The ageing lung under stress

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ABSTRACT   Healthy ageing of the lung involves structural changes but also numerous cell-intrinsic and cell-extrinsic alterations. Among them are the age-related decline in central cellular quality control mechanisms such as redox and protein homeostasis. In this review, we would like to provide a conceptual framework of how impaired stress responses in the ageing lung, as exemplified by dysfunctional redox and protein homeostasis, may contribute to onset and progression of COPD and idiopathic pulmonary fibrosis (IPF). We propose that age-related imbalanced redox and protein homeostasis acts, amongst others (*e.g.* cellular senescence), as a "first hit" that challenges the adaptive stress-response pathways of the cell, increases the level of oxidative stress and renders the lung susceptible to subsequent injury and disease. In both COPD and IPF, additional environmental insults such as smoking, air pollution and/or infections then serve as "second hits" which contribute to persistently elevated oxidative stress that overwhelms the already weakened adaptive defence and repair pathways in the elderly towards non-adaptive, irremediable stress thereby promoting development and progression of respiratory diseases. COPD and IPF are thus distinct horns of the same devil, "lung ageing".

Introduction

Ageing is the major risk for the decline in lung physiological functions and is therefore associated with increased susceptibility to injury and development of lung diseases. Aged human lungs show characteristic airspace enlargement called "senile emphysema" with similarities to tobacco smoke-related emphysema [1]. Several molecular mechanisms are proposed to contribute to this structural change, including increased oxidative stress [2], DNA damage [3], chronic inflammation [2, 4], replicative senescence caused by telomere shortening [5], mitochondrial dysfunction [3, 6], loss of proteostasis [7], decline of antioxidant defence mechanisms [8, 9] and epigenetic alterations resulting in deregulated gene expression [10]. These ageing mechanisms lead to impaired cell–cell communication, deregulated nutrient signalling, defective host defences, decline in immune responses, and altered matrix remodelling [11]. Importantly, this age-related dysregulation contributes to the failure of the lung to respond appropriately to injuries and stressors, causing the eventual demise of cells, defective repair and accumulation of damage over time. Both COPD and idiopathic pulmonary fibrosis (IPF) are diseases of the elderly [12, 13], and eminently associated with an accelerated lung ageing process [14]. In addition, altered lung development such as upon early damage of the immature lung has been demonstrated to predispose to the development of COPD, its impact on IPF, however, is less well understood [15]. It is tempting to speculate that the disruption of normal lung development accelerates the process of lung ageing thereby contributing to the increased susceptibility to the

development of respiratory diseases [16]. Environmental factors, such as cigarette smoke or other gaseous pollutants, accelerate the ageing of the lung through worsening of ageing-related events and may lead to defective resolution of inflammation, impaired regeneration and insufficient repair. These "second hits" may not only accelerate the progression of both diseases, they also represent an increased risk of the aged, vulnerable lung to develop one of these lung diseases and may act as "final elicitors" for the onset of COPD or IPF [17, 18]. However, the distinct mechanisms that predispose the old, vulnerable lung to develop either COPD or IPF after the "decisive injury" are still elusive, but distinct cell types might be involved. COPD lungs show increased endothelial cell and fibroblast apoptosis [19–22], as well as destruction of connective tissue including alveolar septae [14, 23], resulting in an irreversible loss of lung tissue and progressive formation of emphysematous spaces (Figure 1). In contrast, the distortion of the alveolar architecture in IPF is characterised by excessive replacement with fibrotic tissue and abnormal bronchiolar epithelium, indicating generation of abnormally excessive tissue (figure 1) [24, 25]. Despite these (cellular) differences, COPD and IPF share a number of the very same ageing mechanisms in the lungs, among them, the presence of senescent cells and a general decline in "adaptive stress responses". Here we propose that such impaired stress response is no longer able to cope with additional challenges to maintain physiological lung function. Instead, the balance is tipped towards "non-adaptive, irremediable stress" which is associated with accumulating damage, cell death, inflammatory signalling and disease progression. COPD and IPF are thus distinct horns of the same devil "lung ageing".

In this review, we would like to provide a conceptual framework of how impaired stress responses in the ageing lung, as exemplified by redox and protein homeostasis, may contribute to onset and progression of COPD and IPF.

Impaired redox homeostasis in the ageing lung

Cellular redox homeostasis is defined as the maintenance of an oxidant-antioxidant balance in the cell to prevent oxidative stress. The disequilibrium of this balance due to an increased production of oxidants/ reactive oxygen species (ROS) and/or depletion of antioxidants causes oxidative stress in the cell [26]. Antioxidants include enzymatic antioxidants and non-enzymatic, low-molecular weight antioxidant scavengers (*e.g.* vitamins C and E) [27]. The process of healthy lung ageing is associated with a progressive decline in antioxidant defence capacity [8, 9, 28]. One underlying reason might be the impaired activity of the antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) in aged lung cells [8, 9]. NRF2 signalling drives expression of antioxidant/detoxifying genes, such as heme oxygenase-1 (*HMOX1*), NAD(P)H quinone oxidoreductase 1 (*NQO1*) and both subunits of glutamate cysteine ligase (*GCLC* and *GCLM*) *via* the antioxidant response element in their promoter regions [29]. Decreased nuclear NRF2 levels were accompanied with a significant decline in the induction of NRF2-regulated antioxidant genes in primary human bronchial epithelial (HBE) cells from aged donors compared to young controls [9]. Loss of inducibility correlated with an increased expression of the NRF2 inhibitors Bach1 (transcription regulator protein BACH1) and c-Myc [9]. Lung fibroblasts isolated from aged mice also exhibit deficient Nrf2 induction in response to exogenous H_2O_2, as compared to young murine fibroblasts [30]. Similarly, mRNA expression of NRF2-targets *HMOX1*, *NQO1* and *GCLC* was downregulated in H_2O_2-treated senescent human IMR90 lung fibroblasts resulting in a reduced ability to detoxify ROS contributing to increased oxidative stress [30]. Impaired induction of NRF2 and NRF2-regulated genes in older cells may thus underlie the increased
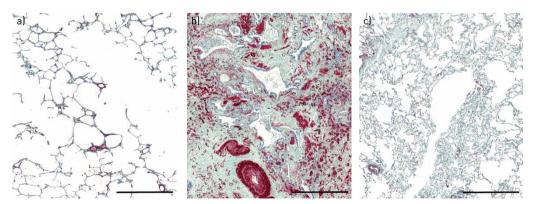

FIGURE 1 Lung histology of a) a 62-year-old patient with COPD, b) a 64-year-old patient with idiopathic pulmonary fibrosis and c) a 61-year-old organ donor. All patients are male and are (former) smokers. Shown is a representative immunohistochemistry for alpha-smooth-muscle-actin (α-SMA). Scale bar=1 mm.

susceptibility of the elderly to oxidative stress and lung disease triggered by ROS [9]. Oxidative stress results in detrimental oxidative modifications and molecular damage to cellular proteins, lipids and RNA/DNA, impairing their function and contributing to shortened life span [26]. Oxidative damage to DNA results in either cell cycle arrest through the p53/p21^{CIP1} pathway or cell death *via* p53-induced apoptotic pathways if the dose and damage is too great [31, 32]. Lipid peroxidation exerts its detrimental effects through two main mechanisms [33]. Extensive peroxidation of lipids alters the assembly, composition, structure and dynamics of lipid membranes including the plasma membrane. Furthermore, lipid peroxides degrade very fast into highly reactive aldehydes, with 4-hydroxynonenal (4-HNE) and malondialdehyde being the best described [34], which react with proteins to create irreversible oxidative protein modifications through formation of covalent carbonyl adducts [33]. The oxidative modifications of proteins, *e.g.* protein carbonylation, which is generated beside lipid peroxide mediated-protein oxidation also through direct oxidation of amino acid side-chains by oxygene radicals, affects the physical and chemical properties of proteins, including conformation, structure, solubility, susceptibility to proteolysis and enzyme activities [35, 36]. This can be detrimental for cellular function and promotes accelerated ageing processes as demonstrated for example for the anti-ageing molecule sirtuin-1, an enzyme involved in mitochondrial biogenesis and antioxidant induction, whose deacetylase activity declines with the ageing process in the liver, kidney, heart and lung due to protein oxidation [37, 38]. Moreover, excessive ROS and oxidation of sirtuin-1 promote its proteasomal degradation [38, 39].

Concomitant with an impaired antioxidative capacity, elevated levels of oxidative stress associated damage have been observed in aged tissue, including the lungs [8, 28, 40, 41]. Among them, BRAIDY *et al.* quantified the levels of oxidative stress in the form of protein carbonyls, lipid peroxidation, and oxidative DNA damage in the liver, heart, kidney and lung from female wistar rats aged 3 to 24 months, thus spanning life stages from young adulthood to old age. Middle-age (12-month-old) rats already exhibited higher levels of oxidatively modified proteins, lipids and DNA than young adult animals aged 3 months. This was further accelerated massively after 12 months of age in all organs [28]. Moreover, accumulating oxidative insults to macromolecules may promote cell death *via* apoptotic and non-apoptotic mechanisms [42, 43]. In accordance, aged organs generally demonstrate significantly lower cell volumes, indicative of cell loss due to increased cell death occurring during the ageing process [28].

Taken together, the aged lung is primed to oxidative stress. This is at least in part caused by an age-related decline in the antioxidative capacity of aged cells due to reduced activity of the antioxidant master regulator NRF2.

Redox imbalance and oxidative stress in COPD
Decline in antioxidant capacity
In aged smokers and COPD patients, redox homeostasis as maintained by NRF2-BACH1 equilibrium is dysregulated in the lung including alveolar macrophages [44]. Consistent with these findings, Nrf2-deficient mice are more susceptible to cigarette smoke exposure (CSE) and develop more severe lung emphysema and apoptosis, and the activity of antioxidant enzymes is repressed [45]. Moreover, other protective antioxidant mechanisms are not sufficiently adapted in elderly smokers and COPD patients as the increase of superoxide dismutase (SOD) expression is lacking, so that oxidants may subsequently take over the leading role [46, 47]. KONDO *et al.* [47] observed that superoxide ($O_2^{\bullet-}$) anion generation (briefly superoxide) in alveolar macrophages of elderly smokers was presumably due to proteolytic degradation of SOD in elderly smokers, while mRNA level were not altered compared to age-matched nonsmokers. The failure of cells to induce antioxidants such as SODs can also be explained by inactivation and loss of the anti-ageing molecule sirtuin-1 in COPD lungs which is known to protect against oxidative stress by upregulating forkhead box O3A (FOXO3A) transcriptional activity and FOXO3A-dependent antioxidant genes, such as catalase and *Mn*SOD (manganese SOD) [38, 39, 46]. FOXO3A expression itself is significantly decreased in the lungs of smokers and patients with COPD [48]. Furthermore, although the expression of various glutathione peroxidases (GPXs) is increased in smokers and COPD patients, GPX activity is significantly reduced in both groups in comparison to nonsmokers [49]. Impaired GPX function can be due to general increase in protein oxidation which has been observed in various cell types of COPD lungs [50].

Chronic oxidative stress
In COPD, the foremost risk factor leading to the development of disease besides ageing is tobacco smoke consumption [17, 51]. Cigarette smoke contains numerous highly reactive chemical compounds causing oxidative stress [52]. For the ageing lungs, cigarette smoke exposure represents a major additional challenge of the already reduced redox balance resulting in chronic oxidative stress. There is documented evidence that superoxide-generating (phagocytic and non-phagocytic) NADPH oxidases (NOX) are involved in mediating the cytotoxic effects of cigarette smoke [53–57]. Thiol-reactive compounds in cigarette smoke-induced endothelial superoxide production *via* NOX2 in cell- and tissue-culture systems,

which was associated with increased peroxynitrite ($ONOO^-$) formation (nitrosative stress), enhanced expression of pro-inflammatory cytokines and endothelial dysfunction [53, 54]. Inhibitors of NOX enzymes reduced cigarette smoke-induced superoxide production and its deleterious consequences in endothelial cells [53, 54]. Importantly, the lung shows high basal expression of NOX enzymes, and nearly all NOX family members are upregulated in response to cigarette smoke exposure *in vitro* and/or in COPD lungs *in vivo* (reviewed in [57]), indicating that ROS derived from NOX enzymes play a crucial role in COPD pathogenesis. In agreement, it was shown that bronchoalveolar lavage (BAL) leukocytes isolated from COPD patients release high levels of superoxide which was linked to increased levels of inflammatory mediators and oxidative stress in the air spaces [55, 56, 58]. Levels of H_2O_2, the dismutation product of superoxide, are much higher in the exhaled breath condensate of COPD patients than of healthy controls, and correlate with disease severity [59]. Oxidative stress and damage in COPD are further represented by increased lipid peroxidation and protein oxidation in both the airway and alveolar epithelia of patients [50], as well as by oxidant-induced DNA damage in bronchial epithelial cells, type-II alveolar epithelial cells (AECII), septal endothelial cells, macrophages and fibroblasts of COPD lungs [21, 22, 60]. DNA oxidation has a crucial role in COPD pathogenesis [61] because it promotes further ROS generation [60, 61], mediates microsatellite instability [62], inhibits DNA methylation [63], impairs DNA repair [60, 64] and accelerates telomere shortening [65], resulting in somatic mutations in patients with COPD [60, 64]. Increased nucleic acid oxidation in RNA and DNA has been reported in alveolar wall cells, in particular in alveolar fibroblasts, in both the mouse model of cigarette smoke-induced emphysema and in COPD lung tissue, and correlated with the severity of disease [21, 22]. *In vitro* studies also underscored that cigarette smoke induces nucleic acid oxidation in lung fibroblasts, inhibits the recruitment and proliferation of lung fibroblasts, and activates apoptosis [22, 66, 67]. Oxidant-mediated injury in alveolar fibroblasts targets destruction of alveolar collagen and elastic fibres required to maintain the architecture of the alveolus, thereby leading to emphysema formation [68]. It has also been suggested that the increase in alveolar wall/septal cell death in COPD likely involves "anoikis", *i.e.* cell death by detachment from the underlying extracellular matrix (ECM) [43]. Other pathogenic mechanisms of cigarette smoke and persistent oxidative stress in the "aged stressed COPD lung" include exaggerated airway and lung inflammation and protease/antiprotease imbalance [51, 69]. The inflammatory response to cigarette smoke and oxidative stress is the major aetiological factor in the pathogenesis of COPD [51, 70]. ROS enhances the inflammation through neutrophil accumulation and the activation of mitogen-activated protein kinases and redox-sensitive transcription factors, such as nuclear factor (NF)-κB and activator protein-1, resulting in increased expression of inflammatory mediators and cytokines (interleukin (IL)-1, tumour necrosis factor (TNF)-α, IL-8, granulocyte-macrophage colony-stimulating factor and inducible nitric oxide synthase (iNOS)), that contribute to chronic inflammation in COPD [70, 71]. Neutrophils are the most abundant inflammatory cells present in the bronchial wall and lumen of patients with COPD [72], and are themselves powerful producers of ROS [73], which is believed to be generated by NOX2 [57]. Neutrophil elastase plays an important role in neutrophil-mediated bacterial and fungal killing, but also degrades almost every ECM component, including collagen, fibronectin, proteoglycans, heparin and cross-linked fibrin thereby contributing to emphysema formation in COPD [74]. Despite smoking cessation, oxidative stress, somatic mutations, neutrophil inflammation and apoptosis, as well as activation of adaptive immune responses, persist in the majority of COPD patients [75–80], which can be attributed to the impaired defence of the stressed aged lung.

Interestingly, despite the established role of NOX2 in exaggerating ROS production and inflammation in response to cigarette smoke exposure, genetic ablation of components of Nox2 ($p47^{phox(-/-)}$ and $gp91^{phox(-/-)}$) in mice, in which the phagocytic cells are incapable of respiratory burst and ROS release, did not protect the lungs against detrimental effects of cigarette smoke [81]. Instead, alveolar destruction, inflammatory cell infiltration, inflammatory gene expression, and mucus secretion were significantly augmented in lungs of both $p47^{phox(-/-)}$ and $gp91^{phox(-/-)}$ mice in response to sub-chronic cigarette smoke exposures, as compared to cigarette smoke exposed wild-type mice [81].

Furthermore, dysregulation of iNOS and nitrotyrosine formation have been proposed as contributing pathomechanisms of COPD [82, 83]. Beside inflammatory IL-6 and IL-8 production, iNOS contribute to increased nitric oxide production and nitrosative stress in COPD, as nitric oxide may react with superoxide ($O_2^{\bullet-}$) to form the potent oxidant peroxynitrite ($ONOO^-$) [84], which in turn reacts very fast with tyrosine residues from many proteins to form nitrotyrosine [82, 83]. Increased iNOS expression and nitrotyrosine levels have been observed in the pulmonary vasculature and AECII of both COPD patients and emphysemateous mice upon cigarette smoke exposure, and correlated with induction of apoptosis in these cells [82]. Interestingly, in the study by SEIMETZ *et al.* [82], pulmonary vascular remodelling and pulmonary hypertension (PH) preceded the development of alveolar destruction/emphysema in chronically cigarette smoke-exposed mice. Mice lacking iNOS (*Nos2*) were protected against cigarette smoke-induced emphysema and PH, and iNOS inhibition both prevented smoke-induced lung damage and reversed PH and fully established emphysema in *wt*-mice [82]. Cigarette smoke-induced iNOS

upregulation and subsequent peroxynitrite formation was also linked to disruption of the soluble guanylate cyclase (sGC) activity in alveolar and endothelial cells in cigarette smoke-exposed rodents and COPD lungs [85]. Stimulators of sGC prevented cigarette smoke-induced PH and emphysema in rodents through inhibition of alveolar and endothelial cell apoptosis, and reduction of inflammatory gene expression and neutrophil numbers in rodents with long-term exposure to cigarette smoke [85].

Taken together, the persistence of oxidative (and nitrosative) stress and inflammation is driven both by the inactivation or depletion of antioxidant defence capacity of the ageing lung as well as the permanent chronic challenge to oxidants and presents a paradigm for a non-adaptive, irremediable stress responses in COPD.

Redox imbalance and oxidative stress in IPF
Decline in antioxidant capacity

Strong evidence suggests that impaired redox balance is also involved in the pathogenesis of IPF. Low expression levels of enzymatic antioxidants, including extracellular superoxide dismutase (EcSOD), MnSOD, catalase and HMOX1 have been observed in the fibrotic areas [86–88], which may result in increased ROS generation and consecutive damage to the overlying epithelium. Glutathione, a major component of the lung antioxidant defence system, is decreased in the epithelial lining fluid, both in the bleomycin model of lung fibrosis and in IPF patients [89]. These effects might be driven by dysregulation of NRF2, the "master regulator" of the antioxidant response. First, $Nrf2^{(-/-)}$ knockout mice are more sensitive to bleomycin- and paraquat-induced pulmonary fibrosis compared to wild-type animals [90]. Secondly, NRF2 expression is largely absent in myofibroblasts within fibroblast foci of IPF lungs [30], and coincides with increased NADPH oxidase (NOX)-4 expression and enhanced H_2O_2 production [30, 91, 92]. In agreement, in an in vitro study using cultured primary IPF fibroblasts, decreased NRF2 expression was associated with an α-smooth muscle actin expressing myofibroblast phenotype, whereas NRF2 activation increased antioxidant defences and abrogated the contractile myofibroblastic phenotype in these IPF fibroblasts [93]. Of particular interest, increased expression of NRF2 has been observed by two independent groups in the AECIIs of IPF lungs [30, 94], the chronic injury of which is regarded as the crucial pathogenetic event in IPF. However, expression of two NRF2 target genes, among them peroxiredoxin 1 (PRDX1) [95], was reduced in AECII of IPF lungs compared to cells from patients with nonspecific interstitial pneumonia, an entity with a better outcome than IPF [96]. NRF2 induction may thus represent an attempt of IPF AECIIs to survive under conditions of severe oxidative stress, but is less able to adaptively increase antioxidant enzymes and thus incapable of restoring the redox balance. In contrast, oxidative stress and ROS generation in IPF fibroblasts appear to have a (survival-related) "physiological" function in myofibroblast differentiation and also confers resistance to apoptosis in these cells, which is in marked contrast to interstitial COPD fibroblasts.

Chronic oxidative stress

Alveolar oxidative stress plays a crucial role in the pathogenesis of IPF and contributes to the fibrotic process by: 1) inducing alveolar epithelial cell injury and apoptosis [97, 98]; 2) increasing the release and activation of profibrotic/inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β [99, 100], or 3) causing impairment of the pulmonary surfactant system [101].

Biomarkers of oxidative stress, such as protein carbonyls or 8-isoprostane, a lipid peroxidation product, are elevated in BAL fluid of patients with IPF [102–104], and positively correlate with poor clinical outcome [105]. Furthermore, superoxide radicals and H_2O_2 are released spontaneously by alveolar macrophages and other BAL cells of IPF patients, which consecutively may result in injury of the alveolar epithelium [86]. In line with this, release and oxidative damage of mitochondrial DNA has been observed in lung epithelial cells of IPF patients [106–108]. This may drive ATP depletion and mitochondrial dysfunction, amplifying ROS generation as the electron transport chain is uncoupled from proton pumping and ROS are released into the cytosol [108]. The profibrotic cytokine TGF-β can augment the production of ROS. First, TGF-β induces prolonged mitochondrial ROS generation by decreasing complex IV activity in lung epithelial cells, with senescent arrest and persistent disruption of mitochondrial membrane potential [109]. Secondly, TGF-β mediated myofibroblast differentiation is associated with the enzymatic production of extracellular H_2O_2 that promotes damage to the overlying alveolar epithelium, a key event in initiating and perpetuating fibrosis in IPF [98]. Thirdly, TGF-β leads to activation of NOX4 expression in various lung cells in vitro and in vivo to produce ROS [91, 92, 110]. In agreement, NOX4 is strongly overexpressed in the fibroblast foci as well as the hyperplastic AECII of IPF patients [91, 92, 111], and Nox4 deficient mice are protected against bleomycin-induced AECII apoptosis and pulmonary fibrosis [111]. Moreover, pharmacologic inhibition of non-phagocytic Nox4 reversed pulmonary fibrosis in aged mice [30], demonstrating that targeting Nox4 is sufficient to correct oxidative stress and to promote resolution of age-associated fibrosis. These data also highlight the crucial pathogenic role of oxidative stress and ageing

in the development of lung fibrosis [30]. The increased levels of protein oxidation in IPF lungs, as well as lungs of ageing mice, including protein carbonylation [104] and protein S-glutathionylation (PSSG) [112], which is generated through covalent attachment of glutathione to reactive protein cysteine residues, could be a potential consequence of NOX4- or mitochondrially derived oxidants as well as of the observed decline in antioxidants as described above. Interestingly, the antioxidant glutaredoxin-1 is itself S-glutathionylated and inactivated in IPF lungs, thereby amplifying the generation of PSSG protein adducts (and perpetuating depletion of glutathione) [112]. Consistent with alveolar oxidative stress, PSSG patterns were found in AECII of IPF patients, but not in normal AECII of control lungs [112]. Another prominent target for PSSG important in pulmonary fibrosis is the death receptor FAS through its role in augmenting epithelial apoptosis. Fas glutathionylation occurs in response to endoplasmic reticulum redox imbalances in lung epithelial cells and subsequently increases caspase-3 and -8 activity [113], and was also shown to be upregulated in experimental lung fibrosis and IPF [112].

Finally, oxidative stress is the most commonly cited underlying mechanism for accelerated telomere shortening [114]. Several studies report more single strand breaks and 8-oxoguanine lesions in telomeres as compared to microsatellite repeats and bulk genomic DNA, after exposures to oxidising agents [115, 116].

In IPF, telomere attrition appears to predominantly affect AECII [117, 118], while no shortening in telomere length is observed in fibroblasts surrounding the injured AECII [117]. Notably, 25% of sporadic cases of IPF (without any identified genetic cause) reveal shortened telomeres comparable to those in familial IPF with identified telomerase gene mutations [118, 119]. Cigarette smoke has been considered to be one of the risk factors for sporadic and familial IPF [18] and is shown to cause telomere shortening in a dose-dependent manner [65]. TSAKIRI et al. [120] reported that smokers with a mutation in TERT or TERC die 10 years earlier than nonsmoking IPF patients. In conclusion, in IPF, both, the reduced antioxidant defence and the chronic oxidative stress, contribute to the myoactivation of lung fibroblasts and their resistance to apoptosis and at the same time drive telomere shortening in AECII cells (through oxidative damage to telomeric DNA) which contributes to disease development.

Decline in proteostasis in the ageing lung

Proteostasis is the synthesis, folding, processing, trafficking and degradation of proteins [121]. Loss of proteostasis contributes to the accumulation of unfolded, misfolded or aggregated proteins and is a hallmark of ageing [122]. The drivers of age-related loss of proteostasis are mainly oxidative stress, endoplasmic reticulum stress, and epigenetic alterations resulting in changed expression profiles of key factors maintaining protein homeostasis [26, 122, 123]. As the result, the activities of the chaperone network and of the two major proteolytic systems, the autophagy-lysosomal system and the ubiquitin-proteasomal system, become significantly deregulated with age in many tissues, especially in liver and lung [124, 125].

Impaired chaperone network

Ageing significantly decreases the levels of molecular chaperones that are required for folding of proteins such as heat shock protein (Hsp)-27, Hsp60 and Hsp70 (includes Hsp72 and Hsc70) in various organs, both at mRNA and protein levels [126–128]. In ageing cells the activity of the master transcription factor heat shock factor (HSF)-1 is reduced as evidenced by diminished binding of HSF1 to heat shock elements (HSE) in HSP promoters in gel shift assays, resulting in an impaired capacity to transcriptionally activate expression of HSPs [126, 129]. Moreover, HSF1 expression itself is significantly reduced in senescent IMR90 lung fibroblasts and skin fibroblasts from older human subjects [129]. In addition to cytosolic HSPs, a decline in protein levels of the endoplasmic reticulum chaperones ERp55 (protein disulfide isomerase (PDI) A1), ERp57 (PDIA3), ERp72 (PDIA4), GRP78, GRP94 and calnexin has been observed in many organs during ageing [130–132]. Interestingly, BOROK et al. [133] observed that AECII isolated from lungs of aged mice showed decreased expression of Grp78, while the endoplasmic reticulum chaperone Grp94 was increased, compared to AECII from 2-month-old mice. As (endoplasmic reticulum) chaperones are progressively oxidised during ageing [134, 135], this may contribute to their functional decline and consequently to impairment in protein folding, disulfide crosslinking and glycosylation. However, there is also evidence of increased expression of HSP and endoplasmic reticulum chaperones (especially GRP78) in some ageing cells [136, 137], and especially under accumulating ROS conditions [138], possibly representing an adaptive response to elevated endogenous oxidative stress in ageing. By using transcriptome profiling, MISRA et al. [138] showed progressive increase in oxidative stress in lungs of healthy ageing DBA/2J mice from 2 months to 20 months of age, which was accompanied by upregulation of stress-response genes including HSP and enzymes involved in xenobiotic detoxification mechanisms. On the contrary, aged C57Bl/6J mouse lungs showed downregulation of HSP genes, thus indicating a mouse strain-specific regulation of stress-response genes during lung ageing [138].

Endoplasmic reticulum-stress

Endoplasmic reticulum-stress results from misfolding of secretory proteins and leads to the upregulation of a signalling pathway called the endoplasmic reticulum stress response or unfolded protein response (UPR) [139]. The UPR starts when GRP78 dissociates from the three endoplasmic reticulum transmembrane sensors PRKR-like endoplasmic reticulum kinase (PERK), activating transcription factor-6-alpha (ATF6α) and inositol requiring enzyme-1-alpha (IRE1α), and gets recruited to the misfolded protein cargo, thereby permitting the activation of these "endoplasmic reticulum-stress sensors" and UPR signalling pathways PERK/eIF2α/ATF4, ATF6α and IRE1α/X-box binding protein-1 (XBP1). The adaptive UPR signalling is characterised by the induction of chaperones, such as GRP78, degradation of misfolded proteins *via* the endoplasmic reticulum-associated degradation (ERAD) pathway, and attenuation of protein translation [139].

Endoplasmic reticulum stress and oxidative stress are tightly interconnected through increased protein oxidation which disrupts protein folding in the endoplasmic reticulum, leading to accumulation of misfolded oxidised protein substrates. This process is further aggravated by ROS through impaired function of oxidatively modified proteins from chaperoning systems or other cellular "quality control" mechanisms [140, 141]. Moreover, NRF2 is closely associated with ROS-dependent UPR activation as PERK kinase directly phosphorylates NRF2 upon endoplasmic reticulum stress, thereby preventing its proteasomal degradation allowing NRF2 translocation and accumulation in the nucleus [142]. When protective UPR action fails to restore folding capacity in the endoplasmic reticulum, the apoptotic arm of the UPR is activated [139, 143–147]. Apoptosis in response to endoplasmic reticulum stress is mediated largely by induction of C/EBP homologous protein (CHOP) [139], which upregulates the transcription of various pro-apoptotic factors [143–146], while it downregulates anti-apoptotic genes such as B-cell leukaemia/lymphoma 2 protein (*BCL2*) [147]. In addition, CHOP has been implicated in exaggerated ROS production by increasing expression of endoplasmic reticulum oxidoreductase alpha, which contributes to H_2O_2 generation during oxidative disulfide bond formation [148]. Furthermore, CHOP leads to intracellular glutathione depletion through upregulation of glutathione-specific gamma-glutamylcyclotransferase-1, which degrades glutathione [149]. Moreover, CHOP is also involved in induction of inflammatory cytokines *IL1B* [150] and *IL6* [151], thereby perpetuating cell injury and death. Endoplasmic reticulum stress-induced inflammation can also be triggered through various pathways induced by hyperactivated p-IRE1α, which may result in NF-κB, JNK (c-Jun N-terminal kinase) but also NLRP3 inflammasome activation [139, 152].

Elevated endoplasmic reticulum stress response and UPR activation has been observed in adipose tissue of old compared to young mice, and contributes to adipose tissue inflammation in ageing [136]. In addition, in ageing cells the adaptive UPR mechanisms are weakened, whereas the proapoptotic UPR arm becomes more active [132, 153]. Ageing deteriorates expression and the kinase activity of the endoplasmic reticulum stress sensor PERK and impairs cytoprotective eIF2α phosphorylation, thereby permitting protein translation and proapoptotic protein expression in the endoplasmic reticulum under ageing conditions [153, 154]. In agreement, Chop expression levels increases in various organs with age [132, 153, 155]. In ageing lungs, endoplasmic reticulum stress and UPR genes were not altered in global transcriptome studies [138], but protein levels of the endoplasmic reticulum chaperones Grp78 were reduced, while Grp94 and proapoptotic Chop were concertedly upregulated in AECII cells of ageing lungs [133].

Dysregulation of proteasome system

The proteasome system is key to cellular proteostasis in ageing as it degrades old and damaged proteins [156]. Mild oxidative stress results in the adaptive activation of proteasomal degradation, whereas at higher oxidant levels proteasomal degradation decreases [157]. In such stress conditions, the misfolded/damaged proteins accumulate in non-degradable aggregates which are dispersed in the cytosol and are able to bind to the proteasome, and which makes the degradation of other misfolded proteins less efficient. Moreover, the proteasome itself is affected by oxidative stress. It has been shown that reactive aldehydes produced during the oxidation of carbohydrates (*e.g.* glyoxal, methylglyoxal) and lipids (*e.g.* 4-HNE, malondialdehyde) are able to cause oxidative adduct formation on several 20S proteasomal subunits which consequently leads to impaired proteasome function [125, 158, 159]. In agreement, the activity of the proteasome system is impaired during ageing, while activation of the proteasome system promotes longevity [160–162]. It has been shown that during proliferative senescence of human MRC-5 fibroblasts the proteasome activity declines while the level of oxidised proteins increases [163]. *In vivo* studies with rats also revealed that proteasome activity declines in several tissues (brain, liver, lung, muscle and heart) during ageing [164]. Healthy ageing of the lung in mice, however, had neither altered expression of the proteasome nor of the main catalytic activity, *i.e.* the chymotrypsin-like activity of the proteasome [165]. In contrast, the immunoproteasome, an alternatively composed 20S proteasome containing a set of inducible catalytic subunits, was significantly increased in aged *versus* young mice [166]. These results are of particular interest, because immunoproteasomes are usually known to be induced under conditions of viral/bacterial infection or interferon-γ stimulation, in which they generate specific peptides that have a

higher affinity to major histocompatibility complex (MHC) class-I mediated antigen presentation, thereby contributing to the efficient elimination of infected cells and an improved immune responses [167]. Moreover, immunoproteasomes have also been proposed to play a protective role against oxidative stress through degradation of oxidised misfolded proteins, which is, however, still a matter of debate [168–171]. Of note, the immunoproteasome is apparently not activated by NRF2 in response to ROS, as neither Nrf2 inducers nor Nrf2 inhibitors altered immunoproteasome expression [95]. Very recent studies in healthy aged mice unequivocally indicate that immunoproteasome induction in ageing lungs is associated with a prominent activation of the adaptive MHC class-I related immune responses and interferon signalling [172].

Dysregulation of autophagy-lysosome pathway

Growing evidence indicates that autophagy activity declines with age [124] as the rate of autophagosome formation and maturation and the efficiency of autophagosome/lysosome fusion are reduced in aged tissues [173]. The so-called autophagy-related (ATG) proteins are central to this process, as they regulate different steps during autophagosome biogenesis, *i.e.* vesicle nucleation, autophagosomal membrane elongation, and autophagosome completion [124]. In mammals, the conversion of microtubule-associated proteins 1A/1B light chain 3B (LC3B) from LC3B-I (free form) to LC3B-II (membrane-bound form, recruited to autophagosomal membranes) is regarded as a key step in the induction of autophagy. The expression of some autophagy-associated genes, including LC3B, ATG5 and ATG7, and beclin-1, declines during ageing in various tissues in humans and rodents [174, 175]. KHALI *et al.* [175] observed that the promoter regions of *Atg5* and *Map1lc3b* (LC3B) are hypermethylated in macrophages from aged, but not young C57Bl/6J mice, which was accompanied by low gene and protein expression of both autophagy markers in macrophages, as well as whole lung tissue of aged mice. Importantly, the lack of a single Atg is sufficient to abort the completion of autophagosomes [124]. Thus, intracellular accumulation of multiprotein aggregates and damaged organelles due to compromised autophagy is common during physiological ageing. Of note, aggregates collected from spleen protein lysates of aged mice revealed >90% of carbonylated proteins, underscoring again the relevance of oxidative stress (and oxidatively damaged proteins) for increased protein aggregation and challenging the autophagy-lysosomal system [176]. Autophagy plays also an essential role in the clearance of damaged mitochondria (mitophagy), and compromised autophagy leads to mitochondrial dysfunction, accumulation of abnormal mitochondria and in turn oxidative stress [177]. The expression of PTEN-induced putative kinase 1 (PINK1), which has an important role in the maintenance of mitochondrial function and in the selective degradation of damaged mitochondria by mitophagy, is reduced in lungs of aged mice [177].

Decline in proteostasis by cigarette smoke and in COPD
Dysregulated chaperone expression and activity

In a comparative proteome analysis, KELSEN *et al.* [178] demonstrated that lungs of middle-aged smokers exhibit significant upregulation of the endoplasmic reticulum chaperones GRP78, PDI and calreticulin, as compared to age-matched ex- and nonsmokers. In another study, increased levels of GRP78 have been reported in BAL fluid of middle-aged smokers compared to age-matched never-smokers [179]. Several HSPs, including HSP10, HSP40 and HSP60, were increased in lung biopsies from COPD patients as compared to aged-matched nonsmokers and smokers with normal lung function, and in particular HSP60 level were expressed in neutrophils and positively correlated with elevated numbers of neutrophils in COPD patients [180]. Furthermore, "global" expression of HSP70 was observed to be upregulated in peripheral lung tissue of COPD patients, and positively correlated with the severity of the disease [181]. Serum levels of HSP27, HSP70 and HSP90 were also elevated in COPD patients and may serve as diagnostic markers for COPD [182].

Taken together, inducibility of endoplasmic reticulum and HSP chaperones is not impaired in the aged, stressed COPD lung, and may by triggered by environmental (excessive) ROS or other stress-stimuli; but their function is presumably compromised, as they are progressively oxidised under conditions of ageing and ROS [134, 135, 183].

Endoplasmic reticulum stress

Induction of endoplasmic reticulum stress including CHOP has been observed in different cell types of end-stage COPD lung tissue (Global Initiative for Chronic Obstructive Lung Disease (GOLD) III/IV), but not in age-matched GOLD0, GOLD1 and GOLD2 patients [184]. Isolated fibroblasts from end-stage COPD patients displayed disorganised endoplasmic reticulum, Golgi and lysosomal structures and a tendency of increased caspase-3 activation in comparison to fibroblasts obtained from age-matched healthy smokers and never-smokers [185]. The induction of the UPR in COPD lungs has been linked to oxidative stress induced by chronic exposure to cigarette smoke [51]. In agreement, cigarette smoke induced a proapoptotic endoplasmic reticulum stress response in HBE cell lines preferentially by two

branches of the UPR, namely through the PERK/eIF2α- and the ATF6-pathways, with upregulation of GRP78, phospho-eIF2α, ATF4 and CHOP [186, 187]. Similarly, increased endoplasmic reticulum stress involving Chop has also been observed in mice exposed to cigarette smoke for 3 months [186, 187]. Interestingly, some reports document the induction of a rather protective UPR in airway epithelial cells in response to 2 4h exposure to CSE, with specific upregulation of endoplasmic reticulum chaperones, adaptive ATF4 genes, and increases in NRF2 and antioxidant genes, but no activation of proapoptotic UPR genes [178, 188]. Gᴇʀᴀɢʜᴛʏ et al. [189] observed that mice exposed to acute levels of cigarette smoke for 4 weeks developed only minimal lung destruction and exhibited only slightly elevated levels of Chop, while long-term chronic CSE for 1 year resulted in emphysema-like tissue destruction with reduced levels of Atf4 and Chop. Interestingly, protein levels of Grp78 were increased both in acute and chronic cigarette smoke-exposed mice [189]. These in vivo data indicate that chronic cigarette smoke is not sufficient to induce proapoptotic endoplasmic reticulum stress and/or COPD-like pathogenesis, but possibly involves additional detrimentally acting environmental as well as endogenous factors. Dysregulated NRF2-signalling and lack of antioxidants in old individuals [8, 9] may predispose to a proapoptotic UPR response induced by cigarette smoke. Similarly, viral and bacterial infections which are well-known inducers of endoplasmic reticulum stress, may amplify existing stress in elderly COPD patients and contribute to pro-apoptotic UPR [190]. The specific contribution of endoplasmic reticulum stress to the pathogenesis of COPD, however, remains to be systematically studied.

Defective proteasome function

Several studies in the past reported impairment of standard proteasome activities by acute cigarette smoke exposure in lung epithelial cells in vitro and in lung tissue of mice in vivo [184, 191, 192]. In contrast, chronic exposure of mice to cigarette smoke (4 months) rather activated proteasome expression and activity, possibly as part of an adaptive stress response [193]. The effect of cigarette smoke on the proteasome in aged mice has not been investigated so far. In a mouse model of experimentally reduced proteasome function, CSE accelerated emphysema-like lung destruction, suggesting that reduced proteasome function is a key driver of COPD pathogenesis [194]. In line with this observation, both standard and immunoproteasome activities are strongly impaired in end-stage COPD lung tissues, and immunoproteasome expression was specifically downregulated in total BAL cells and isolated alveolar macrophages of COPD patients [193]. These results suggest impaired MHC class I-mediated antigen presentation of viral or bacterial antigens to CD8^{+} T-cells in COPD, possibly contributing to impaired clearance of pathogens, sustained infections and exacerbations in the aged COPD patients. Impaired proteasome function in end-stage COPD is in agreement with the results by Mɪɴ et al. [184] who found abnormal accumulation of ubiquitinated (misfolded) proteins concomitantly with aggregation markers widely spread in overall lung tissue of GOLDIII and GOLDIV COPD patients. Thus, it appears that COPD patients, in contrast to mice, have lost their adaptive capacity to activate the proteasome system to cope with the elevated levels of (misfolded) oxidatively modified proteins.

Activation of autophagy

Notably, lungs of COPD patients display a remarkable activation of autophagy [195]. Markers of autophagy, such as the ratio of LC3B-II/LC3B-I, as well as the expression of ATG proteins ATG4B, ATG5-ATG12 and ATG7, were significantly enhanced in COPD lungs from all stages, including GOLD0, when compared to lungs from age-matched nonsmokers [195]. Electron microscopy, a gold standard for determination of autophagy, revealed that autophagic vacuoles were dramatically increased in COPD lung tissues from all stages, whereas little vacuole formation was evident in control tissues [195]. Widespread apoptosis in GOLD3/4 COPD lungs, as observed by many research groups [42, 79, 195], could be the cause of autophagic cell death due to "excessive autophagy" which has been associated with degradation and consumption of bystander normal cellular constituents [196].

Furthermore, increased autophagy has been clearly demonstrated as a general response to CSE in rodent lungs or epithelial cells [195, 197]. Exposure of primary HBE cells to CSE triggered dose- and time-dependent accumulation of the active form of LC3B, LC3B-II, increased autophagosome formation and was accompanied by induction of apoptosis markers [195, 197, 198]. Similarly, lungs of C57Bl/6J mice exposed to cigarette smoke for 4 months indicated increased accumulation of active LC3B-II and apoptosis markers [197]. In contrast, mice with global deficiency of LC3B (Map1lc3b[(−/−)] mice) were protected from epithelial apoptosis and cigarette smoke-induced airspace enlargement, suggesting a pivotal role of LC3B in cigarette smoke-induced apoptosis and emphysema formation [197]. Moreover, cigarette smoke-induced autophagy in wild-type mice involved extensive cilia consumption and shortening, thereby contributing to mucociliary dysfunction in bronchial cells. Mice with impaired autophagy (Becn1[(+/−)] or Map1lc3b[(−/−)] mice) resisted cigarette smoke-induced cilia shortening [196]. In addition, increased mitophagy and necroptosis contributed to cigarette smoke-induced epithelial cell death and emphysema development in

mice [199]. In accordance, the expression of mitophagy marker PINK1 was upregulated in bronchial epithelial cells, as well as whole lung tissue of COPD patients [199, 200]. While induction of autophagy might initially be part of an adaptive stress response to cope with misfolded and accumulating proteins upon oxidative stress, insufficient autophagic clearance may take place as indicated by *in vitro* experiments with CSE treated primary HBE cells isolated from COPD patients and normal HBE cells, where accumulation of p62 and ubiquitinated proteins, both indicators of insufficient autophagic clearance was observed at later time-points of CSE exposure [201]. In light of documented "excessive autophagy and apoptosis in COPD lungs", these additional data indicate that protective autophagy appears to be absent in COPD, presumably due to excessive ROS overwhelming the autophagy-lysosomal system.

Taken together, the proteostasis system is severely imbalanced in COPD, with activation of chaperones, the UPR and autophagy but impairment of the proteasomal protein degradation pathway.

Impaired proteostasis in IPF
Dysregulated chaperone expression and activity

Several studies demonstrated increased expression of various HSP and endoplasmic reticulum chaperones in IPF lung tissue, with localisation in fibroblast foci, abnormal bronchiolar epithelium and/or AECII [202–205]. The multifunctional HSP90 chaperone is upregulated in fibroblast foci and abnormal bronchiolar epithelium, as well as AECII of IPF patients. HSP90 acts as a crucial mediator of TGF-β1 signalling in fibrotic lung diseases, and inhibition of HSP90 by the small molecule 17-AAG (17-allylamino-17-demethoxygeldanamycin) prevented myofibroblast transdifferentiation in response to TGF-β1 exposure from both fibroblasts and AECII-like A549 cells *in vitro* and in bleomycin-challenged mice *in vivo* [202]. Induction and upregulation of HSP27 in abnormal bronchiolar basal cells of IPF lungs might promote their exaggerated proliferation and migration during the aberrant bronchiolisation process of damaged alveoli in IPF [203, 204]. In contrast, both the major inducible (HSP72) and constitutive (HSC70) isoforms of HSP70 are reduced in cultured IPF-fibroblasts *in vitro* and in fibrotic areas of IPF-lungs *in vivo* as compared to normal lungs from age-matched donors [206]. TGF-β1 suppressed HSP70 protein expression in primary human lung fibroblasts and homozygous $Hsp70^{(-/-)}$ knockout mice revealed accelerated development of lung fibrosis in response to bleomycin compared to wild-type control animals [206]. *Vice versa*, overexpression of Hsp70 in transgenic mice protected against bleomycin-induced lung fibrosis [207].

The endoplasmic reticulum chaperone GRP78 is upregulated in AECII and overall lung tissue of old IPF patients *versus* age-matched organ donors, concomitantly with other markers of endoplasmic reticulum stress [204, 205, 208, 209]. Of note, alveolar epithelial GRP78 upregulation in sporadic IPF does not differ from familial IPF associated with *SFTPC* mutations [205] which are reported to result in misfolding and aggregation of the mutant SP-C proprotein [205]. However, there might be context-specific regulation as BOROK *et al.* [133] observed downregulation of GRP78, but concomitant upregulation of GRP94 in AECII of IPF patients as compared to AECII from age-matched donors. Mice with conditional AECII-specific knockout of *Grp78* developed lung fibrosis similar to human IPF after 2 weeks following tamoxifen treatment, with increased susceptibility of aged and male mice [133].

Taken together, it appears that the inducibility of some HSP and endoplasmic reticulum chaperones in response to stress is not impaired in the ageing lung under conditions of IPF, but their function is presumably compromised due to increased protein oxidation [134, 135, 140, 141].

Activation of endoplasmic reticulum stress

A growing body of evidence implicates a non-adaptive pro-apoptotic endoplasmic reticulum stress response which largely involved CHOP as a critical mediator of AECII apoptosis upon injury in both sporadic and familial IPF pathogenesis [133, 204, 205, 208, 210–212], thus representing a common pathomechanistic principle. In familial cases, this may be caused by mutations in the surfactant protein (SP)-C (*SFTPC*) and SP-A2 (*SFTPA2*) genes, which cause misfolding of SP-C and SP-A2 proteins, respectively [205, 213–215]. In sporadic cases the reasons are as yet unclear, but various conditions such as oxidative stress, DNA-damage, ageing, protein overload, viral infections and expression of damaged/mutant proteins may well contribute to protein damage and misfolding, and thus endoplasmic reticulum stress and UPR induction, with AECII being primarily affected [18, 216, 217]. IPF AECII show increased levels of oxidative stress and protein oxidation [104, 112], proteostasis imbalance, genomic instability, telomere attrition [117, 118], deregulated nutrient sensing and mitochondrial dysfunction [18, 177] which are all potent endoplasmic reticulum stress stimuli. This may all (together) contribute to non-adaptive proapoptotic endoplasmic reticulum stress in AECII in the presence of "second hits", especially in the more common sporadic form of IPF without genetic predisposition. In accordance, old C57Bl/6J mice (aged >18 months), but not young mice (aged 2 months) develop lung fibrosis that is associated with increased AECII endoplasmic reticulum stress and apoptosis when challenged with murine γ-herpesvirus 68 [218].

As outlined above, aged mice with an inducible AECII-specific knockout of *Grp78* are highly sensitive to endoplasmic reticulum stress-mediated AECII apoptosis and lung fibrosis compared to young knock-out mice [133]. The tight interconnection between oxidative stress and endoplasmic reticulum stress in IPF pathogenesis is supported by the reported increases in protein oxidation in IPF AECII [104, 112] and the fact that antioxidants reduce endoplasmic reticulum stress and improve protein secretion in an *in vitro* model of protein misfolding [219]. Moreover, it was demonstrated that direct administration of glutaredoxin into airways in mice with TGF-β1- or bleomycin-induced lung fibrosis reduced protein oxidation and attenuated collagen deposition and fibrosis, even when administered to fibrotic, aged mice [112]. Aerosolised administration of the glutathione precursor N-acetyl-L-cysteine also attenuated bleomycin-induced lung fibrosis in mice [220]. All these results support the suggestion that the lack of antioxidant capacity observed in aged individuals [8, 9] and patients with IPF [86–89, 96] also contributes to endoplasmic reticulum stress induction. In addition, DNA damage, genomic instability and aberrant DNA repair induced by shortened telomeres may also cause proteotoxicity through translation of aberrant proteins and consecutive UPR activation in IPF AECII [216, 221]. It is also intriguing to speculate that oxidative stress and defective telomere maintenance in the IPF AECII affects surfactant production and processing of hydrophobic surfactant proteins in these cells [101, 216].

In contrast to proapoptotic endoplasmic reticulum stress in IPF AECII, various studies document that (prosurvival acting) adaptive UPR mechanisms mediated by the ATF6α- and the IRE1α/XBP1 branch in IPF fibroblasts contribute directly to fibrogenesis and ECM production, involving upregulation of endoplasmic reticulum chaperones. GRP78 and XBP1 are significantly overexpressed in myofibroblasts of fibroblast foci in IPF [209, 222]. Similarly, ATF6α, XBP1, GRP78 and calreticulin are upregulated during TGF-β-induced myofibrobast differentiation of primary mouse and human lung fibroblasts [209, 223–225], whereas apoptotic endoplasmic reticulum stress indicators are not induced [209]. RNAi mediated depletion of GRP78 or calreticulin abrogates TGF-β-induced ECM production in mouse, rat and human IPF fibroblasts [209, 223]. A more recent study by GHAVAMI *et al.* [222] describes that the adaptive IRE1α/XBP1 pathway as well as autophagy promote the profibrotic effects of TGF-β1 in isolated IPF fibroblasts *ex vivo*. It is well-known that the UPR may induce cytoprotective autophagy under constant endoplasmic reticulum stress, through the PERK/ATF4 branch [139, 226, 227] and the IRE1α/XBP1 arm [228], to support the degradation of "unwanted" proteins and non-functional organelles to maintain endoplasmic reticulum homeostasis. In line with these observations, TGF-β1-induced autophagy and ECM production is abated by inhibiting IRE1α activity [222]. It thus appears that an adaptive endoplasmic reticulum stress response takes place in contractile protein-expressing myofibroblasts, aiming to maintain endoplasmic reticulum proteostasis under conditions of elevated ECM protein production.

Altered proteasome function

In IPF fibrotic lungs, the expression and activity of the proteasome system is generally activated [204, 229, 230], while expression of the 11S proteasome regulator PSME1, which is involved in degradation of oxidised proteins, is decreased in IPF [95, 96]. Protein degradation of damaged proteins appears to be impaired especially in AECII of IPF patients with familial *SFTPC* mutations. Overexpression of misfolded BRICHOS mutant SP-C proteins leads to the formation of SDS-insoluble proSP-C macroaggregates *in vitro* and intracellular amyloid/aggresome formation *in vivo* [213, 214, 231]. It was associated with defective ERAD and impaired proteasome function [214, 231]. Defective protein degradation is also suggested in AECII of sporadic IPF patients, where increased ROS and protein oxidation occur concomitantly with impaired detoxifying mechanisms [96, 104, 112]. Defects in surfactant processing in IPF AECII [101, 216] together with the propensity of the very hydrophobic SP-B and SP-C proteins to form β-amyloid structures [232] might contribute to the observed upregulation of endoplasmic reticulum and cytosolic chaperones such as GRP78, PDIA4, HSP90β, Hsc70/Hsp90-organising protein and the ERAD component valosin-containing protein (VCP) in these cells [204]. VCP is involved in the export of polyubiquitinated misfolded proteins from the endoplasmic reticulum to the cytoplasm for degradation by the 26S proteasome [233]. VCP is also found in non-degradable polyubiquitinated substrates and abnormal multiprotein aggregates [234, 235]. The abnormal increase in VCP levels in AECII of IPF lungs might thus reflect an impairment of proteasome function in this highly secretory cell-type [204]. On the contrary, collagen-producing IPF fibroblasts/myofibroblasts rely on an increased activity of the 26S proteasome to cope with activated protein turnover [230]. Such activation in proteasomal protein degradation accords very well with the above described pro-survival activity of the adaptive UPR in ECM producing myofibroblasts [209, 222, 223].

Defective autophagy

Autophagy is impaired in lungs of IPF patients as indicated by reduced levels of autophagy markers and rare occurrence of autophagosomes, although the underlying mechanisms have not yet been identified

[236, 237]. In agreement, immunohistochemical evaluation of IPF lung tissue revealed increased level of polyubiquitinated proteins and p62, both markers of insufficient autophagy, in epithelial cells as well as fibroblastic foci [237]. Aggregation markers, such as VCP (see above), are upregulated in AECII and whole lung tissue of IPF lungs [204], suggesting defective clearance by impaired autophagy. Insufficient autophagic clearance of misfolded proteins may also contribute to endoplasmic reticulum stress-induced apoptosis of IPF AECII, a critical event in IPF pathogenesis [208, 210–212]. Accordingly, activation of autophagy by the well-known mTOR (mammalian target of rapamycin) inhibitor rapamycin in mice was demonstrated to attenuate bleomycin-induced lung fibrosis in two independent studies [238, 239]. Furthermore, IPF AECII suffering from endoplasmic reticulum stress also exhibited marked accumulation of dysmorphic and dysfunctional mitochondria and lack of PINK1, indicative of defective mitophagy. Moreover, $PINK1^{(-/-)}$ deficient mice developed similarly dysmorphic, dysfunctional mitochondria in the AECIIs and were vulnerable to apoptosis and development of lung fibrosis [177].

Interestingly, the role of autophagy in fibrotic lung fibroblasts/IPF fibroblasts is controversial. On the one hand, it has been demonstrated that TGF-β1-mediated mTOR activation and concomitant autophagy inhibition was required for myofibroblast differentiation in normal human MRC-5 lung fibroblasts, as rapamycin reversed this effect and blocked myodifferentiation [236]. On the other hand, ARAYA et al. [237] showed increased autophagy and ECM protein production in primary normal lung fibroblasts in response to TGF-β1 treatment, which was augmented by siRNA mediated knockdown of the autophagy-related genes MAP1LC3B and ATG5 in the presence of TGF-β1. Of interest, in cardiac fibrosis, autophagy appeared to be required for fibroblast to myofibroblast phenoconversion, as autophagy was induced by TGF-β1 and inhibition of autophagy by bafilomycin-A1 or chloroquine repressed the fibrogenic response of cardiac fibroblasts [240, 241]. In agreement with a profibrotic role of autophagy in lung fibroblasts, GHAVAMI et al. [222] recently reported that TGF-β1 activated IPF fibroblasts trigger the IRE1α/XBP1-UPR-arm for induction of autophagy to enable the excessive biosynthesis of ECM proteins in the endoplasmic reticulum. Inhibition of IRE1α activity abated the profibrotic effect of activated autophagy in this model, underscoring the crucial interplay of endoplasmic reticulum stress and autophagy in IPF [222].

In conclusion, IPF lungs show signs of severely disturbed proteostasis such as elevated endoplasmic reticulum stress and proteasome function but defective autophagy.

Cellular senescence

The cellular programme of senescence is characterised by the irreversible cell cycle arrest in response to cellular ageing and telomere attrition, oncogenic signalling and stress stimuli, including cytokines, ROS (e. g. cigarette smoke) or DNA damage [242]. This latter form of senescence is also known as stress-induced premature senescence [243]. In contrast to apoptotic cells, senescent cells are metabolically active and may affect neighbouring cells via secretion of multiple inflammatory proteins (IL-1β, IL-6, TNF-α, IL-8, monocyte chemoattractant protein-1), ROS molecules and ECM-degrading enzymes including matrix metalloproteinases (MMPs), which is described as the senescence-associated secretory phenotype (SASP) [244]. Markers for cellular senescence include increased expression of cell-cycle regulators and tumour suppressors p53, $p21^{Cip1}$, and $p16^{INK4a}$, as well as upregulation of β-galactosidase activity. While the p53/$p21^{CIP1}$ pathway seems to play a key role in the initiation of senescence upon DNA damage stress [31], the pathway involving p16 and the retinoblastoma family of proteins (Rb family) appears to have a central role in the maintenance of senescence [245]. Although cellular senescence exhibiting SASP has been considered to protect against tumorigenesis and cancer [246], accumulation of senescent cells over time in ageing mammals affects tissue regenerative capacity and leads to tissue dysfunction and frailty, thereby shortening life span [247].

Cellular senescence in COPD

In support of the concept of accelerated lung ageing in COPD, there is evidence for the accumulation of senescent cells in lungs from patients with COPD [248, 249]. Increased levels of senescence-related markers β-galactosidase, p21 and p16 are observed in numerous cell-types of COPD lungs, including AECII, smooth muscle and endothelial cells and fibroblasts, as compared to age-matched non-COPD controls [248–252]. The senescence phenotype in COPD epithelial cells has been found to be associated with a robust inflammatory SASP repertoire [253]. Senescence of COPD fibroblasts was also linked with abnormal high expression of proinflammatory mediators, dysregulated ECM protein production, decreased contractility and migration, reduced TGF-β1 responsiveness, and an overall impaired repair capacity [254–256]. It is hypothesised that in COPD senescent epithelial and mesenchymal cells with the secretion of proinflammatory SASP factors contribute to chronic inflammation, tissue destruction and emphysema, and impaired wound healing processes in the lung [248, 253, 256]. Of note, in an experimental model of induced cellular senescence by global genetic or lung epithelial cell-specific ablation of p16INK4a p16, mice were not protected from emphysema development, inflammation, and deterioration of lung function when exposed to chronic cigarette
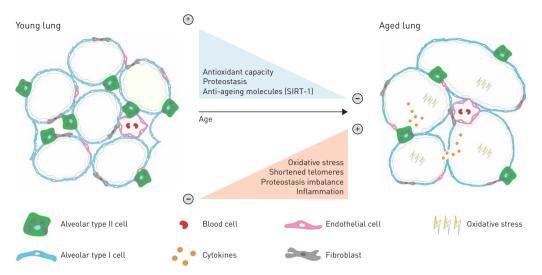
FIGURE 2 Pathogenesis of lung ageing. The decline of major components of overall adaptive homeostasis capacity (*e.g.* antioxidant defences and proteostasis capacity) during ageing increases oxidative stress which further exerts detrimental effects on genomic integrity and proteostasis, leading to a progressive deterioration of pulmonary function and a pro-inflammatory shift. This is accompanied by a structural change designated "senile emphysema", which is characterised by reduced proliferation of alveolar/septal wall cells and eminent enlargement of the alveolar airspaces without alveolar wall destruction.

smoke or elastase [257]. These results indicate that this pathway of cell senescence might not be causal for the development of experimentally induced lung emphysema. However, numerous reports have demonstrated a role for senescence in COPD development [258–260], and chronic exogenous and endogenous oxidative stress is considered as a key driver of cellular senescence in COPD [260]. "Senotherapies" are currently suggested for COPD treatment, either by inhibiting the pathways that lead to cellular senescence (senostatics) or by deleting senescent cells (senolytics) [260, 261]. Senolytic drugs remove senescent cells though induction of apoptosis while having no or little effect on proliferating cells [260]. The combination of dasatinib, a tyrosine kinase inhibitor, and the polyphenol quercetin (both acting as senolytics) reduced the numbers of senescent cells, p16 expression, and the SASP-response in elderly mice and restored age-related cardiovascular changes [262]. For detailed information, the reader is referred to the recent reviews on senotherapies in age-related diseases as well as their potential use as future therapeutic options in COPD [260, 261, 263].

Cellular senescence in IPF
Some studies have suggested that accumulation of senescent epithelial cells and fibroblasts contribute to the fibrogenic process in experimental lung fibrosis and human IPF *via* the action of SASP mediators [217, 264, 265]. These studies demonstrated attenuation of bleomycin-induced lung fibrosis *in vivo* by depletion of senescent cells using senolytic drugs such as dasatanib and quercetin [264, 265], as well as in tissue cultures of fibrotic mouse lungs *ex vivo* [217]. It was also shown that the stress-induced SASP of irradiated senescent IMR90 lung fibroblasts and that of senescent AECII isolated from bleomycin injured lungs are fibrogenic, as it exhibited amongst others increased levels of IL-6, MCP-1, MMP12, PAI-1 and TGF-β1 [217, 264]. However, two contrary reports also exist which report resolution and attenuation of lung fibrosis in bleomycin mice by lung fibroblast senescence [266, 267]. In agreement, myofibroblast senescence has been widely documented to play important roles in fibrosis resolution in the liver, skin, heart and kidney [268, 269]; and the SASP of liver and skin myofibroblasts included reduced levels of collagen-I and TGF-β1 [269, 270]. In support of a cell-type specific role of senescence, it was shown by two independent research groups that systemic induction of telomere attrition (a well-known driver of cellular senescence) in AECII, but not mesenchymal cells, contributed to lung remodelling and fibrosis in mice [271, 272]. This notion is further supported by the regional expression patterns of established senescence markers in IPF lungs. Although increased expression of β-galactosidase, p53, p21 and p16 has been reported in some studies in both fibroblasts and AECII of IPF lung tissue [217, 264, 273–276], other reports document significantly lower level of basal p53 in cultured IPF fibroblasts or no detection of p16 or p21 in fibroblasts, as well as in fibroblast foci of IPF lungs [237, 277, 278]. Furthermore, the reported upregulation of cancer-associated class-I HDAC enzymes in IPF fibroblasts, in particular HDAC2 [279], preclude the existence of high p21 level in this cell-type, since HDAC2 inhibits p53 transcriptional activity through deacetylation and represses p21^{Cip1} expression [280]. Moreover, telomere attrition has been observed in AECII, but not fibroblasts of IPF lungs, and both in sporadic as well as familial IPF cases associated with ubiquitous *TERT* and *TERC* mutations [117, 118]. LEHMANN *et al.* [217] demonstrated
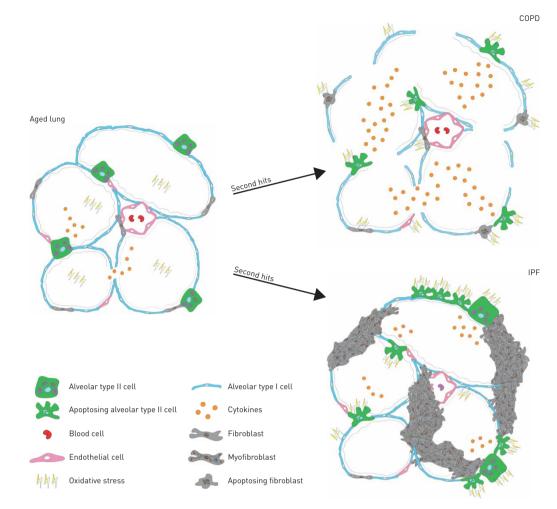
FIGURE 3 Predisposition of the aged lung to chronic lung diseases such as COPD or idiopathic pulmonary fibrosis (IPF). The vulnerable aged lung is primed to oxidative stress, cell injury and disrepair, acting as the "first hit". Environmental challenges such as cigarette smoking, air pollution or viral/bacterial infections may serve as "second hits" which worsen age-related events and contribute to persistently elevated oxidative stress levels, that overwhelms the (weakened) defence and repair pathways. Whereas increased demise of type-II alveolar epithelial cells (AECII) is a common feature in both diseases, in COPD, however, oxidative stress and its detrimental consequences also result in a loss of structural fibroblasts and septal endothelial cells, leading to an irreversible loss of lung tissue and progressive formation of emphysematous spaces. In IPF, death and loss of AECII triggers abnormal fibroblast proliferation, myofibroblast differentiation and irreversible scar tissue generation, with oxidative stress contributing to this process. The "origins" that predispose the ageing lung to develop either COPD or IPF after the decisive injury, remain elusive.

that epithelial cells, in particular AECII, represent the major cell type that is affected by senescence in fibrotic lungs of bleomycin-treated mice as well as IPF-patients, and that profibrotic SASP factors released by senescent AECII of bleomycin mice contribute to the proliferation of neighbouring fibroblasts thus contributing to pulmonary fibrosis. A more recent study demonstrated that p53-induced AECII senescence was able to drive the development of pulmonary fibrosis in mice, and highlighted the importance of senescent AECII as a source of TGF-β and the driving force of epithelial TGF-β for fibroproliferation [281]. However, the SASP of senescent cells does not appear to be the sole driving force for the development of human IPF. It must not be ignored that loss of AECII and alveolar stem cell depletion *per se* represent a critical determinant of IPF. It is still a matter of ongoing research, how senescence contributes to IPF pathogenesis. As outlined in this review, other hallmark mechanisms of ageing, such as endoplasmic reticulum stress and oxidative stress, defective proteostasis and mitochondrial dysfunction also contribute to IPF, through demise of AECII (by cell death) and consecutive loss of alveolar progenitors.

Despite the promising results of senolytic drugs in preclinical studies [264, 265], as well as from a small phase 1 study in IPF patients using dasatanib and quercetin [282], the use of senolytics as a therapeutic option for the treatment of IPF should be carefully reconsidered, because not every cell type and every type of senescence seems to be affected by these drugs [283].

Conclusion

In conclusion, the summarised data provide strong evidence that the age-related decline in redox and proteostasis balance is a major contributing factor to oxidative stress and impaired proteostasis in COPD and IPF. Oxidative stress is indeed one of the central effector pathways which affects several of the ageing pathways, such as genomic instability, telomere attrition, loss of proteostasis, mitochondrial dysfunction and cellular senescence. The stress-induced senescence programme as outlined above may also represent a proxy to further spread cellular damage and activate the immune system.

In COPD and IPF, both the age-dependent decline of central quality control and antioxidant defence mechanisms and the resulting induction of oxidative stress might represent a "first hit" that renders lungs susceptible to injury and disease. Altered lung development may further add to this process. Environmental insults such as smoking, air pollution and/or infections appear to serve as "second hits" which contribute to persistently elevated oxidative stress levels, that overwhelm the (weakened) adaptive defence and repair pathways, thereby promoting respiratory disease development.

It is thus obvious that the steady impairment of protective and adaptive stress responses in the ageing lung causes a different response to additional stress than in the young lung (figure 2). We propose that at some point the balance towards detrimental stress responses is tipped which will then promote an avalanche of detrimental reactions that contribute to development of respiratory diseases, such as COPD or IPF (figure 3). While there are obvious differences in the cell-specific regulation of adaptive stress responses in COPD and IPF (as outlined above) the particular mechanisms that drive one or the other disease still remain elusive. Nevertheless, it is clear from this review that treatment options for COPD and IPF should aim for the activation of overall adaptive homeostasis capacity, such as by augmenting endogenous antioxidant defences and stabilising the proteostasis network, and the suppression of oxidative stress sources in the older patients.

Conflict of interest: None declared.

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