreover, PINK1 or PARK2 inhibition in these cells exacerbated CSE induced-mitochondrial ROS production and senescence in these cells [163]. However, decreased PARK2 expression in IPF lungs AEC has not been reported. Further studies are required to determine the evolution of PARK2 expression in IPF lungs epithelial cells in particular bronchiolar and AECII.

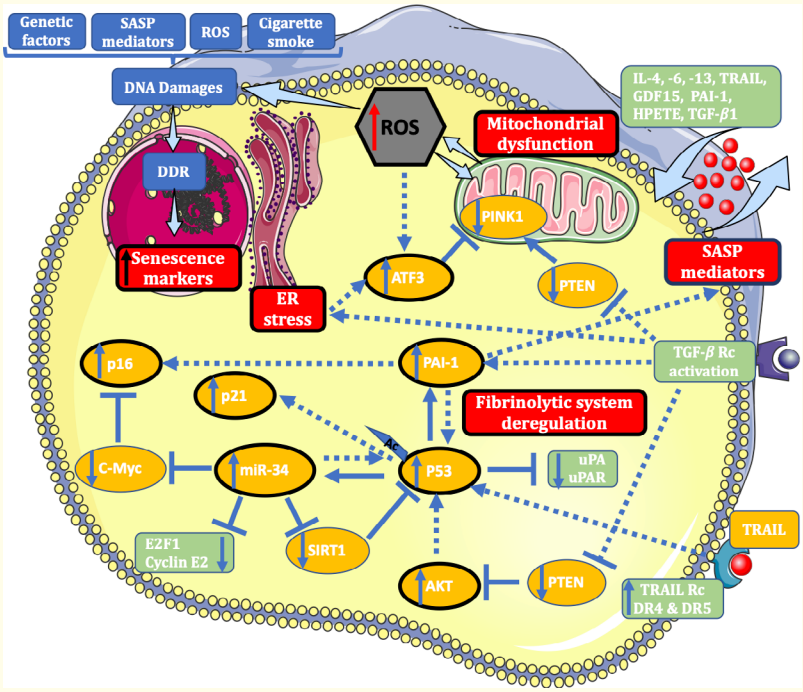

Figure 2: *Summary of the molecular changes supporting AEC senescent phenotype in IPF.*

In IPF, a combination of genetic factors, extracellular ROS, senescence-associated mediator milieu, and cigarette smoke particles may cause AEC DNA damages, leading to a DDR and increased expression of senescence markers (i.e. p16, p21 β-galactosidase) and p53. In addition to some of the pathways presented in figure 1, epithelial cells from IPF lung present an ER stress caused by various factors (e.g. surfactant protein mutation, cigarette smoke) which stimulate the expression of the transcription factor ATF3, the subsequent inhibition of PINK1 expression and impaired mitophagy. As a result, increased mitochondrial dysfunction lead to increased mitochondrial ROS production, which in turn increases DNA damage and cellular senescence. ER stress can also be induced by TGF-β stimulation whose sustained stimulation inhibits the PINK1 inducer PTEN accentuating mitophagy impairment and mitochondrial dysfunction. Another important factor in epithelial cell senescence in IPF is the activation of p53 by DNA damages, but also mediators such as TRAIL, PAI-1, and signalling molecule such as AKT whose activity is increased by PTEN inhibition. p53 activation support PAI-1 and miR-34 expression leading to further p53 activation and post-translational modifications. miR-34a inhibition of SIRT1 favour p53 acetylation and activity, resulting in p21 expression and inhibition of the fibrinolytic system proteins uPA and uPAR. On the other hand, miR-34 favour p16 expression by inhibiting its inhibitor c-Myc, while also favouring cell cycle arrest by inhibiting E2F1 and cyclin E2 expression. Finally, TGF-β-induced epithelial cells senescence has recently been shown to require PAI-1 expression and subsequent activation of p16 leading to a profibrotic SASP including the pro-fibrotic cytokines IL-4 and IL-13.

Lung fibroblasts senescence in IPF

In addition to the detection of p16 in fibroblasts from IPF lung samples, various authors cultured IPF samples-derived fibroblasts (IPF-DF) and age-matched healthy tissue-derived fibroblasts (HT-DF) to study, their expression of senescent markers, the signalling pathways supporting their expression, and the impact of fibroblasts senescence on other cells types phenotype and fibrosis development.

Senescence-associated cell cycle arrest

In the late 1980s, several research teams cultured IPF-DF with contrasting results regarding their proliferative potential. Whereas Jordana., *et al*. observed a faster proliferation of fibroblasts from fibrotic tissues [164], Raghu., *et al*. found a faster proliferation of fibroblasts from early fibrotic areas whereas fibroblasts from dense fibrotic areas had slower growth rates compared to HT-DF [165]. A more recent study by Ramos., *et al*. concluded to a significantly lower growth rate of IPF-DF compare to HT-DF, and found in these cells increased expression of myofibroblast markers such as α-SMA, pro-α1-collagen, gelatinase B, and various tissue inhibitors of metalloproteinases (TIMPs) [166]. The discrepancies regarding IPF-DF proliferation might be due to many factors such as the lack of consensus regarding IPF diagnosis at the time, the heterogeneous pattern of lesions characterising IPF tissues, and technical aspects of fibroblasts recovery (e.g. Enzymatic vs non-enzymatic technics). In recent work, Alvarez., *et al*. showed that compared to HT-DF from age-matched controls, IPF-DF had increased expression of senescence markers such as β-galactosidase, p21, p16, p53, SASP as well as decreased in proliferation and apoptosis rate [167]. The authors further identified shorter telomeres, mitochondrial dysfunction, and transforming growth factor--induced endoplasmic reticulum stress (ER-Stress) as likely contributors to IPF-DF senescence [167].

Senescence-associated oxidative stress and mitochondrial dysfunction

Earlier work by Yanai., *et al*. showed that compared to HT-DF, cultured IPF-DF presented premature RS, high resistance to oxidative-stress-induced-cytotoxicity and cellular senescence, an early senescent-like morphology, and accumulation of α-SMA$^+$ senescent myofibroblasts [168]. In addition to apoptosis resistance, ROS dependent signalling supported other aspects of the IPF-DF phenotype such as their increased α-SMA, Collagen I expression as well as their reduced proliferation rate [169]. However, the authors in this study did not observe increased β-galactosidase activity in IPF-DF compared to HT-DF and failed to assess other senescence markers. The increased ROS production in IPF-DF was later attributed to their higher expression level of NADPH oxidase-4, aka NOX4 and their failure to activate the Nrf2 (NFE2-related factor 2) antioxidant response as suggested by immunostaining of IPF and aged mice lung fibrosis samples [131]. In addition to NOX4, another study pointed to mitochondrial dysfunction mediated by the mammalian target of rapamycin complex 1 as a

source of superoxide production, DNA damage and SASP mediators (i.e. IL-6, CCL2, CCL5 and IGFBP5) production by IPF-DF [170]. Using a model of oxidative stress-induced cellular senescence, Waters., *et al*. identified the IL-6-induced transcription factor STAT3, as a molecular bridge between oxidative-stress and mitochondrial dysfunction, as STAT3 inhibition in this model reduced, mitochondrial dysfunction, p21 level, β-galactosidase activity and IL-6 secretion [171]. In human IPF samples, constitutive STAT3 phosphorylation has been observed in a subset of fibroblasts characterised by a lower proliferation rate and decreased expression of α-SMA, Thy-1/CD90 and β3 integrins [172]. In this study, STAT3 inhibition was found to diminish IPF-DF resistance to Staurosporine-induced apoptosis and responsiveness to TGF-β1 [172]. In addition to STAT-3-mediated mitochondrial dysfunction, Caporarello., *et al*. showed in IPF-DF, a repression of the central mitochondrial biogenesis regulator PGC-1α, resulting in reduced mitochondrial mass and function, enhanced myofibroblastic (i.e. α-SMA, Collagen I, Fibronectin), and senescence markers expression as well as SASP mediators secretion [173]. Reduced PARK2 expression has also been observed in IPF lungs FF, and mice deficient in PARK2 showed exacerbated lung fibrosis following intratracheal administration of bleomycin [174]. Moreover, the authors identified PARK-2 inhibition-mediated mitochondrial ROS production as a critical event in PDGFR receptor activation, subsequent PI3K/AKT activation and myofibroblastic differentiation of primary lung fibroblasts [174].

Senescence-associated apoptosis resistance

Although undergoing premature RS *in vitro*, the resistance of IPF-DF to oxidative stress-induced cellular senescence is consistent with earlier studies highlighting in these cells an excess ROS production due to the activation of an NADPH oxidase-like system and, resistance to oxidative-stress induced cell death [169]. Consistent with a critical role of ROS in lung fibroblasts senescence programme, moderate hyperoxia has been shown to induce senescence in developing human lung fibroblasts [175]. IPF-DF resistance to apoptosis, however, is not restricted to oxidative stress-induced apoptosis, as another study reported resistance to genotoxic stress (i.e. cisplatin-induced cell death), due to a reduced expression of γH2A.X, PUMA, and caspase 3/7 activity coupled to an increased activity of the DNA repair protein XRCC1 as a result of CK2 hyper-activation [176]. However, other DNA repair actors such as DNA protein Kinase catalytic subunit (DNA-PKcs) are downregulated in IPF mesenchymal progenitor cells and their fibroblasts progeny contributing to their senescence [177]. Besides, Im., *et al*. also showed that IPF-DF are resistant to FasL mediated apoptosis due to abnormally high AKT activation and subsequent overexpression of the FasL inhibitor decoy receptor 3 (DcR3) [178]. This high AKT activity in IPF-DF, correlates with suppression of its inhibitor PTEN, as revealed by immunohistochemistry staining of IPF lung sections [179]. Research by Xia., *et al*. suggest that the decreased PTEN activity in IPF-DF result from reduced expression of the integral membrane protein caveolin-1 [156]. IPF-DF could also resist TRAIL-induced apoptosis, as other studies showed by immunohistochemistry staining and qPCR that in addition to FAS, IPF-DF expresses lower levels of the TRAIL receptors DR4 and DR5 [180,181]. However, Akram., *et al*. observed by immunohistochemistry an increase in TRAIL and its cognate receptors DR4 and DR5 in both AEC and fibroblasts, although p53 expression in fibroblasts was lower than in AEC, suggesting a resistance of the later to TRAIL-induced apoptosis [127]. In addition to FASL mediated apoptosis, the high AKT activation in IPF-DF results in higher mTOR activation and resistance to type one collagen matrix-induced cell death [182], a process essential to terminate wound healing and prevent fibrosis [183]. AKT activation has also been involved in IPF-DF resistance to plasminogen-induced apoptosis. Indeed, Chang., *et al*. showed that IPF-DF expressed an increased level of secreted protein acidic and rich in cysteine (SPARC), the fibrinolytic system member plasminogen activator inhibitor-1 (PAI-1), and active β-catenin [154]. It was shown that SPARC controlled IPF-DF PAI-1 expression level, through AKT activation-dependent, GSK-3β inhibition and β-catenin accumulation. Both SPARC and β-catenin inhibition reduced IPF-DF resistance to plasminogen-induced apoptosis [154]. In contrast with Chang., *et al*. report, a more recent publication by Marudamuthu., *et al*. observed a decreased PAI-1 expression in IPF-DF accompanied by an increase uPA at both the mRNA and protein level [147]. Moreover, in their article Chang., *et al*. claimed that both HT-DF and IPF-DF start to show signs of senescence at passage nine (Data not shown) without disclosing the markers used to assess it. Moreover, they failed to detect any difference in proliferation and survival rate between HT-DF and IPF-DF. As shown in table 2, most studies on IPF-DF senescence, reported increased expression of senescence markers in these cells. However, it is essential to notice that in many of them, the senescent phenotype of IPF-DF was weak in freshly isolated cells but increased more dramatically compared to HT-DF in response to TGF-β [167], H_2O_2 [168],

or serial passages [168,180]. Thus, the method of fibroblasts isolation can influence the detection of senescence in the early passages, as fibroblasts isolated by passive chemoattraction would have proliferated much more than fibroblasts isolated through enzymatic digestion.

Healthy Tissue (HT) vs IPF-Derived Fibroblast					
Senescence markers	**Technics**	**Change**	**Fibroblastic markers**	**IPF-DF cultures**	**Ref**
β-Galactosidase*, p16, p21	β-Galactosidase assay, Western-blot.	↑	Phalloidin, α-SMA, Col1a1.	12	[167]
β-Galactosidase#	β-Galactosidase assay.	↑	α-SMA	5	[168]
β-Galactosidase	β-Galactosidase assay.	→	α-SMA, Col1	7	[169]
β-Galactosidase, p21, p16	β-Galactosidase assay, Real-time PCR.	↑	α-SMA, Col1	13	[170]
p16, p21$	Real-time PCR.	↑	NSM	3 - 4	[180]
p21	Real-time PCR.	↑	α-SMA	12 - 14	[198]

Table 2: *Summary of IPF-DF senescence findings in vitro.*

*Abbreviations: Ref: References; NSM: No selective marker used; * In response to TGF-β stimulation; # Very week signal at early passages, increases at passage 13, no comparison with HF-DF at this passage; $: Premature senescence of IPF-DF between passage 7-8.*

Senescence-associated secretory phenotype (SASP)

As stated earlier, cellular senescence can be strengthened and triggered in neighbouring cells (i.e. secondary senescence) by SASP mediators such as IL-6, IL-1 and TGF-β. Besides, Schafer., *et al*. demonstrated that the SASP mediators present in the conditioned medium of IMR90 fibroblasts made senescent by irradiation (10Gy), had a profibrotic effect on naïve IMR90 recipient cells [130]. In a recent article, Wiley., *et al*. observed that in IPF samples, about 50% of the 5-lipoxygenase (ALOX5) positive cells also express the senescence marker $p16^{INK4A}$ [184]. ALOX5 is a critical player in the production of the pro-inflammatory and profibrotic mediator leukotrienes [184], which was shown to be consistently part of the SASP mediators induced by various stress in different cell types including fibroblasts [184]. Interestingly, ALOX5 inhibition in senescent fibroblasts abrogated the pro-fibrotic effect of their SASP conditioned medium on naïve fibroblasts [184]. Moreover, pharmacological or genetic ablation of senescent cells in experimental fibrosis reduced bronchoalveolar lavage fluid (BALF) level of cysteinyl leukotrienes and the overall lung fibrosis [184]. On the other hand, cyclooxygenase-2 (COX-2), the rate-limiting enzyme in the synthesis of prostaglandins, is induced in senescent fibroblasts [185]. However, IPF-DF have been shown to have diminished basal expression of COX-2 and failed to induce its expression or synthesize prostaglandins E2 (PGE2) in response to inducers such as IL-1 and LPS [186], suggesting an imbalance in pro- and anti-fibrotic eicosanoids production in senescent fibroblasts from fibrotic lungs. Interestingly, Feng., *et al*. recently demonstrated that citrus alkaline extracts prevented bleomycin-induced lung fibrosis and inhibited fibroblasts senescence *in vitro* through a COX-2 dependent inhibition of p53 [187]. These results are consistent with studies reporting an exacerbation of bleomycin-induced lung fibrosis in mice with reduced lung expression of PGE2 due to COX-2 [188,189], Microsomal prostaglandin E2 synthase-1 [190], GM-CSF [191] deficiency, or PGD2 due to conditional deletion of the prostaglandin-D synthase in hematopoietic cells [192]. However, Zhang., *et al*. showed that administration of the COX-2 and soluble epoxide hydrolase (sHE) dual inhibitor PTUPB (i.e. from J7 to end at J21) prevented bleomycin-induced lung cell senescence and fibrosis *in vivo*; while preventing bleomycin-induced AEC (i.e. A549) senescence *in vitro* [193]. Unfortunately, the effect of PTUPB on fibroblasts senescence or the secretion of the different prostaglandins are not reported in this article. Further studies are required to understand the specific function of the different eicosanoids and their receptors in AEC and fibroblasts senescence during fibrosis.

In addition to eicosanoids, recent evidence suggests a role for the cytokine IL-18 in IPF. Previous reports showed increased IL-18 expression in the blood, BALF, sputum, and lung tissue of IPF patients, as well as increased expression of the IL-18R by additional lung cells type in IPF [194,195]. Moreover, IL-18 inhibition by intraperitoneal administration of its inhibitor IL-18 binding protein (IL-18BP), during bleomycin-induced lung fibrosis, reduced collagen deposition and TGF-β1 expression [196]. Data recently published by Zhang., *et al*. suggest that IL-18 contribution to IPF and experimental lung fibrosis could be mediated by fibroblasts senescence. Indeed, IL-18 stimulation triggered primary lung fibroblasts senescence, and the release of a pro-fibrotic SASP conditioned medium [197]. IL-18 treatment was found to downregulate the anti-senescence protein klotho, whose overexpression in primary lung fibroblasts blocked, IL-18-dependent senescence and SASP of primary lung fibroblasts [197].

Role of senescent fibroblasts in lung fibrosis

Although IPF-DF resistance to apoptosis might increase their number and promote chronic pulmonary inflammation and fibrosis, one study by using mice deficient in the pro-senescence miRNA miR-34a suggest that, *in vivo*, fibroblasts senescence might protect against lung fibrosis by favouring an antifibrotic (i.e. M1) macrophage phenotype and promote ECM degradation through MMP secretion [198]. This unexpected result might be because, in IPF, fibroblasts become senescent after an extended period of proliferation before acquiring a SASP triggered by a mix of RS and SIPS. Therefore, their contribution to the diseases in the late stages combines a quantitative and qualitative aspect. By preventing fibroblast senescence and favouring their proliferation, the quantitative aspect in this model of miR-34a might overcompensate for the lack of SASP (i.e. Qualitative aspect). Lastly, even though miR-34a is expressed by numerous pulmonary cells type including AECII [138], the model of miR-34a deficiency is not restricted to fibroblasts suggesting that the results might be attributed to effects in others cells type. More importantly, Cui., *et al*. showed using a mouse model of lung fibrosis induced by bleomycin administration to young and old mice, that miR-34a did not increase in the fibroblasts from fibrotic old lungs as it did in AEC despite a global increase in the fibrotic lung [142]. These data are consistent with the authors own data showing a modest increase (1.5 fold <) in miR-34a expression in IPF-DF while in total IPF lung tissue miR-34a expression increases around three-fold compared to healthy lung tissue [198].

In another study, kanaji., *et al*. showed that human foetal and adult lung fibroblasts respond differently to prolonged cigarette smoke extracts (CSE) exposure, with 50 and 33% of cells respectively becoming senescent while the remaining ones become resistant to CSE-induced senescence. Interestingly, the authors found that fibroblasts resistant to CSE-induced senescence developed a stronger profibrotic phenotype characterised by higher TGF-β1 production, migration proliferation and collagen gels contraction. Thus, the authors suggested a differential involvement of both populations in emphysema/COPD and IPF [199]. However, the authors failed to consider the proliferation gap between both populations. Thus, both cells population were separately recovered at day 14 after the start of CSE exposure, and collagen contraction was assessed at day 14 or 19, while TGF-β1 secretion was measured at day 16. The proliferation of CSE-induced senescence resistant fibroblasts between day 14 and 16 or 19 might account for their better performance during the assessment of their fibrogenic potential [199].

In conclusion, there are scarce pieces of evidence of fibroblasts senescence in the lung of IPF patients. However, *in vitro* studies suggest a pre-senescent phenotype of these cells, which is supported by their premature senescence in culture compared to fibroblasts from the lungs of healthy age-matched donors. These studies confirmed their reduced proliferative capacity, resistance to apoptosis and secretion of SASP mediators. The pathologic nature of senescent fibroblasts and their contribution to lung fibrosis and IPF need to be further addressed to determine whether their overall impact leans toward fibrosis development or limitation.

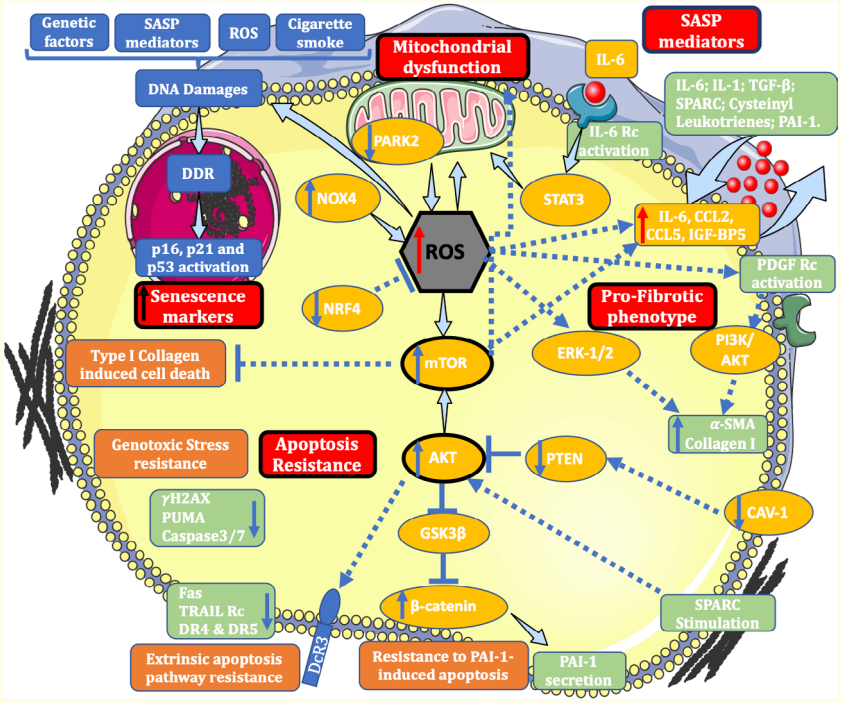

Figure 3: *Summary of the molecular changes supporting senescent fibroblasts phenotype in IPF*

In IPF, a combination of genetic factors, extracellular ROS, senescence-associated mediators milieu and cigarette smoke particles may cause lung fibroblasts DNA damage, leading to a DDR and increased expression of senescence markers (i.e. p16, p21; β-galactosidase) and p53. In addition to the pathways presented in figure 1, the presence in the surrounding milieu of SASP mediators such as IL-6 can activate through its receptor the transcription factor STAT3, which in conjunction with PARK2 inhibition contribute to mitochondrial dysfunction and increase ROS production, ROS accumulation in senescent IPF-DF is also favoured by an increased expression of the ROS producing enzyme NADPH oxidase-4 and a failure to activate the transcription factor NRF2 involved in the expression of antioxidant proteins. The accumulated ROS contributes to further DNA damage and perpetuation of senescence, while also contributing to the activation of mTOR and its associated complex mTORC1, which further contributes to mitochondrial dysfunction. ROS and mTOR/mTORC1 have been shown to regulate the expression of SASP mediators (e.g. IL-6) while ROS also favour IPF-DF expression of myofibroblastic markers such as α-SMA and collagen I through PDGF receptor activation and subsequent PI3K/AKT signalling as well as ERK1/2 signalling. IPF-DF also present a decreased activity of the AKT inhibitor PTEN contributing to high AKT activity. AKT activation further stimulates mTOR and contribute in this way to IPF-DF resistance to collagen I binding-induced apoptosis. Autocrine activation by SPARC is involved in AKT activation and subsequent PAI-1 expression through the inhibition of GSK3β and β-catenin accumulation. Moreover, AKT activation stimulates the expression of the FASL inhibitor DcR3, which coupled with a lower expression of FAS and the cognate TRAIL receptors DR4 and DR5 confers IPF-DF resistance to the extrinsic apoptotic pathway. Lastly, IPF-DF are also resisting genotoxic stress supposedly as a result of a decreased expression of the DDR protein γH2AX and pro-apoptotic proteins PUMA and caspase3/7.

Targeting cellular senescence in IPF

In recent years, senescent cells have attracted much interest as therapeutic targets in various ageing-associated diseases such as osteoarthritis, atherosclerosis, COPD and IPF [24]. This is due to their accumulation in numerous diseased tissues as well as their ability to induce the senescence of neighbouring cells through the release of SASP mediators and subsequent 'bystander effect' [23]. Moreover,

recent animal experiments suggest an overall benefit of senescent cells elimination on ageing-associated disorders [200,201], including COPD [202]. These observations prompted the quest for molecules able to selectively or preferentially eliminate senescent cells (i.e. senolytics) or inhibit the SASP (i.e. senomorphics) [22,203].

The dasatinib/quercetin combination

Thus, Zhu., *et al*. using senescent cells transcript analysis, found a network of upregulated pro-survival genes in which they identified key nodes such as ephrin (EFNB1 or 3), PI3Kδ, BCL-X_L, or PAI-2. Inhibition of these key senescence actors by siRNA resulted in senescent cells death, with minimal effect on proliferating or quiescent differentiated cells [204]. From a screen of 46 compounds inhibiting the targets of interest, the authors identified the multiple tyrosine kinase inhibitors Dasatinib and the natural flavonol with kinases (e.g. PI3K) and serpins inhibitory function Quercetin as potential senolytic drugs [204]. They further showed that Dasatinib was able to eliminate senescent fat cells while Quercetin was more effective in eliminating senescent human endothelial cells and bone marrow-derived mesenchymal cells [204]. The combination of both molecules effectively eliminated senescent MEF *in vitro* and senescent cells from various mouse models of ageing (e.g. progeroid $Ercc1^{-/\Delta}$ mice) or SIPS (e.g. radiation-exposed mice) [204]. Using this combination, Schafer., *et al*. successfully killed IR-induced senescent primary human fibroblasts, and etoposide-induced senescent IMR-90 fibroblasts [130]. Moreover, they showed that administration of the Dasatinib/Quercetin combination (DQC) in the bleomycin-induced lung fibrosis model had similar effects (i.e. Inhibition of SASP mediators, BALF cells recruitment, lung compliance loss) than senescent cells clearance by the AP20187-mediated suicide gene activation (i.e. Ink-Attac mouse model) [130]. Lehmann., *et al*. using the DQC showed its ability to eliminate cultured senescent AECII derived from the lung of bleomycin-treated mice, resulting in decreased fibrotic markers expression and increased epithelial cells markers in the culture [129]. Using a three-dimensional lung tissue cultures system (3D-LTCs) that more closely model the 3D environment and multicellular interactions of the lung, the authors showed that the DQC treatment of 3D-LTCs derived from bleomycin-treated mice, decreased senescence and fibrotic markers expression while increasing apoptosis and epithelial markers [129]. As discussed earlier, the persistence of apoptosis-resistant, senescent fibroblasts/myofibroblasts in IPF, might contribute to the perpetuation of a pathologic repair process in this disease. Interestingly, Hohmann., *et al*. recently showed that Quercetin treatment of IPF-DF abolished their resistance to FasL- and TRAIL-induced apoptosis by increasing their expression of FASL, caveolin-1 while inhibiting AKT activation [180]. Moreover, Quercetin administration in the bleomycin-induced lung fibrosis model, prevented fibrosis development, mortality, weight loss, as well as the accumulation of senescent cells (i.e. $p16^+$ and $p19^+$ cells) and SASP mediators [180]. Recently, Lewinska., *et al*. used Quercetin surface functionalised Fe_3O_4 nanoparticles to treat human foreskin fibroblasts made senescent by H_2O_2 exposure. They found that Quercetin coated nanoparticles eliminated senescent fibroblasts by a non-apoptotic cell death mechanism, and reduced IL-8 and Interferon-β secretion by activating the AMP-activated protein kinase pathway [205].

Finally, 2018 saw the first-in-human, open-label, pilot study using senolytic drugs, that is to say, the DQC for the treatment of IPF. Fourteen patients received 100 mg/day Dasatinib and 1250 mg/day Quercetin, three days a week for three weeks, and their pulmonary and physical functions were evaluated one to two weeks after the last dose [206]. The study showed that the combination therapy was well tolerated by patients, but did not significantly improve lung function [206]. A modest improvement in physical function as assessed by, 6-min walk distance, 4-m gait speed, timed chair stands, and Short Physical Performance Battery score was observed [206]. However, no analysis of senescent cells elimination in the blood or lung tissues of these patients was performed, and the level of circulating SASP mediators did not change significantly [206]. Future blinded clinical trials, with larger cohorts, longer treatments and follow up periods, proper assessment of senescent cells elimination should provide better insight into the therapeutic potential of the DQC and senolytic drugs in general in IPF treatment. In mid-2019, Hickson., *et al*. published the preliminary results from a clinical trial evaluating the therapeutic effect of the DQC in patients with diabetic kidney disease. The patients received 100 mg/day Dasatinib and 1000 mg/day Quercetin during three days, and the number of senescent cells, as well as the circulating level of SASP mediators, were evaluated in blood, skin and adipose tissues sampled before and 11 days after the end of the treatment [207]. The treatment resulted in a significant reduction (i.e. ~ 33%) of senescent cells in adipose and skin tissues as assessed by $p16^{INK4A}$, $p21^{CIP1}$ and β-galactosidase activity staining.

Moreover, the authors observed a significant decreased in circulating SASP mediators such as IL-1α, IL-6, MMP-9 and -12 [207]. This study provided the first proof of principle, of an *in vivo* senolytic effect of the DQC in human and suggest a therapeutic potential in diseases driven by cell senescence. However, previous clinical trials of Dasatinib for chronic myeloid leukaemia revealed several side effects, including haemorrhage in 6% of patients probably as a result of induced thrombocytopenia [208]. Moreover, an *in vitro* study by Hwang., *et al*. showed that Quercetin could have severe toxic effects on non-senescent human adult coronary endothelial cells [209]. These reported sides effects dampen the therapeutic potential of the DQC, particularly for longer-term treatments, and support the development of alternative senolytics.

Inhibitors of the BCL-2 family members

In addition to Dasatinib and Quercetin, Zhu and others have assessed the senolytic potential of drugs targeting the BCL-2 family of anti-apoptotic factors. Thus Chang., *et al*. showed that the BCL-2 and BCL-X_L inhibitor ABT-263 selectively induced senescent cells apoptosis *in vitro* independently of the cell types and species [210]. *In vivo,* ABT-263 administration depleted senescent muscle stem cells (MuSCs) and senescent bone marrow-derived hematopoietic stem cells (BM-HSMs) from normally aged mice and sub-lethally irradiated mice resulting in the rejuvenation of the aged MSCs and HSMs [210]. Zhu., *et al*. using the same molecule also known as Navitoclax, showed it could reduce the viability of senescent human umbilical vein endothelial cells (HUVECs), IMR90 human lung fibroblasts, MEFs, but not human primary preadipocytes [211]. Moreover, ABT-263 treatment eliminated IR-induced senescent AECII *in vitro* and its administration sixteen weeks after exposure to a single dose of ionising radiation (i.e. 17 Gy) on the right side of the thorax, prevented the development of lung fibrosis by selectively killing senescent AECII [212]. Yosef., *et al*. using the BCL-W and BCL-X_L inhibitor ABT-737, showed that it can eliminate senescent lung cells in irradiated mice and senescent epidermal cells in genetically engineered mice in which expression of the human $p14^{ARF}$ gene is inducible in the basal layer of the skin epidermis leading to a p53-dependent senescence of epidermal cells [213]. Inhibition of BCL-2 by its specific inhibitor ABT-199 (Venetoclax), on the other hand, only reduced the viability of OIS cells but has not affected senescent or growing MEF [212]. In addition, ABT-737 administration also eliminated senescent cells and prevented lung destruction in a model of chronic LPS-induced bronchitis [214]. Unlike the DQC, the therapeutic potential of BCL-2 family members inhibitors such as Navitoclax have been previously tested in phase I and II clinical trials for various tumours [215], revealing side effects similar to those of Dasatinib, such as neutropenia and thrombocytopenia [216]. Clinical trials evaluating the efficacy of 150 - 1200 mg ABT199/Venetoclax in Chronic lymphoid leukaemia (CLL) also revealed significant side effects in patients such as diarrhoea and neutropenia [217]. However, no clinical trial evaluating their senolytic potential has been reported so far.

Senolytics Candidate	Targets/Effects	Senescent cells targeted *In vitro*/*In vivo* results	Possible side effects	Ref
Quercetin	Kinases and serpins inhibitor, Antioxidant.	Endothelial cells; BM-MSC; foreskin fibroblasts	Human coronary endothelial cells toxicity *in vitro*.	[204,205,209]
Dasatinib	Multiple tyrosine kinase inhibitor.	Fat cells	Thrombocytopenia; Haemorrhage in CML	[204,208]
Dasatinib/ Quercetin Combination	Kinases, tyrosine kinase, serpins inhibitor Antioxidant.	MEF; primary human fibroblasts; IMR-90 fibroblasts/progeroid $Ercc1^{-/\Delta}$ mice, radiation-exposed mice, Bleomycin-induced lung fibrosis	No side effects reported in clinical trials for usage as senolytic drugs.	[129,130,204]
ABT-263/ Navitoclax	BCL-2; BCL-X_L	Muscle stem cells; BM-HSMs; HUVECs; IMR90; MEFs; AECII/Irradiated mice.	Neutropenia, Thrombocytopenia	[210-212,216]
ABT-737	BCL-W; BCL-X_L	p53-dependent Epidermal cells senescence *in vivo*.	Not Reported	[213]
ABT-199/ Venetoclax	BCL-2	Mainly target OIS cells/LPS-induced bronchitis.	Diarrhoea, Neutropenia in CLL	[213,214,217]

Table 3: *Summary of Senolytic drugs candidate characteristics and experimental outcomes.*

Alternative senolytics and senomorphics molecules.

Apart from the DQC, and inhibitors of the BCL-2 family, heat shock protein 90 inhibitors [218], redox catalysts influencing the $NAD(P)^+$/NAD(P)H ratios [219], as well as the well-known macrolide antibiotics Azithromycin and Roxithromycin [220] have been shown to exert senolytic activities *in vitro*. Moreover, several polyphenols (e.g. Apigenin, Genistein, kaempferol), and non-polyphenol (e.g. resveratrol, curcumin) flavonoids, as well as vitamins (e.g. Vitamin E, D and A) have been shown to exert some form of senolytic and senomorphic activities. See [221] for review.

Finally, targeting immunosenescence, a process characterised by i) a reduced ability to respond to new antigens; (ii) the accumulation of memory T cells; (iii) a lingering level of low-grade inflammation termed "inflamm-ageing" [222]; could offer new therapeutic avenues for the treatment of ageing-associated diseases such as IPF. Indeed, senescent cells accumulation during ageing could be due to the immune system failure in eliminating them. Thus, Ovadya., *et al*. showed that mice deficient for perforin an essential protein in immune-mediated cytotoxicity, present an accumulation of senescent cells and a chronic inflammation [223]. Moreover, a recent article by Pereira., *et al*. showed that senescent dermal fibroblasts evade immune clearance by natural killer and $CD8^+$ T cells by upregulating their expression of the Human Leukocyte Antigen-E [224]. Importantly, the blocking of senescent cells HLA-E interaction with the NK and $CD8^+$ T cells inhibitory receptor NKG2A by a blocking antibody boosted senescent cells elimination *in vitro* [224]. Further studies on immunosenescence and senescent cells immune evasion strategies could allow the development of new drugs targeting senescent cells.

As shown in table 3, several molecules targeting intracellular signalling molecules such as Kinases/tyrosine kinases and BCL-2 family of proteins have shown senolytics activity *in vitro* as well as *in vivo*. Many of these drugs have a broad effect, targeting senescent cells from several cell types and induced by different stimuli. Further clinical trials are required to determine the optimal dosage and regimen at which these drugs could deliver the best benefit/risk in the treatments of ageing-associated chronic inflammatory disease such as IPF.

Concluding Remarks

IPF is a devastating age-related disease whose burden is expected to grow in the coming decades as the world population is ageing. Despite recent progress in its management (i.e. Pirfenidone and Nintedanib marketing), IPF is still associated with poor survival, and new therapeutic options are warranted. In this review, we presented the mechanisms involved in cellular senescence and the way they contribute to their cell cycle arrest, apoptosis resistance and adoption of a SASP. Senescent cells accumulation and persistence in aged and diseased tissues, together with their presumed ability to induce the secondary senescence of neighbouring cells and perpetuate chronic tissue inflammation make them an attractive therapeutic target in many ageing-associated diseases. We showed that epithelial cells, as well as fibroblasts from IPF lung express senescence markers, have reduced proliferation potential, secrete SASP mediators, and in the case of fibroblasts, show high resistance to apoptosis. These phenotypes are supported by substantial changes in cell signalling pathways regulation such as activation of the Akt, NF-κB and TGF-β pathways and repression of the PTEN pathway. We identified cigarette smoking, telomere attrition and telomere functioning genes mutations as possible contributors to senescence in IPF cells. In particular, the presence of a persistent DNA damage response in AECII seems to be a key determinant of their senescence and could play a key role in IPF as suggested by experimental lung fibrosis models.

On the other hand, despite *in vitro* pieces of evidence of a pro-fibrotic effect of their SASP on naïve cells, the contribution of senescent fibroblasts to lung fibrosis *in vivo* remains controversial and more studies are required to determine their overall impact on lung fibrosis development. The enthusiasm around the therapeutic potential of senescent cells targeting in age-related chronic diseases prompted the recent development of senolytic drugs designed to eliminate senescent cells selectively, and senomorphic drugs intended to inhibit the release of SASP mediators. Experimental results and preliminary clinical data suggest a potential benefit of senolytic drugs such as Quercetin, Dasatinib, and Navitoclax in IPF treatment. Additional clinical studies, involving larger cohorts, with extended regimens and a longer-term assessment of cell senescence, SASP mediator level as well as lung function and fibrosis will allow a better evaluation of

senolytic drugs therapeutic potential in IPF. In the future, the scientific community will have to clarify the possible contribution of other cells type (e.g. alveolar macrophages, lung endothelial cells) senescence to IPF development, design senolytic drugs with fewer side effects and better specificity toward a particular senescent cell type to allow their selective depletion if required. Finally, efforts must be made to understand the mechanisms leading to senescent cells accumulation *in vivo*, especially the potential role of immunosenescence in this process.

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