DEFINING AND CHARACTERIZING RESPIRATORY DISEASE IN AN AGING POPULATION

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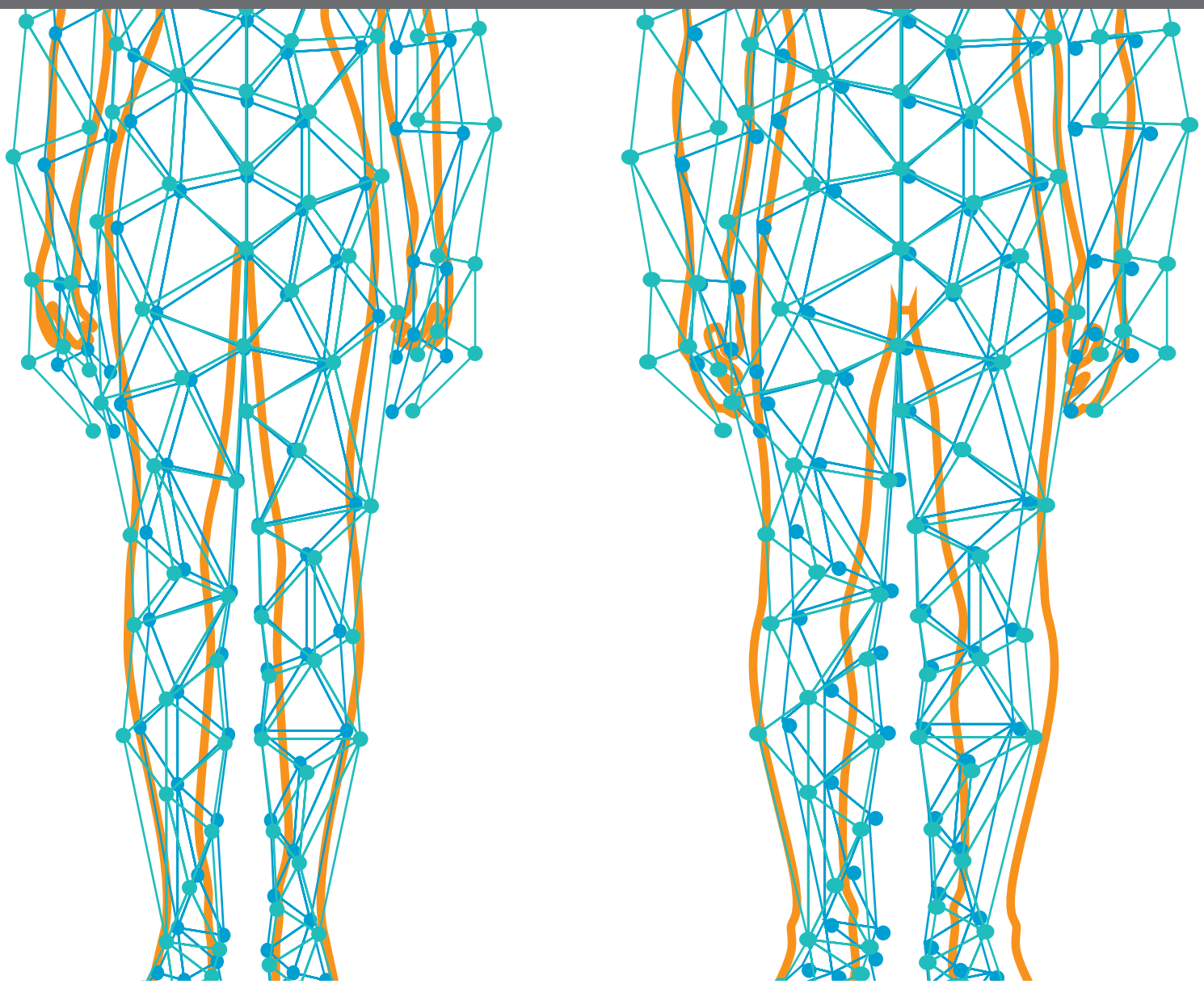

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DEFINING AND CHARACTERIZING RESPIRATORY DISEASE IN AN AGING POPULATION

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MINI REVIEW
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The Impact of Aging in Acute Respiratory Distress Syndrome: A Clinical and Mechanistic Overview

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Acute respiratory distress syndrome (ARDS) is associated with increased morbidity and mortality in the elderly population (≥65 years of age). Additionally, age is widely reported as a risk factor for the development of ARDS. However, the underlying pathophysiological mechanisms behind the increased risk of developing, and increased severity of, ARDS in the elderly population are not fully understood. This is compounded by the significant heterogeneity observed in patients with ARDS. With an aging population worldwide, a better understanding of these mechanisms could facilitate the development of therapies to improve outcomes in this population. In this review, the current clinical evidence of age as a risk factor and prognostic indicator in ARDS and the potential underlying mechanisms that may contribute to these factors are outlined. In addition, research on age-dependent treatment options and biomarkers, as well as future prospects for targeting these underlying mechanisms, are discussed.

Keywords: acute respiratory distress syndrome, acute lung injury, aging, immunosenescence, biomarkers

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a common yet complex syndrome that develops in critically ill patients. Clinically, ARDS presents as acute hypoxemia and the presence of bilateral pulmonary infiltrates that cannot be fully ascribed to heart failure or fluid overload (1–3). This flooding of the alveoli with protein-rich edema is associated with the breakdown of the alveolar-capillary unit and, in many cases, the influx of neutrophils and other immune cells into the air spaces. These immune cells, along with activated epithelial and endothelial cells, release numerous pro-inflammatory mediators, which propagate a profound inflammatory response, and a battery of cytotoxic species, causing damage to the lung parenchyma (4). This endothelial and alveolar cell injury with fluid and cellular exudation has been termed as diffuse alveolar damage (DAD), however, data from biopsy and autopsy studies have shown that only half of those who meet the clinical definition of ARDS present with DAD (5–7). The presence of DAD is associated with increased mortality in ARDS (7). Despite over 50 years of investigation, ARDS is still under-recognized and no specific, effective treatments are available (8, 9). Mortality due to ARDS remains high, with most estimates indicating mortality rates in the region of 30–50% (8, 10, 11). ARDS is a heterogeneous disease process that may be triggered by a variety of direct or indirect pulmonary injuries including pneumonia, aspiration, non-protective mechanical ventilation, chest trauma,

sepsis, and acute pancreatitis. Although ARDS can affect people of all ages, increasing age is a widely reported risk factor and is associated with increased mortality in ARDS patients (12, 13). This relationship has been emphasized by the current Covid-19 pandemic, in which severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a causative agent of ARDS, has exhibited significant mortality among older patients (14–16).

The world's population is aging; over 65s are expected to account for 1 in 6 of the population by 2050, an increase from 1 in 11 in 2019 (17). With a strong association between aging and increased incidence of disease, including respiratory conditions such as chronic obstructive pulmonary disease and acute infection (18), a thorough understanding of age-related changes in the lung and how these impact on disease risk and severity will be important to effectively manage the health of an ever aging population. Age-associated structural changes in the lung mainly encompass increased alveolar spaces, reduced elasticity (19, 20) and a gradual decrease in lung function (21). An accumulation of senescent cells in the lung is observed in the elderly population (22). The release of soluble mediators by senescent cells contributes to persistent, low-level inflammation (23, 24), and impaired regenerative abilities (25). Similarly, the phenomenon of inflamm-aging is apparent in elderly cohorts where heightened levels of inflammatory factors are observed but several cell types including neutrophils and macrophages have attenuated phagocytic capabilities (26, 27). Thus, there are a number of pathophysiological features of the aging lung that may contribute to increased disease severity in elderly populations, which will be discussed in further detail in this review.

CLINICAL CHARACTERISTICS OF AGING IN ARDS

To date, few studies have focused primarily on assessing age *per se* as a risk factor for ARDS. Patients with ARDS often carry multiple risk factors and co-morbidities, some of which may be influenced by age themselves, which makes isolating the influence of age particularly challenging. Epidemiological studies frequently report the mean/median age of patients with ARDS at 55–65 years, making it a disease of late middle-age rather than exclusively old age (8, 10, 11, 28). One approach is to quantify the incidence of ARDS in the general population within different age groups. A study in the United States reported an overall incidence of 64 cases per 100,000 person-years but incidences of up to 306 cases per 100,000 person-years in the 75–84 years age group (10). Studies in Spain (29) and Taiwan (30) have shown similar age-dependent increases in incidence in the general population. The relationship between age and ARDS can also be examined by comparing cohorts of patients with another risk factor (pneumonia, trauma etc.) who did not develop ARDS with patients who had the same risk factor and did develop ARDS. Such analysis reveals that age appears to be a risk factor for ARDS in combination with certain other etiologies or risk factors. For instance, a large study of trauma patients by Johnston et al. demonstrated that the mean age of patients who developed ARDS was significantly higher than that of those who did not, and that the risk of developing ARDS increased up to 60–69 years of age (31). Likewise, patients with Covid-19 pneumonia who develop ARDS are significantly older than those who do not (16). However, in a recent study by Iriyama et al., there was no significant difference between the mean age of patients who developed ARDS following non-pulmonary sepsis and those who did not (32). Similarly, the age of patients who developed ARDS did not vary significantly from those who did not following out-of-hospital cardiac arrest (33), neutropenia after hematologic malignancy (34), or kidney transplant (35). Thus, it appears that while older age may generally be considered a risk factor for ARDS, larger studies are required to assess this relationship in ARDS with particular etiologies, especially indirect ARDS.

Accurately extrapolating the prognostic implication of age in ARDS is challenging. Observational data is derived from retrospective studies, which have inherent methodological flaws whilst elderly patients may frequently be excluded from randomized controlled trials. Consequently, the existing evidence-base may be prone to inaccuracy. Age is associated with worse ICU outcome and is embedded within several critical illness severity scoring systems, such as SAPS (simplified acute physiology score) and APACHE (acute physiology and chronic health evaluation) (36). These scores provide utility in prognostic prediction in the ICU setting, however, they lack specificity (37). There are currently no validated mortality prediction scoring systems in ARDS (36). Villar et al. prospectively evaluated a 9-point scoring tool (APPS) encompassing clinical parameters including age, PaO_2/FIO_2 and plateau pressure at 24 h following ARDS diagnosis ($n = 600$) (38). The authors stratified patients into three severity groups, assigning higher score in accordance with age group and reported a significant association with mortality (38). Subsequent external validation of the APPS score concluded that the model showed moderate-accuracy in predicting all-cause hospital mortality (39). Within adult populations, increased age is generally associated with higher disease severity (29). Several investigators have reported age as an independent predictor of mortality in ARDS (40, 41). It should be emphasized that substantial heterogeneity exists within the syndrome and non-linear associations between age and mortality have been reported within specific ARDS phenotypes [i.e., trauma; (42, 43)]. When considering the trauma-ARDS cohort, the highest burden of mortality has been shown to exist at the extremes of age (42, 43). A large retrospective review of a national trauma database ($n = 1{,}297{,}190$) reported the ARDS-related mortality was highest in those $\geq$80 and $\leq$4 years (42). Other investigators have reported no significant difference in mortality using a dichotomous age cut-off of 65 years, potentially supporting the notion that the relationship between age and ARDS may be more complex (44). While age is associated with ARDS mortality, there is no significant difference between ventilator or ICU free days, length of stay in ICU or length of stay in hospital between patients above 65 and those under 65 (12, 45). Indeed, a number of studies have concluded that co-morbidities rather than age primarily affect prognosis following ICU admission (46–48). Further elucidation of the impact of age upon prognosis in ARDS is required and well-designed prospective observational studies are paramount.

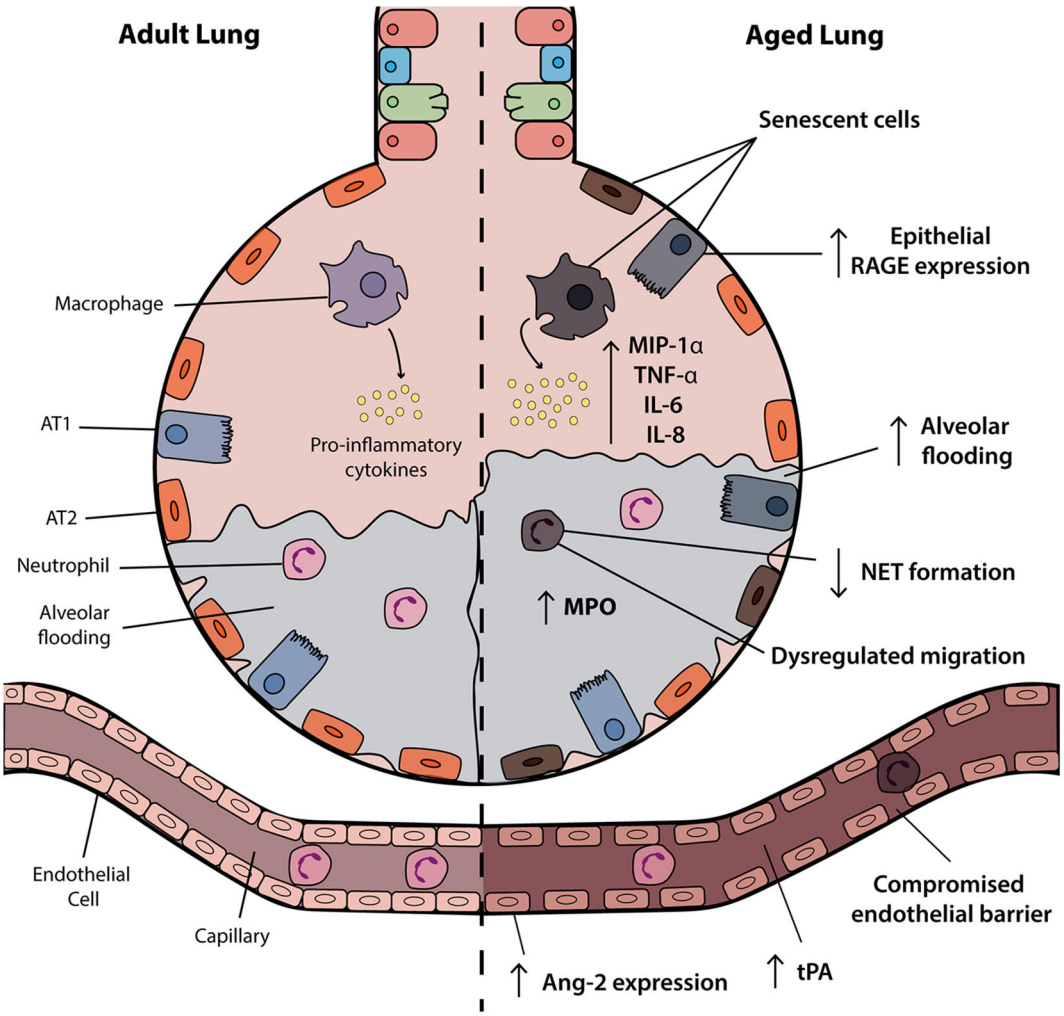

FIGURE 1 | Age-associated changes in the ARDS lung. The aged alveolus in acute respiratory distress syndrome (ARDS) demonstrates a number of age-related changes in the alveolar epithelium, vasculature and immune cells that may contribute to disease pathogenesis in elderly patients. Vascular permeability may be affected by changes in angiopoietin-2 (Ang-2) expression and receptor for advanced glycation end products (RAGE) signaling is associated with inflammation and progression of the exudative and fibroproliferative phases of ARDS. Accumulation of senescent cells and the senescence-associated secretory phenotype (SASP) results in the release of soluble pro-inflammatory mediators such as IL-6 and IL-8. Although increased levels of inflammatory factors are observed, neutrophils and macrophages may have attenuated functional activities. AT1, alveolar type I; ATII, alveolar type II; NET, neutrophil extracellular trap; tPA, tissue plasminogen activator.

MECHANISMS OF AGE-DEPENDENT RISK AND SEVERITY IN ARDS

A number of factors influence disease severity in ARDS including features of the immune system, vasculature and structural components of the airway, as highlighted in **Figure 1**. Research to date has highlighted a number of mechanisms through which age may affect these systems and alter the risk of developing and/or disease severity in ARDS.

The decline in immune function with age is well-documented and is referred to as immunosenescence. Immune cell activation is a major mediator of inflammation in ARDS and immunosenescence may impact on the pathogenesis and outcomes in the aged ARDS subpopulation. Experimental murine models of ARDS induced by endotoxin indicate that aged mice display increased bronchoalveolar lavage fluid (BALF) cell counts, protein concentration, and cytokines such as the neutrophil chemoattractant CXCL1/KC in comparison to younger mice (49–51). Alterations in innate immune cell function, such as macrophage and neutrophils, may contribute to this heightened inflammatory response (52, 53). Alveolar macrophages (AMs) have increased expression of genes associated with lung injury and fibrosis (54) and defective phagocytic ability (26). In one study of lipopolysaccharide (LPS)-induced acute lung injury (ALI), expression of the M1-macrophage markers, CD80 and CD86, were increased in AMs from older mice and *in vitro* these cells had increased propensity to produce MIP-1α in response to LPS (50). Impairment of antigen presentation in aging macrophages has also been observed and resulting loss of bacterial and viral clearance from

the lung could further promote inflammation and damage (55). In addition, aberrant neutrophil responses such as dysregulated chemotaxis (27) and the inability to form neutrophil extracellular traps (56) were observed in elderly cohorts. Studies across a breadth of ages in a small cohort of patients with ARDS suggest a correlation between age and the neutrophil biomarker myeloperoxidase (MPO) in BALF (57). Impaired neutrophil phagocytosis and microbial killing has also been observed in the elderly (58). The output of adaptive immune cells, particularly T cells, reduces over time. However, regulatory T (Treg) cells, which are responsible for regulation of immune responses, increased with age (50, 59). In experimental ARDS, aged groups show concomitant increases in both Tregs and inflammatory markers in comparison to younger groups (50). This suggests that while Treg numbers may be increased in the elderly, their anti-inflammatory function may be repressed. Clinically, there has been no consensus on the role of Tregs in ARDS with both protective (60, 61) and deleterious (62) effects observed. Furthermore, impaired adaptive immunity with decreased $CD8^+$ T cells in advancing age may contribute to poor prognosis in ARDS (50). Recently published research brings together single cell sequencing data and tissue proteomics to document cell specific changes associated with aging (54, 63). Overall, changes in immune cell function may drive age related ARDS pathogenesis and further characterization of these changes may highlight potential targeted therapies that may be beneficial in an elderly population.

In addition to the immune system, age-related changes in structural components of the airway and vasculature may also contribute to ARDS pathogenesis. Within the vasculature, changes in the expression of angiopoietin-2 (Ang-2) have been linked to disease severity in ARDS (64). Ang-2 is a growth factor involved in angiogenesis which also plays a role in modulating endothelial permeability. Increased Ang-2 levels result in endothelial destabilization and increased vascular permeability (65). A positive correlation between Ang-2 and age has been observed in animal models and in the clinical setting (66, 67). Increases in Ang-2 may therefore be associated with ARDS severity in aging patients via increased vascular permeability and alveolar filling. Aging is also associated with an accumulation of senescent cells (68). Senescent endothelial cells (ECs) in ARDS demonstrate exacerbated permeability responses and this permeability was sustained by the upregulation of the reactive oxygen species (ROS)-generating enzyme NADPH oxidase-4 (Nox4) (69). In addition, Nox4 interacts with toll like receptor (TLR)-4 inducing NF-κB activation and inflammation (70). Taken together, these effects may contribute to increased ARDS severity, and inhibition of Nox-4 attenuated ALI in mice (71). Oxidative damage plays a significant role in age-related disease severity. Both aging and ARDS are associated with a cumulative oxidant burden and the generation of oxidized phospholipids has been linked to vascular leakage and inflammation and, as a result, the pathogenesis of ALI (72).

The structural components and mechanics of the lung also play an important role in airway pathophysiology. Aging is associated with changes in lung function and respiratory mechanics. Increased compliance and decreased small airway diameter in the elderly increase the severity of lung injury under mechanical ventilation (73). In addition to vascular ECs, senescence of airway epithelial cells and fibroblasts has also been linked to the pathogenesis of idiopathic pulmonary fibrosis through their expression of pro-inflammatory and profibrotic factors (74). This may also be of relevance in ARDS where a late fibrotic phase is associated with poor prognosis, high mortality and prolonged ventilator dependence (75). Targeting of senescent cells was beneficial in *in vivo* models of fibrosis, improving lung function and physical health (74). A decline in cellular autophagic function has also been observed in the aging lung (18) and induction of autophagy protected against lung permeability in models of ALI (76), highlighting the importance of this process. Another potential mechanism by which aging may contribute to increased ARDS severity is through the upregulation of receptor for advanced glycation end products (RAGE), a marker of type I alveolar epithelial cell injury (77). RAGE signaling likely promotes progression of both the exudative and fibroproliferative phases of ARDS (78, 79). Upregulation of RAGE was observed in mouse models of ALI and in ARDS patients and was associated with increased disease severity including increased mortality and fewer ventilator-free and organ failure-free days (77, 80). Treatment with recombinant soluble (s)RAGE, which acts as a decoy receptor, reduced inflammation in a murine model of LPS-induced ALI, suggesting that sRAGE participates in negative feedback after excessive inflammatory processes (81). Increased RAGE levels in the elderly may represent a potential mechanism through which aging effects ARDS severity, although a better understanding of how RAGE and its ligands impact pulmonary inflammation is needed (82).

IMPACT OF AGE ON TREATMENT STRATEGIES AND SPECIFIC BIOMARKERS

Biomarkers represent an important tool in diagnostics and patient stratification. However, to date, no single specific ARDS biomarker has been validated (83). Analysis of systemic inflammatory mediators and endothelial activation markers in plasma from adults with ARDS found that, compared to younger patients, elderly patients had lower levels of inflammatory mediators and endothelial activation markers (interleukin (IL)-6, IL-8, IL-10, interferon-γ, fractalkine, intracellular adhesion molecule (ICAM)-1, E-selectin) and higher levels of platelet factor-4 and tissue plasminogen activator (tPA) (12). Furthermore, advanced age was found to be independently associated with increased plasma levels of tPA and decreased plasma levels of fractalkine and E-selectin (12). However, only tPA was linked with outcome, accounting for 10% of the association between age and outcome. Although these findings suggest a possible link between aging, fibrinolysis and mortality, further work is needed to determine the role of tPA. More recently, Schouten et al. analyzed age-dependent differences in inflammatory and endothelial activation markers in BALF from ARDS patients of various ages (57). Although no difference was detected between adult and elderly cohorts, higher levels of neutrophil markers were found with increasing age and the association between increasing age and increased levels of MPO,

IL-10, P-selectin, and decreased ICAM-1 remained significant after correction for severity of ARDS (57). These data suggest that plasma may be more useful in identifying age-dependent differences in ARDS patients although longitudinal analysis of BALF and plasma from the same cohort of patients may be required to shed light on the pathophysiology of pulmonary vs. systemic responses and how they interact across the spectrum of ARDS phenotypes. Like other diseases, variations in published data highlight the difficulties in identifying biomarkers that relate to disease severity and outcome in this heterogeneous syndrome, and a broader "omic" (e.g., proteomic) approach may be required. The identification of specific biomarkers would be helpful to identify those in the elderly population most at risk and to guide potential treatment options, although this may be a difficult feat.

Conclusive evidence to support age-dependent differences in treatment responses is lacking. However, there is evidence from both *in vivo* (84, 85) and computational studies (73, 86) to suggest that ventilator induced lung injury (VILI) may be more prevalent in the elderly population as a result of increased lung compliance and stiffness. The use of conservative fluid management and low tidal volume ventilation (LTVV) has been shown to attenuate this age-associated increase in ventilator associated mortality *in vivo* (87). Clinically, the benefits of LTVV are uncertain and adherence among clinicians is low (88, 89). Further study into the clinical benefits of LTVV specifically in the elderly subpopulation would be valuable. Extracorporeal membrane oxygenation (ECMO) has become a valuable therapy to support recovery in ARDS. Data on the use of ECMO in the elderly is limited, with most data coming from retrospective studies. Current data suggests that age is not a contraindication for ECMO, rather, its use should be decided on a case by case basis (90–92). Potential predictors that should be considered before initiation of ECMO support include presence of cardiogenic shock, APACHE II, and SAPS II scores (93). The accumulation of senescent cells has been suggested as a mechanism for increased mortality in elderly ARDS populations. Therefore, the use of senolytic drugs may represent an interesting therapy to improve outcomes in elderly patients with ARDS. Though senolytic drugs have not yet been evaluated clinically in ARDS, they have demonstrated benefits in preclinical models of fibrosis (74, 94). In the future, the identification of those most at risk of developing ARDS, along with the identification of the most effective treatment strategies for phenotypically and demographically distinct groups of patients, will be of paramount importance in reducing mortality in the elderly ARDS population.

CONCLUSIONS AND FUTURE DIRECTIONS

Research to date has highlighted the significant analytical challenges that ARDS presents due to its complex etiology. Although aging may alter the immune system, vasculature, and airway epithelium to promote the pathogenesis of ARDS, further work is needed to elucidate these mechanisms and this will be critical to improving our understanding of the effects of aging on the risk of developing ARDS and its severity. While more remains to be learned, accumulation of senescent cells along with increased oxidative damage, defective autophagy, and increased vascular permeability due to age-related changes are likely to play a role. A better understanding of the contributions of these individual factors along with the potential impact of co-morbidities could help direct future therapeutic strategies. A clearer understanding of the treatment options that are most beneficial for elderly patients with ARDS will be vitally important to reduce mortality in this subpopulation. Cell therapies represent a promising therapeutic option in ARDS (95), and these may be of particular significance in the elderly population where senescence and immune-aging render host cells ineffective and the lungs exhibit age-related structural changes (26, 27, 69). Treatment with mesenchymal stem cells, endothelial progenitor cells, or Tregs may attenuate age-related pathology. Alternatively, slowing the process of aging to mitigate age-related functional decline may be worth exploring in ARDS. Indeed, calorie restriction slowed the rate of aging and reduced ARDS risk in a preclinical model (96). Other potential therapeutics targeting aging including rapamycin (97), mTOR inhibitors (98), and metformin (99) could also be examined in ARDS. These therapies have been discussed in a recent review (100) and represent a number of potential avenues for the development of more targeted therapies for elderly ARDS patients. The stratification of ARDS patients into hypoinflammatory and hyperinflammatory subphenotypes revolutionized the clinical perspective. Studies of several ARDS cohorts identified distinct subphenotypes based on plasma inflammatory markers, level of shock and metabolic acidosis (101–103). These subphenotypes appear to respond differently to treatment (102), and while age does not significantly differ between the subphenotypes in most of these cohorts (101, 103, 104), further studies focusing on the role of age as a determinant of ARDS subphenotypes may be a useful strategy to direct treatment. Alternatively, identification of subphenotypes within elderly ARDS patient populations allowing stratification of these patients may also prove useful.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Long-Term Non-invasive Ventilation: Do Patients Aged Over 75 Years Differ From Younger Adults?

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Background: Noninvasive ventilation (NIV) is accepted as standard of care for chronic hypercapnic respiratory failure (CHRF) and is being increasingly implemented in older subjects. However, little is known regarding the use of NIV on a long-term basis in the very old. The outcomes of this study were: 1/to report the proportion of patients ≥ 75 years old (elderly) among a large group of long-term NIV users and its trend since 2000; 2/to compare this population to a younger population (<75 years old) under long-term NIV in terms of diagnoses, comorbidities, anthropometric data, technical aspects, adherence to and efficiency of NIV.

Methods: In a cross-sectional analysis of a multicenter cohort study on patients with CHRF under NIV, diagnoses, comorbidities, technical aspects, adherence to and efficiency of NIV were compared between patients ≥ 75 and <75 years old (chi-square or Welch Student tests).

Results: Of a total of 489 patients under NIV, 151 patients (31%) were ≥ 75 years of age. Comorbidities such as systemic hypertension (86 vs. 60%, $p < 0.001$), chronic heart failure (30 vs. 18%, $p = 0.005$), and pulmonary hypertension (25 vs. 14%, $p = 0.005$) were more frequent in older subjects. In the older group, there was a trend for a higher prevalence of chronic obstructive pulmonary disease (COPD) (46 vs. 36%, $p = 0.151$) and a lower prevalence of neuromuscular diseases (NMD) (19 vs. 11%, $p = 0.151$), although not significant. Adherence to and efficacy of NIV were similar in both groups (daily use of ventilator: 437 vs. 419 min, $p = 0.76$; $PaCO_2$: 5.8 vs. 5.9 kPa, $p = 0.968$). Unintentional leaks were slightly higher in the older group (1.8 vs. 0.6 L/min, $p = 0.018$).

Conclusions: In this cross-sectional study, one third of the population under NIV was ≥ 75 years old. Markers of efficacy of NIV, and adherence to treatment were similar when

compared to younger subjects, confirming the feasibility of long-term NIV in the very old. Health-related quality of life was not assessed in this study and further research is needed to address this issue.

Keywords: non-invasive ventilation, elderly, prevalence, compliance, chronic obstrucive pulmonary disease, obesity hypoventilation syndrome, ventilator settings

INTRODUCTION

Long-term non-invasive ventilation (NIV) is an accepted treatment for chronic hypercapnic respiratory failure (CHRF). Since the beginning of long-term NIV in the mid 80's, its prevalence has increased substantially, with a European average of $6.6/10^5$ inhabitants in the Eurovent study (2000-1) (1) and reported values of $33–47/10^5$ inhabitants in recent data from Switzerland and Northern Europe (2018) (2). Long-term NIV is indicated in case of CHRF resulting from restrictive disorders (i.e., peripheral or central neurological disorders, myopathies, diseases affecting chest wall and/or pulmonary compliance such as kyphoscoliosis, or morbid obesity), obstructive disorders (such as chronic obstructive pulmonary disease) or sleep-related breathing disorders (3). Chronic obstructive pulmonary disease (COPD) is presently the most frequent cause of CHRF leading to NIV and tends to increase in older subjects (2). It is now well-accepted that NIV is efficient for treating acute episodes of hypercapnic respiratory failure (AEHRF) in older subjects by improving gas exchange and reducing respiratory work (3). However, little is known regarding the use of NIV on a long-term basis in the very old.

Advanced age *per se* may compromise the use of NIV in this population because of functional decline, cognitive impairment, frailty, and other causes of disability including neurological or rheumatological impairment. Appropriate positioning of interface, and thus unintentional leaks and treatment efficacy, could also be a problem, as well as skin sores. Furthermore, a French multicentric cohort study suggested that, although NIV improves arterial blood gas (ABG) and sleepiness in subjects ≥75 years of age, it does not improve health-related quality of life (HRQL, measured by the SF-36), as opposed to what is seen in younger subjects (4). Indeed, there are to date very few published reports of long-term NIV in the very old (4–7).

We recently conducted a comprehensive survey of NIV in the Cantons of Geneva and Vaud (≈ 1,300,000 inhabitants) (2). The outcomes of this study were: 1/to document the proportion of patients ≥75 years old (elderly) among a large group of long-term NIV users and its trend since 2000; and 2/ to compare this population to younger subjects (<75 years old) under long-term NIV in terms of diagnoses, comorbidities, technical aspects (i.e., choice of devices, modes, settings, interfaces, unintentional leaks), adherence to and efficiency of NIV. HRQL was not assessed in this study.

PATIENTS AND METHODS

A detailed description of the methodology of this study has been recently published (2). Briefly, this analysis was based on a cross-sectional observational study performed in 2018 and including all patients under NIV in our area (Cantons of Geneva and Vaud, that is, a population of 1,288,378 inhabitants). Identification, screening, and data collection were performed by two investigators between June 1, 2016 and July 10, 2018.

Ethical approval was granted by the Cantonal Commission for Research Ethics (CCER) in Geneva, Switzerland (n°PB_2016-00925/15-275) in agreement with the amended Declaration of Helsinki. Trial was registered at clinicaltrials.gov (N°: NCT04054570).

The present study focuses exclusively on patients treated by pressure-cycled, multimodal and volume-cycled ventilators at home or in a long-term care facility (not a hospital) for ≥3 months. Patients were excluded if they refused data collection regarding their long-term NIV, or if their pulmonologist refused to participate in the study.

Outcomes

The outcomes of this study were: 1/to report the proportion of patients ≥75 years old (elderly) in a comprehensive database of long-term NIV users in the Cantons of Geneva and Vaud; 2/to compare these results to a similar study performed earlier in our area (data from 2000); 3/to provide a detailed description of diagnoses, comorbidities, technical aspects (i.e.: choice of devices, modes, settings, interfaces, unintentional leaks), adherence to and efficiency of NIV and compare these results with similar data collected from younger subjects (<75 years old) under long-term NIV in the same cross-sectional cohort study.

Data Collected

Anthropometric data, diagnoses leading to NIV, major comorbidities, pulmonary function tests, ABG, nocturnal pulse oximetry, technical aspects of NIV (i.e.: choice of devices, modes, settings, interfaces, unintentional leaks), adherence and relevant items from reports downloaded from device software were collected from medical records. Availability of recent pulmonary function tests, ABG and nocturnal pulse oximetry depended on "real-life" follow-up procedures and medical records. Data recorded were the most recent measurements performed within the 12 months prior to data collection. Tests which had not been performed within the previous 12 months were considered as missing data. We also recorded whether NIV was initiated in an acute setting, or electively, and as an outpatient vs. an inpatient setting (hospital ward). Prevalence was compared to values published in 2000 from the same area.

Diagnostic Categories

For all patients, indication for implementing NIV was based on the 1999 Consensus conference report. There is no "*a priori*"

attitude regarding NIV in older subjects in our area and therefore the selection of patients is not age dependent.

Statistical Analyses

Patients' characteristics, efficiency and technical aspects of NIV were described overall and by age group. Qualitative data were described as simple frequency and percentage, quantitative data were described as median (first quartile, third quartile). Qualitative data were compared between age groups using chi-square tests. Quantitative data were compared between age groups using Welch *t* tests. Statistical significance was assessed at a two-sided 0.05 alpha level for all analyses. No correction for multiple testing was applied. Analyses were performed on R software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Prevalence

The proportion of patients under long-term NIV aged ≥ 75 years of age increased in our area from 17% in 2000 (8) to 31% in 2018 (151/489) (2). In 2000 (8), 8.4% of patients (13/154) under long-term NIV had their treatment initiated at ≥ 75 years and 27/154 (17.5%) of NIV patients were aged ≥ 75 years. In 2018, 109/489 (22%) were ≥75 years of age when NIV was started, and prevalence of subjects aged ≥ 75 was 31% (151/489) (2).

Population Characteristics

Table 1 provides the basic characteristics of the study population by age group. Patients had been under NIV for a similar length of time (median value of ≈ 3 years). In the older group, there was a trend for a higher prevalence of chronic obstructive pulmonary disease (COPD) (46 vs. 36%, $p = 0.151$) and a lower prevalence of neuromuscular diseases (NMD) (19 vs. 11%, $p = 0.151$), although non-significant (**Figure 1A**). Body mass index (BMI) was similar in both groups (**Table 1**, $p = 0.26$). Older subjects had significantly more comorbidities ($p = 0.001$, **Table 1**) and comorbidities such as systemic hypertension (86 vs. 60%, $p < 0.001$), chronic heart failure (30 vs. 18%, $p = 0.005$), or pulmonary hypertension (25 vs. 14%, $p = 0.005$) were more frequently reported in the elderly (**Figure 1B**). Conversely, treatment of central sleep apnea due to opioids was less frequent in older subjects (0 vs. 5%, $p = 0.011$, **Figure 1B**). Modalities of initiation of NIV were similar in both groups: electively (48 vs. 55%, $p = 0.151$) vs after an AEHRF (52 vs. 45%, $p = 0.151$), outpatient (12 vs. 17%, $p = 0.191$) vs. inpatient setting (88 vs. 83%, $p = 0.191$) (**Table 1**).

TABLE 1 | Characteristics of study population according to age group.

	All patients 489 (100)	<75 years 338 (69)	≥ 75 years 151 (31)	*P*-value
Population characteristics				
Gender (male)	272 (56)	203 (60)	69 (46)	0.004
Age (years)	71 (59; 77)	64 (52; 71)	80 (78; 83)	NA
Age (years)*	5 to 94	5 to 74	75 to 94	NA
Age when NIV started (years)	65 (53; 73)	59 (48; 66)	76 (74; 80)	<0.001
Body-mass index (kg/m^2)	31 (24; 39)	31 (23; 40)	31 (24; 37)	0.26
Missing data (n)	1	1	0	
Number of comorbidities	3 (2; 4)	3 (1; 4)	3 (2; 5)	0.001
Missing data (n)	*1*	*1*	*0*	
Time spent under NIV (months)	39 (14; 73)	40 (14; 74)	36 (15; 69)	0.235
Initiation of NIV				
Electively	247 (53)	179 (55)	68 (48)	0.151
Missing data (n)	*22*	*14*	*8*	
After an AEHRF	220 (47)	145 (45)	75 (52)	
Missing data (n)	*22*	*14*	*8*	
Inpatient setting	400 (84)	273 (83)	127 (88)	0.191
Missing data (n)	*16*	*9*	*7*	
Outpatient setting	73 (16)	56 (17)	17 (12)	
Missing data (n)	*16*	*9*	*7*	
Diagnostic groups				
COPD	192 (39)	123 (36)	69 (46)	0.151
Obesity-hypoventilation syndrome	127 (26)	86 (25)	41 (27)	
Neuromuscular disorders	79 (16)	63 (19)	16 (11)	
Restrictive lung disorders	49 (10)	34 (10)	15 (10)	
Sleep-related breathing disorders	42 (9)	32 (9)	10 (7)	

P-values refer to Chi-square tests or Welch Student tests.
number (=n) of missing data, corresponds to the number of patients without the values of interest.
*Values listed are the most recent values obtained. Values expressed as median (interquartile range) or n (%) unless specified otherwise. *Range.*
AEHRF, acute episode of hypercapnic respiratory failure; COPD, chronic obstructive pulmonary disease; NA, not applicable; NIV, non-invasive ventilation.

Interfaces, Adjuncts to and Efficiency of NIV

Table 2 shows the impact of NIV on ABG (values are without NIV) and nocturnal pulse oximetry (NPO, overnight under NIV). Missing values reflect "real life" availability of tests during the study process. Taking into account this caveat, correction of ABG and NPO was similar in both groups (**Table 2**). Choice of interfaces was similar (**Table 3**, $p = 0.170$), with a very high proportion of facial masks in both age groups (79 vs. 72%, $p = 0.170$). Use of supplemental oxygen was more frequent in the older group (54 vs. 36%, $p < 0.001$, **Table 3**). Use of humidifiers was similar in both groups (75 vs. 70%, $p = 0.386$).

Devices, Settings, Unintentional Leaks and Adherence

Use of bi-level positive pressure ventilators (BPPV) in a spontaneous/timed (S/T) mode was by far the most frequent modality of NIV (89 vs. 80%, $p = 0.150$, **Table 3**). None of the older subjects used multimodal devices or volume-cycled modes (**Table 3**). **Table 4** shows basic ventilator settings for BPPV devices in an S/T mode (i.e., 83% of the whole population, 89% of those aged ≥ 75 years). In the elderly, 11% used auto-titrating BPPV devices. No significant difference was noted in pressure settings, back-up respiratory rate or residual respiratory events estimated by ventilator software (**Table 4**). Adherence to NIV (average time spent under NIV) was similar in both groups (**Table 4**). The percentage of patients using their device

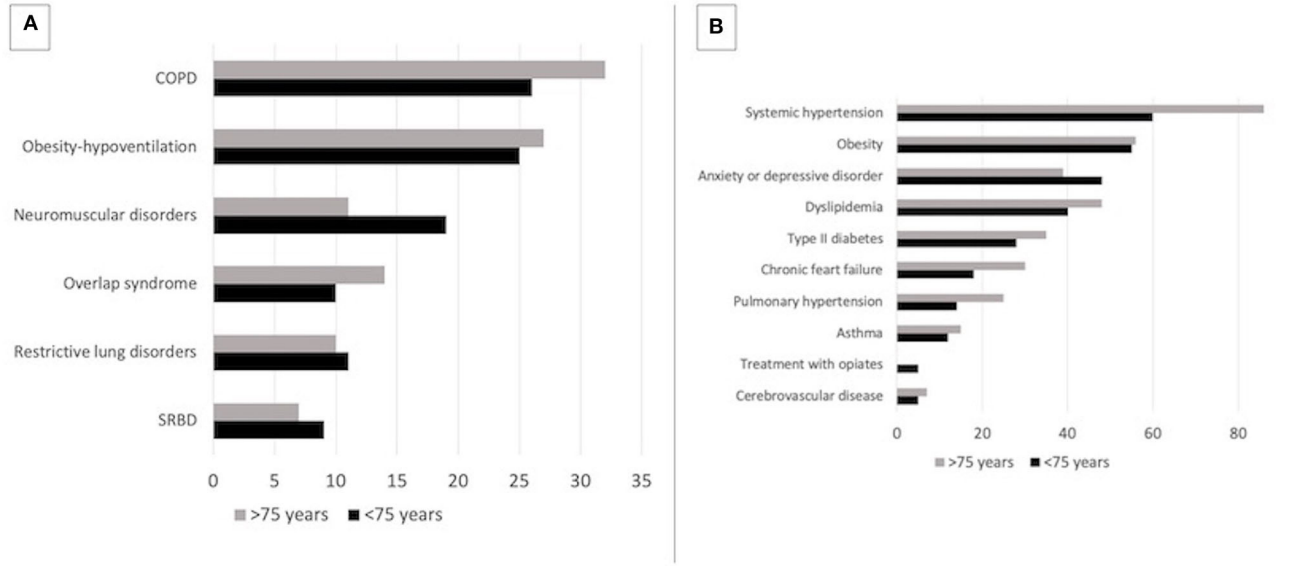

FIGURE 1 | (A) Distribution of indications for long-term NIV according to age group. **(B)** Frequencies of comorbidities according to age group. COPD, Chronic obstructive pulmonary disease; SRBD, Sleep-related breathing disorders; Overlap syndrome, association of COPD and obstructive sleep apnea syndrome.

<03:30 h was 7% in the older group and 9% in the younger subjects (median values). However, unintentional leaks (median and 95th centile values) were significantly higher in older subjects (**Table 4**).

DISCUSSION

This cross-sectional observational study shows that 1/close to one third of the population under NIV is presently aged over 75 years of age in this study area: this is a substantial increase since our previous survey, 18 years ago (8); 2/disorders leading to CHRF and NIV did not differ significantly, with a trend for a higher proportion of COPD in the older group, and a lower representation of NMD; 3/efficiency of NIV (daytime ABG, nocturnal pulse oximetry, residual respiratory events) was similar in both groups, but unintentional leaks were slightly although significantly more important in older subjects; and importantly 4/adherence to treatment did not differ between groups.

The first study focusing on NIV in older subjects in our area had identified 6 patients between 1994 and 1996 in an on-going cohort study in whom NIV had been initiated at or after 75 years of age (8, 9). Efficiency of NIV, tolerance and adherence to NIV and HRQL were all very satisfactory. What was at that time a very rare occurrence is presently standard practice with close to one third of patients under NIV being aged ≥ 75 years. Interestingly, this prevalence is exactly the same as that reported in a French multicentric cohort study (2009-14; $n = 264$) by Tissot et al. (4). Similar trends are also reported in countries which have a national register of NIV such as Norway (Norwegian national registry for long-term NIV) and Sweden (Swedevox data). In 2007 already, 12% of the population on long-term NIV in Sweden was aged over 75 (10).

In the present study, there was a trend for COPD to be more frequent and neuromuscular diseases to be less frequent in older subjects (**Figure 1A**): prevalence of COPD increases with age and is thus a more frequent cause of AEHRF and CHRF (11, 12). Conversely, NMD is rarely an indication for long-term NIV (albeit for ALS) in the elderly and subjects with NMD leading to NIV rarely reach the age of 75. Patients with "historical" causes of CHRF such as sequelae of tuberculosis or post-polio syndrome, have now almost disappeared. Other causes of CHRF in the older population such as chronic heart failure, cerebrovascular disease, or interstitial lung disease seldom lead to long-term NIV (3). Interestingly, the obesity epidemic— and thus obesity hypoventilation syndrome (OHS)— also affects the very old: 27% of our older patients are under NIV for OHS! OHS represented 52% of the elderly population in the French cohort study by Tissot et al. (4). Earlier studies reported lower figures for OHS in this age group (14–20%) (4–7), suggesting that the increase in OHS as a cause of CHRF noted in younger adults may also involve the older population.

Adherence to treatment was excellent in older subjects without any significant difference compared to younger subjects, confirming previous reports that age *per se* does not seem to adversely affect adherence (4, 6, 7). The percentage of subjects using their NIV insufficiently (arbitrarily defined as <03:30 h/day) was 7% in the older group (vs. 9% in younger subjects). However, the cross-sectional structure of our study does not allow us to comment on the discontinuation rate of NIV. The fact that these patients were on long-term NIV for a median duration of 3 years suggests that the treatment was well-tolerated, considered acceptable, and did not adversely affect HRQL.

TABLE 2 | Arterial blood gases without NIV and nocturnal pulse oximetry under NIV according to age group.

	All patients 372 (100)	<75 years 251 (67.5)	≥ 75 years 121 (32.5)	*P*-value
Arterial blood gases without NIV				
pH	7.40 (7.38; 7.43)	7.40 (7.38; 7.42)	7.40 (7.38; 7.43)	0.546
$PaCO_2$ (kPa)	5.8 (5.3; 6.5)	5.9 (5.2; 6.5)	5.8 (5.3; 6.5)	0.968
HCO_3 (mmol/L)	26.9 (24.8; 30)	26.8 (24.6; 29.6)	27.9 (25.3; 30.5)	0.183
PaO_2 (kPa)*	9 (8; 9.9)	9.1 (8; 10.1)	9.1 (8.1; 10.0)	0.856
SaO_2 (%)*	94 (92; 96)	94 (92; 96)	94 (91; 96)	0.536
	All patients 197 (100)	**<75 years 138 (70)**	**≥75 years 59 (30)**	***p*-value**
Nocturnal pulse oximetry under NIV				
Mean SpO_2 (%)	92 (90; 94)	93 (90; 94)	91 (91; 94)	0.184
Time with SpO_2 < 90% (%)	6 (0.4; 34.3)	6 (0.1; 34.2)	5.3 (0.7; 34.5)	0.636
ODI ≥ 3% (events/hour)	7.3 (3.2; 13.8)	7.5 (3.2; 14)	7.3 (3.4; 13.5)	0.538
	All patients 353 (100)	**<75 years 242 (68.5)**	**≥ 75 years 111 (31.5)**	***p*-value**
Pulmonary function tests				
FEV_1 (% predicted)	46 (31; 64)	44 (29; 62)	46 (35; 64)	0.192
FVC (% predicted)	62 (45; 75)	60 (44; 74)	65 (48; 76)	0.129
FEV_1/FVC (%)	83 (63; 98)	83 (64; 98)	84 (63; 97)	0.742

P-values refer to Chi-square tests or Welch Student tests.
Values listed are the most recent values obtained.
Values expressed as median (interquartile range) or n (%) unless specified otherwise.
**PaO_2 and SaO_2 are room air values (PaO_2: n = 92; SaO_2: n = 89).*
FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; HCO_3^-: bicarbonates; NIV: non-invasive ventilation; ODI, oxygen desaturation index (≥3%); $PaCO_2$, arterial partial pressure of carbon dioxide; PaO_2, arterial partial pressure of oxygen; SaO_2, arterial oxygen saturation; SpO_2, pulse oximeter oxygen saturation.

TABLE 3 | Devices used for long-term NIV with modes, interfaces, and adjuncts to NIV according to age group.

	All patients 489 (100)	<75 years 338 (69)	≥75 years 151 (31)	*P*-value
Bi-level positive pressure ventilators (*missing data*: *n* = 3)				
ST mode	407 (83)	272 (80)	135 (89)	0.150
Auto-titrating modes	68 (14)	52 (15)	16 (11)	
Multimodal ventilator modes (*missing data*: *n* = 1)				
VAC, PC or PS	10 (2)	10 (2)	0 (0)	NA
Interfaces				
Facial	359 (73)	240 (72)	119 (79)	0.170
Nasal	91 (19)	66 (20)	25 (17)	
Nasal pillows	40 (8)	31 (9)	7 (5)	
Missing data (*n*)	*3*	*3*	*0*	
Other adjuncts to NIV				
Humidifiers	350 (72)	237 (70)	113 (75)	0.386
Missing data (*n*)	*2*	*2*	*0*	
Oxygen	196 (40)	123 (36)	81 (54)	<0.001

P-values refer to Chi-square tests or Welch Student tests.
number (=n) of missing data, corresponds to the number of patients without the values of interest.
Values listed are the most recent values obtained.
Values expressed as median (interquartile range) or n (%) unless specified otherwise.
NA, not applicable; NIV, non-invasive ventilation; PC, pressure control; PS, pressure support; ST, spontaneous-timed; VAC, volume assist-control.

TABLE 4 | Settings and data provided from ventilator software for bi-level positive pressure ventilators in ST mode according to age group.

	All patients 407 (100)	<75 years 272 (67)	≥75 years 135 (33)	*P*-value
Settings for bi-level positive pressure ventilators in ST mode				
IPAP (cmH_2O)	18 (16; 21)	18 (16; 22)	18 (16; 21)	0.93
EPAP (cmH_2O)	7 (5; 10)	7 (5; 10)	7 (6; 9)	0.27
BURR (cycles/min)	14 (12; 17)	14 (12; 17)	15 (12; 17)	0.08
Data from ventilator software				
Median leaks* (L/min)	1.2 (0; 7.2)	0.6 (0; 7)	1.8 (0; 8.4)	0.018
Median leaks* 95th centile (L/min)	14.4 (3; 30)	12.6 (2.3; 28.3)	16.8 (6; 33)	0.028
AHI (events/hour)	1.5 (0.4; 4.1)	1.3 (0.3; 3.6)	2.1 (0.6; 5)	0.138
Adherence				
Daily use of ventilator (min)	428 (310; 532)	419 (304; 534)	437 (321; 529)	0.76

P-values refer to Chi-square tests or Welch Student tests.
Values listed are the most recent values obtained.
Values expressed as median (interquartile range) or n (%) unless specified otherwise.
**Unintentional leaks.*
AHI, apnea-hypopnea index; BURR, back-up respiratory rate; EPAP, expiratory positive airway pressure; IPAP, inspiratory positive airway pressure; ST, spontaneous-timed.

Because of the cross-sectional nature of this study, we have no information on survival: however, as stated, patients aged ≥ 75 had been under NIV for a median of 3 years, which shows that prolonged acceptance with good adherence is possible at this age. A few studies provide encouraging figures in subjects aged ≥ 75 under long-term NIV: Laub et al. (13) and Duiverman et al. (14) noted a 5-year survival close to 50% in this age group, while Farrero et al. (5) reported a median survival of 58.5 month in non-ALS older patients.

Technical aspects of NIV did not show any major difference in the older group. All patients aged over 75 used BPPV in an S/T mode or auto-titrating devices, following the trend described for all patients since the late 90's (8). None had a volume-cycled device. Pressure settings and choice of interface (predominantly facial masks in our area) were similar in both age groups. Noteworthy are the very satisfactory results on control of ABG and nocturnal pulse oximetry: all patients were initially hypercapnic whether in a stable condition or after an AEHRF.

Unintentional leaks increased with aging: this was expected as a consequence of mispositioning of interface and/or changes in texture of facial subcutaneous tissue associated with aging. However, the median and peak values obtained in older subjects are well within what is clinically acceptable, and in most cases did not compromise the efficiency of NIV.

Study Limitations

There are several limitations to this study. 1/This cross-sectional study describes a selected population which have accepted and adapted to NIV: we do not have information as to prior dropouts or refusals. However, the large group of older subjects described supports the idea that long-term NIV is feasible on a long-term basis in the very old, with a similar efficacy in terms of correction of ABG and nocturnal SpO_2 as in younger subjects; 2/Missing data reflect the "real life" nature of this study; 3/These data are related to the socioeconomic conditions, demographics, and epidemiology prevailing in Switzerland and may not reflect findings in different geographic, economic or ethnic settings; they show however that advanced age *per se* is not a contra-indication to long-term NIV; 4/We could not provide information on burden for care-givers in older subjects: this must be further assessed, since this may be a critical factor in a population which is already affected by several comorbidities.

Clinical and Research Implications

Based on the various results of our study, age itself alone should not be a criterion of exclusion if long-term NIV is considered. However, impact on HRQL and burden placed on caregivers requires further studies.

CONCLUSIONS

In this observational study of patients on long-term NIV, patients aged over 75 years of age represented almost one third of the population treated and had the same benefit in terms of correction of ABG, nocturnal pulse oximetry, as younger adults in the same area. Adherence, residual respiratory events were similar to that of younger subjects, and quite satisfactory. Although unintentional leaks were increased in older subjects, this was within acceptable median and peak values. COPD was the most important diagnostic group, and OHS seems to be increasing. These data confirm the feasibility of long-term NIV in the very old.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Cantonal Commission for Research Ethics (CCER) in Geneva, Switzerland (no. PB_2016-00925/15-275) in agreement with the amended Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CC, PP, and J-PJ contributed to the conception and design of the study. CC and PP organized the database. CC and J-PJ wrote the first draft of the manuscript. CC, PP, DA, and J-PJ wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Idiopathic Pulmonary Fibrosis in Elderly Patients: Analysis of the INSIGHTS-IPF Observational Study

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Background: An association between idiopathic pulmonary fibrosis (IPF) and advancing age is suspected since IPF occurs primarily in patients over 60 years of age. Though, little is known about the disease in the elderly. The aim of this study was to characterize elderly IPF patients using data from the longitudinal, German-wide INSIGHTS-IPF registry.

Methods: Patients were grouped into elderly (≥75 years) and nonelderly IPF (<75 years) at the time of enrollment into the study. Baseline clinical characteristics, comorbidities, health related quality of life (HRQoL), medical therapy and survival were compared between age groups. Effects of antifibrotic therapy on forced vital capacity (FVC) were analyzed over 24 months.

Results: Of 1,009 patients, 350 (34.7%) were ≥75 years old. Elderly IPF patients compared to younger patients had a higher number of comorbidities (3.6 ± 2.5 vs. 2.8 ± 2.3; $p < 0.001$). The mean ± SD EQ-5D score (0.64 ± 0.21 vs. 0.69 ± 0.21; $p = 0.005$), and the overall WHO-5 score (13.1 ± 5.9 vs. 14.3 ± 6.0; $p = 0.015$) were significantly lower while the UCSD-SOBQ (52.6 ± 31.2 vs. 45.5 ± 31.2; $p = 0.030$) was significantly higher in elderly patients, indicating a more impaired HRQoL and more breathlessness. At baseline, 55.4% of elderly and 56.8% of nonelderly patients with IPF were treated with antifibrotic therapy ($p = 0.687$). For FVC decline after initiation of antifibrotic therapy, there was neither a significant difference between age groups at the different time points over 24 months (beta: 0.41; 95%-CI: −0.98 to 1.81; $p = 0.563$) nor over the whole course of time (beta: −0.05; 95%-CI: −0.20 to 0.09; $p = 0.478$). All-cause mortality was higher in elderly patients (49.1 vs. 37.9%; HR 1.65; 95%-CI 1.36–2.00; $p < 0.001$). Antifibrotic therapy was associated with improved survival in IPF patients, independent from age (<75 years: beta 0.76; 95%-CI: 0.59–0.99; $p = 0.049$; ≥75 years: beta 0.71; 95%-CI: 0.51–0.98; $p = 0.043$).

Conclusion: In real life, a significant proportion of IPF patients are ≥75 years old, characterized by higher number of comorbidities and global reduced HRQoL. However, the effect of an antifibrotic therapy was similar between age groups and associated with a survival benefit emphasizing the importance for an early antifibrotic therapy in IPF, independent from age.

Keywords: aging, elderly, antifibrotic therapy, prognosis, multivariate analysis

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease (ILD) characterized by clinical symptoms such as dyspnea, cough and increasing immobility (1). IPF occurs primarily in patients over 60 years of age (1, 2), for which reason a connection with aging processes has been suspected. The aging lung is subject to biological changes which make it more susceptible to disease. Recently, genetic alterations have been identified which are associated with an increased risk for the development of IPF, but may also increase the risk of other lung diseases associated with aging such as chronic obstructive pulmonary disease (COPD) or lung cancer (3). Although the exact relationship between aging and the pathogenesis of IPF is still unclear, there are a number of processes, which can be found in the disease including telomerase shortening, cell aging, mitochondrial dysfunction, and dysregulation of the extracellular matrix (4, 5).

Additionally, the advanced age of patients with IPF in general is associated with a higher number of comorbidities and reduced quality of life. Indeed, studies have shown that patients with IPF often suffer from comorbidities (6, 7), which may also be due to advanced age.

IPF is a disease with dismal prognosis. For only a few years now, there are two antifibrotic drugs approved that, while they cannot cure the disease, can slow down the loss of forced vital capacity (FVC). A subgroup analysis of the INPULSIS trials detected no differences between patients below and above the age of 65 years in the primary endpoint (annual rate of decline in FVC) and key secondary endpoints [change from baseline in St. George's Respiratory Questionnaire (SGRQ) total score or time to first acute exacerbation] in patients treated with nintedanib vs. placebo (8). However, it is unclear if antifibrotic therapy is consistently initiated and effective in IPF patients over the age of 75 years, and if the effect also can be reproduced outside randomized controlled trials, under real-life conditions.

The aim of our study was to characterize elderly patients with IPF (≥75 years) in comparison to younger IPF patients with respect to comorbidities and quality of life. Further, we wanted to evaluate the use of antifibrotic therapy and its effect on lung function and survival in elderly compared to younger patients with IPF.

METHODS

Study Population

The INSIGHTS-IPF ("Investigating significant health trends in idiopathic pulmonary fibrosis") is a German, nationwide, investigator-initiated cohort study (registered at Clinicaltrials.gov NCT01695408). Since November 2012, patients with IPF have continuously been enrolled in routine clinical care in 19 pulmonary specialist centers in Germany. Patients are eligible if they are ≥18 years old, have a study-site diagnosis of IPF following the 2011 international IPF guideline and have provided written informed consent (9). The study was approved by the Ethics Committee of the Medical faculty, Technical University, Dresden, Germany in 2012, and by additional local ethics committees in accordance with local requirements. The methodology and structure of the registry as well as a detailed description of the baseline characteristics have been reported previously (9–11). The ongoing INSIGHTS-IPF study has no explicit exclusion criteria. Clinical data are collected at the initial enrollment, and thereafter every 6–12 months. Clinical events like hospitalization, acute exacerbation (as judged by the treating physician) and death are recorded at these follow-up visits. For the data collection an internet-based, secure case report form (eCRF) was used. For the present analysis, all patients from the INSIGHTS-IPF registry with at least one follow-up assessment were included. The last patient of our analysis was included on December 11, 2019. Data cut-off date was on April 29, 2020. Based on the age at enrollment patients were grouped into "elderly IPF" (≥75 years) and "nonelderly IPF" (<75 years).

Clinical Data

At baseline and the study visits (every 6–12 months), routine pulmonary function tests were performed and different measures were documented, including FVC % predicted, diffusing capacity of the lung for carbon monoxide (DLCO) % predicted, the forced expiratory volume in 1 s (FEV1), and six-minute walk distance (6MWD). Based on lung function, age and gender, the Gender, Age, Physiology (GAP) index was calculated (12). Based on clinical judgement of overall disease course, when possible, the

Abbreviations: CI, confidence interval; COPD, chronic obstructive pulmonary disease; DLCO, diffusing capacity of the lung for carbon monoxide; EQ-5D, EuroQol five-dimensional questionnaire; FEV1, forced expiratory volume in 1; FVC, forced vital capacity; GAP, gender-age-physiology index; HR, hazard ratio; HRQoL, health-related quality of life; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; SD, standard deviation; SGRQ, St. George's Respiratory Questionnaire; UCSD SOB, University of California San Diego Shortness of Breath Questionnaire; WHO-5, World Health Organization-5 Well-Being Index; 6MWD, six-minute walk distance.

treating physician categorized the patient as stable disease, slow progression, rapid progression (10).

A range of comorbidities was documented and recorded at baseline. Based on these pre-selected comorbidities, the individual number of comorbidities was calculated.

Specific therapy, including formerly used immunosuppressants, anticoagulation, antifibrotic therapy, and long-term oxygen were documented at baseline and thereafter during study visits. For the follow-up analysis of FVC decline under antifibrotic therapy only patients, in whom an antifibrotic therapy was newly started, were eligible.

HRQoL

To evaluate health-related quality of life (HRQoL) the St. George's Respiratory Questionnaire (SGRQ) and the University of California San Diego Shortness of Breath Questionnaire (UCSD SOB) were applied as described in detail before (13, 14). In addition, the World Health Organization-5 Well-Being Index (WHO-5) was used. Based on 5 items, the WHO-5 is a short questionnaire evaluating well-being. The total score ranges from 0 to 25, whereby lower scores correspond to a lower level of well-being. A score below 13 can indicate a possible depression. Further, the EuroQol five-dimensional questionnaire (EQ-5D), generated by the EuroQol group, is used for evaluation of general healthcare and cost-utility (15). It consists of five domains (mobility, self-care, usual activities, pain or discomfort, and anxiety or depression) and a visual analog scale (VAS). A sum utility score can be calculated based on the five domain scores, which ranges from 0 to 1 (perfect health state). The scores of the VAS range from 0 (health state equivalent to death) to 100 (best imageable health state).

Data Analysis

Continuous variables are presented as the mean ± standard deviation (SD), and categorical variables are summarized by frequency and percentage. Continuously distributed sociodemographic and clinical parameters were compared by a *t*-test between nonelderly and elderly patients. A Chi-square test was used to compare categorical parameters between the two groups. The risk of mortality was investigated by a multivariable Cox proportional hazard model for overall mortality in the total sample and separately in nonelderly and elderly patients. For survival analysis, patients who underwent lung transplantation were censored at the time of lung transplantation. Potential predictor variables for overall mortality were assessed in univariable analyses. All parameters with a significance level of $p < 0.10$ were included in the multivariable model. All treatment episodes with antifibrotic therapy (nintedanib or pirfenidone) were identified at enrollment and during follow-up in the registry. There was no pre-defined treatment threshold for initiation of an antifibrotic therapy. Patients with a maximum visit of 20 days before and 20 days after the start of antifibrotic therapy were selected for the analyses of the course of FVC % pred. after start of antifibrotic therapy as described previously (16). Since the date of the start of an antifibrotic therapy did not necessarily correspond exactly to the study visit date, the lung function values at the start of antifibrotic therapy were extracted from the visits +/– 20 days before the start of therapy. The change in FVC % pred. up to 24 months after therapy start was analyzed by a generalized linear mixed model. Data management and statistical analyses were conducted with use of STATA 12.1 (StataCorp LP. Stata Statistical Software: Release 12. College Station, TX, USA).

RESULTS

Patient Characteristics

Of 1,009 patients enrolled in the INSIGHTS IPF registry until December 2019, 350 (34.7%) were ≥75 years of age. The most common initial symptoms in elderly and nonelderly patients with IPF were dyspnea (86.3 and 85.7%; $p = 0.811$) and cough (68.3 and 72.8%; $p = 0.128$). Bibasilar crackles were found in 80.0% of elderly and 82.4% of nonelderly patients ($p = 0.350$) on auscultation. Baseline characteristics of elderly and nonelderly IPF patients are shown in **Table 1**. There were marked differences with regard to mean age at enrollment (78.6 ± 3.1 years vs. 65.4 ± 7.4 years; $p < 0.001$), age at symptoms onset (75.4 ± 5.1 years vs. 61.6 ± 8.8 years; $p < 0.001$), age at IPF diagnosis (76.8 ± 4.7 years vs. 63.5 ± 8.3 years; $p < 0.001$) and duration since first IPF symptoms (3.1 ± 3.7 years vs. 3.8 ± 4.3 years; $p < 0.026$) between elderly and nonelderly IPF patients. At baseline, elderly IPF patients had significantly higher FVC (70.6 ± 17.4% pred. vs. 66.2 ± 18.8% pred.; $p < 0.001$) and FEV1 (79.9 ± 18.5% pred. vs. 73.6 ± 19.4% pred.; $p < 0.001$) and lower DLCo (34.3 ± 14.0% pred. vs. 36.8 ± 17.1% pred.; $p = 0.030$) or PaO2 (67.1 ± 10.7 mmHg vs. 69.3 ± 11.7 mmHg; $p = 0.010$), respectively. The mean ± SD BMI of elderly IPF was lower (26.7 ± 3.9 vs. 27.9 ± 4.3; $p < 0.001$) with less patients suffering from obesity (18.6 vs. 27.3%; $p < 0.001$).

UIP pattern was found in 64.3% of elderly and in 60.0% of nonelderly IPF patients. A possible UIP pattern was present in 25.7% of elderly and 29.6% of nonelderly patients and a HRCT inconsistent with UIP was found in 1.1% of elderly and 1.1% of nonelderly patients. In 8.9% of elderly and 9.0% of nonelderly patients no information on the HRCT pattern was provided. The diagnosis of IPF was more often based on HRCT (91.1 vs. 86.3%; $p = 0.025$) in elderly IPF and less patients had undergone surgical lung biopsy/histology (20.0 vs. 41.3%; $p < 0.001$). In elderly patients, there were no difference between patients without surgical lung biopsy and patients with surgical lung biopsy in terms of FVC (70.5 ± 17.7% pred. vs. 71.1 ± 16.2% pred.; $p = 0.821$), DLCO (33.7 ± 14.2% pred. vs. 37.3% pred.; $p = 0.105$), PaO2 (66.8 ± 10.6 mmHg vs. 68.6 ± 10.6 mmHg; $p = 0.302$), number of comorbidities (3.6 ± 2.5 vs. 3.5 ± 2.3; $p = 0.755$), and HRQoL (EQ-5D score: 66.2 ± 24.8 vs. 63.6 ± 20.6; $p = 0.469$; WHO-5: 13.0 ± 5.9 vs. 13.8 ± 6.3; $p = 0.398$; SGRQ: 57.8 ± 20.8 vs. 56.3 ± 26.1; $p = 0.704$). Diagnosis of IPF was based on multidisciplinary discussion in 63.4% of elderly patients and 62.7% of nonelderly patients, while in 15.7% of elderly and 15.8% of nonelderly the diagnosis of IPF was not based on a multidisciplinary discussion. There was no data available if or not a multidisciplinary discussion was performed in 20.9% of elderly and 21.6% of nonelderly IPF.

TABLE 1 | Baseline characteristics of elderly and nonelderly patients with IPF.

		Age group <75 *n* = 659	Age group ≥75 years *n* = 350	*p*-value
Sex, *n* (%)				
	Male	534 (81.0%)	280 (80.0%)	0.693
	Female	125 (19.0%)	70 (20.0%)	
Age in years, mean (sd)		65.4 (7.4)	78.6 (3.1)	<0.001
BMI in kg/m^2, mean (sd)		27.9 (4.3)	26.7 (3.9)	<0.001
	Underweight	5 (0.8%)	6 (1.7%)	<0.001
	Normal weight	148 (22.5%)	109 (31.1%)	
	Overweight	326 (49.5%)	170 (48.6%)	
	Obesity	180 (27.3%)	65 (18.6%)	
Smoking status				
	Never	221 (33.5%)	131 (37.4%)	0.176
	Former stopped	421 (63.9%)	215 (61.4%)	
	Current	17 (2.6%)	4 (1.1%)	
Age at symptom onset, mean (sd)		61.6 (8.8)	75.4 (5.1)	<0.001
Duration since first IPF symptoms				
	In years, mean (sd)	3.8 (4.3)	3.1 (3.7)	0.026
Age at IPF diagnosis, mean (sd)		63.5 (8.3)	76.8 (4.7)	<0.001
Duration since IPF diagnosis				
	In months, mean (sd)	23.1 (33.2)	21.6 (41.3)	0.537
	In years, mean (sd)	1.9 (2.8)	1.8 (3.5)	0.537
	<3 months	200 (31.2%)	114 (33.3%)	0.183
	3 to <6 months	61 (9.5%)	43 (12.6%)	
	6+ months	381 (59.4%)	185 (54.1%)	
IPF diagnosis was based on				
	HRCT	569 (86.3%)	319 (91.1%)	0.025
	Surgical lung biopsy/histology	272 (41.3%)	70 (20.0%)	<0.001
Current NYHA class				0.248
	I	41 (14.2%)	11 (8.1%)	
	II	120 (41.5%)	54 (39.7%)	
	III	117 (40.5%)	65 (47.8%)	
	IV	11 (3.8%)	6 (4.4%)	
Current Borg dyspnea index, mean (sd)		2.2 (2.3)	2.1 (2.2)	0.493
6-min walk distance, mean (sd)		299.3 (196.5)	275.5 (164.6)	0.063
Lung function test, mean (sd)				
	FEV1	73.6 (19.4)	79.9 (18.5)	<0.001
	FVC	66.2 (18.8)	70.6 (17.4)	<0.001
	TLC	70.2 (20.2)	69.4 (14.6)	0.522
	DLCO	36.8 (17.1)	34.3 (14.0)	0.030
	PaO2	69.3 (11.7)	67.1 (10.7)	0.010
Physician's global assessment of clinical course of IPF				
	Stable disease	268 (40.7%)	150 (42.9%)	0.189
	Slow progression	173 (26.3%)	85 (24.3%)	
	Rapid progression	62 (9.4%)	21 (6.0%)	
	No judgement possible	156 (23.7%)	94 (26.9%)	

Data are presented as mean ± SD, or n (%).
BMI, body mass index; IPF, idiopathic pulmonary fibrosis; HRCT, high resolution computed tomography; NYHA, New York Heart Association; FEV1, forced expiratory volume in 1; FVC, forced vital capacity; TLC, total lung capacity; DLCO, diffusing capacity of the lung for carbon monoxide.

Further, elderly IPF patients had a significantly higher number of comorbidities at baseline (3.6 ± 2.5 vs. 2.8 ± 2.3; $p < 0.001$; **Table 2**). Comorbidities that were more prevalent in elderly IPF included left heart failure (10.3 vs. 4.7%; $p = 0.001$), coronary artery disease (36.9 vs. 22.5%; $p < 0.001$), peripheral arterial disease (5.4 vs. 2.6%; $p = 0.020$), atrial fibrillation (15.4 vs.

TABLE 2 | Comorbidities and number of comorbidities in elderly and nonelderly patients with IPF.

	Age group <75 years *n* = 659	Age group ≥75 years *n* = 350	*p*-value
Left heart disease, *n* (%)	31 (4.7)	36 (10.3)	0.001
Coronary artery disease, *n* (%)	148 (22.5)	129 (36.9)	<0.001
Cerebrovascular disease[a], *n* (%)	45 (6.8)	28 (8.0)	0.494
Peripheral arterial disease[b], *n* (%)	17 (2.6)	19 (5.4)	0.020
Atrial fibrillation, *n* (%)	37 (5.6)	54 (15.4)	<0.001
Deep venous thrombosis, *n* (%)	13 (2.0)	10 (2.9)	0.370
Pulmonary arterial embolism, *n* (%)	10 (1.5)	11 (3.1)	0.082
Pulmonary hypertension, *n* (%)	96 (14.6)	59 (16.9)	0.337
Arterial hypertension, *n* (%)	332 (50.4)	222 (63.4)	<0.001
Diabetes mellitus, *n* (%)	140 (21.2)	92 (26.3)	0.070
Emphysema, *n* (%)	65 (9.9)	36 (10.3)	0.832
Lung cancer, *n* (%)	11 (1.7)	5 (1.4)	0.771
Obstructive sleep apnoea, *n* (%)	73 (11.1)	29 (8.3)	0.161
Depression/depressive disorder, *n* (%)	48 (7.3)	9 (2.6)	0.002
Anxiety, *n* (%)	32 (4.9)	6 (1.7)	0.013
Other comorbid diseases, *n* (%)	338 (51.3)	189 (54.0)	0.412
Number of comorbidities, mean (sd)	2.8 (2.3)	3.6 (2.5)	<0.001
None, *n* (%)	89 (13.5)	29 (8.3)	0.001
1–3, *n* (%)	354 (53.7)	165 (47.1)	
≥4, *n* (%)	216 (32.8)	156 (44.6)	

Data are presented as mean ± SD, or n (%).
[a]*Carotid stenosis, stroke.*
[b]*Symptomatic or ankle brachial index <0.8.*

TABLE 3 | Medical therapy at baseline in elderly and nonelderly patients with IPF.

	Age group <75 years *n* = 659	Age group ≥75 years *n* = 350	*p*-value
Prednisone, *n* (%)	136 (20.6)	64 (18.3)	0.372
Other steroid, *n* (%)	9 (1.4)	8 (2.3)	0.280
Azathioprine, *n* (%)	11 (1.7)	3 (0.9)	0.294
Cyclophosphamide, *n* (%)	1 (0.2)	0 (0.0)	0.466
Mycophenolate mofetil, *n* (%)	1 (0.2)	0 (0.0)	0.466
N-Acetylcysteine, *n* (%)	144 (21.9)	69 (19.7)	0.429
Other, *n* (%)	16 (2.4)	9 (2.6)	0.889
Anticoagulation–prophylactic, *n* (%)	51 (7.8)	57 (16.3)	<0.001
Anticoagulation–therapeutic, *n* (%)	63 (9.6)	66 (18.9)	<0.001
Pirfenidone, *n* (%)	256 (38.9)	112 (32.0)	0.032
Nintedanib, *n* (%)	119 (18.1)	82 (23.4)	0.042
Antifibrotic therapy (nintedanib or pirfenidone), *n* (%)	374 (56.8)	194 (55.4)	0.687
Long-term oxygen therapy, *n* (%)	200 (30.4)	109 (31.1)	0.795

Data are presented as n (%).

TABLE 4 | Health-related quality of life in IPF.

		Age group <75 years *n* = 659	Age group ≥75 years *n* = 350	*p*-value
Overall assessment of current health state, *n* (%)				0.067
	Very good	6 (1.2)	1 (0.4)	
	Good	144 (28.5)	48 (19.7)	
	Medium	257 (50.8)	135 (55.3)	
	Poor	89 (17.6)	55 (22.5)	
	Very poor	10 (2.0)	5 (2.1)	
EQ-5D, mean (sd)				
	VAS 0–100	61.6 (19.8)	56.9 (19.4)	0.002
	Score	0.69 (0.21)	0.64 (0.21)	0.005
WHO-5, mean (sd)		14.3 (6.0)	13.1 (5.9)	0.015
	WHO-5 score <13	209 (43.0%)	130 (54.9%)	0.003
SGRQ, mean (sd)				
	SGRQ	47.2 (20.8)	49.4 (20.1)	0.172
	SGRQ symptoms	56.8 (20.9)	57.5 (21.8)	0.665
	SGRQ activity	60.7 (24.7)	64.3 (22.9)	0.062
	SGRQ impacts	37.2 (22.2)	37.4 (20.7)	0.897
UCSD Shortness of breath, mean (sd)		45.5 (31.2)	52.6 (31.2)	0.030

Data are presented as mean ± SD, or n (%).
EQ-5D, EuroQol five-dimensional questionnaire; WHO-5, World Health Organization-5 Well-Being Index; SGRQ, St. George's Respiratory Questionnaire; USCD-SOBQ, University of California San Diego Shortness of Breath Questionnaire.

5.6%; $p < 0.001$), and arterial hypertension (63.4 vs. 50.4%; $p < 0.001$). In contrast, depression and depressive disorder (2.6 vs. 7.3%; $p = 0.002$) and anxiety (1.7 vs. 4.9%; $p = 0.013$) were more often seen in nonelderly IPF. At baseline, lung cancer was present in five (1.4%) elderly and eleven (1.7%) nonelderly patients ($p = 0.771$). During the follow-up, two (0.6%) elderly and five (0.8%) nonelderly patients were newly diagnosed with lung cancer ($p = 0.773$).

Medical therapy at baseline is shown in **Table 3**. Elderly IPF patients were more often on prophylactic (16.3 vs. 7.8%; $p < 0.001$) or therapeutic anticoagulation (18.9 vs. 9.6%; $p < 0.001$) in comparison to nonelderly IPF.

HRQoL was assessed using different standardized questionnaires (**Table 4**), which were available in 752 patients. The EQ-5D score (0.64 ± 0.21 vs. 0.69 ± 0.21; $p = 0.005$), EQ-5D VAS (56.9 ± 19.4 vs. 61.6 ± 19.8; $p = 0.002$), the overall WHO-5 score (13.1 ± 5.9 vs. 14.3 ± 6.0; $p = 0.015$), and the number of patients with WHO-5 scores <13 (54.9 vs. 43.0%; $p = 0.003$) showed significantly reduced HRQoL in elderly patients in comparison to nonelderly IPF. Breathlessness was more commonly reported by elderly patients (mean ± SD UCSD-SOBQ 52.6 ± 31.2 vs. 45.5 ± 31.2; $p = 0.030$). The overall assessment of current health status as well as the overall SGRQ and its subdomains symptoms, activity and impacts did not show significant differences between elderly and nonelderly patients.

Antifibrotic Therapy

At baseline, 55.4% ($n = 194$) of elderly patients and 56.8% ($n = 374$) of nonelderly patients were on antifibrotic therapy, with more elderly patients on nintedanib (23.4 vs. 18.1%; $p = 0.042$) but more nonelderly patients treated with pirfenidone

(32.0 vs. 38.9%; $p = 0.032$; see **Table 3**). Of the 568 patients with antifibrotic therapy at baseline, 20.1% elderly and 20.9% nonelderly patients discontinued antifibrotic therapy during the follow-up ($p = 0.868$). While significantly more elderly had to discontinue antifibrotic therapy due to lack of tolerability (79.5 vs. 52.6%; $p = 0.019$), reasons for discontinuation of antifibrotic therapy were less often due to efficacy failure (15.4 vs. 34.6%) and other reasons (5.1 vs. 12.8%) compared to nonelderly patients. Dose distribution of pirfenidone and nintedanib did also not differ significantly between elderly and nonelderly IPF.

TABLE 5 | Multivariable model of FVC decline after initiation of antifibrotic therapy in patients with IPF.

	Beta	95%CI	*p*-value
Time	−0.18	−0.26 to −0.10	<0.001
Age group 75+ years	0.41	−0.98 to 1.81	0.563
Time × Age group 75+ years	−0.05	−0.20 to 0.09	0.478
Female	−0.37	−1.69 to 0.95	0.581
Death during Follow-up	−1.90	−2.93 to −0.86	<0.001
FVC at treatment start	0.94	0.91–0.97	<0.001
6MWD	0.00	0.00–0.01	0.041
Physician's global assessment of clinical course of IPF			
Slow progression	−2.83	−4.11 to −1.54	<0.001
Rapid progression	−3.23	−5.69 to −0.78	0.010
Number of comorbidities	0.23	0.00–0.46	0.046

FVC, forced vital capacity; 6MWD, 6 minutes walking distance; IPF, idiopathic pulmonary fibrosis.

Pirfenidone was taken in full dose (2,403 mg/day) by 61.7% of elderly patients who took pirfenidone and 67.2% of nonelderly patients ($p = 0.352$) who took pirfenidone. The full dose 300 mg/day nintedanib were taken by 80.5% elderly patients who took nintedanib and 83.5% nonelderly patients ($p = 0.537$) who took nintedanib.

Follow-up data after initiation of antifibrotic therapy was available in 148 elderly (42.3%) and 294 nonelderly (44.6%) patients. In elderly patients, FVC% pred. was significantly higher in comparison to nonelderly patients at the time of initiation of antifibrotic therapy [71.7% (95%-CI: 68.1–75.2) vs. 66.5 (95% CI: 64.1–68.9) $p = 0.002$]. Using a model analyzing FVC decline after initiation of antifibrotic therapy in patients with IPF, on average, there was no significant difference between age groups at any time point over 24 months (beta: 0.41; 95%-CI: −0.98 to 1.81; $p = 0.563$; **Table 5** and **Figure 1**). Additionally, over the course of time, there was no significant difference between elderly and nonelderly patients (beta: −0.05; 95%-CI: −0.20 to 0.09; $p = 0.478$; **Table 5**). Other associations between FVC decline under antifibrotic therapy and different clinical variables are shown in detail in **Table 5**.

Survival

In elderly IPF patients, the mean ± SD follow-up was 1.9 ± 1.3 years (median 1.5; IQR: 0.7–3.0) and in nonelderly patients 2.3 ± 1.4 years (median 2.1; IQR: 1.0–4.0). During the follow-up, 172 (49.1%) elderly and 250 (37.9%) nonelderly patients died. Reasons for death are shown in detail in **Table 6**. The all-cause mortality was higher in elderly IPF (49.1 vs. 37.9%; HR 1.65; 95%-CI 1.36–2.00; $p < 0.001$; **Figure 2**). While all other reasons for death did not show significant differences between elderly

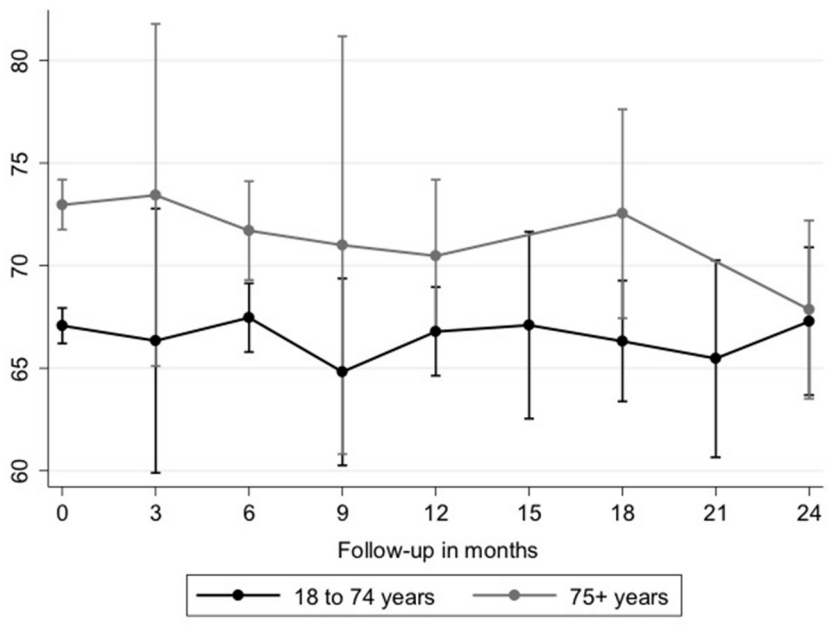

FIGURE 1 | Longitudinal course of FVC (% pred.). FVC decline is shown in elderly (gray line, $n = 148$) and nonelderly (black line, $n = 294$) patients with IPF. Differences were not statistically significant. FVC, forced vital capacity; IPF, idiopathic pulmonary fibrosis.

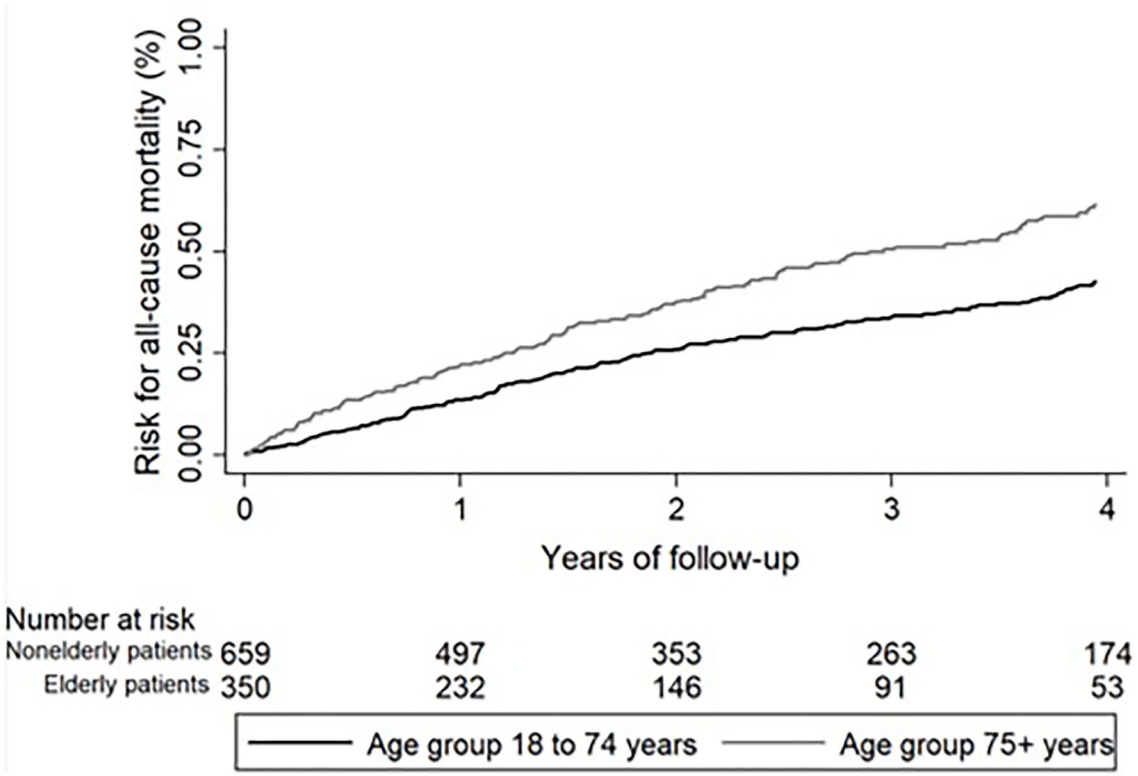

FIGURE 2 | All-cause mortality over 4 years in patients with IPF. All-cause mortality was significantly higher in elderly (black line) compared to nonelderly patients with IPF (gray line). IPF, idiopathic pulmonary fibrosis.

TABLE 6 | Reasons for death in elderly and nonelderly patients with IPF.

	Age group <75 years *n* = 659	Age group ≥75 years *n* = 350	HR	95%CI	*p*-value
All cause mortality	250 (37.9%)	172 (49.1%)	1,65	1.36–2.00	<0.001
Death related to IPF	105 (15.9%)	57 (16.3%)	1,28	0.93–1.77	0,131
Death by respiratory failure	54 (8.2%)	25 (7.1%)	1,06	0.66–1.71	0,809
Death by acute exacerbation	20 (3.0%)	7 (2.0%)	0,77	0.32–1.86	0,566
Death by right heart failure	4 (0.6%)	1 (0.3%)	0,70	0.07–6.71	0,754
Death by respiratory infection/pneumonia	18 (2.7%)	14 (4.0%)	1,81	0.89–3.70	0,102
Death related to IPF but unknown reason	29 (4.4%)	17 (4.9%)	1,44	0.79–2.63	0,230
Death by complicating comorbidity	22 (3.3%)	9 (2.6%)	0,96	0.44–2.09	0,921
Death by other cause not related to IPF	24 (3.6%)	15 (4.3%)	1,51	0.80–2.86	0,201
Reasons for death unknown	99 (15.0%)	91 (26.0%)	2,23	1.68–2.96	<0.001
Lung transplantation	42 (6,4%)	0 (0.0%)			
Combined endpoint (all cause mortality/lung transplantation)	283 (42.9%)	172 (49.1%)	1,47	1.22–1.78	<0.001

IPF, idiopathic pulmonary fibrosis.

and nonelderly IPF, more elderly patients died due to unknown reasons for death (26.0 vs. 15.0%; HR 2.23; 95%-CI: 1.68–2.96; $p < 0.001$). During the follow-up one (0.3%) elderly patients with IPF and three (0.5%) nonelderly patients died due to lung cancer (0.90; 95%-CI: 0.11–7.66; $p = 0.926$).

For the mortality analysis, patients who underwent lung transplantation were censored at the time of lung transplantation [elderly: $n = 0$ (0.0%); nonelderly: $n = 42$ (6.4)]. Choosing a combined endpoint of all-cause mortality and lung transplantation also resulted in a higher transplant-free mortality in elderly IPF (49.1 vs. 42.9%; HR 1.47; 95%-CI 1.22–1.78; $p < 0.001$).

Multivariable analysis for all-cause mortality identified the age group ≥75 years (HR 1.49; 95%-CI: 1.20–1.85; $p < 0.001$), FVC

TABLE 7 | Multivariable model assessing the effect on survival in patients with IPF.

	Total ($n = 1,009$)			Age group <75 years ($n = 659$)			Age group ≥75 years ($n = 350$)		
	HR	**95%CI**	***p*-value**	**HR**	**95%CI**	***p*-value**	**HR**	**95%CI**	***p*-value**
Age group 75+ years	1.49	1.20–1.85	<0.001						
Female	1.04	0.81–1.33	0.752	1.12	0.83–1.51	0.475	0.84	0.55–1.28	0.416
Duration since first IPF symptoms (years)	0.98	0.95–1.00	0.088	0.99	0.96–1.02	0.623	0.92	0.86–0.98	0.009
BMI	0.99	0.97–1.02	0.438	1.01	0.98–1.04	0.723	0.95	0.91–1.00	0.059
Number of comorbidities	1.03	0.99–1.08	0.136	1.05	0.98–1.11	0.153	1.02	0.96–1.08	0.578
FVC	0.98	0.98–0.99	<0.001	0.99	0.98–0.99	0.002	0.98	0.97–0.99	0.004
GAP index									
Stage I	1.00			1.00			1.00		
Stage II	1.91	1.34–2.74	<0.001	2.11	1.40–3.18	<0.001	1.83	0.81–4.15	0.149
Stage III	3.51	2.35–5.23	<0.001	4.13	2.60–6.57	<0.001	1.28	0.45–3.62	0.644
Physician's global assessment of clinical course of IPF									
Stable disease	1.00			1.00			1.00		
Slow progression	1.38	1.08–1.76	0.009	1.23	0.90–1.69	0.192	1.61	1.10–2.34	0.014
Rapid progression	1.76	1.22–2.55	0.002	1.48	0.95–2.30	0.081	2.34	1.20–4.54	0.012
Antifibrotic therapy	0.74	0.60–0.90	0.004	0.76	0.59–0.99	0.049	0.71	0.51–1.98	0.043

IPF, idiopathic pulmonary fibrosis; BMI, body mass index; FVC, forced vital capacity; GAP, gender-age-physiology index; HRCT, high resolution computed tomography; NYHA, New York Heart Association.

(HR 0.98; 95%-CI: 0.98–0.99; $p < 0.001$), GAP stage II (HR 1.91; 95-CI: 1.34–2.74; $p < 0.001$) and III (HR 1.76; 95%-CI: 1.22–2.55; $p < 0.01$), the categorization into slow (HR 1.38; 95%-CI: 1.08–1.76; $p = 0.009$) and rapid progressive (HR 1.76; 95%-CI: 1.22–2.55; $p = 0.002$) and no antifibrotic therapy (HR 1.35; 95%-CI: 1.10–1.65; $p < 0.01$) as being significant predictors for mortality in the whole study population (**Table 7**). Antifibrotic therapy was associated with a significantly better survival independent from age groups (<75 years: beta 0.76; 95%-CI: 0.59–0.99; $p = 0.049$; ≥75 years: beta 0.71; 95%-CI: 0.51–0.98; $p = 0.043$).

When only considering elderly patients, in contrast to the entire cohort, GAP stage II and III were no significant predictors but the duration since first IPF symptoms (HR 0.92; 95%-CI 0.91–0.99; 0.009). Analyzing only nonelderly patients, slow progression and rapid progression were no significant predictors for mortality.

DISCUSSION

Under clinical practice conditions, derived from our data from the INSIGHTS IPF registry, over one third of IPF patients are 75 years or older at registry enrollment. Our study identified marked clinical differences between elderly patients and nonelderly patients with IPF including a higher number of comorbidities and reduced HRQoL. At baseline, patients received equally often antifibrotic therapy without differences in the effectiveness of therapy among age groups. All-cause mortality and unknown reasons for death were more often seen in elderly IPF. Antifibrotic therapy was a significant predictor for a better survival in both elderly and nonelderly patients.

Although our study showed that a significant number of patients with IPF are 75 years or older, data about this patient group and its clinical characterization are sparse. It is known that pulmonary function and oxygenation are declining with aging (17). While our elderly patients with IPF had a better FVC and FEV1 at baseline, the gas exchange and oxygenation were more impaired in comparison to nonelderly patients. This is in contrast to a study with a smaller sample size which identified no differences in lung function, gas exchange and oxygenation between patients with IPF ≥70 years and <70 years (18). Interestingly, duration since first IPF symptoms was shorter in elderly patients. Possible explanations are that, on the one hand, elderly patients have more comorbidities, which might lead to more frequent physician consultations with subsequent medical work-up and diagnosis of lung disease. On the other hand, time to diagnosis might be longer in nonelderly patients since differential diagnosis are more likely and diagnostic work-up is more thorough compared to elderly patients. Most likely due to risk assessment, less elderly patients underwent surgical lung biopsy and the diagnosis of IPF was more often based on HRCT in elderly patients. In our study, the number of comorbidities was higher in elderly IPF, mostly driven by a higher prevalence of cardio-vascular diseases such as left heart disease, coronary artery disease, peripheral arterial disease, atrial fibrillation and arterial hypertension. IPF is known to be associated with various cardiac comorbidities (6, 19, 20). Further, all of the comorbidities we identified are known to be more prevalent in an aging population (21, 22). The prevalence of cardiovascular comorbidities (i.e., coronary artery disease and arterial hypertension) was not only high in elderly, but also in nonelderly patients with IPF. It has been shown before, that arterial hypertension can be found in 14–71% of patients with IPF (6). The prevalence of coronary artery disease was reported to range between 4 and 68% in patients with IPF (6), with higher numbers in studies including patients on the waiting list for

transplantation (23). The majority of patients in the INSIGHTS IPF registry have a history of smoking (10). Given the association between smoking and cardiovascular disease (24), this might partially explain the higher prevalence of coronary artery disease and arterial hypertension. There is also data showing that there is an association between patients with pulmonary fibrosis and coronary artery disease (25). Depression and anxiety are also frequently observed in patients with IPF, as patients with chronic lung disease are prone to psychological distress. In patients with ILD, depression and anxiety were found to be present in up to 23–49% and in 31%, respectively (26, 27). While in these studies with a smaller sample size, depression was independent from age, we found depression and anxiety to be less present in elderly IPF (26, 27). Perhaps elderly patients can cope better with the diagnosis of a fatal disease than nonelderly patients. Hence, comorbidities have to be acknowledged individually in the management of elderly patients with IPF. Overall, HRQoL was more reduced in elderly patients. The SGRQ, which was originally designed for COPD, showed no differences between age groups, which is in line with another study which identified the SGRQ independent from age in IPF (28). However, it must be noted that the EQ-5D and the WHO-5 used in our study are not IPF- or ILD-specific questionnaires. Still, we have shown recently in patients from the INSIGHTS IPF registry, that EQ-5D VAS and WHO-5 are associated with IPF disease progression as both parameters decrease significantly over time indicating a worsening HRQoL and patients with a decrease of >10% FVC over time have lower EQ-5D scores in the follow-up (14). Further, associations were seen between lower EQ-5D VAS and WHO-5 scores and mortality and also hospitalization (14).

A major focus of our study was antifibrotic therapy in an elderly IPF cohort. Recently, an US registry study identified age being negatively associated with antifibrotic therapy use (pirfenidone and nintedanib) (29). In contrast, in our study, more than half of elderly and nonelderly patients were treated with antifibrotic therapy at the time of enrollment, with no differences in age groups. Of note, there were significant differences concerning the drug distribution between age groups: while pirfenidone was more often used in nonelderly, nintedanib was more common in elderly IPF which might be explained by different side effect profiles and tolerability of the individual drugs. Recently, it has been shown that over 1 year follow up patients with IPF ≥75 years are more likely to discontinue pirfenidone and have a higher incidence of gastrointestinal disorders (30). In our study, dose reduction and discontinuation of therapy occurred equally often in both age groups. Lack of tolerability was more often a reason for discontinuation in elderly patients, while nonelderly discontinued more often due to efficacy failure or other reasons. Further, in our study, the course of FVC under antifibrotic therapy was independent from age groups, which is in line with data from a subgroup analysis of the two INPULSIS trials (nintedanib vs. placebo) (8). Taken together, this points out that the two antifibrotic therapies, which are currently the only approved medical treatment option in IPF (31), are effective in elderly patients and IPF patients shall therefore be treated with an antifibrotic therapy (32), regardless of the patients' age.

Another finding of our study was that all-cause mortality and death with unknown reasons were higher among elderly patients. This is in line with previous findings, where age has been identified as risk factor for mortality in IPF (12, 33). Interestingly, death related to IPF was not increased in elderly patients. In the present study, we identified several risk factors for early death in the entire cohort including the age group ≥75 years and no antifibrotic therapy. In 2018, data from the European IPF registry (eurIPFreg) suggested that patients with IPF and antifibrotic therapy have a better survival in comparison to patients without antifibrotic therapy (34). Recently, a positive effect of antifibrotic therapy on all-cause mortality was also identified using a large U.S. insurance data base without differences between pirfenidone and nintedanib (35). In line with these results, we also described a significantly better survival in IPF patients with antifibrotic therapy in the here analyzed INSIGHTS-IPF registry (16). In the present analysis of INSIGHTS-IPF, which is based on a considerably larger cohort and a longer follow-up, we could show that in elderly and nonelderly patients, antifibrotic therapy was significantly associated with better survival, which underlies again the benefit of antifibrotic therapy in patients with IPF independent from age. Beyond common predictors of mortality, there were also differences between the age groups. For example, duration since first IPF symptoms was a predictor for mortality, which was only identified in the elderly age group, but neither in the entire nor in the nonelderly age group. Further, slow and rapid progressions were significant predictors in elderly patients with IPF but not in nonelderly which underlines the importance of clinical assessment in the prognosis of disease in elderly patients. Interestingly, GAP stages II and III were no predictors for survival in elderly patients with IPF. This could be potentially caused by the fact, that all patients were ≥75 years, which qualifies every patient for the maximum of points in the "age" domain and leaves less discrimination (12). But more studies are needed to evaluate the usefulness of the GAP index in elderly patients.

Our study has strengths and limitations. The main strength is the large cohort of eligible patients; further strengths are the real-world setting, the prospective and multicentre data acquisition, and the comparatively long follow-up period. As limitations, first, owing to the (non-interventional) study time, the data collection varies in different study sites including documented visits and visit time-points depending on clinical routine schedules. Second, data were collected in highly experiences sites (possible selection bias). Third, transplantation was not included in the survival analysis since none of the elderly patients would have qualified for this intervention. Another limitation might be, that less elderly patients underwent surgical lung biopsy and the histological confirmation of an UIP pattern would strengthen the diagnostic confidence of IPF. However, over two thirds of elderly (and nonelderly) patients without surgical lung biopsy had UIP pattern on the HRCT scan. According to the guidelines, in patients with newly detected ILD with clinically suspected IPF and a HRCT pattern of UIP surgical lung biopsies is not essential (1, 36). In addition, the diagnosis of IPF was based on a multidisciplinary discussion in the majority of elderly and nonelderly patients, respectively. Further, a significant

amount of data is missing in this retrospective analysis. The incompleteness of data especially affected the evaluation of HRQoL questionnaires, which were not available for all patients and the lung function over time under antifibrotic therapy. We could only include a limited number of patients in the longitudinal analysis of antifibrotic therapy effects, as we only included patients in whom an antifibrotic therapy was newly initiated and a sufficient follow-up was present. Further, we only included patients in this analysis if the antifibrotic therapy was started not earlier than 20 days before and not later than 20 days after a specific study visit, as described before (16). Using 20 days, it can be assumed that the FVC value will not be too strongly influenced by the onset of the therapeutic effect after the start of therapy. Choosing only 15 days as inclusion criteria would have clearly reduced the number of cases resulting in less robust results. Nevertheless, to our knowledge, this is the first study analyzing antifibrotic therapy in elderly patients longitudinally. The analyses were focused on the comparison of elderly and nonelderly patients including the outcomes of antifibrotic therapy. The comparison of patients treated and not treated with antifibrotic therapy was not adjusted by a propensity score. However, the association between antifibrotic treatment and mortality was analyzed in a multivariable Cox regression model including the parameters that were used to estimate the propensity score in Behr et al. resulting in comparable results (16). Concerning HRQoL questionnaires, currently, there are IPF-specific HRQoL questionnaires available such as an adaption of the SGRQ (37) or the Kings' Brief Interstitial Lung Disease health status (K-BILD) (38), but these were not included in the INSIGHTS-IPF registry. Finally, the cut-off of 75 years may seem arbitrary. Still, similar results in comorbidities, HRQoL, the effects of antifibrotic therapy and survival were seen when using a cut-off of 80 years in our study cohort, but due to a more limited number of patients and less robust data we decided to use the cut-off of 75 years for elderly vs. nonelderly patients. Further, the cut-off of 75 years has been used for the definition of "elderly" patients before (39).

In conclusion, a significant proportion of IPF patients are ≥75 years old and the management of these elderly patients requires consideration of the general health situation of elderly people. This includes more comorbidities, global reduced HRQoL and a higher all-cause mortality in elderly in comparison to nonelderly patients with IPF. The effects of an antifibrotic therapy do not differ between elderly and nonelderly patients with IPF and no antifibrotic therapy is a significant predictor for mortality in both age groups, emphasizing the importance for an early antifibrotic therapy in IPF, independent from age.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data will only be available upon reasonable request. Requests to access the datasets should be directed to Gabriela Leuschner, gabriela.leuschner@med.uni-muenchen.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical faculty, Technical University, Dresden. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GL, JK, JB, and NK analyzed and interpreted the data. MK, DP, AP, JK, HW, and JB were study steering committee members and contributed to the design and/or analysis of the data. All authors were involved in collecting the data, in writing the manuscript, and approved the final manuscript.

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OPINION
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Senescent Cells in IPF: Locked in Repair?

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INTRODUCTION

Cellular senescence has been recognized since the 1960s as a cell biological program of aging. It also serves physiological functions in organismal development, regeneration and tissue repair. Senescence is triggered by replicative telomere attrition but also stress such as DNA damage, hypoxia, nutrient deprivation, mitochondrial impairment and oncogene activation (1, 2). In response to macromolecular damage, senescent cells enter a presumably permanent cell cycle arrest characterized by activation of the p53 DNA-damage response pathway and transcriptional induction of the cell cycle inhibitors p21WAF1/Cip and p16INK4A, which becomes reinforced by heterochromatin changes (3). Senescent cells are metabolically active, produce senescent-associated β-galactosidase and acquire a senescent-associated secretory phenotype (SASP) with secretion of pro-inflammatory cytokines, proteases and growth factors (3, 4).

The diverse biological functions of senescence in aging, developmental processes, tissue repair, cancer growth and chronic diseases suggest that the senescent state reflects a dynamic cellular stress program depending on the cell type, nature of inducer and extent of senescence with highly variable SASP composition (2, 3, 5). Data from chemo-resistant tumors demonstrated re-entry of senescent cells and their reprogramming into cancer stem cells (6, 7). Similarly, novel single cell RNA sequencing (scRNAseq) analyses of lung repair identified activation of the senescence program in stem cell-like repair cells in mice (8, 9). These data thus challenge our traditional understanding of senescence in repair and disease. They also question whether the term "senescence" is still appropriate or whether we should rather specify the distinct physiological states associated with senescence of different cell types.

SENESCENCE IN LUNG HEALTH AND DISEASE

Senescence in Repair and Development

The timely and spatially controlled induction of senescence is part of a developmental program where the secretory function of senescent cells serves to fine-tune cell fate determination and tissue patterning (10). In highly regenerative organisms, e.g., salamanders and zebrafish, senescent cells contribute to the regeneration of complex structures (11). A recent report suggests the presence of senescent fibroblasts in the developing and adult lung supporting epithelial progenitor cell function (12).

In tissue remodeling and repair, senescence is induced locally in a well-controlled manner (10, 11). The inducing factors are not fully defined. While activation of the senescence program restricts cellular reprogramming in inducible pluripotent stem cells (iPSCs) (13), the senescent cells activate proliferation and reprogramming in neighboring cells via secretion of SASP factors

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(10, 13, 14). The local induction of cellular stemness by senescent cells contributes to tissue repair and wound healing. It also represents a trade-off of senescence in cancer and might further promote cancer growth and resistance toward chemotherapy (2, 15). In this mode of reparative senescence, the SASP producing senescent cells are cleared by the immune system such as natural killer (NK) cells or macrophages (2, 10, 16, 17) preventing the spread of senescence within the tissue, known as secondary senescence (5).

Senescence in Aging and Disease

In aging and in chronic diseases, senescent cells accumulate and persist in the tissue (2). Senescence is induced by repeated or chronic exposure to stress over time. Moreover, senescent cells are ineffectively cleared by an aging or dysfunctional immune system in disease (18, 19). Senescence restricts stem cell and progenitor functions in aging and disease (20). It further aggravates inflammation and tissue fibrosis via secretion of pro-inflammatory and pro-fibrotic SASP (4, 21). The senescence program is further spread though-out the tissue upon activation of secondary senescence (5). As SASP factors can be secreted via extracellular vesicles, this might allow senescent cells to signal not only locally but also systemically (22, 23). Age-related or stress-induced senescence of immune cells impairs clearing of senescent cells and further aggravates accumulation of senescent cells thereby contributing to the vicious cycle that promotes aging and chronic diseases (19). In contrast, enhanced clearance of senescent cells delays age-related disorders in mice (24). All of the above mentioned features of senescence have also been demonstrated in lung aging and age-related lung diseases (25–27). While chronic lung diseases as for example IPF and COPD present pathologically very different diseases, they share aging-associated hallmarks such as changes in ECM, aberrant repair processes and cellular senescence (28). Defining the specific phenotypes of these hallmarks might help to shed light on why they give rise to different pathological diseases.

Senescence in Lung Repair and Fibrosis

Senescence is a well-recognized feature of lung fibrosis. Patients with mutations in telomere genes, which causes replicative senescence, develop familial forms of idiopathic pulmonary fibrosis (IPF) indicating that senescence causally contributes to the development of lung fibrosis in this subgroup of familial IPF (29). Cellular senescence was also observed in non-familial IPF, namely in lung myofibroblasts, in hyperplastic bronchial epithelial cells and in alveolar epithelial cells (27, 30–35). While different cell types display senescent features in IPF and might exert cell-specific effects (27), we here focus our discussion on epithelial cells as the injured epithelium represents an early trigger for disease development (36). Activation of senescence was confirmed in several mouse models of lung injury and fibrosis (27, 34, 37). Importantly, clearing of senescent cells in mice protected from lung fibrosis (33, 34). Available evidence thus supports the above outlined concept that while senescence is a crucial feature of physiological lung repair, it promotes lung fibrosis upon dysregulation and accumulation of senescent cells in the aging lung. These detrimental effects have been mainly attributed to the paracrine pro-fibrotic effects of the SASP and an impaired clearing of accumulating senescent cells in the lung, as also suggested for other tissues (2, 27, 38).

RE-THINKING SENESCENCE

The recent scRNA seq data from mouse models of lung repair fundamentally challenge the traditional view that senescent cells are irreversibly growth arrested and act mainly in a paracrine fashion to promote proliferation and reprogramming in neighboring cells. Strunz et al. identified a transitional stem cell state involved in alveolar repair of bleomycin-injured mouse lungs (9). These Keratin8+ (Krt8+) alveolar repair cells are characterized by activation of cell senescence and wound healing programs as well as of the p53, MYC and TNFα/NFκB pathways. The cells originate from either AT2 or activated Club cells and transition toward AT1 cells. EdU pulse labeling of lineage-traced cells indicated that these Krt8+ alveolar repair cells were actively proliferating as confirmed by cell cycle regression analysis and Ki67 co-staining. Very similar, Kobayashi et al., identified a transitional stem cell-like cell en route from AT2 to AT1 cells in mouse organoids and models of lung repair (8). These cells were similarly enriched for cellular senescence, TGFβ signaling and p53 activation signatures as also demonstrated by marker expression on protein level. A similar intermediate repair cell type was identified during alveolar regeneration after bleomycin induced lung injury and inflammatory signaling in mice (39). These studies thus demonstrate the dynamic existence of alveolar stem cell-like cells in physiological lung repair, which are de-differentiated and proliferate but at the same time show markers of senescence and activation of SASP. However, the distinct overlap of proliferation and cellular senescence markers within the same cells remains to be carefully demonstrated on the protein level. While the causal contribution of these cells to adaptive lung repair remains to be determined, these findings contradict our conventional understanding of senescence. The cellular senescence program in these alveolar repair cells involves re-entry into the cell cycle, de-differentiation with stem cell-like properties and SASP secretion. This suggests that the senescent cell is capable of doing the repair job itself and does not (only) act as the sentinel to alert neighboring cells by paracrine SASP signaling. Most probably, senescence is induced as part of the tissue repair program to facilitate cellular de-differentiation and stemness involving autocrine SASP signaling. Importantly, as these cells transition into AT1 cells, there is no need for clearing them by immune cells. These findings extend our current view on senescent cells in repair and regeneration (2, 10). They are fully in line with the observed reprogramming of tumor-derived senescent cells into plastic cells which exhibit features of cancer stemness (7). Escape of such senescent tumor cells from their cell cycle arrest transformed them into "super" cancer stem cells with high tumor initiating potential (15).

Remarkably, a very similar signature of de-differentiation, stemness, and senescence was detected in aberrant basaloid cells of irreversibly remodeled IPF lungs (40, 41). These scRNA seq studies dissected the cellular composition of

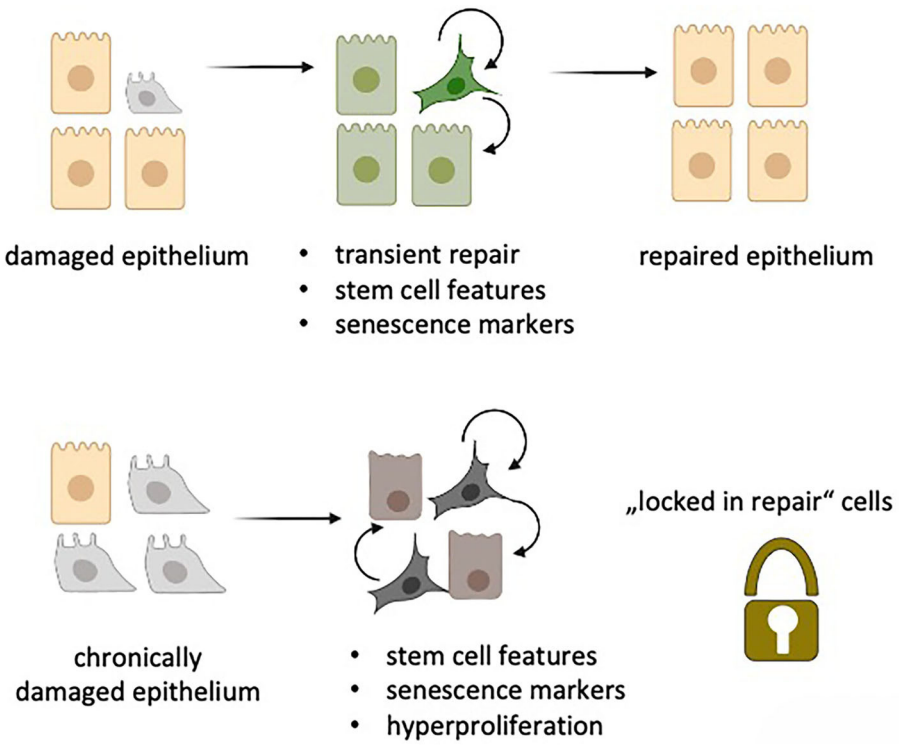

FIGURE 1 | "Locked in repair" concept: Upon damage of the lung epithelium, transient repair cells (green) obtain features of senescence with stem cell like activities thereby facilitating repair of the damaged epithelium via autocrine and paracrine SASP secretion. Upon chronic epithelial damage, a similar process is induced. The senescent repair cells, however, are unable to repair the epithelium but rather promote hyperproliferation by maintaining stem cell like features via autocrine and paracrine SASP signaling as well as by escaping immune cell-mediated clearing. (Created in Biorender.com).

fibrotic IPF lungs in unprecedented detail. Their RNA and protein data confirmed that the previously described aberrant bronchial cell-derived cells show prominent expression of senescence markers (30, 40, 41). In addition to activation of the senescence program, these newly termed basaloid cells showed enriched gene expression for wound healing programs, activation of p53 and integrin signaling pathways, together with the induction of SOX9-controlled genes, which strongly suggests activation of a distal airway development and repair program (42). It remains to be established whether these IPF-specific basaloid cells are permanently cell cycle arrested and have fully lost their proliferative potential. Importantly, the presence of these cells in irreversibly damaged and remodeled IPF lungs strongly indicates that they have lost their repair capacity.

Given this astonishing overlap of gene expression programs between aberrant basaloid cells in IPF and the newly identified alveolar repair cells, i.e., de-differentiation, stemness and senescence, it is tempting to speculate that the IPF-specific basaloid cells are "locked in repair" (**Figure 1**). Locking of lung epithelial cells in a de-differentiated, plastic and senescent state would promote uncontrolled spreading of cellular plasticity and senescence to neighboring cells. Further, it would initiate maladaptive repair by continuous secretion of SASP-related inflammatory mediators and pro-fibrotic molecules. As senescent cells are inherently resistant to apoptosis they will not be cleared by cell intrinsic death programs (2). Moreover, senescent cells might also escape immune cell clearance by NK and $CD8^+$ T cells (43). Together with defective immune surveillance in aging and chronically inflamed lungs (18) this will further increase the number of senescent cells thereby closing the vicious cycle that drives irreversible lung fibrosis in IPF. It might also contribute to an increased tumor burden observed in IPF patients (44).

DISCUSSION

Our concept predicts that repair-locked senescent cells represent an Achilles heel for the development of pulmonary fibrosis. Novel therapeutic concepts should then aim at targeting these cells in IPF by either eliminating them or putting them back on the right repair track. Such approaches could have both cell-autonomous as well as non-autonomous effects due to the expected modulation of the SASP.

The key issue is to understand what locks these cells in their senescent repair modus. Is this an irreversible state? Are there any means to unlock them? Comparison of these basaloid cells in IPF that seem to be permanently locked in repair with their repair competent counterpart in the bleomycin mouse model might help to shed light on possible approaches, including epigenetic signatures, to unlock these cells.

As the senescence program is characterized by extensive chromatin remodeling with predominant $H3K9me^3$ histone signatures (2, 3, 15) it is well-feasible that distinct epigenetic changes mediate the repair-locked phenotype of aberrant basaloid cells. This hypothesis can be tested by investigating the chromatin landscape of basaloid cells and analyzing the effects of inhibitors of histone modifying enzymes on their phenotype. In addition, one should investigate the interaction with other cell types of the lung such as myofibroblasts and immune cells to distinguish cell-autonomous vs. non-autonomous effects. Moreover, the senescent program of aberrant basaloid cells in IPF might be specifically targeted by anti-senescent therapies such as senotherapeutics (45). Among these are senolytic compounds that aim to overcome apoptosis-resistance in senescent cells (46). Indeed, first in human trials demonstrated initial tolerability in IPF patients (47). However, these drugs do not discriminate reparative from aberrant senescence. A more specific approach might involve the development of engineered T-cells to selectively ablate senescent cells in a disease specific manner (48). This approach illustrates the heterogeneity of cellular states which is lumped together as "senescence" (5). For the development of a specific anti-senescent therapy in IPF, the lack of deep knowledge on the specific senescence phenotype including the SASP is the main hurdle that needs to be overcome. Moreover, any successful therapeutic approach would most probably be applicable to also familial forms of IPF and even other age-related lung diseases such as COPD (27). It's time to re-think targeting of senescent cells for therapy of chronic lung diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Non-coding RNAs as Regulators of Cellular Senescence in Idiopathic Pulmonary Fibrosis and Chronic Obstructive Pulmonary Disease

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Cellular senescence is a cell fate implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD). Cellular senescence occurs in response to cellular stressors such as oxidative stress, DNA damage, telomere shortening, and mitochondrial dysfunction. Whether these stresses induce cellular senescence or an alternative cell fate depends on the type and magnitude of cellular stress, but also on intrinsic factors regulating the cellular stress response. Non-coding RNAs, including both microRNAs and long non-coding RNAs, are key regulators of cellular stress responses and susceptibility to cellular senescence. In this review, we will discuss cellular mechanisms that contribute to senescence in IPF and COPD and highlight recent advances in our understanding of how these processes are influenced by non-coding RNAs. We will also discuss the potential therapeutic role for targeting non-coding RNAs to treat these chronic lung diseases.

Keywords: COPD–Chronic Obstructive Pulmonary Disease, IPF–Idiopathic Pulmonary Fibrosis, cellular senescence, non-coding RNA, micro-RNA (miRNA), long-non coding RNA

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) are chronic lung diseases that disproportionately affect the elderly and impose a significant global health burden. IPF is characterized by chronic and progressive lung scarring while COPD is characterized by heterogenous manifestations of emphysema, small airway disease, and chronic bronchitis. Despite their distinct pathologic features, both diseases are epidemiologically and biologically associated with aging (1–3). The prevalence of COPD amongst individuals aged ≥75 years is approximately 10% compared with 3–4% in those 25–44 years (1, 4) and the prevalence of IPF amongst individuals aged ≥75 years is 0.2–0.3% compared to 0.004–0.012% cases in those aged 35–44 years (5). Additionally, the pathogenesis of IPF and COPD involve biologic "hallmarks of aging" (1, 2, 6–10). These "hallmarks of aging," first described by Lopez-Otín et al., are cellular processes that occur more frequently with age, contribute to aging-related functional decline, and can be experimentally manipulated to accelerate or slow aging in model organisms (11). One biologic "hallmark of aging" that has emerged as a therapeutic target for ILD, COPD, and other age-related disorders is cellular senescence.

Cellular senescence is a cell fate that occurs in response to diverse causes of cellular stress, such as DNA damage, oxidative stress, telomere shortening, and oncogene activation (12, 13).

Cellular senescence is characterized by permanent cell cycle arrest due to persistent activation of $p16^{INK4a}$-RB (retinoblastoma) and p53-$p21^{CIP1/WAF1}$ pathways (14, 15). However, senescent cells frequently have altered cellular metabolisms, reorganized chromatin, and activated damage sensing pathways (e.g., p38 MAPK and NF-κB), and are apoptosis resistant. They also adopt a senescence associated secretory phenotype (SASP) and secrete high levels of cytokines, chemokines, and matrix metallopeptidases (MMPs). The biologic consequences of cellular senescence are complex because senescence has both beneficial and detrimental effects. Cellular senescence is critical for embryogenesis, promotes wound healing, and mitigates malignant transformation. However, the accumulation of senescent cells with age also causes chronic inflammation, extracellular matrix alterations, a decline in tissue regeneration, and an increased risk for many aging-related disorders (16).

Cellular senescence is just one of many potential cell fates. Cells maintain diverse stress responses that can resolve cellular stress or activate alternative cell fate pathways such as programmed cell death (e.g., apoptosis/necroptosis), quiescence, or differentiation. While cell fate is influenced by the type, magnitude, and duration of cellular stress, microenvironmental and intracellular factors also influence cell fate "decisions" through modulation of intracellular signaling networks. Consequently, susceptibility to cellular senescence varies across cell/tissue types and with age and in disease (17). There is increasing recognition for the important role of non-coding RNAs in the regulation of signaling networks that influence susceptibility to cellular senescence (18, 19). In this review, we will highlight non-coding RNAs that regulate senescence-associated molecular pathways in the context of IPF and COPD pathogenesis and discuss current approaches and challenges for therapeutically targeting non-coding RNAs for these diseases.

CELLULAR SENESCENCE AND ITS CAUSES IN THE PATHOGENESIS OF IPF AND COPD

Cellular senescence is now considered an important mechanism of IPF and COPD pathogenesis (20, 21). In IPF, cellular senescence markers are increased in epithelial and mesenchymal cells within remodeled areas of fibrotic lung, and eliminating senescent cells using genetically modified mice or pharmacologic agents decreases disease severity in animal models of pulmonary fibrosis (22–26). Singe-cell RNA sequencing studies suggest epithelial senescence in IPF occurs in a unique subpopulation of cells that reside adjacent to myofibroblasts and may arise from the persistence of a transitional alveolar epithelial cell state (27, 28). These cells express p16, p21, certain basal cell markers, developmental and epithelial-mesenchymal transition markers, and may be a source of pro-fibrotic SASP signaling (29, 30). Additionally, while fibroblast senescence is important for normal wound healing, IPF pathogenesis may involve the persistence of senescent fibroblasts that secrete pro-fibrotic mediators and senescent myofibroblasts that are apoptosis resistant (23, 31). Lungs of patients with COPD demonstrate increased markers of cellular senescence in epithelial cells, fibroblasts, and endothelial cells and many (albeit not all) studies have demonstrated genetic or pharmacologic inhibition of cellular senescence can mitigate disease severity in animal models of COPD (32–37). It has also been postulated that chronic inflammation and airway remodeling in COPD may arise from the production of pro-inflammatory SASP factors (38). Additionally, impaired tissue repair may be the result of reduced replicative capacity in senescent progenitor cells.

Cellular senescence in IPF and COPD is commonly caused by oxidative stress and DNA damage (39). Oxidative stress refers to an imbalance between reactive oxygen/nitrogen species (ROS/RNS) and cellular antioxidants. While ROS can arise from many exogenous sources (e.g., cigarette smoke) and chronic inflammation, one of the most abundant sources of ROS are mitochondria. There is increased mitochondrial ROS production and mitochondrial dysfunction with age and in IPF and COPD (40–42). In addition, IPF and COPD are associated with increased oxidative biomarkers, and consequences of oxidative stress including macromolecular damage (e.g., protein, DNA, and organelle damage), inflammation, cellular senescence and cell death (26, 32, 43). DNA damage is another important cause of cellular senescence, through activation of p53, increased p21 and p16 transcription, and stabilization of GATA4 (12, 15, 44). DNA damage is increased in IPF and COPD, and inadequate DNA repair capacity may contribute to disease progression in COPD (45–47). Telomere shortening can also activate DNA damage responses (17, 48). Normally, a shelterin complex protects telomeric strands from being recognized by DNA damage responses. However, telomere shortening can cause loss of the shelterin complex, telomere "uncapping," DNA damage response activation, and cellular senescence. In IPF, shortened telomeres and mutations in telomere maintenance genes are well-described risk factors for disease (49–51). Similarly, telomerase mutations are risk factors for early onset emphysema and telomere length is associated with severity of airflow limitation, increased risk for acute exacerbations, and increased mortality (34, 52–55).

To combat cellular stress, cells maintain a repertoire of cellular stress responses, but many of these adaptive responses wane with age or are decreased in IPF and COPD. For example, NRF2 is a transcription factor that promotes the production of cellular antioxidants and detoxifying enzymes. However, NRF2 activity decreases with age and impaired NRF2 activity is implicated in the pathogenesis of IPF and COPD (31, 56–59). Another adaptive stress response is autophagy, a process in which cells degrade and "recycle" damaged proteins and organelles through lysosome-dependent pathways. With age, there is reduced autophagy and mitophagy, a selective type of autophagy for the specific degradation of mitochondria. Consequently, there is a reduced capacity to alleviate consequences of oxidative stress and increased susceptibility to cellular senescence (60, 61). In IPF, reduced autophagy in epithelial cells and fibroblasts increase susceptibility to cellular senescence and disease pathogenesis (62–66). Similarly, deficient mitophagy and its mediators, including PINK1 and SIRT3, impair mitochondrial function, increase mitochondrial ROS production, and contribute to

progressive fibrosis in IPF (9, 10, 67). While autophagy is an adaptive response, persistent autophagy can cause activation of cell death and cell senescence pathways as well (68, 69). Autophagy and mitophagy are increased in severe COPD, and both insufficient and excess autophagy and mitophagy are implicated in COPD pathogenesis (70–74).

Collectively, these findings underscore the increasing evidence that the pathogeneses of IPF and COPD involve cellular senescence and dysregulation of stress responses that mitigate cellular senescence. Therefore, regulatory factors that increase or reduce susceptibility to cellular senescence may represent novel therapeutic targets for these diseases.

NON-CODING RNAs IN AGING, IPF, AND COPD

Non-coding RNAs lack protein coding capacity but still regulate diverse cellular processes including those implicated in aging biology, cellular senescence, and the pathogeneses of IPF and COPD. Non-coding RNAs are mainly classified into two groups, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). miRNAs are small 18–25 base single stranded RNA molecules (75). They are initially transcribed as primary-miRNA (pri-miRNA) molecules that fold into a stem loop structure. Subsequently these pri-miRNAs undergo sequential processing by enzymes Drosha and Dicer to generate miRNA strand duplexes. The mature miRNA strand of the duplex is then loaded into a miRNA-induced silencing complex (miRISC) where it binds complementary mRNA sequence to inhibit mRNA translation or promote mRNA degradation. Typically, miRNAs bind the 3′ untranslated region (UTR) of mRNA but can bind other regions as well. Because one single miRNA can target hundreds of mRNAs, miRNAs can modulate complex biologic processes including those related to lifespan and aging (76, 77). In humans, age-related changes in miRNA expression have been identified in lung, peripheral blood mononuclear cells (PBMCs) and serum (18, 78–81).

LncRNAs are a diverse group of non-coding RNAs longer than 200 nucleotides (82, 83). Certain lncRNAs are transcribed from intergenic regions (long intergenic non-coding RNAs or lincRNAs), while others are derived from excised introns. Sense lncRNAs are located in proximity to a coding gene on the sense strand while antisense lncRNAs are transcribed from the opposite strand of a coding gene. Certain lncRNAs undergo capping, splicing, and polyadenylation much like mRNAs, while others undergo alternative post-transcriptional processing, such as forming circular molecules or processing by RNase P to form stabilizing triple helix structures at their 3′ ends (84). LncRNAs are also functionally diverse. They can act as cis- or trans- regulatory elements to enhance or inhibit mRNA transcription and/or mRNA translation. LncRNAs can mediate their regulator effects by affecting chromosomal architecture, modulating the recruitment of chromatin modifiers, binding DNA directly to form complex structures that interfere with transcriptional machinery, or binding complementary mRNA transcripts to regulate their stability, splicing, or post-transcriptional modification (84–86). Similar to microRNAs, lncRNAs have also been implicated in aging biology (87, 88).

Non-coding RNAs are implicated in the pathogeneses of IPF and COPD and are emerging targets for therapeutic intervention. Approximately 10% of miRNAs are significantly changed in IPF lungs, including a decrease in miR-29, miR-30, let-7, miR-96, and miR-17–92 family members and an increase in miR-154, miR-155, miR-34, miR-26, miR-200 and miR-21 family members (89–91). miRNA expression can be altered by cigarette smoke or by the presence of COPD (92, 93). Studies profiling miRNAs in COPD lung tissue samples have demonstrated increased expression of miR-34a, miR-146a, miR-144, miR-15b, miR-570, and decreased expression of miR-24 and mir-218 (94–99). Other studies evaluating the expression of miRNAs in serum and sputum samples have found differential expression of miR-21, let-7c, miR-610, miR-34 a/b/c, let-7c, miR-146a, miR-125b, and miR-199a with COPD (93, 95, 97, 100–102). The differentially expressed lncRNAs in IPF or animal models of lung fibrosis include MEG3, TERRA, SIRT1-AS, MALAT1, FENDRR, and DNM3OS (103–108). Studies of lncRNAs in COPD lung tissue have identified differential expression of MEG3, ANRIL, SAL-RNA, and SCAL1 with COPD (97, 109, 110). Many of these differentially expressed non-coding RNAs in IPF and COPD have been shown to regulate various aspects of aging biology and cellular senescence. Below, we provide examples of such non-coding RNAs and discuss how their regulation of aging biology and cellular senescence may contribute to disease pathogenesis (**Tables 1**, **2**).

Non-coding RNAs in COPD and IPF

miR-34 and miR-570 Regulation of Sirtuins in IPF and COPD

The miR-34 family consists of three members: miR-34a, miR-34b, and miR-34c. They are direct transcriptional target of p53 and therefore can be induced by oxidative and genotoxic stress (111). Members of the miR-34 family are encoded by two different genes; miR-34a is encoded within chromosome 1 whereas miR-34b and miR-34c are encoded within chromosome 11. Studies show miR-34a expression increases with age and can promote cellular senescence in part through negative regulation sirtuins, particularly SIRT1 and SIRT6 (111–113). Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent molecules that promote longevity by regulating diverse cellular processes including: cellular senescence, inflammation, DNA repair, autophagy, mitochondrial generation and mitochondrial ROS production (114, 115). For example, SIRT1 can function as a histone deacetylase to negatively regulate NF-κB, mitochondrial biogenesis, p53, p21, and p16 (116, 117). In the lung, miR-34a is expressed in type II alveolar epithelial cells (AECs) and fibroblasts, and increased miR-34a expression coupled with reduced SIRT1 and SIRT6 expression are associated with IPF and COPD (118–123).

Previous studies demonstrate miR-34a is increased in type II AECs from patients with IPF and in murine models of lung fibrosis (124, 125). Both *in vitro* and *in vivo* experiments demonstrate miR-34a promotes cellular senescence in part

TABLE 1 | Aging related non-coding RNAs in IPF.

Non-coding RNAs		Expression in IPF	*In vivo* role in fibrosis	Senescence related mechanisms
microRNAs	mir-34a	↑Lung	Antifibrotic (young) Profibrotic (aged)	Inhibits cellular senescence through SIRT1 in AECs and fibroblasts
	mir-29	↓Lung	Antifibrotic	Increases AEC antioxidants (SOD2, MnSOD, catalase) Inhibits apoptosis in AECs by regulating FOXO3A
	mir-17∼92	↓Lung	Antifibrotic	Inhibits mTOR, promotes autophagy, decreases cellular senescence
	mir-200 family	↓Lung	Antifibrotic	Inhibits AEC cellular senescence and epithelial mesenchymal transition
lncRNAs	SIRT1-AS	↓Lung	–	Inhibits miR-34a-mediated targeting of SIRT1
	TERRA	↑Lung	–	Promotes telomere maintenance
	MALAT1	↓Lung	Antifibrotic	Stabilizes the antioxidant NRF2
	LincRNA-p21	↑Lung	–	Promotes cellular senescence through activation of p53 and p21

AEC, alveolar epithelial cells.

TABLE 2 | Aging related non-coding RNAs in COPD.

Non-coding RNAs		Expression in COPD	*In vivo* role in COPD	Senescence related mechanisms
microRNAs	mir-34a	↑Lung ↓BAL	Increases susceptibility to emphysema	Promotes cellular senescence by inhibition of SIRT1/6
	mir-570	–	–	Promotes cellular senescence by inhibition of SIRT1
	mir-24	↓Lung	Protects against emphysema	Inhibits DNA repair and apoptosis
	mir-126	↓Blood outgrowth endothelial cells	–	Inhibits DNA damage response
	mir-218	↓Lung and Sputum	Protects against cigarette smoke induced inflammation	Inhibits cellular senescence via BMI1
lncRNAs	ANRIL (CDKN2B-AS1)	↓Plasma	–	Inhibits p16 expression and SASP cytokine production
	SCAL1	↑Airway epithelium	–	Activates antioxidant responses downstream of NRF2
	MEG3	↑Lung	–	Promotes p53 activity

through inactivation of SIRT1 and increased mitochondrial dysfunction (118, 119, 124). Interestingly, the consequences of miR-34a genetic deletion in mice are age-dependent. miR-34a protects against lung fibrosis by increasing fibroblast susceptibility to cellular senescence in young mice, while miR-34a promotes lung fibrosis by increasing alveolar epithelial susceptibility to cellular senescence and apoptosis in old mice (119, 124). The divergent roles for miR-34a in young and old mice underscore the complex temporal- and cell type-specific roles for cellular senescence in disease pathogenesis.

miR-34a is also increased COPD lungs. In airway epithelial cells and lung tissue, miR-34a expression is induced by oxidative stress and inversely correlates with SIRT1 and SIRT6 expression (99, 126). In a murine model of COPD, miR-34a inhibitors increase SIRT1 and SIRT6 expression and reduce NF-κB signaling, matrix metalloproteinase expression, cellular senescence, and emphysema severity. (121–123, 126). Another sirtuin regulator in COPD is miR-570, which is located at chromosome 3 and targets the 3′-UTR of SIRT1 mRNA for degradation. miR-570 expression is induced by oxidative stress and increased in lung tissue and airway epithelial cells from patients with COPD (98). Inhibition of miR-570 reduces cellular senescence and the secretion of SASP factors such as IL-6, IL-1, and CXCL8. Together, these data demonstrate the important roles of miR-34a and miR-570 in regulation of cellular senescence and susceptibility to IPF and COPD through modulation of sirtuins.

miR-29 and IPF

The roles of miR-29 family members in IPF are context dependent and underscore the complex interactions of microRNAs, aging biology, and disease pathogenesis. There

are three mature members of the miR-29 family, miR-29a, miR-29b, and miR-29c, which are encoded within two bicistronic clusters (miR-29a/miR-29b-1 located on chromosome 7 and miR-29b-2/mir-29c located on chromosomes 1) (127, 128). In the lung, miR-29 is largely expressed in mesenchymal and epithelial cells where its expression is associated with oxidative stress, DNA damage, and cellular senescence (129–131). However, miR-29c is decreased in IPF lung tissue samples and experimentally induced fibrosis in mouse lungs (124, 132). miR-29c deficiency in type II AECs increases susceptibility to apoptosis and reduces their capacity for epithelial renewal while miR-29c mimics protect type II AECs from apoptosis by regulating FOXO3A and increasing expression of ROS-neutralizing enzymes such as SOD2, MnSOD and catalase (133). miR-29b mimics can inhibit bleomycin-induced lung fibrosis, fibroblast production of extracellular matrix, expression of IGF-1 and production of inflammatory cytokines such as IL-4 and IL-12 (128, 134). Therefore, an increase in miR-29 with oxidative stress, cellular senescence, or with age may be an endogenous response that protects against fibrosis, and a loss of this adaptive response may contribute to the pathogenesis of IPF.

miR-17∼92 Cluster and miR-200 Family in IPF

Both the miR-*17∼92* cluster and miR-200 family regulate susceptibility to cellular senescence in IPF. The miR-*17∼92* cluster encodes 6 miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a) on chromosome 13 and is frequently decreased in multiple tissue types with age and in senescent cells (135). miR-17∼92 decreases susceptibility to cellular senescence through diverse mechanisms including targeting cell cycle proteins, inhibition of the mechanistic target of rapamycin (mTOR), and activation of autophagy (136). Members of the miR-17∼92 cluster are hypermethylated in lung tissue samples and fibroblasts from IPF patients, and the use of epigenetic methylation inhibitors to promote expression of the miR-17∼92 cluster attenuates fibrosis in bleomycin-murine models (90). Similarly, mice overexpressing miR-17 have highly proliferative, albeit poorly differentiated, epithelial cells and decreased number of senescent cells in their lung (137, 138).

The miR-200 family consists of five members within two clusters, miRs-200a/b/429 on chromosome 1 and miRs-200c/141 on chromosome 12. These microRNAs can regulate oxidative stress, DNA repair, and cellular senescence, although the direction of effect can be context dependent (139, 140). Levels of miR-200a and miR-200c are significantly decreased in IPF lungs and in the lungs of mice with experimental lung fibrosis (141). Transfection of AECs with miR-200a and miR-141 reduces epithelial mesenchymal transition (EMT) and the expression of cellular senescence markers including p16 and p21, but does not improve AEC proliferation capacity. In contrast, transfection with miR-200b/c increases differentiation of senescent type II AECs into type I AECs, decreases EMT, and reduces disease severity in animal models of pulmonary fibrosis (142–145).

miR-24 and miR-126 Regulate DNA Damage Responses in COPD

miR-24 is a member of a poly-cistronic miR-23∼27∼24 miRNA clusters that occur in two genomic loci in humans. The miR-23b-27b-24-2 cluster is located in an intronic region of chromosome 9 while the miR-23a∼27a∼24-1 cluster is located in an intergenic region of chromosome 19 (146, 147). Dysregulation of miR-23∼27∼24 signaling has been identified in multiple age-related disorders including diabetes and Alzheimer's disease, and both oxidative and genotoxic stress have been shown to modulate expression of these miRNAs, although the direction of effect is context dependent (148, 149). In COPD, miR-24 expression inversely correlates with COPD disease severity as measured by FEV_1 percent predicted and radiographic emphysema (150). miR-27a and miR-23a expression also inversely correlates with disease severity, albeit it to a lesser degree that miR-24. In a mouse model, inhibition of miR-24 increases susceptibility to cigarette smoke-induced emphysema. Others have found that inhibition miR-24-27-23 cluster in T-cells increases allergic airway inflammation and goblet metaplasia (151). miR-24 can inhibit the expression of p16 by targeting its 3′ UTR to inhibit cellular senescence (152). However, miR-24 can also inhibit DNA repair and the translation of DNA repair genes including H2AX, TOP1, and BRCA1, which can promote cellular senescence in certain contexts (148). Interestingly, another miRNA that inhibits DNA damage responses and is decreased in COPD is miR-126 (153–155). These collective findings suggest that microRNA inhibition of DNA damage responses may protect against COPD pathogenesis, although whether this occurs by changing susceptibility to cellular senescence remains to be determined.

miR-218 and COPD

The mature form of miR-218 can be transcribed from intronic regions of SLIT2 and SLIT3 located on chromosomes 4 and 5, respectively (156). miR-218 is decreased in bronchial epithelial cells of smokers and in lungs and sputum from COPD patients (93, 96). In a murine model of COPD, inhibition of miR-218 increases susceptibility to emphysema and airway inflammation with increased production of IL-8 and CCL2 (96). Notably, one of the downstream targets of miR-218 is BMI-1, a polycomb repressive group protein which inhibits p16 expression and cellular senescence (157). This raises the possibility that decreased miR-218 expression promotes cellular senescence and disease progression in COPD, although further studies are warranted.

LncRNA

Lnc ANRIL (CDKN2B-AS1) and Lnc SIRT1-AS

ANRIL is transcribed from the antisense strand of CDKN2A/2B, the genes that encode cyclin-dependent kinase inhibitors p15 and p16, on chromosome 9 (158). ANRIL mediates transcriptional repression of these antisense genes through RNA-RNA interactions, as well as histone methylation and chromatin remodeling of polycomb repressive complexes (PRC) (158, 159). ANRIL activity is highly variable and dependent on tissue type. There are 21 ANRIL splice variants, including linear and circular isoforms, and ANRIL activity is highly influenced by methylation activity in its promoter region (158). In addition to its role in regulating p15 and p16, ANRIL suppresses NF-κB and can inhibit chronic inflammation (160). In one study, ANRIL expression in plasma was decreased during acute exacerbations of COPD and ANRIL expression negatively correlated with SASP

related cytokines such as TNF-α, IL-1β, IL-8 and LTB-4 in stable COPD patients (161). LncRNA SIRT1 antisense (SIRT1-AS), is transcribed from the antisense strand of SIRT1 and can form RNA hybrid double strands with SIRT1 mRNA to increase its stability (162). SIRT-AS protects SIRT1 mRNA degradation by inhibiting miR-34a binding to the 3′UTR of SIRT1 (162). In one study of bleomycin-induced lung fibrosis, SIRT1-AS overexpression inhibited TGF-β-mediated EMT (163). Despite these data, more studies will be necessary to confirm the roles of ANRIL and SIRT1-AS in COPD and pulmonary fibrosis.

TERRA (Telomere Repeat-Containing RNA)

TERRAs are important for telomere maintenance and characterized by 5′-(UUAGGG)-3′ repeats (164, 165). These lncRNAs are commonly transcribed from the subtelomeric 20q locus in humans in response to cellular stress and telomere shortening, the later as a consequence of reduced methylation marks and loss of telomeric heterochromatin (166). TERRAs are recruited to telomeres where they form DNA-RNA hybrid R-loops. This R-loop formation regulates telomere maintenance through interactions with chromatin modifiers, telomerase, and promoting DNA repair (166, 167). TERRAs also facilitate telomere replication and promote the assembly of shelterin proteins (168, 169). However, TERRA expression is increased in the PBMCs from IPF patients and inversely correlated with the percentage of predicted force vital capacity (106). While not well-defined, TERRAs may have an important role in IPF pathogenesis.

MALAT1 (Metastasis Associated in Lung Adenocarcinoma Transcript-1) and SCAL1 (Cancer-Associated lncRNA-1)

Both MALAT1 and SCAL1 are lncRNAs that regulate cellular responses to oxidative stress and cellular senescence. MALAT1 is an 8.7kbp lncRNA transcribed from human chromosome 11 and is ubiquitously express in almost all human tissue (170). MALAT1 is frequently found in nuclear "speckles" and can interact with pre-mRNA splicing factors to modulate alternative mRNA splicing (171, 172). Consequently, MALAT1 can regulate the expression of cell cycle genes and can also stabilize NRF2 to attenuate oxidative stress and DNA damage (173). MALAT1 is decreased in senescent cells and in bleomycin-induced murine fibrosis where myeloid deletion of MALAT1 increases susceptibility to fibrosis and the number of profibrotic M2 macrophages (19, 108). SCAL1, a lncRNA located on chromosome 5, can be induced by oxidative stress through NRF2-mediated transcriptional activity and is increased in the airway epithelium of smokers compared to nonsmokers (97, 174). Inhibition of SCAL1 in airway epithelial cells augments cytotoxicity induced by cigarette smoke extract in vitro, suggesting SCAL1 may act downstream of NRF2 to mediate protective antioxidant responses.

LincRNA-p21 (Long Intergenic Non-coding RNA p21) and MEG3 (Maternally Expressed Gene 3)

Both lincRNA-21 and MEG3 are downstream targets of p53 and mediate many p53-dependent transcriptional responses. LincRNA-p21 is a transcriptional target of p53 located approximately 15 kb upstream from CDKN1A (175). LincRNA-p21 functions as a repressor of p53-dependent transcription by binding to hnRNP-K (heterogeneous nuclear ribonucleoprotein K) and interacting with PRC1 and PRC2, although these same interactions also promote p53 activity at the p21 promoter to increase p21 transcription (176, 177). In one study, lincRNA-p21 inhibited fibroblast collagen expression through downregulation of THY1 expression (178). Maternally expressed gene 3 (MEG3) is a maternally imprinted gene located on chromosome 14, and increases with age in human lung tissue and PBMCs due to changes in promoter methylation (87, 179). Like lincRNA-21, MEG3 also promotes p53 activity. MEG3 interactions with p53 inhibit p53 ubiquitination and MDM2-mediated degradation. MEG3 can also selectively upregulate certain p53 target genes, such as GDF15, and interact with PRC1/2 to mediate p53-dependent gene silencing (180–182). Intriguingly, there are 27 known splice variants of MEG3, and changes in the relative abundance of these slice variants in response to cellular stress can modulate p53 activity (183). MEG3 is increased in the lungs of patients with COPD (184, 185). Additionally, epithelial MEG3 expression has been shown to be induced by cigarette smoke, correlate with disease severity, and promote inflammation and apoptosis through a mechanism involving miR-218 (186). MEG3 expression is also increased in atypical IPF epithelial cells and can impair basal cell differentiation, which may contribute to abnormal tissue remodeling (105). Notably, p53 can induce both cellular senescence and apoptosis in a context-dependent manner, but the role of lincRNA-p21 and MEG3 in regulating p53-mediated cell fate responses in the lung remain unknown. Additionally, while p53 is implicated in the pathogenesis of IPF and COPD, more studies are necessary to determine the roles of lincRNA-p21 and MEG3 in these diseases (187).

Therapeutic Targeting of Non-coding RNAs

There is a growing interest in targeting non-coding RNAs to treat chronic lung diseases due to their regulatory functions and roles in disease pathogenesis (85, 188). Therapeutic approaches for RNA targeting utilize nucleotides with complementary sequences to prevent RNA transcription, promote RNA degradation, or interfere with post-transcriptional processing of target RNAs. Catalytically dead RNA-guided CAS9 endonucleases that target specific DNA sequences can be used to hinder RNA transcription. Single-stranded antisense oligonucleotides (ASOs) that bind RNA molecules through complementary sequences promote RNA degradation through RNAase-H dependent cleavage, although newer ASOs inhibit mRNA translation through steric hindrance or interfering with normal mRNA splicing. Similarly, double-stranded RNA molecules, including small interfering RNA (siRNA) or miRNA mimics, utilize the RISC complex to inhibit transcription or promote RNA degradation.

Nucleotide-based approaches for targeting non-coding RNAs are attractive for a variety of reasons. Many non-coding RNAs, particularly lncRNAs, are expressed in a tissue- or cell-specific manner (189). Therefore, augmenting or inhibiting their expression in a cell- or tissue- specific manner can reduce off-target effects and increase the therapeutic window. Additionally,

generating oligonucleotide sequences complementary to their target sequence is a much easier task using currently available technologies than identifying small-molecule inhibitors or antibodies that can specifically target proteins of interest. Even if targeted antibodies or small molecules are identified, they commonly reduce rather than augment target molecule activity. In contrast, oligonucleotide therapies can increase the concentration of target molecule production through inhibition of negative regulators such as miRNAs. Finally, many therapeutic targets, while pathologic in certain contexts, also have important homeostatic functions. For example, oxidative stress is deleterious, but ROS are critical intracellular signaling molecules. Similarly, cellular senescence promotes aging related disorders but also prevents malignant transformation. Rather than inhibiting such integral pathways completely, a more effective therapeutic strategy may be to focus on modulating these pathways by targeting regulatory non-coding RNAs.

However, nucleic acid-based therapies are not without challenges (190). First, oligonucleotides are susceptible to degradation by extracellular and intracellular nucleases. To overcome this challenge and increase oligonucleotide stability, researchers have used chemically modified phosphate backbones. For example, antagomirs are ASOs that commonly contain 2′-O-methyl or phosphonothioate modifications to improve stability. Locked nucleic acids are another commonly used ASO that utilizes a modified RNA-DNA-RNA backbone to increase binding affinity and improve stability. Certain oligoribonucleotides possess targeting moieties that can deliver nucleic acid-based therapies to specific tissue. Another challenge is that nucleotides are large negatively charged molecules and therefore do not easily cross the cell membrane. Therefore, lipid-, peptide-, and polymer-based nanoparticles have been used to deliver oligonucleotides to the cytosol. Some of these nanoparticles promote the specific uptake of oligonucleotides into the lung or increase retention within the lung following inhalation. (191, 192). Nucleic acid-based therapies are capable of promoting inflammation through toll-like receptors and other innate immune receptors for foreign DNA and RNA, although this problem can be mitigated through assays to test for immune activation and reducing CpG elements (85). Finally, non-coding RNAs can target hundreds of genes and/or function through diverse mechanisms, and therefore targeting non-coding RNAs may cause unwanted effects.

Several oligonucleotide therapies that target mRNAs have already been approved by the U.S. Food and Drug Administration for treating disease, and there are currently multiple clinical trials targeting non-coding RNAs. For example, Remlarsen, a first-generation miR-29 mimic, is currently being evaluated in a Phase 2 clinical trial assessing its safety and efficacy in skin fibrosis (193). MRX-34 is a liposomal nanoparticle formulation of a miR-34 mimic that was under investigation in a Phase I trial for cancer, but the study was stopped short because of serious adverse events (194). While lncRNAs have not been tested in clinical trials, therapeutic manipulation of MALAT1 and MEG3 have shown benefit in preclinical transgenic and xenograft models of cancer (195–197). Lin et al. reported that the knockdown of lncRNA MALAT1 via tail injection of RNAi can improve septic lung injury in mice (198). Additionally, intraperitoneal administration of ASO targeting lncRNA DNM3OS, a regulator of the TGF-β pathway, attenuated bleomycin-induced lung fibrosis in mice (104). These pre-clinical and clinical studies suggest the possibility that non-coding RNAs may have a potential therapeutic role for treating lung diseases such as IPF and COPD.

CONCLUSION

Diverse cellular processes implicated in aging biology, including cellular senescence, contribute to the pathogenesis of IPF and COPD. In these diseases, cellular senescence can occur from oxidative stress, DNA damage, telomere shortening, or mitochondrial dysfunction. While these processes occur commonly with age, their impact on cell fate and disease susceptibility are influenced by diverse regulatory factors. Additionally, many of the cellular responses to these stressors, including senescence, have homeostatic functions and are not universally pathologic. Therefore, nuanced therapeutic approaches will be required to target these processes. Such approaches may need to be cell- or tissue- specific or have modulatory rather than inhibitor effects on key pathways. Because of the fundamental regulatory role of non-coding RNAs, and the growing capacity for cell-specific targeting, non-coding RNAs may emerge as ideal therapies to target chronic lung disease and other age-related disorders.

AUTHOR CONTRIBUTIONS

NO and MS wrote the manuscript and built the tables, which were original. All authors have read and approved the submitted manuscript version.

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Transcriptional and Proteomic Characterization of Telomere-Induced Senescence in a Human Alveolar Epithelial Cell Line

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Cellular senescence due to telomere dysfunction has been hypothesized to play a role in age-associated diseases including idiopathic pulmonary fibrosis (IPF). It has been postulated that paracrine mediators originating from senescent alveolar epithelia signal to surrounding mesenchymal cells and contribute to disease pathogenesis. However, murine models of telomere-induced alveolar epithelial senescence fail to display the canonical senescence-associated secretory phenotype (SASP) that is observed in senescent human cells. In an effort to understand human-specific responses to telomere dysfunction, we modeled telomere dysfunction-induced senescence in a human alveolar epithelial cell line. We hypothesized that this system would enable us to probe for differences in transcriptional and proteomic senescence pathways *in vitro* and to identify novel secreted protein (secretome) changes that potentially contribute to the pathogenesis of IPF. Following induction of telomere dysfunction, a robust senescence phenotype was observed. RNA-seq analysis of the senescent cells revealed the SASP and comparisons to previous murine data highlighted differences in response to telomere dysfunction. We conducted a proteomic analysis of the senescent cells using a novel biotin ligase capable of labeling secreted proteins. Candidate biomarkers selected from our transcriptional and secretome data were then evaluated in IPF and control patient plasma. Four novel proteins were found to be differentially expressed between the patient groups: stanniocalcin-1, contactin-1, tenascin C, and total inhibin. Our data show that human telomere-induced, alveolar epithelial senescence results in a transcriptional SASP that is distinct from that seen in analogous murine cells. Our findings suggest that studies in animal models should be carefully validated given the possibility of species-specific responses to telomere dysfunction. We also describe a pragmatic approach for the study of the consequences of telomere-induced alveolar epithelial cell senescence in humans.

Keywords: telomerase, SASP, secretome, IPF, mass spectrometry, biomarker, A549, aging

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, fibrosing lung disease whose incidence increases with age (1, 2). The average age at presentation is 66 years, and two thirds of all diagnoses are made after age 60 (3). It is an uncommon disease that will almost certainly become more common as our population ages (4). Currently, the prognosis for IPF is often worse than many cancers (5, 6), and the two drugs that exist to treat this disease have only modest effects on disease progression (7, 8). A more complete understanding of the pathogenesis of this disease is essential to the development of novel therapeutics for IPF. Recently, a greater emphasis has been placed on the contribution of the alveolar epithelial cell to the development of this disease (9–14).

Alveolar epithelial cells, while incapable of forming scar tissue themselves, are held to play a causal role in IPF pathogenesis (15–19). Epithelial cell senescence as a result of telomere dysfunction is one component of the alveolar epithelial cell theory of IPF (9). Short telomeres have been identified as a risk factor for IPF (20–28), and IPF is the most common clinical manifestation of patients with mutations in telomere maintenance genes (29). Approximately half of sporadic and >60% of familial cases of IPF have short telomeres (20). When telomeres reach a critically short length, affected cells will either apoptose or become senescent (30). We previously developed a murine model of telomere-dysfunction and found that type 2 epithelial cells (AEC2s)—the principal alveolar progenitor cells—preferentially become senescent in the setting of telomere dysfunction (9). The AEC2s lost their regenerative capacity and rendered the host exquisitely sensitive to pulmonary injury. Additionally, secondary mesenchymal effects were seen *in vivo* that were hypothesized to be due to AEC2s adopting the senescence-associated secretory phenotype (SASP) (9)—a set of characteristic, paracrine signaling pathways (31) that is believed to play a role in the progression of fibrotic lung disease (32). However, while these cells displayed many of the characteristic findings of senescence, few SASP genes were upregulated in this study (9).

The absence of this phenotype led us to hypothesize that the response to cellular senescence may be species-specific, and we sought to develop a human lung epithelial cell model to examine the consequences of telomere dysfunction in a more clinically relevant cell type. We selected the p53 competent, alveolar epithelial-like cell line, A549 (33). As in our prior mouse model, we chose telomeric repeat-binding factor 2 (TERF2) as our target of intervention. As a component of the shelterin complex, TERF2 serves to prevent telomeric ends from being recognized as double-stranded DNA breaks (34). Given the known poor correlation between mRNA and protein levels in human tissues (35), we also employed a novel endoplasmic reticulum (ER) targeted biotin ligase to characterize the secreted protein (secretome) changes induced by cellular senescence. We hypothesized that this combined approach would enable the most complete characterization of human telomere-induced alveolar epithelial senescence and provide the greatest opportunity to identify relevant secreted proteins.

Herein we show that telomere dysfunction in a human alveolar epithelial-like cell line leads to cellular senescence and an upregulation in transcriptional and proteomic SASP. Our results suggest that the consequences of telomere dysfunction may be species-specific and perhaps cell-type specific. We also introduce a set of adaptable tools for the induction of senescence and study of its effects on protein secretion in a variety of cell types.

METHODS

Tissue Culture and Generation of Stable Cell Lines

A549 cells were acquired from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum and penicillin (120 U/mL), streptomycin (100 mcg/mL), and L-glutamine (2 mM). A construct for conditional induction of telomere dysfunction was generated by cloning a truncated version of human TRF2 protein that lacks the N-terminal basic domain and C-terminal Myb domains (36) into the lentiviral vector pCW57-GFP-2A-MCS, a gift from Adam Karpf (Addgene plasmid #71783) (37). Lentiviral particles were generated as described previously (38) and used to transduce low-passage A549 cells. Following transduction, individual clones of cells were selected that showed strong expression of the transgene in the presence of 2 μg/mL doxycycline. Proliferation studies were carried out by plating three independent cultures of each cell line and enumerating cells at each passage. The total number of cells were log2 transformed and plotted against time. Fresh doxycycline (2 μg/mL final concentration) was added at each passage. Clonogenic assays were performed by plating 1,000 cells in 10 cm dishes and enumerating colonies following staining with crystal violet after 12 days in culture. Media was replaced with fresh doxycycline (2 μg/mL final concentration) every 48 h during the course of the experiment. Senescence-associated beta-galactosidase (SA-βgal) was stained according to manufacturer's protocol (Cell Signaling Technologies). Proximity ligation experiments were carried out by expressing a modified biotin ligase (BioID2) (39) that had been targeted to the endoplasmic reticulum (ER) by addition of a N-terminal IgK signal sequence and C-terminal ER retention sequence (KDEL) (40).

Western Blots and Immunoprecipitation

Western blots were performed following standard procedures and employed antibodies specific for Flag epitope (M2, Millipore Sigma), V5 (Thermo Fisher), HA (Millipore Sigma), p21 (Cell Signaling Technologies), and GAPDH (BioRad). Briefly, cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (MiniComplete, Roche). Following protein quantitation, 20–40 μg of protein or 18 μL of media were separated under reducing conditions using SDS-PAGE and transferred to PVDF membranes. Proteins were blotted with antibodies specific for the desired protein and visualized on a ChemiDoc MP gel documentation system (BioRad). Immunoprecipitation of V5-tagged proteins was accomplished by incubating media containing V5-tagged proteins with Anti-V5 agarose (Millipore Sigma) according to the manufacturer's instructions.

Transcriptional Profiling and Analysis

Total RNA was isolated from biologic replicates ($n = 3$) of cultured cells using RNAeasy kits (Qiagen) according to manufacturer's protocol and sent for library preparation, sequencing, quality control, alignment, differential expression analysis, and preliminary enrichment analysis at Novogene (Sacramento, CA). Approximately 20 million paired-end fragments were sequenced for each sample. The raw data have been deposited in NCBI's Gene Expression Omnibus (41) GSE155941. Expression data from senescent murine AEC2s were obtained from GSE56892 (9). Additional enrichment analyses were conducted using Ingenuity Pathway Analysis (Qiagen), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG). Differential expression of several genes was confirmed using quantitative real-time PCR with primers specific for the selected genes.

Proximity Labeling and Mass Spectrometry

Validation of the BioID2 targeting and function was accomplished by transfecting cells stably expressing ER-targeted BioID2 with a plasmid encoding V5-tagged human *SFTPA2* cDNA (pCDNA3-V5-SFTPA2) (14). Eighteen hours after transfection, media was supplemented with biotin (100 μM). The next day, cells and media were collected for western blot analysis. V5-tagged SFTPA2 was immunoprecipitated with anti-V5 resin (Millipore). Detection of biotinylated proteins was accomplished by incubating membranes with streptavidin conjugated to horseradish peroxidase (Strep-HRP) and developing the membranes according to the manufacture's protocol (Vector Laboratories). The unbiased proteomic screen of telomere dysfunction-induced senescence-related changes was carried out by comparing TRF2-DN-BioID2 and TRF2-DN-BioID2+Doxycline. Four days after addition of doxycycline, biotin was added to the media. Eight hours later, cells were washed to remove excess biotin and fresh media was added. Twenty-four hours later, the supernatant was collected, and biotinylated proteins were purified by incubating media with streptavidin coated beads according to the manufacturer's protocol (Dynabeads MyOne Streptavidin C1; Invitrogen). Half of the sample was eluted at 95°C for 10 min in loading buffer and run on a 4–15% SDS-PAGE gel to evaluate yield of recovered protein. The remainder of the protein coated beads were sent to MS Bioworks (Ann Arbor, MI) for mass spectrometry analysis where they were eluted, gel separated, split into 10 samples based on molecular weight, and digested samples were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced with a ThermoFisher Q Exactive mass spectrometer. A single sample was submitted for each condition. Data were searched using Mascot (Matrix Science) and parsed into Scaffold™ (Proteome Software Inc.) for validation, filtering and to create a non-redundant list per sample. Data were filtered using a 1% protein and peptide level false discovery rate (FDR) and by requiring at least two unique peptides per protein.

Immunostaining and Imaging

Cells were grown on coverslips and fixed in 2% PFA for 10 min. Following fixation cells were washed, permeabilized with Triton X-100, and blocked with goat serum. Coverslips were incubated with primary antibodies including rat anti-HA (Millipore Sigma) and rabbit anti-calnexin (Cell Signaling). Proteins were visualized with secondary antibodies conjugated to Alexa 594 and Alexa 647 (Thermo Fisher). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were obtained at the Center for Biologic Imaging at the University of Pittsburgh on an Olympus FluoView Confocal microscope. Brightfield photomicrographs were captured on an Observer A.1 (Zeiss) equipped with AxioCam MRc camera.

Multiplex Screen of Serum Biomarkers

Transcriptional and proteomic data were used to rationally select 17 candidate biomarkers for evaluation in a discovery cohort of control ($n = 30$) and IPF ($n = 50$) patients. Plasma samples from these patients were evaluated using Luminex® panels purchased from R&D systems. Candidates biomarkers were selected based on their differential expression in our current study, the availability of compatible commercial assays to simultaneously measure several proteins, and their dilution compatibility with the chosen assay. For this initial study, we only evaluated proteins that had previously been reported to be detectable in human plasma samples. Panels were analyzed on a Bio-Plex reader (Bio-Rad) according to the manufacturer's protocol. Biomarkers selected from the discovery round were evaluated for correlations with baseline pulmonary function studies in IPF patients.

Human Subjects

All studies were approved by the University of Pittsburgh Institutional Review Board and the Committee for Oversight of Research and Clinical Training Involving Decedents. All subjects provided written, informed consent before enrollment in the research study. IPF subjects were recruited from the Simmons Center for Interstitial Lung Diseases at the University of Pittsburgh Medical Center. Clinical, physiologic, and high-resolution computed tomography studies of these patients supported the diagnosis of IPF. Patients fulfilled the criteria of the American Thoracic Society and European Respiratory Society for the diagnosis of IPF at the time of diagnosis (3, 42). Patients with known causes of interstitial lung disease were excluded. Control patients consisted of unrelated healthy subjects, randomly recruited from the University of Pittsburgh Medical Center, and had no self-reported advanced lung diseases.

Statistical Analysis

All cellular images shown are representative of multiple experiments. RNA-seq differential expression analysis was performed using the DESeq2 R package (43). Fisher's exact test was used for differential expression analysis of mass spectrometry identified proteins. Simple linear regression was used for differential protein vs. RNA correlation. Control vs. IPF plasma protein levels were evaluated using Welch's *t*-test of significance. The Benjamini-Hochberg procedure was used for all corrections of multiple testing. Pearson correlation coefficients for IPF patient baseline PFT values were calculated using square-root-transformed protein levels.

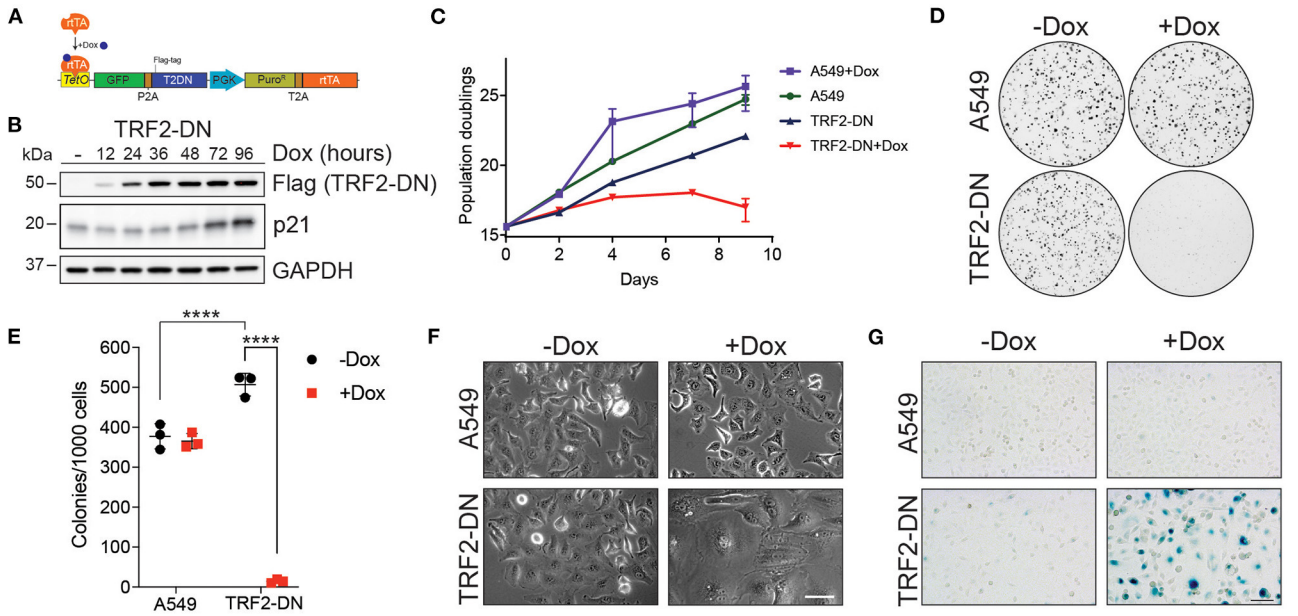


FIGURE 1 | Induction of telomere dysfunction causes senescence of a human lung epithelial cell line. **(A)** Schematic of all-in-one lentivirus expressing green fluorescent protein (GFP), dominant negative TRF2 (T2DN; TRF2-DN hereafter), puromycin N-acetyl transferase (Puro^R), and reverse Tet repressor (rtTA). GFP and TRF2-DN are expressed by a doxycycline-inducible promoter while Puro^R and rtTA are expressed by the constitutive phosphoglycerate kinase promoter (PGK). **(B)** Time course of protein expression following induction with doxycycline (dox). Relative protein levels of TRF2-DN (flag), p21, and GAPDH (load control) are shown. **(C)** Relative proliferation of A549 and A549 cells stably expressing TRF2-DN. Viable cells were counted with trypan blue staining following induction with doxycycline. Mean and standard error of the mean are shown for each count ($n = 3$). **(D)** Representative images of crystal violet stained clonogenic assays. Media was changed every 2–3 days after plating 1,000 cells and plates were imaged after 12 days. **(E)** Quantitation of visible colonies from **(D)**. Mean and standard deviation are shown. **(F)** Photomicrograph of A549 and TRF2-DN cells using phase-contrast microscopy. Images were taken 9 days after induction of TRF2-DN with Dox. **(G)** SA-βgal activity of cells shown in **(F)**. Scale bar in **(F,G)** is 100 microns. ****$P < 0.0001$, one-way ANOVA and Tukey *post hoc* test.

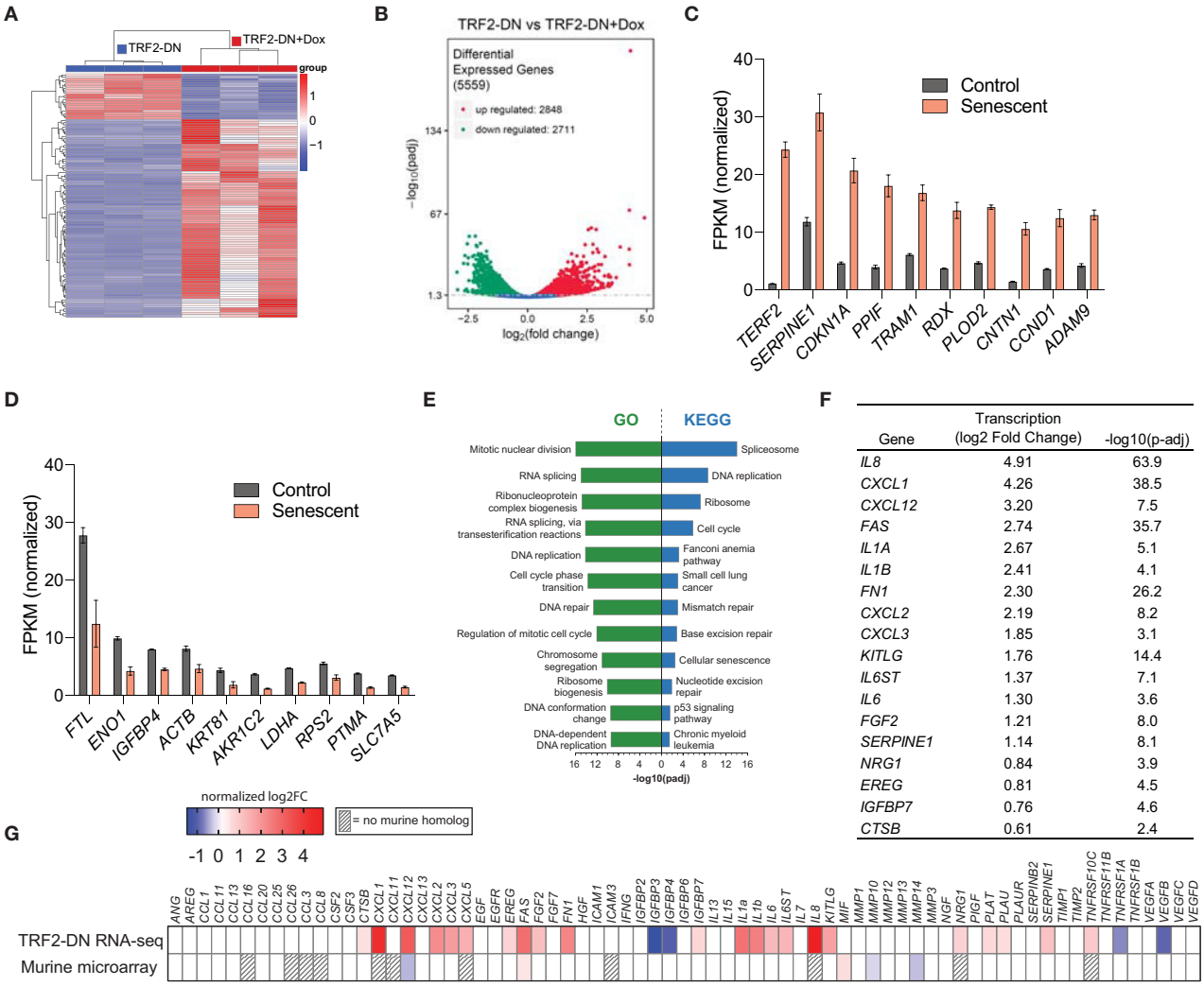
RESULTS

Induction of Telomere Dysfunction Drives Senescence of Human Lung Epithelial-Like Cells

In order to create a model of human, telomere dysfunction-induced, alveolar epithelial cell senescence, we generated a stable A549 cell line that conditionally expressed a dominant negative form of human TRF2 (TRF2-DN) (**Figure 1A**). Expression of TRF2-DN disrupts shelterin function (36) and leads to telomere uncapping and a subsequent DNA damage response. Conditional induction of TRF2-DN protein led to upregulation of cyclin dependent kinase inhibitor CDKN1A (p21) and halted proliferation (**Figures 1B,C**). We noted that cells that expressed the TRF2-DN transgene consistently proliferated at a lower rate compared to untransduced cells, likely due to low-level baseline expression of the transgene. TRF2-DN expression limited the clonogenic potential of A549 cells and triggered morphologic changes consistent with the induction of senescence (**Figures 1D,F**). The apparent increase in colony number for untreated TRF2-DN in **Figure 1E** is due to smaller, unmerged colonies (data not shown). Consistent with the above findings, cells expressing the TRF2-DN stained strongly for SA-βgal (**Figure 1G**). Together, these data suggest that disruption of shelterin function is sufficient to drive cellular senescence in A549 cells.

Comprehensive Transcriptional Profile of Senescent A549 Cells

We hypothesized that expression of TRF2-DN would result in a DNA damage response and additional transcriptional changes associated with senescence. Therefore, bulk RNA sequencing was performed on control A549 cells and TRF2-DN cells 9 days after addition of doxycycline. Examination of the cluster analysis of differentially expressed genes, Pearson correlation coefficients, and principal component analysis confirms the creation of a transcriptionally distinct population of cells after the induction of TRF2-DN expression (**Figure 2A** and **Supplemental Figures 1A,B**). Consistent with previous reports focused on the effects of doxycycline (44, 45), we identified a cluster of genes that were differentially expressed due to the addition of doxycycline (not shown). We also found a significant number of genes that were differentially expressed in A549 vs. TRF2-DN in the absence of doxycycline, suggesting that low baseline expression of TRF2-DN was causing significant transcriptional changes in these cells. We focused our analysis on TRF2-DN cells to identify genes that were upregulated when these cells transitioned into senescence. Nearly 22% of all detected genes were significantly



Gene	Transcription (log2 Fold Change)	-log10(p-adj)
IL8	4.91	63.9
CXCL1	4.26	38.5
CXCL12	3.20	7.5
FAS	2.74	35.7
IL1A	2.67	5.1
IL1B	2.41	4.1
FN1	2.30	26.2
CXCL2	2.19	8.2
CXCL3	1.85	3.1
KITLG	1.76	14.4
IL6ST	1.37	7.1
IL6	1.30	3.6
FGF2	1.21	8.0
SERPINE1	1.14	8.1
NRG1	0.84	3.9
EREG	0.81	4.5
IGFBP7	0.76	4.6
CTSB	0.61	2.4

FIGURE 2 | Dysfunctional telomeres drive senescence-associated transcriptional changes in A549 cells. **(A)** Hierarchical cluster analysis of differentially expressed genes identified using RNA-seq of TRF2-DN and TRF2-DN+Dox 9 days after induction ($n = 3$ per group). Red indicates up-regulated transcripts; blue indicates down-regulated transcripts. The fold-change based on color is shown in the key. **(B)** Volcano plot depicting 5,559 differentially expressed genes. Significance defined as $-\log_{10}$(p-adj) > 1.3. The 10 most upregulated **(C)** and downregulated **(D)** transcripts between TRF2-DN (control) and TRF2-DN+Dox (senescent) cells. Mean and standard deviation are shown. All differences are statistically significant, but *P*-values are not shown for clarity. **(E)** Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes identified pathways involved in cell cycle, DNA repair, gene expression, and cellular senescence as the most significantly altered pathways. **(F)** Table of several canonical SASP genes (31) that were differentially expressed in senescent A549 cells. Significance defined as $-\log_{10}$(p-adj) > 1.3. **(G)** SASP transcriptional heat map of senescent A549 with telomere dysfunction and primary AEC2s from mice with AEC2-specific telomere dysfunction (9). The $\log_2$-fold change is shown. Gray stripes indicate genes with no mouse homolog.

differentially expressed in this context (**Figure 2B**). *TERF2* (our overexpressed target) experienced the greatest increase in gene expression, followed by *SERPINE1* and *CDKN1A*—two canonical senescence genes (31) (**Figure 2C**). The most downregulated genes are shown in **Figure 2D** and include *FTL*, *ENO1*, and *IGFBP4* among others. Pathway analysis of our bulk RNA-seq data revealed an enrichment in pathways consistent with telomere dysfunction and disruption of the cell cycle. Notably, there was also enrichment in the cellular senescence KEGG pathway (**Figure 2E**). An upregulation in the canonical SASP components was also seen (**Figure 2F**). We validated several of the differentially expressed genes using quantitative real-time PCR with primers specific for the genes of interest and found excellent correlation with our RNA-seq data (**Supplemental Figure 2**). We compared our results to primary senescent murine AEC2s and found 127 (13% of upregulated murine genes) genes were upregulated in both datasets and 123 (14% of downregulated murine genes) were downregulated in both datasets (**Supplemental Figure 1C**). Unlike the upregulation seen in our RNA-seq dataset, very few SASP genes were upregulated in senescent murine type II alveolar epithelial cells (**Figure 2G**).

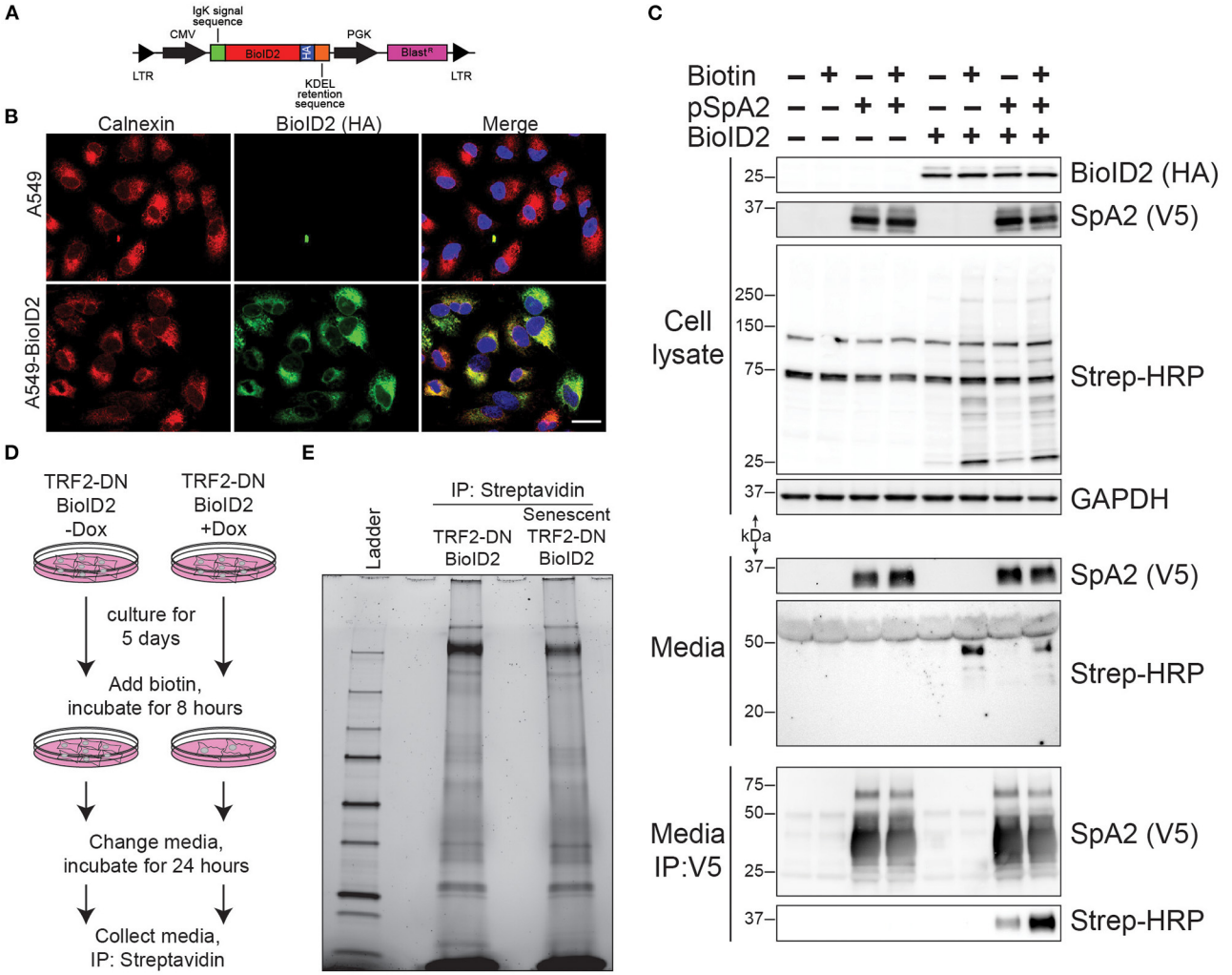
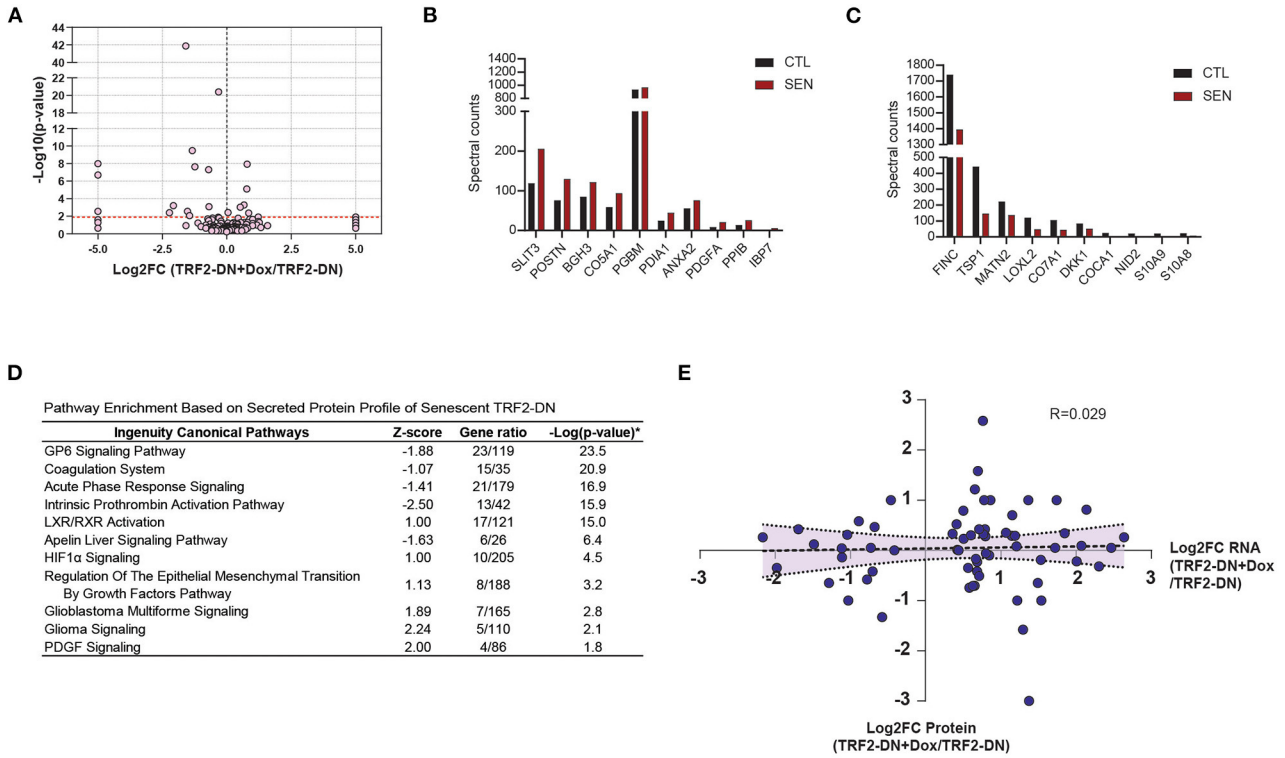


FIGURE 3 | ER-targeted BioID2 to label secreted proteins. **(A)** Schematic of lentivirus expressing ER-targeted BioID2 construct. **(B)** Photomicrographs of A549 and A549-BioID2 cells showing co-localization of the BioID2 (HA-tagged; green) and the ER marker calnexin (red). Nuclei are stained with DAPI (blue). Scale bar is 25 microns. **(C)** Proof of concept demonstrating that secreted proteins are biotinylated by ER-targeted BioID2. A549 or A549 cells stable expression ER-BioID2 were transfected with a plasmid encoding a V5-SFTPA2 construct. Eighteen hours after transfection, biotin was added to some samples and the media and cell lysates were examined the next day by western blotting for SFTPA2 (V5), BioID2 (HA), or biotinylated proteins (Strep-HRP). GAPDH was a load control for cell lysates. Biotinylated proteins were detected in the media only in cells that expressed ER-BioID2 and were cultured in excess biotin. Biotinylated SFTPA2 was detected in media only when ER-BioID2 was present. **(D)** Experimental procedure for unbiased proteomic analysis of secreted proteins. Cells were cultured for 5 days in the presence of Dox to induce senescence followed by incubation with excess biotin for 8 h. After biotin labeling, cells were washed and fresh media was added. Twenty-four hours later, media was collected and biotinylated proteins were isolated by incubation with streptavidin-coated beads and analyzed by stain-free SDS-page **(E)**.

Biotinylation of the Secretome

In an effort to identify senescence-associated changes in protein secretion in an unbiased manner, we next employed an endoplasmic reticulum (ER)-targeted biotin ligase (BioID2) capable of biotinylating proteins that traverse the classical secretion pathway. A lentiviral vector system was again used to stably express the ER-targeted BioID2 (**Figure 3A**). Confocal microscopy confirmed ER-localization of our HA-tagged BioID2 (**Figure 3B**). We next performed a proof-of-concept experiment to test if the ER-targeted BioID2 system was indeed functioning as anticipated. A V5-tagged surfactant protein A2 (SpA2) plasmid that has been reported to be successfully secreted by A549 cells (14) was introduced via transfection into our A549-BioID2 cell line. We first confirmed the presence of SpA2 in the transfected cell lysates. We then verified an increase in biotinylation in lysates from the BioID2 line and a further increase in biotinylation in the lysates of these cells when cultured in the presence of excess biotin. Upon blotting of the cell supernatants for the V5 epitope, SpA2 was readily detectable in the transfected lines (**Figure 3C**, upper panels). We probed the supernatants with streptavidin-HRP and found an increase in biotinylated proteins in the BioID2 cell lines when grown



Ingenuity Canonical Pathways	Z-score	Gene ratio	-Log(p-value)*
GP6 Signaling Pathway	-1.88	23/119	23.5
Coagulation System	-1.07	15/35	20.9
Acute Phase Response Signaling	-1.41	21/179	16.9
Intrinsic Prothrombin Activation Pathway	-2.50	13/42	15.9
LXR/RXR Activation	1.00	17/121	15.0
Apelin Liver Signaling Pathway	-1.63	6/26	6.4
HIF1α Signaling	1.00	10/205	4.5
Regulation Of The Epithelial Mesenchymal Transition By Growth Factors Pathway	1.13	8/188	3.2
Glioblastoma Multiforme Signaling	1.89	7/165	2.8
Glioma Signaling	2.24	5/110	2.1
PDGF Signaling	2.00	4/86	1.8

FIGURE 4 | Unbiased proteomic characterization of senescent secretome. **(A)** Volcano plot showing relevant abundance of proteins in supernatant from TRF2-DN+Dox compared to TRF2-DN cells. The 10 most significantly upregulated **(B)** and downregulated **(C)** proteins between TRF2-DN (control) and TRF2-DN+Dox (senescent) cells (data are from a single experiment). **(D)** Ingenuity Canonical Pathway analysis of differentially expressed secreted proteins shows and enrichment in pathways associated with coagulation, non-specific defense, adhesion, and lipid metabolism. **(E)** Correlation of the fold-changes of RNA and protein from RNA-seq and proteomic data shows limited correlation between the two datasets.

in the presence of excess biotin (**Figure 3C**, middle panels). Immunoprecipitation of SpA2 from supernatants followed by blotting with streptavidin-HRP demonstrated that SpA2 was being biotinylated uniquely in our A549-BioID2 system and to a greater extent when grown in the presence of excess biotin (**Figure 3C**, lower panels).

We next conducted an unbiased screen to identify changes in the secretome as a result of cellular senescence. We utilized our TRF2-DN-BioID2 line ± doxycycline with the addition of excess biotin to the media 5 days after the induction of senescence. After a biotin incorporation period, the media was replaced and later collected for affinity purification with streptavidin beads (**Figure 3D**). A portion of the streptavidin beads were eluted and evaluated using SDS-PAGE to verify protein abundance and to identify qualitative differences in protein secretion (**Figure 3E**). The remainder of the biotinylated protein was then analyzed via mass spectrometry in order to identify quantitative differences in secretion as a result of cellular senescence.

Senescence-Related Changes in the Secretome

Cumulatively, 170 unique secreted proteins were identified by LC/MS/MS for the senescent and non-senescent groups (**Figure 4A**). The most significantly upregulated proteins are shown in **Figure 4B**, which includes the SASP protein, IBP7. Fibronectin 1 (FINC) and Thrombospondin 1 (TSP1) exhibited the greatest decrease in protein expression (**Figure 4C**). Ingenuity Canonical Pathway analysis of the secretome revealed multiple significantly enriched pathways. Of note, these included pathways associated with coagulation, non-specific defense, adhesion, and lipid metabolism (**Figure 4D**). We next analyzed the relationship between transcriptional fold change and corresponding proteomic fold change in the secretome and observed a poor correlation between the two datasets (**Figure 4E**).

Candidate Markers of Senescence in Idiopathic Pulmonary Fibrosis

Once we had established that our system closely mirrored human SASP, we next sought to evaluate its utility in identifying novel biomarkers in human plasma. Our transcriptional and proteomic data were used to rationally select 17 candidate biomarkers for evaluation in a discovery cohort of control ($n = 30$) and IPF ($n = 50$) patients. Of the 17 selected potential biomarkers, S100A9, stanniocalcin-1, contactin-1, tenascin C, periostin, and total inhibin were found to be differentially expressed between

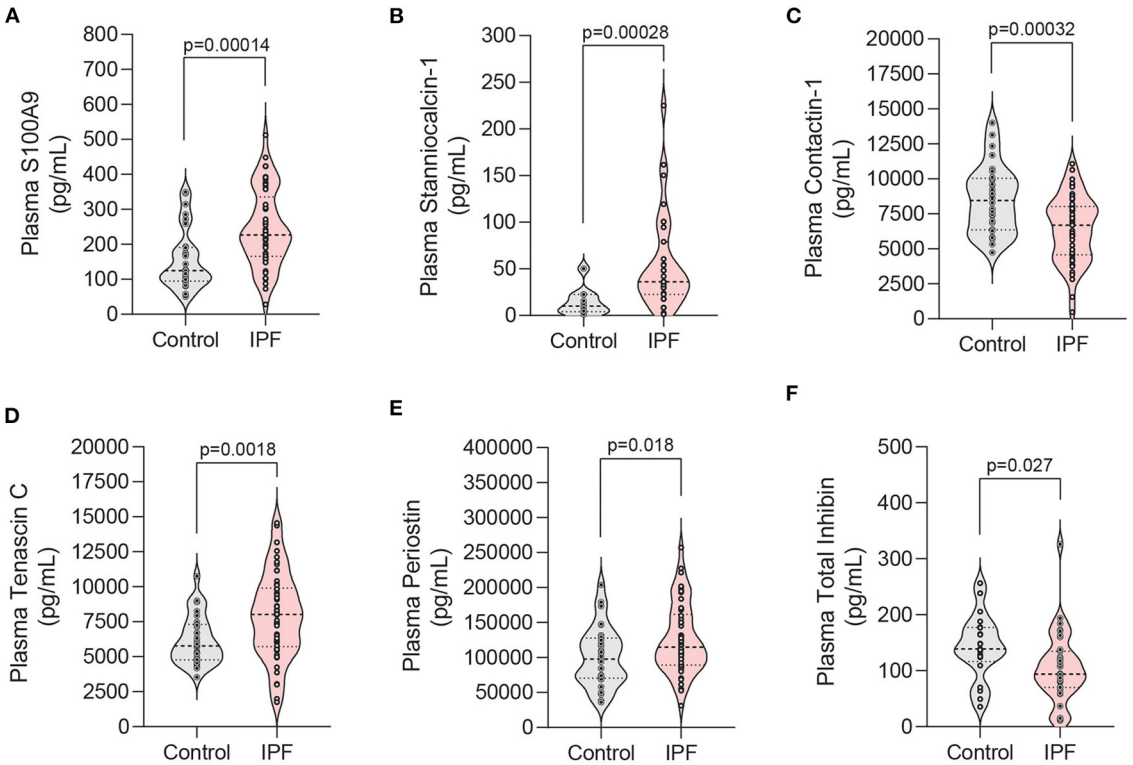


FIGURE 5 | Analysis of candidate biomarkers in plasma from IPF patients and controls. **(A–F)** Violin plots of candidate biomarkers identified in RNA-seq and proteomic analyses that displayed statistically significance differences in patient plasma samples. Plasma from patients with IPF ($n = 50$) and controls ($n = 30$) was analyzed using a custom Luminex panel. Median and interquartile range is shown. Significance defined as $P < 0.035$ by Welch's *t*-test using the Benjamini-Hochberg procedure to correct for multiple testing while utilizing a pre-defined 10% false discovery rate.

control and IPF patients (**Figure 5** and **Supplemental Table 1**). Four of these markers (stanniocalcin-1, contactin-1, tenascin C, and total inhibin) have not been previously associated with IPF. Stanniocalcin-1 elevation displayed a trend toward an association with a lower baseline DLCO percent predicted among IPF patients, but did not reach statistical significance ($p = 0.07$) (**Supplemental Table 2**).

DISCUSSION

In an effort to better understand the pathogenesis of age and short-telomere mediated disease in the lung, we generated a human model of alveolar epithelial cell senescence. We previously investigated the transcriptional response to telomere dysfunction in primary murine AEC2s and found that few of the canonical SASP markers were expressed (9). We hypothesized that this may be due to differences in the species-specific response to telomere dysfunction and cellular senescence (36). We selected human A549 cells due to their origin in the lung epithelium and intact p53 signaling pathway. Instead of deleting TRF2, we conditionally expressed a TRF2 dominant negative (TRF2-DN) protein that has been previously reported to disrupt shelterin function (36). Consistent with disruption of telomere dysfunction and induction of a DNA-damage response, TRF2-DN expression led to accumulation of p21 and ensuing cell cycle arrest with morphologic changes consistent with the induction of cellular senescence. Despite the limitations of the A549 cell line, we reasoned that this system may provide an opportunity to explore the consequences of telomere dysfunction and cellular senescence in human alveolar epithelial cells.

We comprehensively characterized the transcriptional and secretome changes that occurred in our human telomere-induced senescence model system. Our RNA analysis demonstrated that induction of senescence in TRF2-DN leads to enrichment in pathways consistent with a telomere-based injury and to the adoption of a transcriptional SASP phenotype. When we compared our current studies to previously published findings from primary murine AEC2s, we found that in the setting of telomere-induced senescence, both human and murine cells upregulated genes related to cell cycle arrest and the DNA damage response; however, expression of SASP genes was strikingly different. Of the 60 canonical SASP genes with murine homologs, only 2 were found to be differentially expressed in the expected direction in murine cells. In contrast, 27 of the 71 canonical SASP genes were differentially expressed in the anticipated direction in human cells and several were among the top upregulated genes. Only *FAS* was similarly upregulated in both murine and human cells. These data suggest that modeling senescence and telomere dysfunction in mice may not fully

recapitulate the biology of human cells and animal findings should be carefully cross-validated to ensure their translatability.

Given that previous studies have reported relatively poor correlations between human transcriptional and proteomic datasets (35, 46–48), we reasoned that an isolated transcriptional analysis of our conditionally senescent cell line would likely inadequately predict the extracellular protein and pathway changes brought on by senescence. We therefore endeavored to characterize the secretome of senescent A549 cells and adopted a recently reported system to target a biotin ligase to the endoplasmic reticulum where it would label proteins passing through the classical secretory pathway (40). We demonstrated the feasibility of the ER-targeted BioID2 system by showing that SFTPA2 is biotinylated and secreted and can be purified from cell supernatant. We then utilized our model to carry out an unbiased analysis of the secretome from senescent cells. This analysis identified 170 secreted proteins that coalesced into thematic pathways of coagulation, cholesterol homeostasis, and response to injury. A comparison of our pathway analyses following the induction of telomere dysfunction shows that transcriptional pathways primarily highlight the mechanism of injury while secretome pathways point toward downstream and paracrine effects. Consistent with prior studies, a poor correlation was found between our differential RNA and differential protein datasets (49, 50). One potential explanation is that the enrichment seen in the ubiquitin-mediated proteolysis pathway in our senescent RNA-seq dataset (data not shown) may facilitate more rapid intracellular protein degradation. Differential kinetics of protein translation and subsequent secretion in the setting of senescence is an alternative explanation. Nevertheless, the negligible interdependence between these two datasets is also not unexpected given that gene expression and protein abundance are largely uncoupled in pulmonary tissues (51). Taken together, our data suggest that transcriptional profiling alone is not sufficient to predict the secretome profile of senescent cells. Likewise, an evaluation of the secretome does not allow the inference of the intracellular signaling pathways and transcriptional aberrations initiated by telomere-mediated senescence.

Recent studies with an emphasis on aging have attempted to better characterize the many senescence-associated genes, proteins, and pathways in human disease (52, 53). Efforts have been made to highlight the varied context and cell-type specific responses to senescence (53). Similarly, we sought to utilize our telomere-mediated, alveolar epithelial cell senescence model to identify novel plasma markers of the aging associated disease, IPF. To our knowledge, this is the first report of stanniocalcin-1, contactin-1, tenascin C, and total inhibin as being differentially expressed in IPF patient plasma. Periostin (POSTN) was identified in our proteomic screen and was selected as a positive control for our study given that it had been previously reported to be upregulated in IPF patient serum (54). S100A9 was decreased in our senescent secretome data, but it had previously been shown to trend toward upregulation in IPF serum (55). We chose to evaluate this apparent discrepancy and found it to be upregulated in IPF patient plasma. Granulocytes and monocytes are reported to be the principal source of S100A9 (56), and our results suggest that senescent lung epithelial cells are not a significant source. Contactin 1 (CNTN1) was the only non-classically secreted protein evaluated in our plasma study. It was upregulated >6-fold and had the 4th most significant *p*-value for differential expression in our RNA-seq dataset, yet it was found to be downregulated in IPF plasma. Our model system correctly predicted the directionality for the remaining three differentially expressed plasma proteins lending support to its value in identifying novel classically secreted markers in IPF.

There are multiple limitations to our approach, but the data we present here highlight its value and the importance of considering potential species-specific responses to aging and telomere dysfunction. A549 is an epithelial carcinoma cell line. These cells almost certainly do not faithfully represent primary human alveolar epithelial cells and their transcriptional and secretory responses likely do not completely overlap with those of primary cells. Nevertheless, we were able to use this tractable model system to identify several candidate secreted proteins that were validated in patient plasma samples. We recognize that bronchoalveolar lavage would be a more direct measure of the epithelial secretome, but this is not clinically feasible and is an unrealistic source for potential biomarker validation. We specifically designed our experimental system to be portable to facilitate its use in studying the cell-type specific responses to telomere dysfunction in other cell lineages such as fibroblasts where telomere-based pathology has been described (57). Additionally, we expect that future studies will further delineate not only species and cell-type specific, but also context-specific responses to telomere dysfunction. Given the multiplicity of cell types in the lung and that aging and environmental factors contribute to cellular responses, additional investigations are warranted to understand how each of these cell types contribute to age-associated lung disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The RNA-seq data have been deposited to https://www.ncbi.nlm.nih.gov/geo/, GSE155941. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (58) partner repository with the dataset identifier PXD023381 and 10.6019/PXD023381.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Pittsburgh Institutional Review Board and the Committee for Oversight of Research and Clinical Training Involving Decedents. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JA and DS conceived of the project, planned the experiments, and drafted the manuscript. DS, MJ, AH,

MR, and HB performed the experiments. MN and YZ analyzed the data. JL and TF provided essential reagents and protocols. JA, DS, JM, RM, and DK interpreted the findings. All authors gave feedback on the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.600626/full#supplementary-material

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ORIGINAL RESEARCH
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Weak Handgrip at Index Admission for Acute Exacerbation of COPD Predicts All-Cause 30-Day Readmission

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Rationale: Identifying patients hospitalized for acute exacerbations of COPD (AECOPD) who are at high risk for readmission is challenging. Traditional markers of disease severity such as pulmonary function have limited utility in predicting readmission. Handgrip strength, a component of the physical frailty phenotype, may be a simple tool to help predict readmission.

Objective(s): To investigate if handgrip strength, a component of the physical frailty phenotype and surrogate for weakness, is a predictive biomarker of COPD readmission.

Methods: This was a prospective, observational study of patients admitted to the inpatient general medicine unit at the University of Chicago Medicine, US. This study evaluated age, sex, ethnicity, degree of obstructive lung disease by spirometry (FEV_1 percent predicted), and physical frailty phenotype (components include handgrip strength and walk speed). The primary outcome was all-cause hospital readmission within 30 days of discharge.

Results: Of 381 eligible patients with AECOPD, 70 participants agreed to consent to participate in this study. Twelve participants (17%) were readmitted within 30 days of discharge. Weak grip at index hospitalization, defined as grip strength lower than previously established cut-points for sex and body mass index (BMI), was predictive of readmission (OR 11.2, 95% CI 1.3, 93.2, $p = 0.03$). Degree of airway obstruction (FEV_1 percent predicted) did not predict readmission (OR 1.0, 95% CI 0.95, 1.1, $p = 0.7$). No non-frail patients were readmitted.

Conclusions: At a single academic center weak grip strength was associated with increased 30-day readmission. Future studies should investigate whether geriatric measures can help risk-stratify patients for likelihood of readmission after admission for AECOPD.

Keywords: chronic obstructive pulmonary disease, patient readmission, frailty, hand strength, grip strength

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of re-hospitalizations in the US, and over 20% of patients hospitalized for acute exacerbations of COPD (AECOPD) are readmitted within 30 days of discharge (1). Lowering 30-day readmission rates is a specific target of the Centers for Medicare and Medicaid Services, in part because re-hospitalization is expensive—the cost of all-cause re-hospitalization was estimated to be 17.4 billion in 2004 (2). On October 1, 2012, penalties began to be imposed on hospitals with high rates of unplanned readmission in six condition or procedure groups, including COPD (3). COPD is common among Medicare beneficiaries–12% of beneficiaries age 65 years and older have COPD (2); therefore reducing AECOPD readmission rates is an important goal for health systems.

The first step toward readmission reduction is identifying patients at risk of readmission. A number of retrospective studies have found that non-modifiable factors, such as severity of disease at the time of index admission, age, or socioeconomic factors like insurance type, can predict readmission among adults with COPD (4, 5). Several potentially modifiable risk factors have also been identified, such as low baseline physical activity (6) and physical frailty (7), both of which are extra-pulmonary attributes. Unfortunately, a previous meta-analysis of readmission reduction programs found no consistent benefit in readmission reduction interventions (8), though most risk reduction programs did not target modifiable extra-pulmonary risks.

Geriatric assessments, such as physical frailty evaluations and other functional assessments, are attracting interest in COPD readmission risk stratification because these extra-pulmonary factors impact all-cause readmission and mortality (9–13). Physical frailty is a geriatric syndrome of multisystem dysregulation leading to impaired physiologic and psychologic resilience. It is manifest clinically by reduced physiologic function, reduced endurance, and decreased strength (14). In the general population, frailty predominantly affects older adults and confers increased risk of hospitalizations, readmissions, and death (15, 16). Data from the National Health and Nutrition Evaluation Survey found a frailty prevalence of almost 60% in people with COPD (17). In a separate study of community-dwelling people with concomitant COPD and frailty, mortality was three-times that of non-frail patients with or without COPD. Further, frailty better predicted increased mortality better than FEV_1 (18).

Given the high prevalence of frailty among people with COPD and the potentially intervenable nature of this syndrome, frailty may be an important extra-pulmonary risk factor that identifies patients at high risk for hospitalizations and readmissions. A 2017 study of 103 patients hospitalized for AECOPD found that frailty, measured by the Reported Edmonton Frail Scale, predicted 90-day readmission (7). Further, previous work has demonstrated that frailty is modifiable, and can improve following behavioral interventions, physical therapy or pulmonary rehabilitation (6, 19, 20). Improvements in frailty may improve disability and quality of life, as has been demonstrated in patients following lung transplantation for cystic fibrosis (21).

Handgrip strength, a component of the classic physical frailty phenotype (15), has been demonstrated in many settings to be associated with outcomes such as disability and hospital length of stay (22, 23). Handgrip weakness indicates dynapenia, which means loss of strength, and is a component of sarcopenia. Sarcopenia may be found alone or as part of the frailty syndrome (24–28). Handgrip strength is obtained via a simple bedside measurement of isometric grip strength using a commercially available handheld dynamometer. Previous work has demonstrated that low handgrip strength is predictive of the number and severity of COPD exacerbations in non-hospitalized adults, COPD mortality, and poor inhaler technique in older adults (29–31). While an alternate upper-extremity strength measure predicted all-cause COPD readmissions in a small pilot study (32), handgrip strength at index hospitalization for AECOPD has not been evaluated as a biomarker of readmission risk. The objective of this sub-study of a larger study on frailty was to test the hypothesis that handgrip strength, a feasible and simple objective assessment, could predict all-cause 30-day readmission in patients admitted for AECOPD.

MATERIALS AND METHODS

Study Design

We conducted a prospective observational study of patients admitted to the inpatient general medicine unit at the University of Chicago Medicine, US. All participants provided written informed consent and the study was approved by the University of Chicago Institutional Review Board (14–848). This manuscript's reported findings are a subanalysis of a larger study on the interaction between frailty and COPD readmission risk.

Study Participants

From July 2016 to January 2019, research staff screened the electronic health record (Epic Systems Corporation, Verona, WI, "EHR") on weekdays (Monday-Friday) for patients admitted to the general medicine floor for AECOPD. The structure of care at the University of Chicago is as follows: patients with AECOPD are typically admitted from the emergency room, and cared for in the intensive care unit (which follows a closed intensive care model, in which critically ill patients are cared for by critical care physicians), or on the general medicine floor (where patients are cared for by general medicine physicians). Patients who met all inclusion criteria and met none of the exclusion criteria were considered eligible to participate (see **Figure 1**). Inclusion criteria included admission to a general medicine service for AECOPD and age over 18 years. Exclusion criteria were current admission to the intensive care unit and inability to give informed consent using the teachback method. Patients admitted to the ICU could become eligible for inclusion during their hospitalization upon transfer to the general medicine unit. Patients who provided written informed consent were enrolled. Patients were enrolled as they were identified but assessments did not occur at a standardized timepoint in their AECOPD hospitalization.

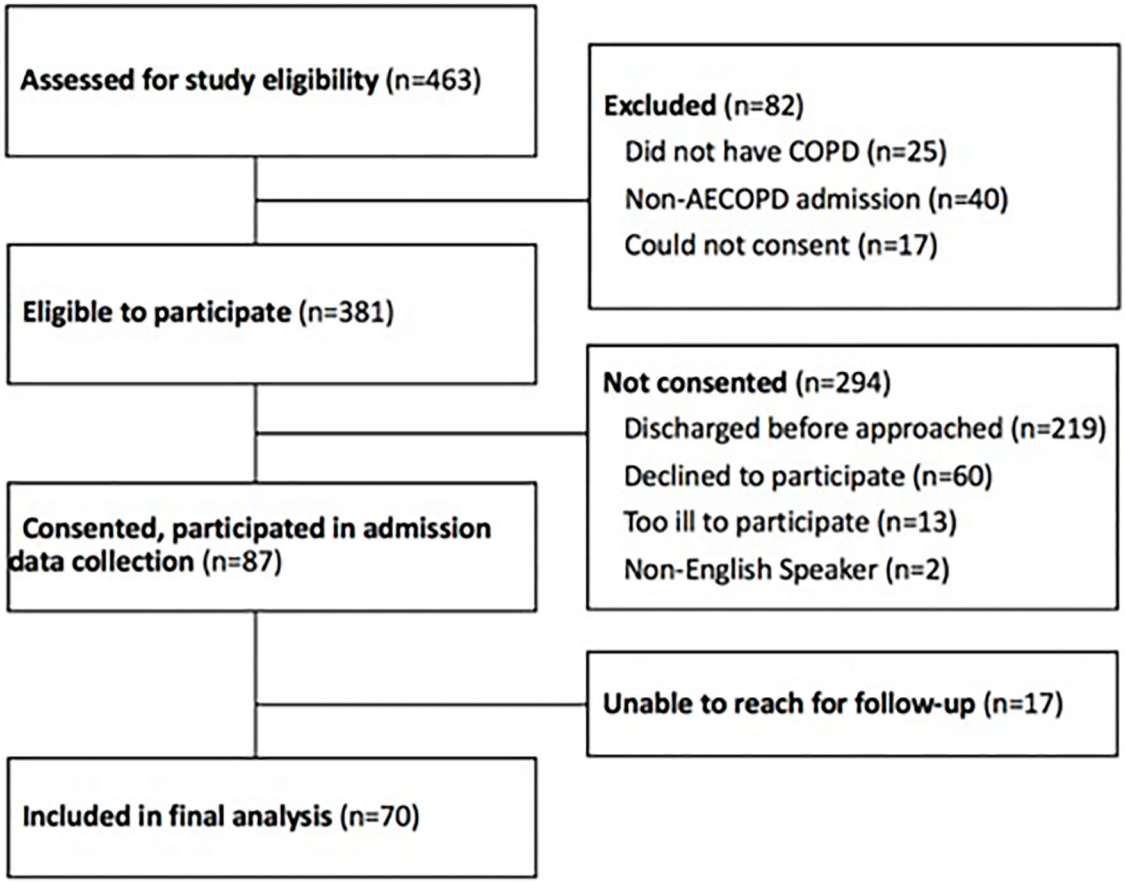

FIGURE 1 | Study participants. COPD, chronic obstructive lung disease; AECOPD, acute exacerbation of chronic obstructive lung disease.

Data Collection

Participant information such as comorbidities and length of stay were derived from medical records (see **Supplementary Table 1**). Forced Vital Capacity (FVC) and Forced Expiratory Volume in 1 s (FEV_1) were obtained via bedside spirometry (KoKo PFT System, version 4.3, nSpire Health Inc.) by a research coordinator trained in accurate spirometry collection (33). In the larger frailty study, participants underwent two assessments for frailty and function: physical frailty phenotype (15) and the Short Physical Performance Battery (SPPB) (34). This study reports only the physical frailty phenotype results. The physical frailty phenotype is a composite score of five domains (see **Supplementary Table 1**): 1. handgrip strength, assessed at beside using a handheld dynamometer (Jamar Technologies Plus+, Sammons Preston, Bolingbrook, IL), and measured using the average of three isometric grip attempts (kilograms) using the dominant hand, one point for "weak grip" is assigned if the subject's grip strength is below previously published cut-points for the lowest 20th percentile for sex and BMI (15); 2. Usual gait speed, measured as the average time to complete three, 15-foot walks at usual pace, one point for "slow gait" is assigned if the subject's gait speed is below previously published cut-points for the lowest 20th percentile for sex and height subgroups (15); 3. Level of exhaustion, as determined by answers to two questions from the Center for Epidemiologic Students Depression Scale, one point for "exhaustion" is assigned if the subject answers either exhaustion question affirmatively as "a moderate amount of the time" or "most of the time"; 4. Physical activity, assessed using the six-Item Minnesota Leisure Time Physical Activity Questionnaire, one point for low physical activity is assigned for a kilocalorie expenditure <383 kcal/week for men or <270 kcal/week for women (35); and 5. Weight loss, one point for weight loss is assigned if the subject lost $\geq 5\%$ of his or her body weight or 10 pounds unintentionally in the year prior to presentation (36, 37). The frailty components are binary, and one point is assigned to designate frailty in each domain. An individual is assessed to be "frail" if 3 or more domains are positive.

The primary outcome was all-cause hospital readmission within 30 days of discharge from the index AECOPD admission. Readmission data were obtained from medical record review and corroboration with follow-up phone calls if necessary.

Statistical Analyses

Descriptive statistics were utilized to summarize the participants' characteristics. We identified associations between categorical

variables using Chi-squared tests or Fisher's exact tests. Differences between groups were assessed using Student's *t*-tests or Mann-Whitney U tests for continuous variables.

Univariable logistic regression was used to assess the relationship between handgrip strength and all-cause readmission at 30 days. Univariable logistic regression was also used to assess the outcome variable of all-cause readmission at 30 days with the explanatory variables of the individual frailty components and FEV_1 percent predicted. The models were not adjusted for age or sex, because these demographic features are already used to determine "normal" values for grip strength, gait speed, and FEV_1 percent predicted. Data analysis and graphical analysis was conducted using STATA V.15.1 (College Station, TX). A two-tailed *p*-value of 0.05 was considered statistically significant.

RESULTS

Of 463 patients screened, 381 were eligible to participate based on our inclusion and exclusion criteria, and 87 consented for participation in the study (**Figure 1**). Of 87 participants who consented to participate in the study, 17 were unable to be reached for follow-up. The 70 remaining participants completed the study and were included in the data analysis (**Figure 1**).

The majority of participants were African American (91%, $n = 64$) and female (56%, $n = 39$) with a median age of 63.5 years (Quartile 1: 58.1 years; Quartile 3: 71.3 years) (**Table 1**). Of the participants who completed the physical frailty measures ($n = 55$), 67% were frail ($n = 37$). Of the participants who completed the grip strength maneuvers ($n = 63$), 55% had weak grip strength ($n = 37$) (**Table 2**).

Readmissions

Of the 70 enrolled participants, 12 (17%) were readmitted for any cause within 30 days of discharge. There were no differences in baseline characteristics among those participants who were readmitted vs. not readmitted. For instance, the average length of initial hospitalization was similar between the participants who were not readmitted and who were readmitted within 30 days (4.3 vs. 5 days, $p = 0.3$) (**Table 1**).

Grip strength was obtained in 66 of 70 enrolled patients, and weak grip strength was found among 91% ($n = 10$) of participants who were readmitted ($n = 11$) compared to 47% ($n = 26$) of those participants who were not readmitted ($n = 56$). For the primary outcome, weak grip during index admission predicted all-cause readmission at 30 days (OR 11.2, 95% CI 1.3, 93.2, $p = 0.03$).

None of the other individual physical frailty components were correlated with readmission in unadjusted models (**Table 2**). Readmission was not predicted by slow walk as measured by the 15-foot walk time assessment in an unadjusted model (OR 1.8, 95% CI 0.2, 16.4, $p = 0.58$). Readmission was not predicted by degree of airway obstruction (FEV1 percent predicted) (OR 1.0, 95% CI 0.95, 1.1, $p = 0.7$).

Of the 70 enrolled participants, 55 (78%) completed all of the Fried Frailty measures; 47 were not readmitted and 8 were readmitted. All readmitted participants were frail ($n = 8$, 100%) ($p = 0.04$). Of those not readmitted ($n = 47$), 29 participants (61%) were frail.

DISCUSSION

In this prospective single center study of patients hospitalized with AECOPD, weak grip strength at time of index admission predicted increased all-cause 30-day readmission. Severity of obstructive lung disease, as assessed by FEV_1 percent predicted, was not associated with readmission. Our findings suggest that weak grip strength measured during an index admission for AECOPD may be a useful measure to identify admitted patients who are at increased risk of readmission within 30 days. This is important because many of the current tools for readmission prediction have been developed for use post-discharge, limiting the ability to act early to avoid readmissions. This simple assessment can be obtained during admission and used to triage resources to directly impact discharge planning and reduce 30-day readmissions. We also found that no non-frail participants were readmitted.

Our results have two potential implications. First, handgrip strength could be used clinically to risk-stratify patients admitted with AECOPD, as performing this assessment is simple and has more feasibility than multicomponent frailty assessments. In this study, for example, we had significant missing data in the frailty assessments due to challenges obtaining these physical measures, as one-fifth of participants did not complete all of the frailty assessments; this is in contrast to missing data from only 5% of participants for the grip strength measure. Second, use of handgrip strength to risk-stratify patients may allow for more individualized discharge planning and implementation of targeted limited resource interventions such as geriatric evaluation, outreach calls, disease education and pulmonary rehabilitation. Interventions that might modify frailty may be particularly useful in this regard.

Unfortunately most previously described readmission risk scores are underutilized for many reasons, including that some are complicated with difficult to ascertain clinical data or are validated using post-discharge data and are not validated for use during hospitalization (38–41). We hypothesize that grip strength would allow for important resources to be triaged to patients at risk during and immediately after discharge—not down the road. Identifying a just-in-time simple assessment tool to predict readmission is the goal of health systems and insurers.

Health systems aim to identify those at highest risk for readmission and implement targeted interventions that might impact readmission with the dual goals of improving patient care and garnering health care cost-savings (1, 42). Few studies have demonstrated successful interventions to reduce readmission rate after admission for AECOPD. Successful interventions include pulmonary rehabilitation, use of a discharge coordinator and interventions to teach correct inhaler use (43, 44). Unfortunately, many other interventions have had mixed success (38, 42), and others, like a comprehensive

TABLE 1 | Baseline characteristics of total sample.

Baseline characteristics	Total population ($n = 70$)	No readmission ($n = 58$)	Readmission ($n = 12$)	*p*-value
Age (years)	63.5 (58.1, 71.3)	64.3 (58.3, 71.4)	60.2 (56.2, 66.7)	0.23
Female, n (%)	39 (56%)	34 (59%)	5 (42%)	0.28
African American, n (%)	64 (91%)	52 (90%)	12 (100%)	0.58
Current smoker, n (%)	29 (41%)	24 (41%)	5 (42%)	0.99
Length of stay (days)	4.3 (2.8, 6.2)	4.3 (2.5, 6)	5 (3.3, 7.7)	0.3
Charlson comorbidity index	2 (1,3)	2 (1,3)	2 (2, 3.5)	0.21

Data expressed as median (25th, 75th centiles).

TABLE 2 | Univariable logistic regression predicting all-cause readmission at 30 days.

Characteristics	Overall	No readmission	Readmission	*p*-value
Physical frailty phenotype				
Frail[a*]	37 (67%)	29 (62%)	8 (100%)	**0.04**
Slow walk[b]	53 (84%)	44 (83%)	9 (90%)	1.00
Weak grip[c]	36 (55%)	26 (47%)	10 (91%)	**0.009**
Weight loss[d]	17 (29%)	14 (29%)	3 (33%)	1.00
Low physical activity[e]	29 (45%)	23 (43%)	6 (55%)	0.47
Self-reported exhaustion[f]	53 (78%)	45 (80%)	8 (67%)	0.44
Spirometry				
FEV_1 (percent predicted)[g]	32.8 (27.5, 46.3)	32.8 (27.5, 46.3)	36.3 (26.3, 57.5)	0.80

FEV_1 = forced expiratory volume in 1 s.
**frail = physical frailty score of 3 or greater.*
Missing data:
[a]frailty score n = 15 (21%).
[b]slow walk n = 7 (10%).
[c]weak grip n = 4 (6%).
[d]weight loss n = 12 (17%).
[e]low physical n = 5 (7%).
[f]exhaustion n = 2 (3%).
[g]FEV_1 % predicted n = 48 (69%). The bold values indicate significant results that are less than a p of 0.05.

care management program and transitional care/long-term self-management support, have unexpectedly found harm (45, 46). This may be at least partially due to the fact that many readmission risk factors either are not modifiable, such as person-specific characteristics (e.g., age, insurance type) or disease severity such as low FEV_1, or are only determined well after discharge when the patient is no longer available for immediate intervention (4, 5).

In contrast, dynapenia, for which handgrip strength is a surrogate, may be modifiable with targeted therapy interventions such as adherence to pulmonary rehabilitation, an underutilized multidisciplinary educational and exercise program for people with chronic lung disease that improves quality of life and activities of daily living, increases exercise tolerance, and reduces exacerbations (20, 47). Further, recent work demonstrates that pulmonary rehabilitation, initiated within 3 months of hospital discharge, lowers risk of mortality at 1 year among Medicare beneficiaries (48). Unfortunately, despite the strong evidence-base, pulmonary rehabilitation is not widely available in rural or resource-limited settings (49), and adherence to rehabilitation programs may be limited by factors such as patient willingness, transportation, and other social issues. Recent evidence supports more accessible web-based pulmonary rehabilitation tools to increase access for people with COPD, even those with low health efficacy (50). Home-based programs are feasible and have been demonstrated to lead to improvements in walk distance and breathlessness symptoms (51). Addressing this health disparity is critical to achieving the best care for people with COPD and perhaps for reducing readmissions for those at highest risk.

Slow gait has been demonstrated to be predictive of readmission in a 2015 study of 213 patients hospitalized for AECOPD (9). We were unable to replicate this finding in our study; this may be due to our small sample size. We also did not find an association between degree of airflow obstruction and readmission. One potential explanation for this finding is that most of our cohort had severe airflow obstruction (GOLD class 3 or 4), and few subjects had an $FEV_1 > 50\%$ predicted. While some studies have shown that low FEV_1 is associated with readmission, degree of airflow obstruction in general has been of limited use in identifying patients at risk for exacerbation or burdened by symptoms that impact quality of life (52). Indeed, the GOLD

classification system has evolved beyond staging by FEV_1 alone and categorizes disease severity by number of exacerbations in the prior year and degree of breathlessness (53). Additionally, prognostic calculators such as the BODE or ADO indices use degree of obstruction as only one of several inputs for mortality prediction (54, 55).

We have identified limitations to our study. Our study site was a single academic hospital, and our conclusions may not apply to non-academic hospital settings. Our sample included more African-American, women and young participants than are typically included in COPD research studies. Further research is needed to understand if findings in these understudied populations are generalizable. We believe that representation of these patients is a potential study strength. African-American populations are typically understudied, and culturally tailored interventions have been found to be effective in other chronic disease studies (56, 57). The prevalence of COPD is rising among women (58), and several of our team's previous research studies have found a higher proportion of women enrolled, which may be reflective of our study population (59, 60). Our study population was also younger than expected, which again may be reflective of the demographics of our single study site.

Due to our recruitment strategy, the frailty assessments were conducted at variable time points throughout the admission, as weekend admissions could not be evaluated until the weekday. Additionally some assessments were obtained after transfer to a general medicine floor from the intensive care unit. It is possible that those who were in the hospital for 72 h or the intensive care unit prior to undergoing a frailty assessment had experienced muscle loss due to their hospital stay. It is noteworthy that we found a significant relationship between grip strength and readmission despite this flexible enrollment. However, replication of this study with standardized timing of the frailty assessment would be helpful. Further, our response rate for enrollment was low and about 20% of consented participants were discharged before completing all of the study assessments, which may have led to a sampling bias. Because our response sample size was small, we may have been unable to detect true differences in individual component measures such as walk time and spirometry. With respect to grip strength, we did not have an objective measure of sarcopenia, such as cross-sectional area of muscle mass by CT scanning, and could not rule out that handgrip strength was confounded by conditions such as arthritis. Finally, this was a sub-analysis of a larger study of frailty markers, which is not powered to date to compare the utility of grip strength to a full frailty assessment. Larger, multi-center studies are needed to confirm our findings, and future studies should include community-based hospitals as well to determine applicability of these findings across settings.

Most readmissions following index admission for AECOPD are not due to COPD, and half are not respiratory-related (61). This period of vulnerability is referred to as post-hospital syndrome, a phenomenon of decreased function and independence following hospitalization (62). Therefore, identifying extra-pulmonary factors of global vulnerability are critical for improving care for patients with COPD; integration of geriatric and palliative care principles into COPD care would help to achieve this aim (63). Handgrip strength is a surrogate measure of overall muscle strength and conditioning, and we hypothesize that interventions that increase strength may lead to fewer AECOPD events, improved daily function, reduced hospitalizations/readmissions and prevention of post-hospital syndrome. These hypotheses require additional study.

DATA AVAILABILITY STATEMENT

The summary data supporting the conclusions of this article will be made available by the authors, upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Chicago Biological Sciences Division Institutional Review Board (Protocol #14-0848). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VP, VA, SW, and MH-S contributed substantially to the conception and design of this work. LW and AS contributed to the acquisition of the data. KC heavily contributed to the analysis of these data. KC, VP, LW, AS, and MH-S contributed to the interpretation of the data for this work. LW, AS, and VP contributed to the drafting the work. VA, SW, MH-S, and KC revised the manuscript critically for important intellectual content and accuracy. All authors gave final approval of the version to be published, gave agreement to be accountable for all aspects of the work, including the integrity of this work as a whole, from inception to published article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.611989/full#supplementary-material

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REVIEW
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Collagen Biosynthesis, Processing, and Maturation in Lung Ageing

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In addition to providing a macromolecular scaffold, the extracellular matrix (ECM) is a critical regulator of cell function by virtue of specific physical, biochemical, and mechanical properties. Collagen is the main ECM component and hence plays an essential role in the pathogenesis and progression of chronic lung disease. It is well-established that many chronic lung diseases, e.g., chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) primarily manifest in the elderly, suggesting increased susceptibility of the aged lung or accumulated alterations in lung structure over time that favour disease. Here, we review the main steps of collagen biosynthesis, processing, and turnover and summarise what is currently known about alterations upon lung ageing, including changes in collagen composition, modification, and crosslinking. Recent proteomic data on mouse lung ageing indicates that, while the ER-resident machinery of collagen biosynthesis, modification and triple helix formation appears largely unchanged, there are specific changes in levels of type IV and type VI as well as the two fibril-associated collagens with interrupted triple helices (FACIT), namely type XIV and type XVI collagens. In addition, levels of the extracellular collagen crosslinking enzyme lysyl oxidase are decreased, indicating less enzymatically mediated collagen crosslinking upon ageing. The latter contrasts with the ageing-associated increase in collagen crosslinking by advanced glycation endproducts (AGEs), a result of spontaneous reactions of protein amino groups with reactive carbonyls, e.g., from monosaccharides or reactive dicarbonyls like methylglyoxal. Given the slow turnover of extracellular collagen such modifications accumulate even more in ageing tissues. In summary, the collective evidence points mainly toward age-induced alterations in collagen composition and drastic changes in the molecular nature of collagen crosslinks. Future work addressing the consequences of these changes may provide important clues for prevention of lung disease and for lung bioengineering and ultimately pave the way to novel targeted approaches in lung regenerative medicine.

Keywords: collagen, extracellular matrix, ageing, crosslinking, chronic lung disease, advanced-glycation end products, lysyl oxidase

INTRODUCTION

The extracellular matrix (ECM) is a highly dynamic non-cellular component of tissues that provides a complex structural network which serves as a scaffold for adherent and migrating cells. It is mainly composed of collagens, glycoproteins, proteoglycans, glycosaminoglycans, and several other components. By virtue of sequestered growth factors and ECM binding receptors at the surface of adherent cells, the ECM affects a plethora of cellular processes including cell proliferation, differentiation and migration and thus acts as a critical regulator of cell function (1, 2). It is well-established that the ECM plays an important role in the pathogenesis and progression of chronic lung disease (3, 4). Many chronic lung diseases, e.g., chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) primarily manifest in the elderly, suggesting increased susceptibility of the aged lung or accumulated alterations in lung structure over time that favour disease. How the ECM changes during ageing, however, has not been comprehensively assessed or discussed.

Constituting between 30 and 70% of ECM protein in all tissue types, collagen is the main component of the ECM (5) and even forms the most abundant human protein class in general. Mutations and polymorphisms in genes encoding structural collagen chains as well as collagen biosynthetic proteins are associated with disease affecting the full age range. Some cause fatal congenital disorders leading to death very early in life, others result in premature or accelerated ageing in adolescents and adults, and yet others only become evident in the elderly where they lead to a high burden of multi-morbidity, reduced quality of life, and lower life expectancy (6). Probably the most widely known collagen-related disorders result in drastic bone and cartilage abnormalities, as e.g., brittle-bone *(osteogenesis imperfecta)* or Caffey disease, characterised by increased bone fragility or episodes of excessive bone formation, respectively (1). Other frequent effects of collagenopathies are skin alterations, visual defects and hearing loss, muscle weakness, vessel abnormalities and kidney disease (1).

Pulmonary manifestations of such collagen mutations and polymorphisms have received less attention, probably because the most severe lung abnormalities in such patients are caused by defects in chest formation and rib fractures, i.e., are of origin secondary to bone and cartilage defects (1, 6, 7). Nevertheless, altered collagen synthesis or turnover by other than genetic causes are frequent hallmarks of chronic lung disease and contribute considerably to disease progression, severity, morbidity, and mortality (3, 4). In lung cancer, for instance, dysregulated collagen expression and crosslinking appear to favour tumour progression by providing a permissive, pro-invasive, and pro-inflammatory environment (8). In pulmonary fibrosis, irrespective of disease aetiology, excessive collagen deposition in the alveolar space is the ultimate pathological feature leading to increasing dyspnoea and progressive lung function decline (9–14). In contrast, COPD/emphysema is characterised by increased degradation of ECM proteins by matrix metalloproteinases (MMPs) and neutrophil elastase, targeting primarily collagens and an unrelated major ECM protein, elastin, respectively (15).

Given that collagen is the most abundant protein type in the body, it is not surprising that collagen has been a subject of research for about 100 years by now. What is striking, however, is how little we know, nonetheless. The latest member of the collagen protein family, type XXVIII collagen encoded by COL28A1, has only been reported in the year 2006 (16). Similarly, new proteins acting in collagen biosynthesis and modification have only been discovered and characterised in the last two decades (17–21). Also, even though collagen is known to undergo excessive post-translational modification (PTM), both intra- and extracellularly, these PTMs have not been comprehensively mapped and the biological function of the majority of the PTMs remains unclear (22). Equally, it is poorly understood how collagen biosynthesis and turnover change during normal ageing and how such changes may affect the function of adherent cells, lung repair, susceptibility to disease, disease progression and comorbidities.

This review aims to draw attention to the complexity of collagen synthesis, processing, and degradation and the importance of these processes in lung ageing and chronic lung disease. To set the stage, we first provide an overview of collagen types and key steps of collagen biosynthesis, processing, and maturation. We then summarise what is known about collagen alterations in the ageing lung and what can be tentatively inferred from studies in other organs. Ultimately, we believe that a better understanding of these mechanisms may provide important clues for prevention of lung disease and for lung bioengineering and pave the way to novel targeted approaches in lung regenerative medicine.

COLLAGEN TYPES AND STRUCTURE

The collagen suprafamily comprises to date 28 known highly diverse collagen types in vertebrates, characterised by the presence of triple-helical collagenous domains. Collagens are divided into several subtypes depending on their domain structure and their macromolecular assembly (**Figure 1**): (A) Fibril-forming collagens (I, II, III, V, XI, XXIV, XXVII), (B) fibril-associated collagens with interrupted triple helices (FACITs, IX, XII, XIV, XVI, XIX, XX, XXI, XXII), (C) network-forming collagens (IV, VIII, X), (D) transmembrane collagens (XIII, XVII, XXIII, XXV), (E) endostatin-producing collagens or multiplexins (XV, XVIII), (F) anchoring fibrils (VII), and (G) beaded-filament-forming collagen (VI). Types XXVI and XXVIII do not fit well in any category (16, 23–25). Depending on the collagen type, collagens can assemble as homotrimers or heterotrimers. For instance, type III collagen is a homotrimer of three identical α1 chains (encoded by *COL3A1*), type I collagen typically assembles from two α1 chains (*COL1A1*) and one α2 chain (*COL1A2*) and type VI collagen from even three distinct chains (*COL6A1*, *COL6A2*, and *COL6A3* or *COL6A5* or *COL6A6*) (22, 26–28). Interestingly, altered chain stoichiometry has been described for several pathologies including fibrosis and may affect biochemical and biophysical properties of collagen (29–34).

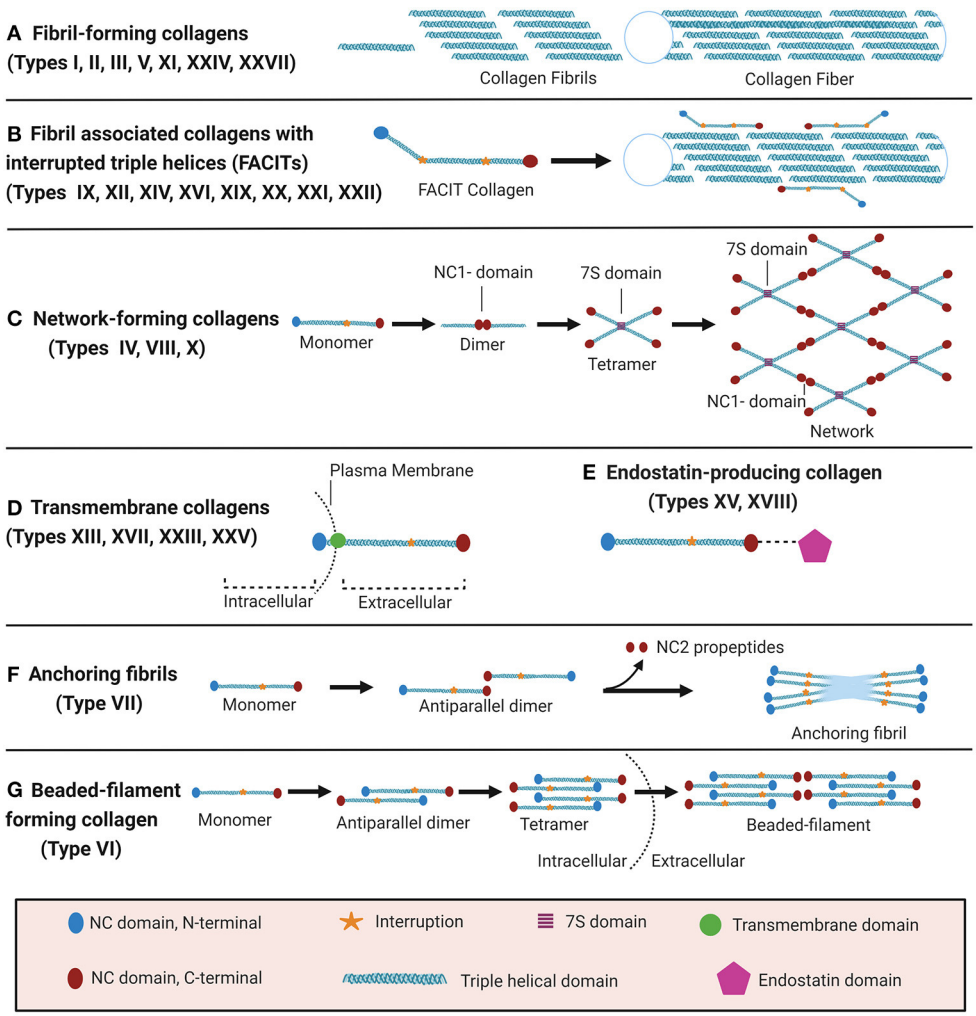

FIGURE 1 | Classification of collagens based on supramolecular assembly. Schematic overview of the major forms of supramolecular assembly of collagen. For some non-collagenous (NC) domains, specific names are established in the literature and therefore specified in the figure: In the collagen IV network (C), the so-called 7S and NC1 domains represent critical nodes. In anchoring fibrils (F), the C-terminal domain which is cleaved off upon fibril formation is termed NC2. Figure was created with Biorender.com.

Collagen Domains and Macromolecular Assembly

The unifying feature of all collagens is the **triple-helical collagenous domain**, which is composed of three so-called α-chains consisting of amino acid repeats of $(Gly\text{-}X\text{-}Y)_n$. The smallest amino acid glycine (Gly) can face the interior part of the triple helix while still allowing for a close association of the three chains. X and Y are often proline (Pro) or hydroxylated proline, 3-hydroxyproline (3-Hyp) or 4-hydroxyproline (4-Hyp), respectively (35, 36). While 4-Hyp in position Y of the Gly-X-Y repeat is frequently found in all collagen types and well-established as a major contributor to collagen thermodynamic stability (37–41), 3-Hyp has so far only been unambiguously detected in very few defined X positions of Gly-X-Y in collagen chains of type I, II, IV, and V, and 3-Hyp function is much less understood (42–46).

Frequent non-collagenous (NC) domains, e.g., in FACIT, beaded-filament forming and anchoring fibril collagens, are fibronectin type III, von Willebrand, thrombospondin (TSP) and Kunitz domains. The physiological function of these domains is incompletely understood. Fibronectin type III and von Willebrand domains seem to facilitate protein-protein interactions between collagens and other structural ECM molecules or growth factors in the extracellular space (47, 48). TSP domains mediate heparin and metal ion binding and may provide protection against blood clotting e.g., in subendothelial basement membranes (49, 50). The C-terminal Kunitz domains in type VI and VII collagens are cleaved off in the extracellular space (51–53). For type VI collagen, this cleavage product, endotrophin, stimulates tumour growth and angiogenesis, mediating many of the tumour-promoting effects of type VI collagen (54–56). Endotrophin is thus classified as a

matricryptin or matrikine, a class of biologically active peptides derived from proteolytic processing of extracellular matrix proteins (57).

Even though all collagens are multi-domain proteins, the extent to which a collagen consists of collagenous and NC domains can differ drastically and the overall collagen domain architecture determines the macromolecular assembly (**Figure 1**). Type I collagen, like all **fibril-forming collagens**, contains a central uninterrupted collagenous domain as a major part of the polypeptide, flanked by relatively short NC domains, the N- and C-terminal telo- and propeptides (25). In contrast, **FACIT**s can contain <10% collagenous domains and associate to the surface of collagen fibrils but do not form such fibrils by themselves (23, 27). **Membrane collagens** comprise an NC cytoplasmic domain, a transmembrane domain, and extracellular repeats of triple-helical collagenous domains (27). Membrane collagen types XIII, XXIII, and XXV are also collectively referred to as membrane-associated collagens with interrupted triple helices (**MACIT**s) (58, 59). Notably, the C-terminal ectodomains of all membrane collagens can be proteolytically shed off the cell surface (23, 27, 60), and are classified as matrikines or matricryptins (27, 57, 61). Another collagen family that serves as a source for matricryptins are the **multiplexins** or endostatin-producing collagens. This family comprises collagen types XV and XVIII which generate restin and endostatin, respectively, upon proteolytic cleavage, both known for their potent antiangiogenic properties (24, 25, 27, 57, 58, 61, 62). The prototypical **network-forming collagen** type IV, a major basement membrane constituent, is encoded by in total 6 genes (*COL4A1-COL4A6*) the products of which form heterotrimers consisting of the chain combinations $(\alpha1)_2\alpha2$(IV), $\alpha3\alpha4\alpha5$(IV), or $(\alpha5)_2\alpha6$ (IV) (23, 24, 27). The so-called 7S and NC1 domains are critical nodes in the collagen IV network and stabilised by covalent bonds, namely by LOXL2-mediated crosslinks between lysines in the 7S domain and the unique sulfilimine crosslink (-S=N-) in the NC1 domain (63–65). Also collagens VIII and X can assemble to form networks in tissues, but the molecular determinants have not yet been studied in similar detail (27). Type VII collagen is the major component of **anchoring fibrils** that are essential for the integrity of the dermo-epidermal junction in skin (24, 25, 27). It consists of two adjacent collagenous triple-helical domains which are flanked by a rather long (140 kDa) NC domain harbouring von Willebrand domains and fibronectin type III repeats at the N-terminus (NC1) and a much shorter (30 kDa) NC domain at the C-terminus (NC2) (27, 66). The initial formation of these anchoring fibrils from homotrimeric type VII collagen molecules involves antiparallel alignment of two collagen VII molecules at the level of their C-termini (NC2 domains), followed by enzymatic cleavage of the NC2 domain and stabilisation of the dimer by disulfide bonds (66). Finally, **beaded-filament-forming collagens** are strictly only represented by type VI collagen. Suprastructural assembly occurs as follows: Two heterotrimeric type VI collagen molecules dimerize in an antiparallel fashion *via* interaction of their central triple-helical domains. The protruding N-and C-terminal globular domains on both sides register in parallel with another dimer, forming a tetramer which is stabilised by disulphide bonds Finally, tetramers assemble end-to-end to generate the beaded-filaments (**Figure 1**) (23, 67). The collagen types XXVI and XXVIII are sometimes mentioned within the context of this class, even if they do not fit well in any category (16, 23–25). Type XXVIII collagen shares some sequence homology with type VI collagen (16). Type XXVI collagen, however, is uncharacteristically small for a collagen, comprises only two very short collagenous domains, and shares few similarities with any of the other described collagens (24).

COLLAGEN BIOSYNTHESIS

Collagen biosynthesis is a highly complex process starting with transcription of collagen genes followed by translation and translocation of the nascent polypeptide chain to the rough ER (rER), co-translational modification and folding, trafficking across the Golgi network, secretion, and finally, extracellular processing and maturation (36, 68) (cf. **Figure 2**). This process is best described for type I collagen which will be used as an example for synthesis of a heterotrimeric fibril-forming collagen in the following.

Translation and Co-translational Modification of the Nascent Polypeptides

Following transcription of *COL1A1* and *COL1A2* genes, the mRNA is translated into the polypeptide at the rER where the nascent polypeptide chain is extended into the lumen of the rER. Proper folding in the rER requires several enzymes and molecular chaperones essential for post-translational modifications (PTMs) and the formation of triple-helical procollagen molecules (36). First, numerous PTMs are introduced into the nascent unfolded polypeptide chain in a co-translational fashion. These include lysyl and prolyl hydroxylations and hydroxylysyl glycosylations, modifications that are mediated by several collagen-specific enzymes which exist in defined multiprotein complexes with other chaperones and protein folding catalysts [for an excellent review, see Ishikawa and Bächinger (36)]. Many of these PTMs are essential for proper stability, assembly and secretion of procollagen, as well as for the final supramolecular structure of these molecules (24, 25, 27, 36). Numerous enzymes catalysing PTMs have been identified. In vertebrates, prolyl-4-hydroxylation is mediated by one of three **prolyl-4-hydroxylases** (encoded by *P4HA1*, *P4HA2*, and *P4HA3*) which form complexes with protein disulphide isomerase (PDI). In the lung, at least one of these prolyl-4-hydroxylases, P4HA3, is upregulated in IPF and has been put forward as a potential drug target (69). Most members of the collagen **prolyl-3-hydroxylase** (P3H1-P3H4) family equally form multimeric complexes with other collagen-modifying proteins and chaperones. Of these, P3H1, P3H2, and P3H3 have been shown to exert prolyl-3-hydroxylase activity, while P3H4 (also termed SC65) forms a trimeric complex with P3H3 and lysyl hydroxylase 1 (LH1), but has no prolyl-3-hydroxylase activity of its own (19, 20). **Lysyl hydroxylases** comprise three enzymes termed LH1-LH3 or procollagen-lysine,2-oxoglutarate 5-dioxygenases 1 to 3 (PLOD1-PLOD3, e.g., Uniprot database) (36). LH1, as already mentioned above, exists in a complex with P3H3 and P3H4, and preferentially hydroxylates lysines in the triple-helical collagenous regions

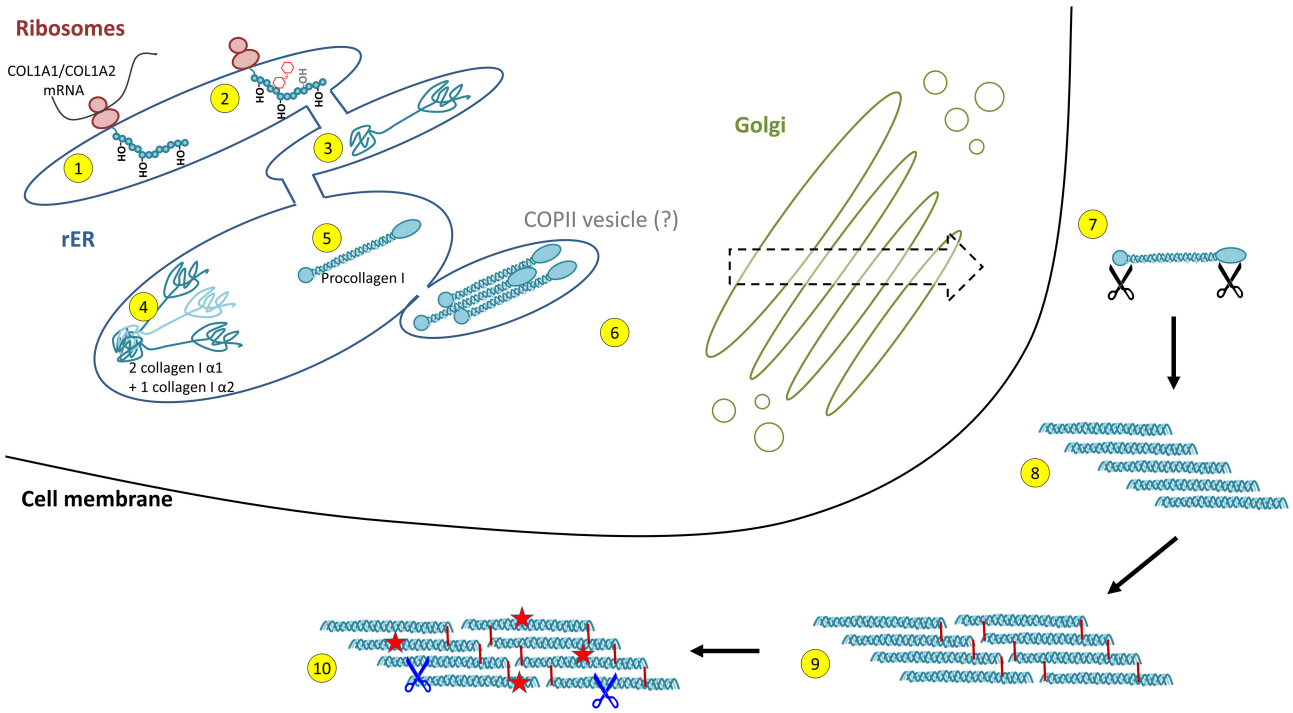

FIGURE 2 | Intracellular collagen biosynthesis and extracellular maturation of collagen I. (1) Cotranslational prolyl-4- and lysyl-hydroxylation of the nascent collagen polypeptide chain in the rough endoplasmic reticulum (rER) is followed by (2) glycosylation and prolyl-3-hydroxylation and (3) folding of the C- and N-terminal propeptides. (4) For collagen I, two properly folded α1 chain C-propeptides assemble with one α2 chain C-propeptide, forming the triple helix nucleus. (5) Triple helix formation occurs in a zipper-like fashion and is dependent on peptidyl-prolyl isomerases and collagen chaperones. (6) Collagen triple helices are transported via the trans-Golgi network and finally secreted into the extracellular space. This was believed to occur *via* COPII vesicles, a concept that has been challenged recently. (7) In the extracellular space, propeptide cleavage involving at least three proteases (black scissors) triggers (8) auto-assembly of collagen fibrils. (9) Finally, fibrils are stabilised by crosslinking. (10) The mature collagen fibres are subject to insults (red stars) and degradation by extracellular proteases (blue scissors, see **Figure 4**).

(70). The resulting 5-hydroxylysine residues may be subject to O-glycosylation by glycosyl transferases (36, 70, 71). In contrast, LH2, which associates with FKBP10 (FKBP65) is responsible for hydroxylation of lysines in the non-collagenous telopeptide regions of fibrillar collagens (70, 72). Notably, telopeptide lysines and hydroxylysine are subject to extracellular lysyl oxidase (LOX)-mediated crosslinking and the hydroxylation status of the involved lysines strongly affects nature and stability of the crosslink (for selected examples for crosslinks, see **Figure 3**) (36, 71–73). LH3 is known to hydroxylate lysines in type IV and V collagens but also exerts glycosyl transferase activity (36, 70, 71). Finally, in addition to LH3, two collagen **glycosyl transferases,** GLT25D1 and GLT25D2 mediate O-glycosylation of hydroxylysines *via* the 5-hydroxyl group. Here, hydroxylysines can be either O-glycosylated with a monosaccharide (β-d-galactopyranose, Gal) or a disaccharide (α-d-glucopyranosyl-(1->2)-β-d-galactopyranose, GlcGal), resulting in galactosylhydroxylysine (GHyl) or glucosylgalactosyl-hydroxylysine (GGHyl), respectively (36, 70, 71).

Triple Helix Formation

Triple helix formation is preceded by folding of the N- and C-terminal propeptides and chain selection *via* the trimerization domains (36, 68, 74, 75), a process supported by a plethora of general ER-folding folding catalysts including Grp78 (BiP), Grp94, PDI, calreticulin, calnexin, and CypB (36). Following chain selection, triple helix formation is initiated and proceeds in a zipper-like fashion. Given the proportionally high number of proline residues in collagen, it is not surprising that one of the rate-limiting steps in triple helix formation is the *cis-trans* isomerization of proline residues catalysed by rER-resident **peptidyl-prolyl isomerases** (PPIases) (36, 68). Only the *trans*-proline conformation allows a linear prolongation of the triple helix (76). The PPIases FKBP10 (FKBP65), CypB as well as FKBP14 (FKBP22) appear to play critical roles in that context (36, 68, 77–79). In addition, **heat shock protein 47** (HSP47 or SERPINH1) functions as an important collagen-specific chaperone in collagen modification, triple helix formation, and export from the ER to the Golgi (36, 68, 80–82).

Trafficking From the rER via the Trans-Golgi Network to the Extracellular Space

Procollagen secretion is dependent on coat protein complex II (COPII) vesicle-mediated transport from the rER to the Golgi. Typically, COPII vesicles are not bigger than 60–80 nm

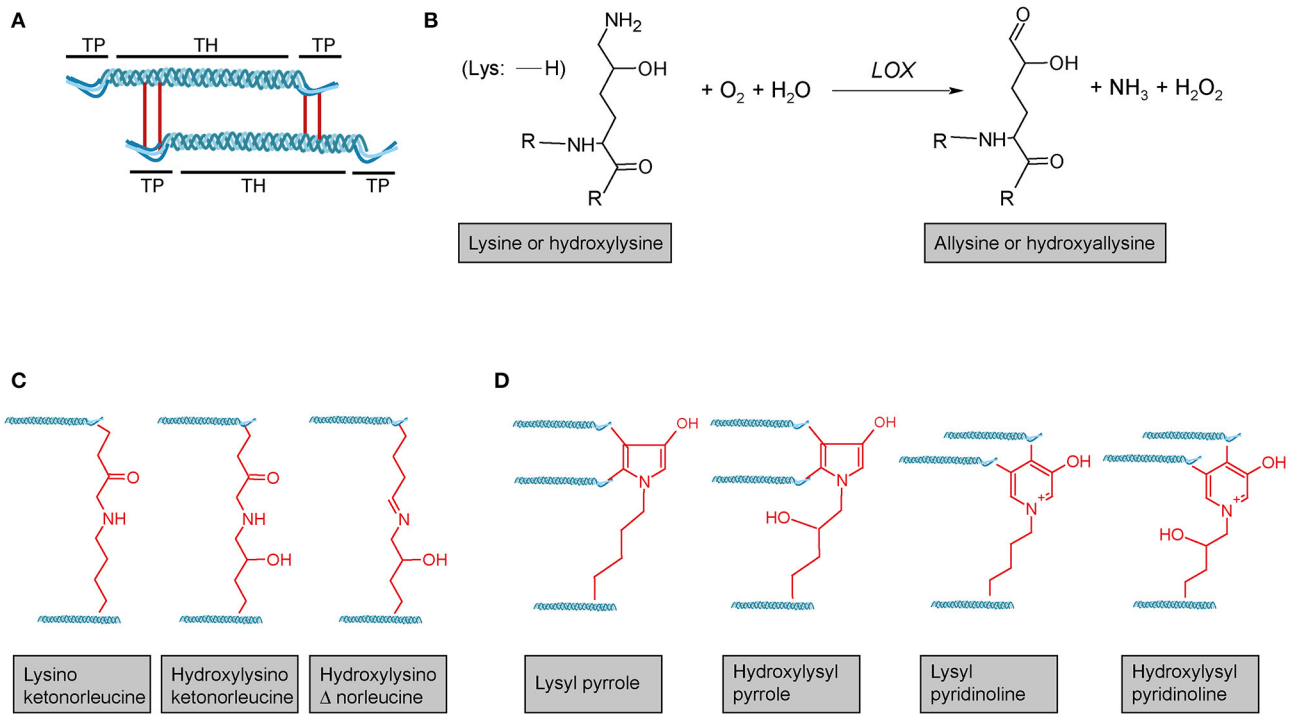

FIGURE 3 | Lysyl oxidase-mediated crosslinking. **(A)** Schematic representation of telopeptide (TP) and triple-helical (TH) crosslink sites of two adjacent tropocollagen molecules in a collagen fibril. **(B)** Lysyl oxidase (LOX) enzymes initially catalyse the oxidative deamination of the ε-amino group of a lysine or hydroxylysine, yielding a highly reactive aldehyde group. This entails subsequent reactions with primarily other (hydroxy)lysines and rearrangements, ultimately resulting in different **(C)** divalent and **(D)** trivalent collagen crosslinks. R = continued polypeptide chain. Structures were generated using ACD/Chemsketch freeware.

in diameter (83), while a completely folded and fairly rigid procollagen molecule can be up to 500 nm in length (84–88). Therefore, a long-held concept involved the transport of procollagen molecules *via* specialised, enlarged, COPII vesicles (85, 89–93). This concept, however, has been challenged recently: Analysis of endogenous and engineered GFP-tagged procollagen by live-cell imaging did not provide any evidence for dynamic large carrier vesicles between the ER and the Golgi but instead rather supports a model of direct interconnections between organelles or the presence of less well-characterised intermediate carriers (88, 94).

Extracellular Processing and Maturation of Collagen

Extracellular processing and maturation of collagen depends very much on the collagen type and the nature of supramolecular assembly (cf. **Figure 1**) but is best described for type I collagen and other fibrillar collagens. Here, secretion is followed by the cleavage of the N- and C-terminal propeptides by specific procollagen proteinases, including bone morphogenetic protein 1 (BMP1), members of the ADAMTS protease family, and the more recently discovered meprins (23, 25, 27, 95). Notably, enzymatic cleavage of propeptides occurs for many non-fibrillar collagens, too, including the above-mentioned membrane collagens and multiplexins, also involves MMPs and cathepsins, and is the source of matrikines or matricryptins, collagen-derived fragments with diverse biological activities (57, 61). For fibrillar collagens, propeptide cleavage is followed by spontaneous, entropy-driven, quarter-staggered assembly of the tropocollagen molecules into fibrils (96, 97). Finally, intra- and intermolecular crosslinks, catalysed by enzymes of the lysyl oxidase (LOX) and transglutaminase (TGM) family, stabilise fibrillar collagens in the extracellular space (98–101). In particular the enzymes LOX and LOX-like 2 (LOXL2) have been shown to crosslink fibrillar collagens both intra- and inter-molecularly (101) and LOXL2 has been put forward as a potential therapeutic drug target in IPF (102). Further collagen-associated proteins like the antioxidant proteins extracellular superoxide dismutase or glutathione peroxidase 3 (GPX3) may protect these long-lived molecules from oxidative damage (103–106).

COLLAGEN TURNOVER

In normal tissue homeostasis, the ECM is subject to a constant and dynamic, albeit typically slow, turnover, in which collagen is degraded and newly synthesised (107). The rate of collagen turnover differs drastically between tissue types. For instance, human cartilage collagen has a half-life of about 117 years, invertebral disc collagen 95 years, and skin collagen 15 years (108, 109). Even if, to our knowledge, the half-life of human lung collagen has not been determined, numerous studies have addressed lung collagen turnover in human and experimental animal lung tissue. Most studies point toward a remarkable level of constant *de novo* collagen synthesis and the presence

of two collagen pools with different degradation rates; a pool of probably newly synthesised collagen which is subject to considerable degradation, and a pool of heavily crosslinked stable collagen which is more resistant to degradation (110–117). Considering the tightly wound triple-helical structure as well as the complex supramolecular structures described above, it is not surprising that only members of two protease families are capable of collagen degradation, namely MMPs and cathepsins. The so far described collagen degradation pathways can be categorised in extracellular and intracellular pathways. These processes are regarded as different from propeptide cleavage which is part of the normal collagen maturation pathway and reflects rates of *de novo* collagen synthesis rather than collagen turnover. Nevertheless, a strict distinction may represent an oversimplification. Notably, both processes can liberate biologically active peptide fragments, i.e., matricryptins or matrikines (57, 61).

Extracellular Collagen Degradation

Extracellular collagen degradation is mainly attributed to **matrix metalloproteinases (MMPs)** and cathepsin K. MMPs are extracellular Proteolytic Zinc-dependent endopeptidases which collectively degrade all major ECM molecules. Collagenolytic activity has been observed for MMP1, MMP2, MMP7, MMP8, MMP9, MMP13, MMP14, and MMP19 (118, 119). MMP-mediated degradation of interstitial collagens, in particular of types I-III is best-described, but several MMPs (e.g., MMP2, MMP7, MMP9) also degrade basement membrane type IV collagen (118, 120–122). For a comprehensive overview of MMPs and their collagen substrates, the interested reader is referred to Visse and Nagase (123) and Jobin et al. (124). The C-terminal hemopexin domain of MMPs is crucial for collagen degradation as it not only recognises and binds the substrate but also unwinds the collagen structure in order to access the cleavage site (125, 126). In contrast, the cysteine protease **cathepsin K** (CatK) is probably the most effective protease for the degradation of extracellular fibrillar collagen (127, 128) because it can also target triple-helical collagen directly, without the need for unwinding of the helix (127).

Uptake of Collagen and Intracellular Collagen Degradation

Uptake of collagen into the cell can occur by phagocytosis of an intact collagen fibril (129) or by macropinocytosis or receptor-mediated endocytosis of already cleaved collagen particles. In order to pass the cell membrane, collagen fibrils are recognised by integrins which triggers the process of phagocytosis (130). The so far observed integrins being involved are α1β1- and α2β1-integrin (131), as well as α10β1- and α11β1-integrins (132, 133). Initial fragmentation of fibrillar collagen is mediated by membrane-bound MMP14 (also termed MT-MMP1) (134).

Smaller collagen fragments, e.g., derived by extracellular MMP- or CatK-mediated degradation, can be taken up by two main pathways, macropinocytosis and receptor-mediated endosomal uptake. In macropinocytosis, solubilized collagen particles are internalised within actin-mediated endocytosis resulting in collagen-containing vacuoles (135, 136). Receptor-mediated endosomal uptake of collagen fragments is dependent on the urokinase plasminogen activator receptor-associated protein (uPARAP/Endo180 or C-type mannose receptor 2, MRC2). This endocytic mannose receptor mediates the internalisation of MMP-cleaved collagen fragments into clathrin-coated vesicles into fibroblasts (137–139). Receptors mediating endosomal collagen uptake in macrophages are macrophage mannose receptor 1 (MRC1) and lactadherin (MFGE8) (140, 141).

For intracellular collagen degradation, both pathways converge at the level of fusion with lysosomes. As to collagen-degrading proteases, lysosomes contain a range of **cathepsins**, including cathepsins B, D, K, and L, which cleave collagen into low-molecular-weight peptides (142–144). An overview of their known collagen substrates is given in **Table 1**. Notably, the same pathway can be used for newly synthesised collagen prior to secretion, for instance, when collagen is misfolded or otherwise "defective" (149, 150). In this so-called lysosome-dependent macroautophagy, lysosomes may fuse with ER- or Golgi-derived vesicles (151, 152).

TABLE 1 | Cathepsins and their collagen substrates.

Cathepsins			
Gene name	**Protein name**	**Collagen substrate(s)**	**References**
CTSB	Cathepsin B	Collagen type IV	(145)
CTSD	Cathepsin D	Collagen type I	(146)
CTSK	Cathepsin K	Collagen type I and II	(143, 147, 148)
CTSL	Cathepsin L1	Collagen type I	(148)

For MMPs, the reader is referred to excellent reviews by Visse and Nagase (123) and Jobin et al. (124), and references therein. Gene and protein nomenclature is according to UniProt.

BASELINE CHANGES IN COLLAGEN SYNTHESIS AND MATURATION DURING MOUSE LUNG AGEING

In order to examine baseline changes, i.e., changes in the absence of environmental stimuli, in collagen biosynthesis, maturation and degradation (**Figures 2**, **4**) upon ageing, we took advantage of a recently published single-cell RNA-Seq and multi-omics analysis data set on the ageing mouse lung (**Figure 5**, **Table 2**) (160). Following extraction of data for all relevant proteins, we observed no significant changes for proteins of the ER-resident machinery of collagen biosynthesis, modification, and triple helix formation (e.g., P4H and PLOD enzymes, SERPINH1, FKBP10, PPIB, **Figure 5A**). In contrast, we found expression of several collagen subtypes deregulated upon normal ageing: The type VI collagen chains COL6A5 and COL6A6, as well as COL14A1, were significantly downregulated, while levels of COL4A3 and COL16A1 were significantly increased. Furthermore, levels of the collagen crosslinking enzyme lysyl oxidase (LOX) were decreased (**Figures 5A,B**). **Table 2** provides a summary of these findings

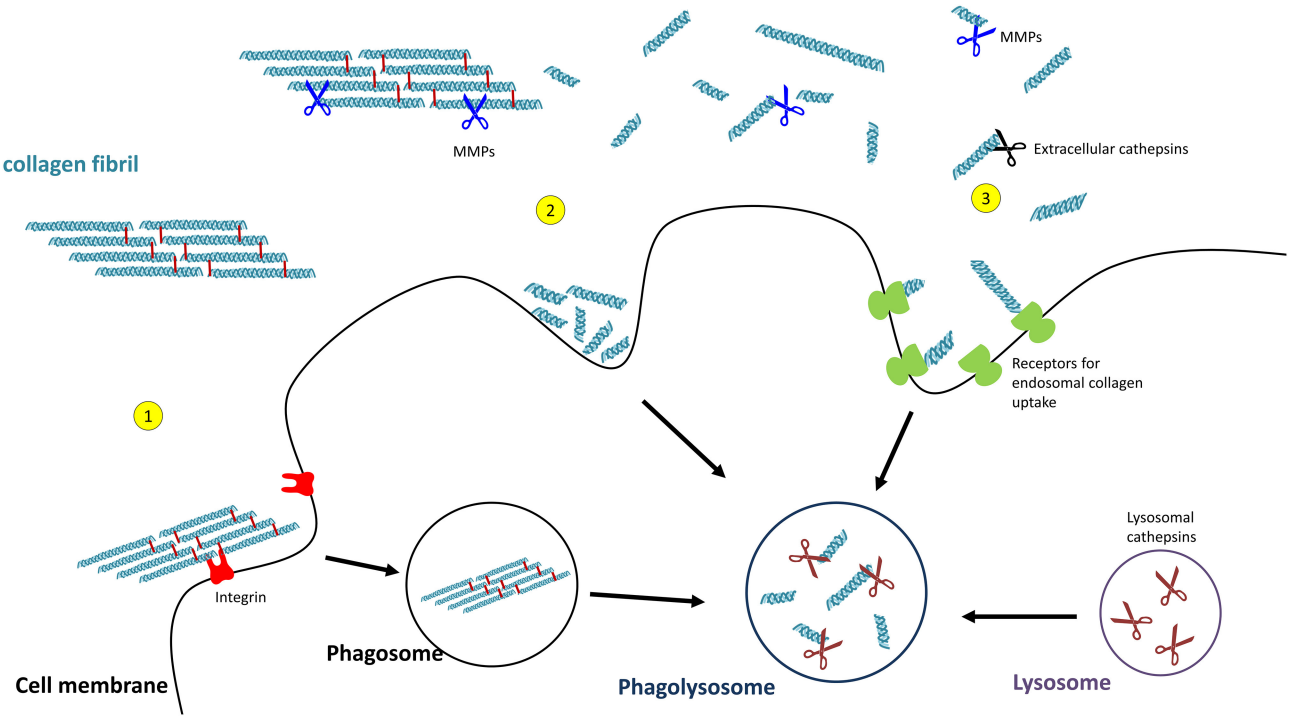

FIGURE 4 | Pathways of collagen degradation. Both extracellular degradation by MMPs and extracellular cathepsins on the one hand and intracellular degradations by (1) collagen phagocytosis, (2) macropinocytosis, or (3) receptor-mediated endocytosis occurs.

including a comparison with what is currently known about corresponding changes in human tissues upon ageing.

Downregulation of Type VI Collagen Chains COL6A5, COL6A6

Type VI collagen differs from most other collagens by its unique supramolecular assembly, the formation of characteristic beaded microfilaments in the ECM, a property which it only shares with type XXVI (COL26A1) and type XXVIII collagen (COL28A1) (23) (**Figure 1**). Type VI collagen is a component of the basement membrane in lung, muscle and skin (165), but can also localise to the pericellular matrix, e.g., in tendon (166, 167). A number of studies, including our own, indicate an important role of type VI collagen for adhesion, migration, proliferation, death, and dysfunction of adherent cells (77, 166–174). Typically, type VI collagen consists of the α1(VI), α2(VI), and α3(VI) chains, which assemble in a 1:1:1 ratio and are encoded by *Col6a1*, *Col6a2*, and *Col6a3*, respectively (166). These three chains are found in all connective tissues, they are by far the most abundant type VI collagen chains, including in the lung, and their levels are not altered during lung ageing in the mouse (160).

Expression of the comparatively recently discovered type VI collagen chains α4(VI), α5(VI), and α6(VI), in contrast, is more restricted, but at least two of these chains are consistently expressed in foetal, new-born, and adult lung (28, 175). An important difference between the murine and human genes is that the human *COL6A4* gene is disrupted and not functional. In mice, expression of *Col6a4* is mostly observed in new-born tissue—including lung—but lost in all adult tissues except for uterus and ovaries (28, 175, 176). The complete loss of COL6A4 from young to old mouse lungs may therefore correspond to remnant *Col6a4* from developmental expression, which is increasingly degraded during normal collagen turnover in a mouse's lifetime, becoming undetectable in the old lung (160). In contrast, *COL6A5* and *COL6A6* are both expressed in adult human lung, even at higher levels than in foetal lung, arguing for a role in adult lung function (28).

Based on sequence similarities, co-purification and colocalization analysis, both COL6A5 and COL6A6 are predicted to assemble with COL6A1 and COL6A2 as an alternative chain to COL6A3 (28, 175, 177). However, strong biochemical evidence in support of this hypothesis is still lacking. Even though some of the α5(VI) (COL6A5) and α6(VI) (COL6A6) chains may be engaged in type VI collagen triple helix formation, in the lung the α3(VI) chain is more abundant by several orders of magnitude, and expression of *Col6a3* is unchanged during ageing. Thus, an α5/α6(VI) -> α3(VI) chain switch from young to old lung tissue is unlikely to represent a major event affecting the general function of type VI collagen. Interestingly, type VI collagen, in particular the α3(VI) chain (COL6A3), has been attributed a role in stemness and promotion of tumour growth. Therefore, local replacement of α5/α6(VI) by α3(VI) chains during ageing may modulate stem cell niches, maybe even contribute to stem cell exhaustion, a major hallmark of ageing (178, 179). But also presence of non-triple-helical (NTH) COL6A5 and COL6A6 polypeptides is conceivable, as evidence for alternative NTH

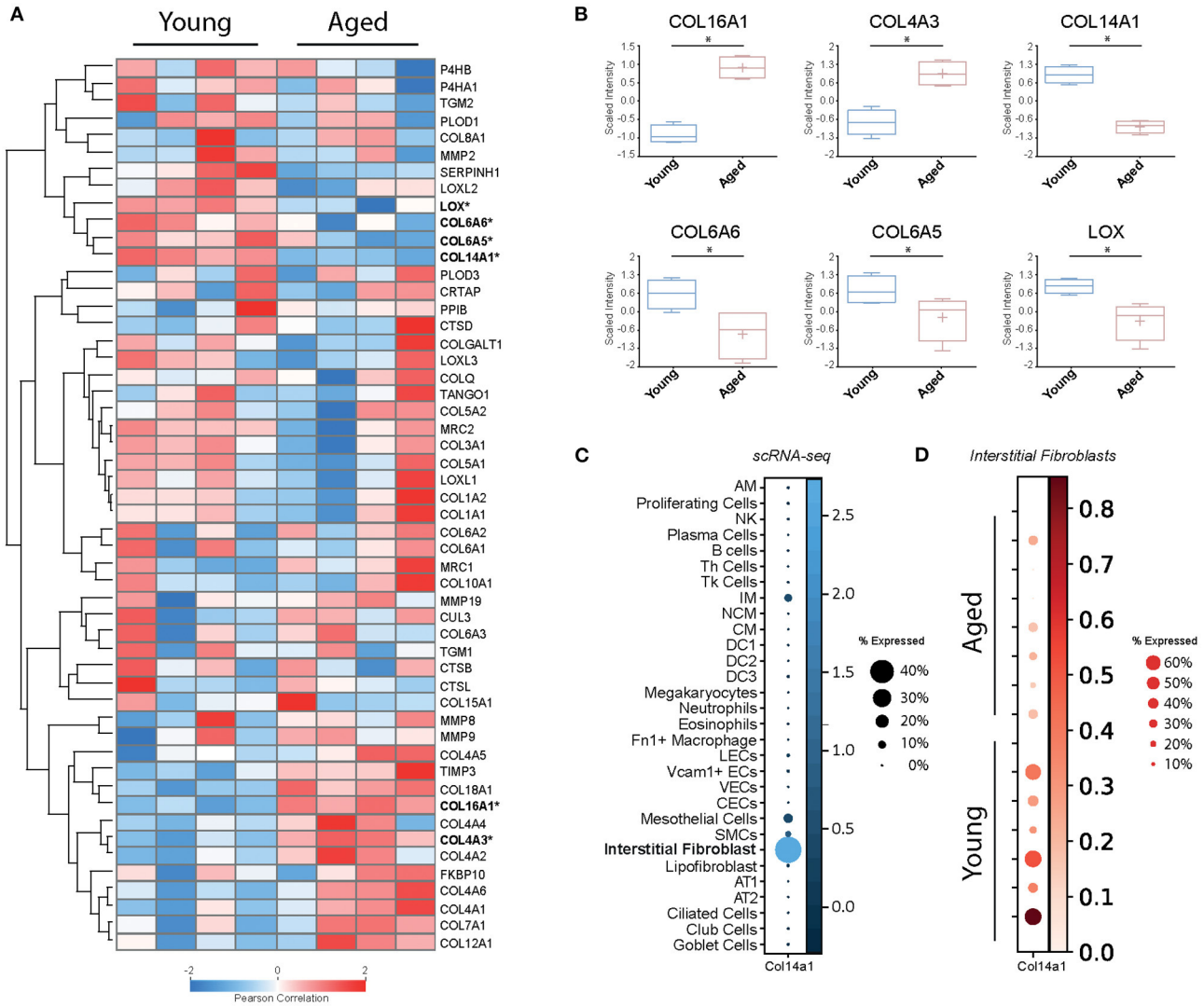

FIGURE 5 | Baseline changes of proteins involved in collagen biosynthesis, trafficking, processing, and degradation. **(A)** Heatmap of normalised mean intensity values of 52 regulated extracellular matrix proteins (whole lung proteome, mass-spec) grouped by unsupervised hierarchical clustering and *Z*-scored (Pearson's correlation). **(B)** Boxplots of 6 significantly regulated proteins with age. **(C)** The dot plot shows mRNA expression specificity of Col14a1 in scRNA-seq data of whole lung homogenate. **(D)** Dotplot indicating mRNA expression levels between aged and young mice within the lung interstitial fibroblasts cluster (* = Student's *t*-test significant, FDR<10%).

collagen gene products is emerging in the literature, e.g., for COL6A1 (22, 180) and COL4A1 (181, 182).

Downregulation of Type XIV Collagen (COL14A1)

Type XIV collagen belongs to the FACITs (**Figure 1**) and plays an important role in fibrillogenesis of type I collagen, in particular during development (183–187). Similar to downregulation of COL14A1 in the ageing mouse lung, downregulation of COL14A1 has also been described for human skin ageing (153). Several studies support a role of COL14A1 in connecting the basement membrane to the subepithelial interstitial matrix (185, 188, 189). Like COL6A4 mentioned above, COL14A1 is developmentally regulated in some tissues, e.g., in tendon or the heart (189–192).

Interestingly, several reports in the past have independently argued for an important role of COL14A1 in the maintenance of normal epithelial cell turnover and differentiation in adult tissues. For instance, a missense mutation in COL14A1 at a highly conserved amino acid residue (Pro1502Leu) leads to punctate palmoplantar keratoderma, a skin disease characterised by aberrant squamous cell differentiation leading to hyperkeratosis in the cornified layer (156, 157). In renal cell carcinoma (RCC), which arises from epithelial cells, frequent hypermethylation of COL14A1 has been observed which resulted in transcriptional silencing; knockdown of COL14A1 increased the growth rate of

TABLE 2 | Overview on collagens and collagen biosynthetic proteins observed to be regulated with age in the mouse lung.

Collagen	FC[a] (old/young) mouse lung	major expressing cell type(s)[b]	Altered in human ageing tissue?	Relevance to human disease
COL4A3	+2.9	AT1	No evidence for COL4A3 COL4A2 ↓ with age in adipose tissue COL4A5 ↑ with age in skin COL4A6 ↑ with age in skin (153)	Goodpasture syndrome (154) Alport syndrome (155)
COL6A4	−15	Not enough data	Pseudogene	Unknown
COL6A5	−3.9	(Myo)fibroblasts, pericytes, smooth muscle cells	No evidence	Unknown
COL6A6	−3.4	(Myo)fibroblasts, smooth muscle cells	No evidence	Unknown
COL14A1	−2.7	Fibroblasts, pericytes, smooth muscle cells	↓ with age in skin (153)	Missense mutation causes punctate palmoplantar keratoderma (156, 157)
COL16A1	+19	Fibroblasts, basal cells, ciliated cells	↑ with age in skin (153)	Unknown
LOX	−4.6	Fibroblasts, mesothelial cells	↓ with age in skin (153)	Loss of functions mutation in *LOX* predispose to thoracic aortic aneurysms, dissections, and ruptures (158, 159)

[a] *FC, Fold Change, extracted from (160).*
[b] *According to recent scRNA-Seq data (160–164).*
Evidence for similar change in human tissues and relevance of these genes and proteins for human disease.

RCC cell lines (193). COL14A1 is also notably downregulated in oesophageal squamous cell carcinoma (194). Collectively, these observations point toward an anti-proliferative or tumour suppressor function of COL14A1.

With this documented role on epithelial cell survival and differentiation, it is at first glance surprising that recent single-cell RNA-Seq data consistently show expression of COL14A1 by interstitial fibroblasts, not epithelial cells (160–164) (**Figure 5C**). A study on fibroblast heterogeneity even revealed that expression of COL14A1 marks a specific matrix-producing fibroblast subtype which increases in cell number in murine lung fibrosis (195). As lung fibrosis is characterised by loss of normal alveolar type I and type II cells and atypical epithelial differentiation (196), it may be speculated that overexpression and extracellular deposition of fibroblast-generated COL14A1 contributes to these epithelial events and therefore to loss of normal alveolar structure. In addition, loss of Col14a1 in the ageing mouse lung correlates with changes in cell type composition in mouse airway epithelial cells (160). Overall, it seems that deregulation or loss of paracrine COL14A1 has profound consequences on epithelial survival and differentiation and may therefore contribute to the observed changes during ageing. These observations warrant future studies on the exact distribution and molecular function of COL14A1 in lung ageing and disease.

Upregulation of Type IV Collagen (COL4A3)

Type IV collagen is a major structural component of the basement membrane and represents the prototypical network-forming collagen. Six genes (Col4a1-Col4a6) encode for six distinct type IV collagen chains which can assemble into three heterotrimeric molecular isoforms, namely $\alpha1(IV)_2\alpha2(IV)$, $\alpha3(IV)\alpha4(IV)\alpha5(IV)$, and $\alpha5(IV)_2\alpha6(IV)$ (197). At least the first two heterotrimeric forms have been reported in the lung, the major one being $\alpha1_2\alpha2(IV)$ (22, 198). Interestingly, Angelidis et al. (160) found that, even if only COL4A3 passed the threshold for significance, protein levels of all six type IV collagen chains were increased in the ageing lung. These changes went in parallel with alterations of other basement membrane-specific proteins, like decrease of FRAS1, FREM1, FREM2, and COL14A1 discussed above. Intriguingly, the authors also found that transcript levels for all type IV collagen genes anti-correlated with protein levels, i.e., were downregulated with ageing. While downregulation of type IV collagen chain transcripts occurs consistently upon ageing of human tissue and cells (153, 199, 200), the post-transcriptional mechanisms that control levels of type IV collagen protein may be tissue-specific: While, similar to the results in ageing mouse lung, Karttunen et al. reported type IV collagen protein to be increased in human kidney upon ageing (201), in skin, type IV collagen protein was found to decrease upon ageing, in parallel to transcript levels (200). Interestingly, a decrease of serum type IV collagen has been described upon ageing (202) which may in part reflect increased type IV collagen retention and deposition in tissue. Overall, type IV collagen turnover, however, as measured by the formation product P4NP7S and the degradation product C4M in serum, seems to be stable during ageing (203).

Upregulation of Type XVI Collagen (COL16A1)

Just like type XIV collagen, also type XVI collagen belongs to the FACIT family of collagens and forms homotrimeric triple helices (23, 204). It is a minor collagen component which shows a tissue-dependent versatility for incorporation into different collagen suprastructures (205, 206). Its distribution in lung ECM

is unknown, but judging from recent scRNA-Seq data, where COL16A1 is found expressed by all fibroblast subtypes as well as a broad range of epithelial cells, it is likely to be a constituent of both the basement membrane and the interstitial matrix (160–164). Even though COL16A1 has been observed to be increased upon ageing also in human skin (153), its potential role in the ageing process has not received much attention. Tajima et al. found that increased COL16A1 expression in dermal fibroblasts correlated with cell growth arrest of these cells (207). In contrast, induction of COL16A1 expression promotes proliferation and invasion of cancer cells (205, 208–210). Similar to our speculations on COL14A1, there may be analogous paracrine effects by fibroblast-generated COL16A1, albeit in reverse directions: Loss of fibroblast-generated COL14A1 on the one hand, as well as induction of COL16A1 deposition by fibroblasts on the other, may lead to increased cell proliferation and aberrant differentiation of adjacent epithelial cells and thus contribute to tumorigenesis and tumour invasion. Notably, this is reminiscent of a proposed link between ageing and cancer where senescent fibroblasts secrete factors, including extracellular matrix components that promote epithelial tumorigenesis (211, 212). Clearly, future studies are needed to decipher the role of COL14A1 and COL16A1 in this context.

Downregulation of Lysyl Oxidase

Lysyl oxidase (LOX) is a copper-dependent protein-lysine-6-oxidase, activity of which is critical for stabilisation of extracellular fibrillar collagen (71, 213). During fibril formation, LOX catalyses the oxidative deamination of specific lysine and hydroxylysine residues in the N- and C-telopeptides of fibrillar collagens, resulting in the corresponding aldehyde forms (71) (**Figure 3B**). These reactive intermediates trigger a series of subsequent condensation reactions between triple-helical subunits of collagen fibres, ultimately leading to divalent or trivalent crosslinks.

Several studies have shown that downregulation of LOX in the context of ageing is not restricted to the lung. For instance, *LOX* expression is reduced with age in human, rat and monkey skin (153, 214, 215), in urogenital tissues of female mice (216) and in rat aorta (217). Notably, loss of function mutations in *LOX* predispose to aortic aneurysms and age is the most important risk factor for aortic aneurysms (158, 159), supporting the concept that loss of LOX upon ageing may have direct effects on ECM integrity and tissue stiffness. Downregulation of LOX upon ageing appears to correlate with fewer reducible difunctional LOX-derived crosslinks in skin, aorta and lung (218, 219), even though, to our knowledge, no study has assessed this correlation directly. This contrasts with non-enzymatic collagen crosslinking which increases with age and will be discussed in more detail below (220). Overall, also taking into account recent findings that the same collagen lysines are targeted by LOX-mediated and non-enzymatic collagen crosslinking events (221), there seems to be a shift from LOX-mediated to advanced-glycation end product (AGE) crosslinks upon ageing.

In cancer research, LOX has received considerable attention, both owing to tumour growth and invasion-promoting properties on the one hand and to tumour suppressor functions on the other hand (222, 223). LOX is synthesised as a pro-enzyme and activation requires removal of the pro-peptide, notably by the same enzymes that cleave off the C-terminal pro-peptide of type I collagen (BMP1/Tolloid-like proteinases) (71). The mature protein is overexpressed in various cancer types including lung adenocarcinoma, typically correlates with poor prognosis, and has been described to create a stiffer microenvironment which supports tumour growth and metastasis (222, 224–230). In contrast, the tumour-suppressing activities of LOX are attributed to the released pro-peptide (222, 223, 231, 232).

The Impact of Ageing on Collagen Degradation

Few studies have directly assessed changes in collagen synthesis and degradation upon ageing. Several early studies, measuring the collagen synthesis rate in tissues of young and aged experimental animals, including lungs, as well as *ex vivo* culture of human skin biopsies, consistently found evidence for a decreased synthesis rate with ageing tissue (233–235). Mays et al., in addition, studied how much of the newly synthesised collagen is degraded and found that, in aged rats, a larger percentage was directly subjected to degradation than in young rats (233). Thus, in older age not only less collagen would be synthesised, but also newly synthesised collagen would be more prone to direct degradation. This concept, however, has been challenged by a recent study on old *vs*. young mouse lungs where the authors demonstrate that aged mice have higher overall levels of total collagen and lower levels of a collagen endocytic receptor termed mannose receptor, C-type 2 (MRC2), the major receptor for fibroblast-mediated intracellular degradation of collagen (236). Notably, in the proteomics data set presented here, we found lower levels of MRC2 in three out of four aged animals, albeit without reaching statistical significance (**Figure 5A**), which may reflect the limited statistical power of the proteomics data set. Furthermore, levels of most major lung collagens (types I, III, IV) remained unchanged or were even increased in old *vs*. young mouse lungs (**Figure 5**) (160). Overall, this indicates that mature collagen is very stable in the adult lung.

Partially supporting this concept, we observed no consistent changes for collagen-degrading proteases in the data set on mouse lung ageing presented here (**Figure 5A**) (160). Neither levels of the detected MMPs and TIMPs (MMP2, MMP8, MMP9, MMP19, TIMP3) nor levels of the detected cathepsins (CTSB, CTSD, CTSL) were significantly altered (160). Of course, this is only circumstantial evidence as (a) this study did not provide a comprehensive quantification of all lung collagenases and their inhibitors, (b) levels *per se* cannot be equated with enzymatic activity, and (c) accessibility of cleavage sites in the collagen substrate itself can be masked or altered upon ageing. As to enzymatic MMP activity, Calabresi et al. have shown that natural ageing of rat lungs is accompanied by decreases in MMP1 and MMP2 activity in parallel with moderately increased levels of the MMP inhibitors TIMP1 and TIMP2 levels (237), suggesting even a loss of collagenase activity upon ageing. Furthermore, and maybe even more importantly, there is evidence suggesting that the degradability of collagen is affected by ageing-related collagen

modification and crosslinking (e.g., non-enzymatic crosslinking by advanced glycation end-products, AGEs, discussed below) and addition or removal of glycosaminoglycans (238–243). Thus, protease activity by itself may not be the decisive factor after all, but in fact the accessibility of the corresponding collagen cleavage sites.

It is to date unclear, whether these findings reflect the human situation. Collagen degradation upon ageing in the human lung has not been directly assessed. A number of studies have measured circulating levels of MMPs and TIMPs and deduced changes in ECM turnover upon ageing. However, the reported findings are not consistent (244–248) and even if such changes may be of importance for collagen degradation within the vascular wall, it is unclear how peripheral levels of MMP and TIMPs correlate with the integrity of the ECM in other tissues. Obviously, tissue-resident levels and activities of proteases in correlation with ECM integrity of the studied human tissue are more relevant to examine in that context, but such studies are much scarcer. An immunohistochemical study of human lungs found an age-dependent increase of $TIMP2^+$ cells, but not $MMP2^+$ cells, in the lung (249), providing some evidence for decreased MMP-mediated collagen degradation in the aged lung. Notably, in a recent pioneering study of human skin ageing, the authors correlated matrisome changes with the assessment of ECM integrity by single harmonic generation and two-photon autofluorescence imaging (214). Among other changes, they found decreased MMP2 and increased TIMP3 levels in aged skin. The mechanisms underlying skin ageing, however, involve environmental insults, e.g., exposure to UV light, microorganisms, and mechanical stress that do not apply to the lung.

Undoubtedly, more research is warranted to elucidate mechanisms of collagen and ECM degradation upon ageing of the lung. Nevertheless, taken together, the current evidence supports an age-dependent decline of lung collagen degradation, suggesting that, also in the lung, collagen is a long-lived molecule and thus susceptible to the accumulation of damage with time.

NON-ENZYMATIC COLLAGEN CROSSLINKING—ADVANCED GLYCATION ENDPRODUCTS

In addition to LOX- and TGM-mediated, i.e., enzymatic crosslinking mentioned above, also non-enzymatic crosslinking of collagen occurs. The probably most important one in the context of human disease and ageing is the reaction of collagen with sugars or sugar metabolites which results in the formation of so-called advanced glycation endproducts (AGEs). These products are principally not restricted to collagen. In the best-described classical Maillard reaction, any free amino group, e.g., the ?-amino group of a lysine or the protein N-terminus, reacts with the carbonyl group of a reducing sugar to form a Schiff base. This unstable Schiff base is spontaneously rearranged into a more stable keto amine intermediate, the so-called Amadori product (**Figure 6A**). This early glycation product is in equilibrium with glucose and still reversible. However, a small part of these Amadori products undergo subsequent, predominantly oxidative, events resulting in the irreversible formation of different protein adducts and protein crosslinks, collectively termed AGEs. As the latter reactions occur over months and years, such adducts and crosslinks accumulate in long-lived proteins like extracellular collagens, including in the lung, and strongly correlate with age (220, 250–253). **Figure 6A** shows the classical Maillard reaction and some of the best-characterised and most often analysed AGEs are depicted in **Figure 6B** [N_ε-(1-carboxyethyl)lysine, CEL; hydroimidazolone, MG-H1] and **Figure 6C** (lysine-arginine cross-links pentosidine and glucosepane; methylglyoxal lysine dimer, MOLD).

Notably, while the above-described reactions of proteins with reducing sugars are the best-described, AGEs can also be formed by the reaction of lysines and arginines with other carbonyl compounds, in particular highly reactive dicarbonyl compounds (254, 255). Accumulation of such compounds and its consequences is also referred to as "dicarbonyl stress" in the literature. Methylglyoxal (**Figure 6B**) is an abundant representative of these dicarbonyl compounds. Methylglyoxal is generated during the formation of AGEs in the context of the above-described glycation of proteins by reducing sugars, but also, in fact more importantly, endogenously as a result of spontaneous degradation of triosephosphates, degradation of glycated proteins, oxidation of aminoacetone in threonine catabolism, and peroxidation of lipids (256–258). In the glycation reaction (**Figure 6B**), methylglyoxal is about 20.000 times more reactive than glucose. It is thus not surprising that an enzymatic detoxification system has evolved to control intracellular methylglyoxal levels: The glyoxalase system, which consists of the enzymes glyoxalase 1 and 2 (Glo1 and Glo2) and a catalytic amount of reduced glutathione, catalyses the formation of lactate from methylglyoxal (256–259). Interestingly, evidence is emerging that methylglyoxal plays an important role in ageing and age-related disease: In the nematode *Caenorhabditis elegans*, a model organism frequently used in fundamental research on ageing and lifespan-extending mechanisms, glyoxalase activity is markedly reduced upon ageing and overexpression of the *C. elegans* orthologue of Glo1 increased lifespan (260, 261). Glyoxalase expression and/or activity has been reported to decrease upon ageing in human arterial tissue (262), in red blood cells (263), in human brain (256, 264), as well as in mouse lung (160). Methylglyoxal-protein adducts are increased in ageing human lens and skin (265, 266). Also, studies in rats have shown that overexpression of Glo1 protects from age-related renal dysfunction (267). Importantly, even if generated intracellularly, methylglyoxal can easily target the extracellular matrix because it is freely membrane-permeable (268) and has a relatively long range of diffusion—its tissue levels are therefore critically dependent on local intracellular glyoxalase activity (257). In summary, it is highly plausible that methylglyoxal and the glyoxalase system also contribute to molecular alterations of the ECM and to lung dysfunction upon ageing, even though direct evidence for this is lacking to this day.

Identification and quantification of AGEs are difficult tasks. First of all, AGEs are derived from many different precursor molecules and represent a highly heterogeneous

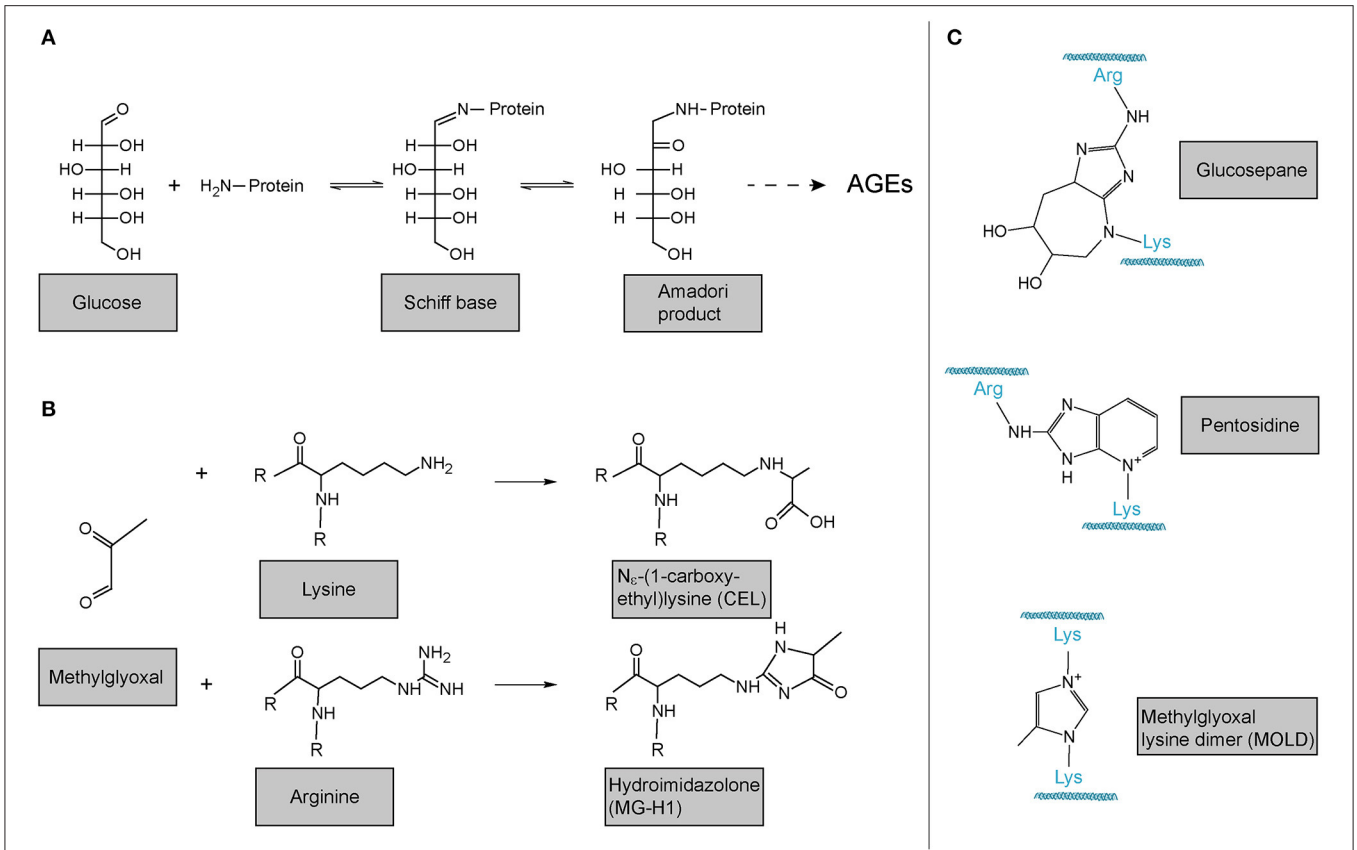

FIGURE 6 | Advanced glycation endproducts (AGE)-mediated collagen modification and crosslinking. **(A)** In the classical Maillard reaction, a reducing sugar (here: glucose) reacts with an amino group in a protein, e.g., the protein's amino terminus or amino groups of the side chains arginine or lysine. The resulting Schiff base rearranges to a ketoamine, the so-called Amadori product. Subsequent reactions of this initial product result in the irreversible formation of different protein adducts and crosslinks, collectively termed advanced glycation endproducts (AGEs). **(B)** Reactions of the dicarbonyl methylglyoxal with protein lysines or arginines results in the formation of protein adducts as e.g., N_ε-(1-carboxyethyl)lysine (CEL) or the hydroimidazolone MG-H1. **(C)** Structures of some of the most common AGE-mediated crosslinks. Structures were generated using ACD/Chemsketch freeware.

group of compounds (253, 269). New variants of AGEs are still being discovered (270) and it is likely that not all AGE-protein adducts and crosslinks are known in molecular detail. For the analysis of clinical samples, early detection methods took advantage of the fluorescent properties of many AGEs: Collagen from connective tissue was extracted and solubilized by collagenase digestion and fluorescence measured [≈370 nm excitation/440 nm emission (271)]. More recently, autofluorescence of non-pigmented skin has been shown to correlate with collagen-linked fluorescence, concentrations of the fluorescent AGE pentosidine (**Figure 6C**), concentrations of the non-fluorescent AGEs N_ε-(1-carboxymethyl)lysine (CML) and N_ε-(1-carboxyethyl)lysine (CEL, **Figure 6B**), as well as with age, diabetes duration, and diabetic complications (272–278). Even though clearly a convenient non-invasive technique, any technique based on fluorescence will be limited in specificity and sensitivity, as not all AGEs are fluorescent including major representatives like CML and CEL and interference by other fluorescent molecules can occur, e.g., by NAD(P)H. Numerous attempts have been made to establish immunochemical detection of AGEs in tissue, albeit yielding inconsistent results due to lack of specificity, sample processing artefacts, and contamination by glycated blocking proteins (279–282). Therefore, quantitative results obtained by enzyme-linked immunosorbent assays (ELISAs), for instance, must be interpreted with caution.

The state-of-the-art detection method for AGEs in tissue and clinical samples is high performance or ultra-high performance liquid chromatography combined with mass spectrometry (279). New trending approaches, collectively referred to as "Maillard proteomics" are expected to revolutionise our understanding of glycation site specificity of AGE adducts, a prerequisite to gain an understanding of their function (269).

Even though the association between increases in AGE-mediated crosslinking, increased collagen stiffness, and decreased solubility with ageing is well-established (251), to the best of our knowledge surprisingly few studies have assessed AGEs and collagen AGE adducts in the lung. This may reflect the technical difficulties of specific AGE quantification pointed out above. Nevertheless, measurements of AGE-related fluorescence in the context of two animal studies support the concept that also in

lung collagen AGE-load increases with age and anti-correlates with enzymatic solubility (220, 283). Interestingly, a study using the bleomycin-induced mouse model of lung fibrosis suggests that inhibition of AGE formation may protect from lung fibrosis (284). In reverse, accumulation of AGE load in the lung upon ageing may therefore represent one of the reasons why idiopathic lung fibrosis predominantly occurs in the elderly. Resonating with this concept, several reports indicate that diabetes mellitus, where increased blood glucose levels lead to increased tissue levels of AGEs, increases the risk for pulmonary fibrosis (285–288).

The Consequences of "AGEd" Collagen

But what can be the consequences of non-enzymatically glycated—or "AGEd"—collagen? There is evidence that AGEs increase collagen fibril stiffness and attenuate collagen turnover by, on the one hand, inhibiting degradation by MMPs and cathepsin K (239, 243, 289), and, on the other hand, inhibiting phagocytosis pathways (290). Furthermore, experiments with *in vitro* generated AGE-crosslinked collagen, by e.g., incubating collagen with ribose or methylglyoxal and measuring mechanical properties, indicate that in particular molecular sliding of collagen fibrils and fibres is affected by this type of crosslinking (291, 292). Hence, most likely "AGEd" fibrillar collagen contributes to the generally observed AGE-induced reduction of viscoelasticity of connective tissues accompanied by increased mechanical fragility (254, 291, 293–295).

In addition to changes in collagen turnover and biomechanics, AGE-mediated modification and crosslinking of collagen has the potential to mask important collagen-cell or collagen-protein-interaction sites with direct effects on adherent cell behaviour and function. Indeed, several studies with diverse cell types support this concept. For instance, non-enzymatically glycated collagen exhibits reduced affinity to heparin and keratan sulphate proteoglycans, resulting in diminished adhesion of B cells and reduced migration of endothelial cells (296). Similarly, methylglyoxal-modified type IV collagen displays reduced affinity to integrins, leading to attenuated adhesion of renal glomerular cells, and reduced attachment, viability, and angiogenic activity of endothelial cells (297, 298). Also neurons cultured on glycated type I collagen, show altered morphology and function in comparison to culture on normal type I collagen including reduced neuron interconnection and increased release of pro-inflammatory stimuli like nitrite and TNF-α (299). Finally, it has been reported that AGE-modified collagen fails to interact with discoidin domain receptor 2 and thus loses the capacity to upregulate expression of LOX in osteoblasts (300). Notably, even if direct evidence is lacking, this provides a potential mechanism underlying the above-described loss of LOX upon ageing in the lung and highlights a potential direct relationship between accumulation of AGE-mediated modifications/crosslinks and downregulation of LOX-mediated crosslinks.

Interestingly, molecular modelling studies using a collagen type I microfibril have put forward a number of candidate inter- and intramolecular Lys-Arg pairs that fulfil the requirements in terms of configuration and distance (301) and change in enthalpy (302) to allow for the formation of glucosepane crosslinks. In both studies, the authors identified more potentially AGE-crosslinked arginines and lysines within binding sites for integrins, proteoglycans, MMPs and other proteins. Collectively, these findings emphasise that "AGEd" collagen has a major impact on adherent cell function in many different tissues and clearly contributes to disease, warranting similar studies in the lung.

Effects Mediated Through the Receptor for Advanced Glycation Endproducts

AGEs are ligands for the receptor for advanced glycation endproducts (RAGE), a membrane-bound receptor of the immunoglobulin family (303, 304). Originally discovered in 1992 as an AGE-binding receptor (305), several additional ligands have been identified including S100/calgranulins, amyloid fibrils, amphoterins, and Mac-1. RAGE can thus be viewed as a relatively promiscuous pro-inflammatory pattern recognition receptor (303, 304). RAGE is abundantly expressed in the lung, at baseline predominantly in alveolar type I cells toward the basal membrane, but it can be activated in many other cell types upon exposure to RAGE ligands (304). Numerous studies have established associations of RAGE and its soluble decoy receptor sRAGE with lung injury and disease (303, 304, 306, 307).

RAGE-mediated downstream signalling depends on the identity and oligomerization state of the ligand and the tissue context. Ligand-engaged RAGE can trigger oxidative stress and multiple signalling cascades including p21 ras, erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, rho GTPases, phosphoinositol-3 kinase, and the JAK/STAT pathways. This leads predominantly to sustained activation of NF-κB- and STAT-dependent gene transcription (304, 308–310). As, typically, studies have been performed using soluble and globular glycated proteins such as serum albumin or ovalbumin (305, 311–313), it is not entirely clear whether "AGEd" collagen is capable of inducing these pathways. Studies using glycated collagen have established a RAGE-dependent link between "AGEd" collagen and apoptosis of mesenchymal cells. For instance, N_{ϵ}(1-carboxymethyl) lysine (CML)-collagen (derived from reaction of collagen with glyoxal instead of methylglyoxal, **Figure 6B**), but not control collagen, induces fibroblast and osteoblast apoptosis *via* the RAGE/p38/JNK axis, but largely independent of NF-κB signalling (314, 315). Interestingly, some evidence indicates that RAGE promotes adhesion to collagen without the need for AGE-mediated modification, even though the glycation status of the collagen used was not reported in these studies (316, 317).

CONCLUSIONS

There can be no doubt that collagen plays an important role in lung physiology and pathology. Even though the collagen family of proteins has been the subject of studies for many decades it is striking how little we still know about

collagen composition changes and molecular alterations in lung ageing and disease. Here, we provided a comprehensive review on mechanisms of collagen biosynthesis, processing, modifications and crosslinking and how these change upon ageing. Collectively, a picture emerges where ageing (1) leaves the normal ER-resident collagen biosynthesis machinery largely unaffected, but (2) results in distinct changes in collagen composition and (3) changes in the molecular nature of collagen crosslinks. Clearly, these observations warrant future work to address the mechanistic consequences of these changes in the context of lung ageing and disease. Ultimately, such studies may be critical to the field of lung regenerative medicine.

AUTHOR CONTRIBUTIONS

CO, ED, and CS-W wrote the manuscript and prepared figures. HS and IA provided data on lung ageing and prepared **Figure 5**. All authors proofread and edited the manuscript.

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Early Changes and Indicators Characterizing Lung Aging in Neonatal Chronic Lung Disease

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Infants suffering from neonatal chronic lung disease, i.e., bronchopulmonary dysplasia, are facing long-term consequences determined by individual genetic background, presence of infections, and postnatal treatment strategies such as mechanical ventilation and oxygen toxicity. The adverse effects provoked by these measures include inflammatory processes, oxidative stress, altered growth factor signaling, and remodeling of the extracellular matrix. Both, acute and long-term consequences are determined by the capacity of the immature lung to respond to the challenges outlined above. The subsequent impairment of lung growth translates into an altered trajectory of lung function later in life. Here, knowledge about second and third hit events provoked through environmental insults are of specific importance when advocating lifestyle recommendations to this patient population. A profound exchange between the different health care professionals involved is urgently needed and needs to consider disease origin while future monitoring and treatment strategies are developed.

Keywords: neonatal chronic lung disease, bronchopulmonary dysplasia, lung aging, inflammation, mechanical ventilation, oxygen toxicity, lung function, preterm infant

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DISEASE CHARACTERISTICS AND PREDISPOSITIONS

As the most common chronic lung disease in infants, Bronchopulmonary Dysplasia (BPD) is associated with long-term sequelae that persist into adulthood (1, 2). Despite significant improvements in perinatal care, i.e., surfactant and antenatal corticosteroid treatment together with improved ventilation strategies, the incidence of BPD has remained unchanged or even increased amongst the most immature infants (3). This is presumably due to a significant reduction of mortality rates together with an increase in the overall number of treated infants born significantly premature. The varying incidence of BPD between newborn care centers closely reflects differences in patient population and infant management practices (4–7). Recent publications report an incidence of BPD of up to 68% in very low birth weight infants (401–1,500 g) born prior to 29 weeks of gestation, or up to 77% in infants born at <32 weeks of gestation with a birth weight below 1 kg (5, 8, 9), with numbers predominantly derived from high-income countries.

The disease is classified into three different severity grades (mild, moderate, severe) according to the need for supplemental oxygen and/or ventilator support for >28 days of life, or beyond 36 weeks postmenstrual age (PMA) (2). Environmental insults associated with preterm birth sum up to sustained inflammation and extensive matrix remodeling resulting in substantial changes to the scaffold provided for the developing organ in concert with functional abnormalities as a consequence of diffuse fibrotic changes and increased smooth muscle hypertrophy in small pulmonary arteries and airways (10). The characteristic histopathologic changes of impaired alveolarization and vasculogenesis (2) are clinically mirrored by signs of impaired respiratory gas exchange, i.e., alveolar hypoventilation with resultant hypercapnia and hypoxemia leading to a mismatch of ventilation and perfusion (11).

Large clinical trials have identified numerous risk factors for the development of BPD, including congenital and nosocomial infections, mechanical ventilation, and oxygen toxicity (12–17). Poor nutritional support, vitamin deficiency as well as insufficient adrenal and thyroid hormone release in the very premature infant further increase the risk after birth (18–20). Prenatal risk factors influence the capacity of the developing lung to respond to these injuries. Preeclampsia is known as an independent risk factor not only for preterm delivery, but more importantly also for BPD, despite its underlying molecular mechanisms remaining elusive (21, 22). Intrauterine growth retardation increases the risk of BPD 3- to 4-fold (23–27), most likely through their impact on altered growth factor signaling and subsequently impaired alveolar and vascular development (28). Exposure to prenatal smoke, although largely underestimated, has been shown to significantly contribute to disease development, potentially beyond growth restriction (29, 30), and even the prenatal application of established therapeutic measures has to be critically reviewed. Here, antenatal betamethasone, despite its broad prenatal application to enhance lung maturation and to prevent respiratory distress while reducing BPD rates (31, 32), has been shown to increase indicators of lipid membrane peroxidation (33). This word of caution is in line with observations on behalf of postnatal dexamethasone treatment, where adverse effects on cardiac function, life expectancy, and neurologic development have been observed (34, 35). The broad use of antenatal maternal antibiotic treatment on the other hand not only significantly affects the bacterial flora of the child (36) but leads to sustained alterations of immune functions, e.g., in the response of invariant natural killer T cells, as indicated by studies in mice (37). Although prenatal exposure to smoke and antibiotics were shown to provoke lung changes on the molecular level as shown by studies in mice, further investigations are needed in order to establish these risk factors clinically, as prior published data from preterm infants are inconsistent (38, 39).

With regard to the genetic background impacting on the clinical course, prior work demonstrates that gene polymorphisms account for 53% of the variance in BPD (40). Identified genetic abnormalities include mutations in genes associated with surfactant biogenesis, innate immune response (41, 42), and superoxide dismutase (43), with details of the possible pathophysiology explained in the paragraphs below. The higher risk for the development of BPD and pulmonary arterial hypertension (PAH) in male preterm infants (44) has been associated with differences in hormonal regulation (45), although longer term, females with a history of BPD seem to be more severely affected (46).

FROM CAUSE TO CONSEQUENCE: BIOLOGICAL PROPERTIES, PULMONARY STRUCTURE, AND LUNG FUNCTION

Inflammation and Oxidative Stress Response

The sustained inflammatory processes that characterize BPD are caused by pre- as well as postnatal mechanisms with key players highlighted in the paragraph above. Both, infections as well as the corresponding immature immune response play an important role in the initiation and perpetuation of inflammatory processes characterizing and driving BPD development (14, 47–49). Perinatal processes, e.g., the fetal inflammatory response syndrome (FIRS), chorioamnionitis, or their development into congenital and nosocomial infections results in neutrophil influx into the immature lung, with an increased number of monocytes and macrophages during the so called "second wave" of inflammation (27, 50, 51). Here, specific pathogens such as Gram-negative bacteria play an important role, clearly increasing the risk for neonatal chronic lung disease (52). Later nosocomial infections are caused by a different, "non-maternal" spectrum of pathogens (e.g., Staphylococcus epidermidis, *Escherichia coli*) that are likewise associated with BPD development (53, 54).

Postnatally, non-infectious causes such as baro- and volutrauma during mechanical ventilation in concert with the effects caused by moderate or severe hyperoxia further contribute to or even initiate the inflammatory processes locally and on a systemic level (55–58). Ongoing studies addressing the debate, whether recruitment of inflammatory cells to the injured lung or activation of resident cells are primarily responsible to start and drive the vicious circle of inflammation (59–62) will give important insight for mechanistic understanding and development of targeted therapies. At the same time, extracellular matrix (ECM) remodeling itself further promotes lung inflammation through the release of proteases and inflammatory mediators such as transforming growth factor beta (63–65). The regulation of NF-kB signaling in inflammatory processes (66) and its lately discovered role in alveolo- and vasculogenesis that may even bear therapeutic potential (67, 68) demonstrate the close relation of lung inflammation during organ development to fundamental and long-lasting structural changes.

The degree of lung inflammation and ECM remodeling as well as early alveolar epithelial dysfunction is directly related to the cellular capacity to respond to postnatal environmental challenges. The relative deficiency of antioxidants and inhibitors of proteolytic enzymes render the immature lung especially vulnerable to the effects of inflammatory mediators and toxic oxygen metabolites (69–72). Different markers have been

investigated to indicate enhanced oxidative stress in the preterm infant. Elevated urinary malondialdehyde concentrations in the first week of life, generated by peroxidation of lipid membranes after oxidant-mediated injury, were correlated with the risk for oxygen radical diseases including BPD (33). In a murine model, reduced superoxide dismutase 3 (SOD3) in reaction to postnatal hyperoxia was associated with alveolar injury, whereas overexpression of SOD3 attenuated hyperoxic injury in an alveolar epithelial cell line (73). Decreased pulmonary antioxidant concentrations have also been measured in the lavage of preterm infants (74). In line with this, intratracheal application of recombinant human CuZn superoxide dismutase at birth improved pulmonary outcome in high-risk premature infants at 1 year corrected age (75). Studies indicated that adolescent BPD patients have evidence of heightened oxidative stress in the airway, suggesting that long-term respiratory abnormalities after preterm birth align with sustained alterations of the oxidative stress response (76). These effects might even translate into altered responses to viral infections in later life (77).

The increased susceptibility of the developing organ to environmental challenges and the induction of long-term consequences are supported by the observation that significant maturational differences exist between neonatal and adult lung cells in response to lung injury. While chronic oxygen exposure (60% for 14 days) enhances lung vascular and airway smooth muscle contraction and reduces nitric-oxide relaxation in the neonatal rat lung, the opposite occurs in adults (78). In line with the observations obtained in neonatal mice, long-term effects of hyperoxia exposure in the first week of life (100% for 4 days) include increased mortality associated with pulmonary vascular disease and the development of right ventricular strain and PAH in mice (79). The alteration of bone morphogenic protein (BMP) signaling likely contributes to this adult lung phenotype. Other mechanisms explaining the increased susceptibility to injury and the subsequent likelihood of long-term effects observed in the newborn lung are suggested from studies by Balasubramaniam et al. showing that hyperoxia reduces bone marrow derived, circulating, and lung endothelial progenitor cells in the developing organ in contrast to adult mice (80), indicating early exhaustion of repair and regeneration capacities. The risk for long-term effects is furthermore mitigated by the affection of central processes such as cell cycle regulation, i.e., upregulation of P21 by hyperoxia exposure together with decreased histone deacetylase activity (81). The studied effects of excessive oxygen exposure on DNA methylation further contribute to the picture of accumulating damage in the face of reduced compensatory mechanisms in the immature lung (82).

The outlined injury effects ultimately result in the impairment of lung growth based on significantly imbalanced growth factor signaling. Orchestrating the interaction of the epithelial, mesenchymal, and endothelial cell compartment during the fine-tuned development of the gas exchange area, Notch and Wingless Int-1 (Wnt), the fibroblast and platelet derived growth factor (FGF, PDGF) as well as the BMP and the vascular endothelial growth factor (VEGF) play a critical role (83–89).

Morphogenetic Changes to the Pulmonary Cellular and Extracellular Matrix

The release of cytokines such as the transforming growth factor (TGF) –β, tumor necrosis factor (TNF) alpha and interleukins, e.g., IL-1beta in response to lung inflammation and subsequent events such as ECM remodeling and cellular injury significantly contributes to the imbalance in growth factor signaling and leads to the activation of different transcription factors enhancing apoptosis in numerous cell types (90–92). The interference with these transcription factors disrupts normal lung morphogenesis (67) and drives the onset of chronic bronchial inflammation and subsequent pulmonary emphysema in the adult organ (93). In the developing organ, the altered regulation of transcription leads to long-term effects such as the impairment in alveolar and vascular development resulting from nuclear factor kappa B (NF-kB) suppression (66, 68, 94), unequivocally linking key processes in BPD development such as infection and inflammation with altered growth factor signaling and transcriptional regulation. The characteristic co-existence of defective alveolar and capillary formation is determined by impaired angiogenic growth factor signaling (67) and ultimately results in sustained vascular disease, in many cases presenting as PAH and/or impaired lung lymphatic drainage (95, 96). The typical reduction in pulmonary expression levels of VEGF and its receptors (97–99), accompanied by diminished endothelial nitric oxide synthase (eNOS) and soluble guanylate cyclase (sGC) in lung blood vessels and airways (100, 101) reflects the expression pattern observed in aged mice (102) and likely contributes to the reduced plasticity of lung capillaries (103). Treatment with recombinant human VEGF during or after hyperoxia exposure improved not only vessel growth but also alveolarization in the lungs of newborn rats (104).

Both direct effects of shear stress, oxygen toxicity, inflammation, and hormonal regulation as well as subsequent impairment of growth factor signaling alters critical events in endoderm to mesoderm transition and myofibroblast proliferation and leads to severe alterations of the pulmonary scaffold (105, 106). Increased matrix remodeling characterized by the greater abundance and abnormal distribution of elastin together with the deformation of collagen scaffolding has been demonstrated in humans and animal models (107–109). The degradation of lung elastin is indicated by e.g., increased urinary excretion of desmosine, preceded, and paralleled by increased elastase activity (110–112). Desmosine, a breakdown product of the mature elastic fiber was found to predict disease severity and outcome in adult patients with acute respiratory distress syndrome (ARDS) (113), indicating the importance of the delicate balance of proteases and their inhibitors. Complicating the definition of friend or foe in the developing organ, the presence of elastases including metalloproteinase activity is crucial as evidenced by studies showing that complete matrix-metalloproteinase deficiency promotes lung remodeling resembling BPD (114).

The significant changes to the structural integrity of the ECM not only affect its function as a scaffold for the formation of alveoli and capillaries but as well-reveal a significant memory function through defining the fate of cells populating the

developing organ (115, 116). The sustained and irreversible reorganization will furthermore result in long term effects with regard to the lung's repair and regeneration capacity, its potential for immune cell interaction, thereby determining its coping with environmental challenges, exacerbation episodes and physiologic aging (117, 118).

Short and Long-Term Pulmonary Function in Preterm Infants With BPD

With increasing survival of infants with BPD, attempts to minimize long-term pulmonary impairment (and associated neurologic complications) has become the main focus of perinatal care (119, 120). Nonetheless, respiratory symptom presentation and suboptimal lung function are manifesting in adult life, and where detected, can be misclassified as more common respiratory diagnoses such as asthma or COPD, particularly if the early life events are not known or asked about (121, 122). In many cases, respiratory disease is not detected until acute presentation or much later in life. As respiratory function serves as a good predictor of later morbidity and mortality (123), knowledge about early changes seems crucial.

After birth, early pulmonary dysfunction is characterized by diminished lung compliance, tachypnea, and increased minute ventilation resulting in increased work of breathing with or without subsequent oxygen dependency. This clinical picture can be accompanied by an increase in lung microvascular filtration pressure that may lead to interstitial pulmonary edema as shown in animal experiments (124). The increased lung vascular resistance, typically associated with impaired responsiveness to inhaled nitric oxide and other vasodilators, can progress to reversible or sustained PAH and right heart failure (95, 96). Early measurement of lung function provides prognostic information and has shown that postnatal development of severe lung disease more likely develops into chronic disease at term (125). At this time point BPD infants present with increased respiratory tract resistance and hyper-reactive airways (126), subsequently leading to frequent episodes of bronchoconstriction and cyanosis after discharge often resulting in hospital readmission. The proportion of underlying vascular disease playing a role in these clinical manifestations beyond the effects caused by the Euler-Liljestrand mechanism (hypoxic pulmonary vasoconstriction) often remains unclear as sensitive diagnostic tools are missing (127).

In the later course following discharge, infants with BPD may remain oxygen dependent for months or years, although only a minority remains oxygen dependent beyond 2 years of age (128, 129). Oxygen dependency indicates the most severe lung disease, as these infants require hospital readmission twice as often in comparison to infants without home oxygen therapy. However, even after having outgrown oxygen dependency, patients with moderate or severe BPD still require outpatient clinic visits, readmissions, and medication in up to 70% of the cases and 30% need three readmissions in the first 2 years of life (130). A major predictor for readmission due to respiratory causes or the need for subsequent mechanical ventilation is the pCO_2 at discharge (131). After the second year of life, hospitalization rates decline (132). Related to prematurity beyond BPD status, lower respiratory tract infections resulting from respiratory syncytial virus remain the major cause for readmission amongst preterm infants (133).

In the later course of disease, BPD is a significant risk factor for persistent wheeze and the need for inhalation therapy (odds ratio 2.7 and 2.4, respectively) affecting about 20–30% of infants with BPD at 6 and 12 months of age (134, 135). Respiratory symptoms remain common at preschool and school age (128, 136). Up to 80% of preterm infants, particularly those who presented with wheezing, demonstrate airway obstruction in early childhood and adolescence, the majority of whom are symptomatic (137–139). Important data on long-term pulmonary function in BPD patients were generated by the EPICure study (140), showing significantly lower peak oxygen consumption, forced expiratory volume at 1 second (FEV_1) and gas transfer for those born extremely premature at school age when compared to age matched controls, not considering BPD status. Mean difference in FEV_1 sum up to a total of 600 ml when comparing infants with extreme prematurity at birth and the respective healthy controls. Significantly lower peak workload and higher respiratory rates in combination with lower tidal volumes during peak exercise and increased residual capacity in these infants may reflect the effect of hyperinflation due to airway obstruction and/or altered pulmonary chemoreceptor function, and suggest the presence of persistent airflow limitations and reductions in alveolar surface area.

In most severe cases symptoms either persist into adulthood (141) or show transient "improvement" reflected by the absence of symptoms, later resulting in the "reappearance" of disease as a consequence of lung function decline below a clinical (or individual) threshold when the disease associated reduction in lung volume and function is met by aging processes or a newly occurring mismatch of the lung-body mass ratio and/or energy expenditure. Altered lung volumes and decreased gas mixing efficiency in BPD has been confirmed by various studies, reflecting abnormalities in lung growth (142, 143), resulting in suboptimal airway function (judging from impaired FEV1 and FEV1/forced vital capacity) in young adults (144, 145), also manifesting in suboptimal exercise capacity (145). While a diverging path in lung growth during adolescence according to spirometric measures was reported by one group (144), the EPICure study revealed no catch-up of the suboptimal lung growth from 11 to 19 years in adolescents following extreme prematurity irrespective of BPD status and even showed significant impairment in all lung function parameters in 19 year old patients born extremely premature (145). Meanwhile, Vollsaeter et al. reported parallel trajectories of lung function in early adult life (146), reflecting the overall need for more robust and contemporaneous studies.

Taken together, the functional data available suggest that early lung injury in preterm infants leads to abnormalities in lung function (and immunity) in infancy and early childhood with significant pulmonary problems persisting in the most severely affected infants. The predisposition for lung function decline in adult survivors of preterm birth is suggested by data obtained in early and later adulthood with lung function measurements in infancy being an important predictor for later

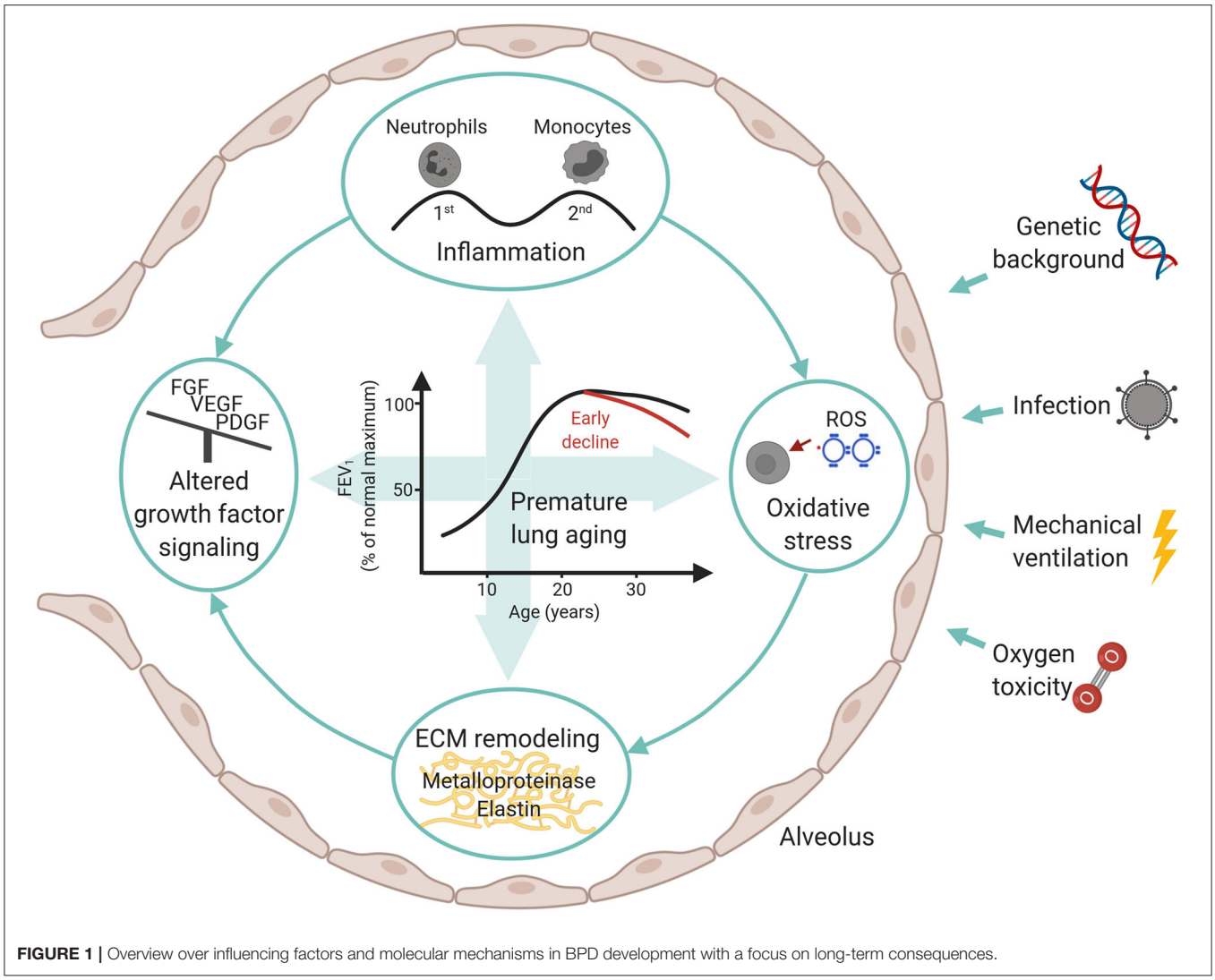

FIGURE 1 | Overview over influencing factors and molecular mechanisms in BPD development with a focus on long-term consequences.

lung disease and the risk for (early) lung function decline. If performed during infancy and childhood, pulmonary function tests have the potential to identify individuals at risk of long-term respiratory sequelae and will help to elucidate the impact of secondary injuries, e.g., first and second hand smoke as well as viral infections on this trajectory (147). Importantly, lung function has to be interpreted in the context of the era in which BPD was diagnosed, thus taking both the underlying BPD definition as well as the standards of perinatal care into account (2, 148, 149).

CONCLUSION

To conclude, the unique response of the developing lung to early postnatal injury is characterized by sustained inflammatory processes, ECM remodeling and a pronounced alteration in growth factor signaling that ultimately results in the characteristic histopathologic picture of impaired alveolar and vascular development. These processes are critically determined by the pulmonary capacity to respond to and compensate for environmental challenges inducing oxidative stress and imbalance in growth factor signaling (**Figure 1**). We now understand that the effects provoked through early organ injury lead to a characteristic response to challenges later in life and an altered lung function trajectory due to differences in the pulmonary aging process. Subsequently, treatment strategies and life-style recommendations advocated to this patient population need to acknowledge the pulmonary "memory" effects that results from early injury as well as later disease characteristics and co-morbidity development (150, 151). Therefore, pediatricians, primary care physicians and adult pulmonologists need a close and iterative knowledge exchange to adequately address these topics, including the role of second and third hits in lung function decline (152, 153).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Construction of a Nomogram for Predicting Survival in Elderly Patients With Lung Adenocarcinoma: A Retrospective Cohort Study

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Elderly patients with non-small-cell lung cancer (NSCLC) exhibit worse reactions to anticancer treatments. Adenocarcinoma (AC) is the predominant histologic subtype of NSCLC, is diverse and heterogeneous, and shows different outcomes and responses to treatment. The aim of this study was to establish a nomogram that includes the important prognostic factors based on the Surveillance, Epidemiology, and End Results (SEER) database from 2010 to 2015. We collected 53,694 patients of older than 60 who have been diagnosed with lung AC from the SEER database. Univariate and multivariate Cox regression analyses were used to screen the independent prognostic factors, which were used to construct a nomogram for predicting survival rates in elderly AC patients. The nomogram was evaluated using the concordance index (C-index), calibration curves, net reclassification index (NRI), integrated discrimination improvement (IDI), and decision-curve analysis (DCA). Elderly AC patients were randomly divided into a training cohort and validation cohort. The nomogram model included the following 11 prognostic factors: age, sex, race, marital status, tumor site, histologic grade, American Joint Committee for Cancer (AJCC) stage, surgery status, radiotherapy status, chemotherapy status, and insurance type. The C-indexes of the training and validation cohorts for cancer-specific survival (CSS) (0.832 and 0.832, respectively) based on the nomogram model were higher than those of the AJCC model (0.777 and 0.774, respectively). The CSS discrimination performance as indicated by the AUC was better in the nomogram model than the AJCC model at 1, 3, and 5 years in both the training cohort (0.888 vs. 0.833, 0.887 vs. 0.837, and 0.876 vs. 0.830, respectively) and the validation cohort (0.890 vs. 0.832, 0.883 vs. 0.834, and 0.880 vs. 0.831, respectively). The predicted CSS probabilities showed optimal agreement with the actual observations in nomogram calibration plots. The NRI, IDI, and DCA for the 1-, 3-, and 5-year follow-up examinations verified the clinical usability and practical decision-making effects of the new model. We have developed a reliable nomogram for determining the prognosis of elderly AC patients, which demonstrated excellent discrimination and clinical usability and more accurate prognosis predictions. The nomogram may improve clinical decision-making and prognosis predictions for elderly AC patients.

Keywords: non-small-cell lung cancer, adenocarcinoma, nomogram, elderly patients, survival prediction

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INTRODUCTION

Lung cancer is the second common cancer worldwide and the leading cause of cancer deaths (1). Non-small-cell lung cancer (NSCLC) accounts for ∼85% of all lung cancer cases, with a 5-year relative survival rate of 23% (2). The elderly make up 76% of lung cancer survivors, with the median age at the diagnosis of lung cancer being 70 years (2). Age is associated with the prognosis of NSCLC patients, such as tumor recurrence and metastasis (3–5). Elderly NSCLC patients exhibit worse tolerance to surgery, radiotherapy, and chemotherapy, and therefore have worse compliance and increased side effects of anticancer treatment. The aging of organs accompanied by a decline in immune function in elderly patients increases the probability of tumor recurrence.

Adenocarcinoma (AC) is the predominant histologic subtype of NSCLC, accounting for 48.2% of cases (6). AC patients benefit from therapies targeted against specific tumor mutations (7), such as angiogenesis inhibitors, epidermal growth factor receptor inhibitors, anaplastic lymphoma kinase inhibitors, and immunotherapy drugs (2, 8, 9) but their 5-year overall survival (OS) rates remain low (2, 10). The diversity and heterogeneity of AC is related to different outcomes and responses to treatment (11–14), and so distinct therapeutic approaches and management strategies should be provided to elderly AC patients. The TNM (Tumor-Node-Metastasis) staging system was employed mainly for deciding treatment option in clinical practice. At present, the TNM (Tumor-Node-Metastasis) staging system is also a tool generally employed by oncologist for prediction tumor prognosis (15). Although TNM as a tool predicting tumor prognostication is not as common as treatment decision-making, it is a gold standard for prognostication in oncology (16). Moreover, the TNM system has several drawbacks since different factors influence the course of cancer treatment and predicting survival (16). A comprehensive prognostic prediction model therefore needs to be established, including TNM system, to more accurately predict the prognosis of patients.

Nomograms have been accepted as reliable tools for visualizing risk by incorporating and illustrating important clinical oncology factors (17). Nomograms have been demonstrated to generate more precise predictions for several types of cancer when compared with the traditional TNM staging system (18–21). The aim of this study was to establish a comprehensive prognostic evaluation model of elderly lung AC patients by constructing a nomogram that includes significant risk factors and improves AC prognoses, based on patient data from the Surveillance, Epidemiology, and End Results (SEER) database.

MATERIALS AND METHODS

Patient Selection and Data Processing

Patient data were extracted from the latest version of the SEER database (which covers 18 registries) using SEER*Stat (version 8) software. We extracted the data of patients older than 60 years who had been diagnosed with lung AC from 2010 to 2015, totaling 103,681 cases. The evaluated variables were age, sex, race, marital status, tumor site, side (lateral or bilateral), histologic grade, AJCC stage, tumor size, metastasis site, surgery status, radiotherapy status, chemotherapy status, insurance type, follow-up time, tumor-specific death, and all-cause death. Cases without data on the above variables were excluded. Our selection criteria identified 53,694 patients who met the research conditions. The selected patients were randomly divided into training and validation cohorts with a ratio of 7:3 to construct and validate the nomogram (22). **Figure 1** displays a flow diagram of the patient selection process. All data were obtained free of charge from the SEER database, and this study abided by the Declaration of Helsinki and was approved by the medical ethics committee of Xi'an Jiaotong University Hospital. Informed consent was considered unnecessary for this study by the institutional review board due to its retrospective design.

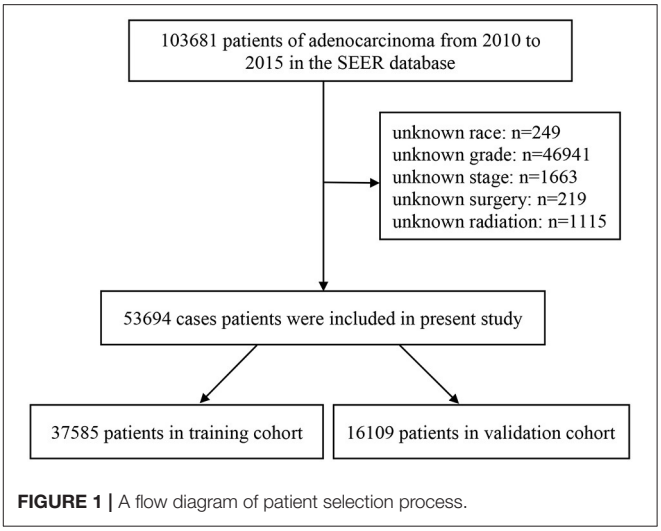

FIGURE 1 | A flow diagram of patient selection process.

Nomogram Establishment and Statistical Analyses

Differences in the baseline characteristics between the training and validation cohorts were determined using Pearson's χ^2 or Fisher's exact test. The variables influencing cancer-specific survival (CSS) and OS in both groups were identified using univariate and multivariate Cox proportional-hazards regression analyses. The prognostic factors identified in the multivariate analysis were used to construct the nomogram, which was tested internally and externally using the training and validation cohorts, respectively, for its ability to predict the 1-, 3-, and 5-year survival rates of NSCLC patients.

The concordance index (C-index) is the area under the receiver operating characteristic (ROC) curve (AUC) that plots the sensitivity against one minus the specificity of the nomogram. Hence, the C-index or AUC (which are often used interchangeably) for lung AC ranges from 0.5 to 1.0, with 0.5 indicating random chance and 1.0 indicating that the model was perfectly concordant with the data set. Discriminability is the accuracy in distinguishing between patients who did and did not experience an event. C-indexes and ROC curves were used to determine the discriminability of the nomogram. Calibration

curves were used to evaluate the actual outcome and the predicted probability based on C-indexes. The predictive power of the model was determined using C-indexes and calibration plots. Discrimination and calibration were both evaluated using 1,000-resample bootstrapping. The net reclassification index (NRI) and integrated discrimination improvement (IDI) were measured to compare the accuracy of the nomogram with the AJCC staging model. Decision-curve analyses (DCAs) tested the clinical value of the predictive models based on their threshold probabilities. The threshold probability was used to obtain the net benefit (defined as the proportion of true positives minus the proportion of false positives, weighted by the relative harm of false-positive, and false-negative results).

All statistical analyses were performed using SPSS or R software, with $P \leq 0.05$ indicating statistical significance.

RESULTS

Patient Characteristics

This study included 53,694 patients older than 60 years with lung AC between 2010 and 2015. The 53,694 cases were divided into a training cohort (37,585 patients) and a validation cohort (16,109 patients) using random-split sampling with a ratio of 7:3. Patients aged 60–80 years, female patients, and white patients accounted for 80, 53, and 82% of the sample, respectively. The main tumor sites were the upper and lower lobes of the lung, and almost all of the lesions (98%) were unilateral. The tumors were mostly at histologic grades II and III, while the AJCC stages were mostly advanced (48.4%) with distant metastases (37%). The proportions of patients who received surgery, radiotherapy, and chemotherapy were 50, 30, and 35%, respectively. Most patients had medical insurance. The median survival time was 14 months (range 4–31 months). Half of the patients died during the follow-up period. **Table 1** provides detailed information about the training and validation cohorts. In AJCC stage I -II patients, with increasing age, the ratio of surgical treatment gradually decreased, and that of radiotherapy gradually increased. From AJCC stage II to stage IV patients, the ratio of chemotherapy gradually reduced with age rise. **Supplementary Table 1** shows treatment information for elderly patients with lung AC.

Prognostic Factors for CSS and OS of Elderly AC Patients

The univariate and multivariate Cox proportional-hazards regression analyses selected 11 prognostic factors for screening in the training cohort. Among these factors, a higher risk of CSS in AC patients was associated with age at diagnosis (70–79 years, HR = 1.115, $P < 0.001$; ≥80 years, HR = 1.261, $P < 0.001$), male sex (HR = 1.354, $P < 0.001$), histologic grade (II, HR = 1.372, $P < 0.001$; III, HR = 1.921, $P < 0.001$; and IV, HR = 1.818, $P < 0.001$), AJCC stage (II, HR = 2.637, $P < 0.001$; III, HR = 4.318, $P < 0.001$; and IV, HR = 8.141, $P < 0.001$), no surgery (HR = 2.833, $P < 0.001$), and no chemotherapy (HR = 1.877, $P < 0.001$), while the risk was lower for Asian or Pacific Islander race (HR = 0.749, $P < 0.001$) and tumor sites of the upper lobe (HR = 0.697, $P < 0.001$), middle lobe (HR = 0.709, $P < 0.001$), lower lobe (HR = 0.752, $P < 0.001$), and no otherwise specified lung cancer (NOS) (HR = 0.812, $P < 0.001$) (**Table 2**). **Supplementary Table 2** lists the prognostic factors associated with OS in elderly AC patients.

Nomogram Construction

A nomogram was constructed for predicting the 1-, 3-, and 5-year CSS of lung AC patients according to the prognostic factors selected from the training cohort (**Figure 2**). The CSS nomogram indicated that AJCC stage was the strongest prognostic factor, followed by surgery status, histologic grade, and chemotherapy status with a greater impact on nomogram. Patients in AJCC stages I and II, or who received surgery, or who received chemotherapy had longer CSS and OS (**Figure 3**). Other significant prognostic factors were tumor site, race, sex, age, marital status, radiotherapy status, and insurance type. Patients older than 80 years at diagnosis had poor CSS and OS (**Figure 3**).

Each level of each factor was given a score on the points scale of the nomogram. The final risk score was calculated by the sum of the score of each selected factor using the nomogram, as depicted in **Figure 2**, which estimated the 1-, 3-, and 5-year CSS probabilities for individual patients based on a vertical line from the total-points row. The OS nomogram was developed using the same method, as shown in **Supplementary Figure 1**.

Nomogram Performance

The C-indexes [nomogram C-indexes >0.70 indicate a high predictive accuracy for CSS (23)] were higher for the nomogram model (0.832 and 0.832 in the training and validation cohorts, respectively) than the AJCC staging model (0.777 and 0.774, respectively). The CSS discrimination performance as indicated by the AUC was better in the nomogram model than the AJCC staging model at 1, 3, and 5 years in both the training cohort [0.888 vs. 0.833, 0.887 vs. 0.837, and 0.876 vs. 0.830, respectively (**Figure 4**)] and the validation cohort [0.890 vs. 0.832, 0.883 vs. 0.834, and 0.880 vs. 0.831, respectively (**Figure 5**)]. The predicted 1-, 3-, and 5-year CSS probabilities corresponded with the actual observations in both the training (**Figure 4**) and validation (**Figure 5**) cohorts in calibration plots of the nomogram. The related results for OS are shown in **Supplementary Figures 2, 3**.

In the training cohort, the NRI values for the 1-, 3-, and 5-year CSS follow-up examinations were 0.424 (95% CI = 0.401–0.447), 0.496 (95% CI = 0.471–0.538), and 0.294 (95% CI = 0.254–0.317), respectively. The corresponding NRI values in the validation cohort were 0.446 (95% CI = 0.400–0.496), 0.484 (95% CI = 0.426–0.562), and 0.301 (95% CI = 0.229–0.355), respectively. Similarly, the IDI values for 1-, 3-, and 5-year CSS follow-up examinations were 0.060 ($P < 0.001$), 0.050 ($P < 0.001$), and 0.040 ($P < 0.001$), respectively, in the training cohort, and 0.060 ($P < 0.001$), 0.042 ($P < 0.001$), and 0.067 ($P < 0.001$) in the validation cohort. These results indicate that our model greatly improves the accuracy of prognostic predictions over the AJCC staging model.

The DCAs of CSS compared the net benefits of the new model with those of the AJCC staging model. As shown in **Figure 6**, 1-, 3-, and 5-year outcomes of our nomogram were superior to those of the AJCC staging model across various death risk factors in the training and validation cohorts. This verifies the clinical usability

TABLE 1 | Patients' demographics and clinicopathological characteristics.

Variable	Total cohort, *n* (%)	Training cohort, *n* (%)	Validation cohort, *n* (%)	*P*-value
	53,694 (100%)	37,585 (70%)	16,109 (30%)	
Age, years				0.551
60–69	21,078 (39.26%)	14,730 (39.19%)	6,348 (39.41%)	
70–79	22,011 (40.99%)	15,462 (41.14%)	6,549 (40.65%)	
≥80	10,605 (19.75%)	7,393 (19.67%)	3,212 (19.94%)	
Sex, *n*				0.427
Female	28,589 (53.24%)	20,054 (53.36%)	8,535 (52.98%)	
Male	25,105 (46.76%)	17,531 (46.64%)	7,574 (47.02%)	
Race, *n*				0.828
White	44,185 (82.29%)	30,954 (82.36%)	13,231 (82.13%)	
Black	5,064 (9.43%)	3,516 (9.35%)	1,548 (9.61%)	
Asian or Pacific Islander	4,244 (7.90%)	2,973 (7.91%)	1,271 (7.89%)	
American Indian/Alaska Native	201 (0.37%)	142 (0.38%)	59 (0.37%)	
Marital status, *n*				0.428
Married	29,172 (54.33%)	20,419 (54.33%)	8,753 (54.34%)	
Single	22,370 (41.66%)	15,686 (41.73%)	6,684 (41.49%)	
Unknown	2,152 (4.01%)	1,480 (3.94%)	672 (4.17%)	
Tumor site, *n*				0.310
Main bronchus	753 (1.40%)	524 (1.39%)	229 (1.42%)	
Upper lobe	30,190 (56.23%)	21,119 (56.19%)	9,071 (56.31%)	
Middle lobe	2,660 (4.95%)	1,873 (4.98%)	787 (4.89%)	
Lower lobe	16,520 (30.77%)	11,623 (30.92%)	4,897 (30.40%)	
Overlapping lesion	526 (0.98%)	369 (0.98%)	157 (0.97%)	
NOS	3,045 (5.67%)	2,077 (5.53%)	968 (6.01%)	
Lateral, *n*				0.336
One side	52,957 (98.63%)	37,081 (98.66%)	15,876 (98.55%)	
Bilateral	737 (1.37%)	504 (1.34%)	233 (1.45%)	
Grade, *n*				0.066
I	10,366 (19.31%)	7,284 (19.38%)	3,082 (19.13%)	
II	21,484 (40.01%)	15,126 (40.24%)	6,358 (39.47%)	
III	21,411 (39.88%)	14,864 (39.55%)	6,547 (40.64%)	
IV	433 (0.81%)	311 (0.83%)	122 (0.76%)	
AJCC Stage, *n*				0.376
I	21,592 (40.21%)	15,155 (40.32%)	6,437 (39.96%)	
II	6,114 (11.39%)	4,238 (11.28%)	1,876 (11.65%)	
III	8,650 (16.11%)	6,096 (16.22%)	2,554 (15.85%)	
IV	17,338 (32.29%)	12,096 (32.18%)	5242 (32.54%)	
Tumor size, *n*				0.396
≤3 cm	27,429 (51.08%)	19,296 (51.34%)	8,133 (50.49%)	
3–5 cm	12,525 (23.33%)	8,752 (23.29%)	3,773 (23.42%)	
5–7 cm	4,891 (9.11%)	3,391 (9.02%)	1,500 (9.31%)	
≥7 cm	4,309 (8.03%)	2,986 (7.94%)	1,323 (8.21%)	
Unknown	4,540 (8.46%)	3,160 (8.41%)	1,380 (8.57%)	
Bone metastasis, *n*				0.922
Yes	6,272 (11.68%)	4,404 (11.72%)	1,868 (11.60%)	
No	46,731 (87.03%)	32,697 (86.99%)	14,034 (87.12%)	
Unknown	691 (1.29%)	484 (1.29%)	207 (1.28%)	
Brain metastasis, *n*	14 (4–31)	14 (4–31)		0.224
Yes	4,388 (8.17%)	3,023 (8.04%)	1,365 (8.47%)	
No	48,558 (90.43%)	34,032 (90.55%)	14,526 (90.17%)	
Unknown	748 (1.39%)	530 (1.41%)	218 (1.35%)	

(Continued)

TABLE 1 | Continued

Variable	Total cohort, *n* (%)	Training cohort, *n* (%)	Validation cohort, *n* (%)	*P*-value
Liver metastasis, *n*				0.396
Yes	2,246 (4.18%)	1,583 (4.21%)	663 (4.12%)	
No	50,634 (94.30%)	35,416 (94.23%)	15,218 (94.47%)	
Unknown	814 (1.52%)	586 (1.56%)	228 (1.42%)	
Lung metastasis, *n*				0.623
Yes	5,828 (10.85%)	4,050 (10.78%)	1,778 (11.04%)	
No	47,018 (87.57%)	32,946 (87.66%)	14,072 (87.35%)	
Unknown	848 (1.58%)	589 (1.57%)	259 (1.61%)	
Surgery, *n*				0.142
Yes	27,305 (50.85%)	19,191 (51.06%)	8,114 (50.37%)	
No	26,389 (49.15%)	18,394 (48.94%)	7,995 (49.63%)	
Radiation, *n*				0.363
Yes	15,612 (29.08%)	10,972 (29.19%)	4,640 (28.80%)	
No	38,082 (70.92%)	26,613 (70.81%)	11,469 (71.20%)	
Chemotherapy, *n*				0.211
Yes	18,625 (34.69%)	12,974 (34.52%)	5,651 (35.08%)	
No	35,069 (65.31%)	24,611 (65.48%)	10,458 (64.92%)	
Insurance, *n*				0.209
Yes	48,031 (89.45%)	33,662 (89.56%)	14,369 (89.20%)	
No	5,663 (10.55%)	3,923 (10.44%)	1,740 (10.80%)	
Vital status, *n*				0.140
Alive	27,190 (50.64%)	19,111 (50.85%)	8,079 (50.15%)	
Dead	26,504 (49.36%)	18,474 (49.15%)	8,030 (49.85%)	
Median follow-up time (Months, 25–75th percentile)	14 (4–31)	14 (4–31)	13 (4–31)	0.525

AJCC, the American joint committee for cancer; NOS, not otherwise specified lung cancer.

and practical decision-making effects of the new model. The results of DCAs of OS are shown in **Supplementary Figure 4**.

DISCUSSION

AC is the dominant pathologic subtype of NSCLC (6, 24), and is diverse and heterogeneous. Most elderly patients have already reached an advanced cancer stage at the time of diagnosis, resulting in a poor prognosis. Although the introduction of many antitumor drugs has improved patient survival, the 5-year survival rate remains very low. TNM staging system is often applied in clinical treatment decision-making, simultaneously, is also the gold standard for survival prediction for patients (16). But TNM staging system to predict the prognosis of lung cancer patients neglects independent prognostic factors such as sex, age, histologic grade, and treatment-related factors that could improve individualized survival predictions (18). Nomograms for predicting the survival outcomes of elderly AC patients are rare. It is therefore necessary to establish a prognostic prediction model that can assist clinicians in making treatment regimens for elderly AC patients. This was the first retrospective study that we know of that used an integrated index derived from the SEER database to establish a prognostic nomogram for predicting the survival rates of elderly AC patients.

There are some unique disease characteristics for elderly AC patients. Most of them were 60–80 years old and white. The main tumor sites were the upper and lower lobes of the lung, and most lesions were unilateral. The histologic grades of tumors were mostly II and III, while the AJCC stages were mostly advanced with distant metastases. A previous study (2, 20) similarly found that 76% of lung cancer patients were old and white, more than 90% of tumors were in the upper or lower lobes of the lung, and 80–90% of AC patients among the known pathological types had poorly or moderately differentiated histologic grades (25, 26). These characteristics are similar to those in our study. Moreover, AC patients of stage I–III accounted for 67%, and ∼50% received surgery in **Table 1**, which indicated that a higher proportion of patients with early stage adenocarcinoma undergone no surgical treatment. Elderly patients of stage I–II, from 60 to 79 years old, the surgery rates are more than 80%, while that of 80 years old or above is only 45.4% for stage I patients and 56.9% for stage II patients, respectively (**Supplementary Table 1**). For early stage patient surgical resection is the treatment of choice providing the best opportunity for cure and long-term survival. Still there is reluctance to recommend surgery for the elderly, partly based on the expectation that the rate of complications will be higher and elderly patients currently receive far higher rates of palliative care (27). Elderly patients are less likely to undergo curative surgery than younger patients for early-stage lung

TABLE 2 | Cox regression analysis based on all variables for cancer-specific survival (Training Cohort).

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age, years				
60–69	Reference		Reference	
70–79	1.061 (1.022–1.101)	0.002	1.115 (1.073–1.157)	<0.001
≥80	1.494 (1.432–1.560)	<0.001	1.261 (1.205–1.320)	<0.001
Sex, *n*				
Female	Reference		Reference	
Male	1.418 (1.373–1.465)	<0.001	1.354 (1.309–1.401)	<0.001
Race, *n*				
White	Reference		Reference	
Black	1.182 (1.119–1.247)	<0.001	0.936 (0.886–0.989)	0.019
Asian or Pacific Islander	0.905 (0.850–0.964)	0.002	0.749 (0.703–0.798)	<0.001
American Indian/Alaska Native	1.402 (1.103–1.783)	0.006	1.070 (0.841–1.361)	0.582
Marital status, *n*				
Married	Reference		Reference	
Single	1.142 (1.105–1.181)	<0.001	1.093 (1.055–1.132)	<0.001
Unknown	0.938 (0.859–1.026)	0.161	0.970 (0.887–1.060)	0.500
Primary Site, *n*				
Main bronchus	Reference		Reference	
Upper lobe	0.290 (0.262–0.322)	<0.001	0.697 (0.628–0.773)	<0.001
Middle lobe	0.280 (0.246–0.317)	<0.001	0.709 (0.625–0.805)	<0.001
Lower lobe	0.288 (0.259–0.320)	<0.001	0.752 (0.677–0.836)	<0.001
Overlapping lesion	0.452 (0.379–0.538)	<0.001	0.948 (0.795–1.130)	0.553
NOS	0.763 (0.680–0.855)	<0.001	0.812 (0.722–0.913)	<0.001
Lateral, *n*				
One side	Reference		Reference	
Bilateral	3.178 (2.851–3.542)	<0.001	0.949 (0.842–1.070)	0.395
Grade, *n*				
I	Reference		Reference	
II	1.582 (1.496–1.673)	<0.001	1.372 (1.296–1.451)	<0.001
III	3.466 (3.286–3.656)	<0.001	1.921 (1.818–2.029)	<0.001
IV	3.295 (2.809–3.866)	<0.001	1.818 (1.549–2.135)	<0.001
AJCC Stage, *n*				
I	Reference		Reference	
II	2.411 (2.246–2.588)	<0.001	2.637 (2.454–2.834)	<0.001
III	4.966 (4.690–5.257)	<0.001	4.318 (4.053–4.601)	<0.001
IV	12.774 (12.154–13.427)	<0.001	8.141 (7.654–8.658)	<0.001
Surgery, *n*				
Yes	Reference		Reference	
No	6.566 (6.314–6.828)	<0.001	2.833 (2.692–2.982)	<0.001
Radiation, *n*				
Yes	Reference		Reference	
No	0.530 (0.513–0.548)	<0.001	1.084 (1.045–1.124)	<0.001
Chemotherapy, *n*				
Yes	Reference		Reference	
No	0.588 (0.569–0.607)	<0.001	1.877 (1.809–1.948)	<0.001
Insurance, *n*				
Insured	Reference		Reference	
Uninsured	1.312 (1.247–1.380)	<0.001	1.092 (1.037–1.151)	<0.001

AJCC, the American joint committee for cancer; HR, hazard ratio; NOS, not otherwise specified lung cancer.

cancer although the cancer-related survival and OS are similar between older and younger patients (28). Octogenarians have poor surgery outcomes and should therefore prefer non-surgical treatments (24). 30% of the AC patients received radiotherapy in **Table 1**. In particular, the older and advanced patients more likely received radiation therapy (**Supplementary Table 1**). Stereotactic body radiation therapy is a reasonable option for high-risk surgical patients (28, 29). 35% of elderly patients with lung AC received chemotherapy in **Table 1**, which is lower than the data reported in the literature (30). Lung AC patients who underwent complete resection benefited from adjuvant chemotherapy (12, 31) or systemic chemotherapy with better survival (30). However, the older the patient, the less willing to receive chemotherapy (**Supplementary Table 1**). The median survival time was 14 months (range 4–31 months). Univariate and multivariate analyses identified 11 variables including age, sex, race, marital status, tumor site, histologic grade, AJCC stage, surgery status, radiotherapy status, chemotherapy status, and insurance type. CSS was worse in patients who had higher AJCC stages, no surgical treatment, no chemotherapy treatment, poor histologic grade, advanced age, male and single, while patients without main bronchus as the tumor site and who were Asian or Pacific Islander had longer CSS. The prognostic factors influencing OS were similar to those of CSS, which were TNM stage, no surgery, histologic grade, age, and sex.

A nomogram is a convenient graphical representation of a predictive model. This study established a new and comprehensive nomogram that combines various patient risk factors to improve prognosis predictions for elderly AC patients (**Figure 2** and **Supplementary Figure 1**). Compared to the traditional AJCC staging model, our nomogram was capable of providing more accurate assessments and predictions for lung AC patients (**Figures 4**, **5**). Our newly established model indicates that AJCC stage makes the greatest contribution to the prognostic score, which was similar to previous research where the 5-year overall survival rate of AC ranged from 79% for disease stage IA to 6% for stage IV (25). AC was associated with a higher risk of developing bone (32) and brain metastases (33). Our analysis indicated that surgery status, chemotherapy status, and histologic grade had greater impacts on patient survival. Surgical treatment benefits octogenarians with AC patients (34), especially for those in stage I and II (35). Chemotherapy significantly improved patient prognoses and prolonged the survival of elderly patients (30, 36). Integration of geriatric assessments can improve risk stratification and

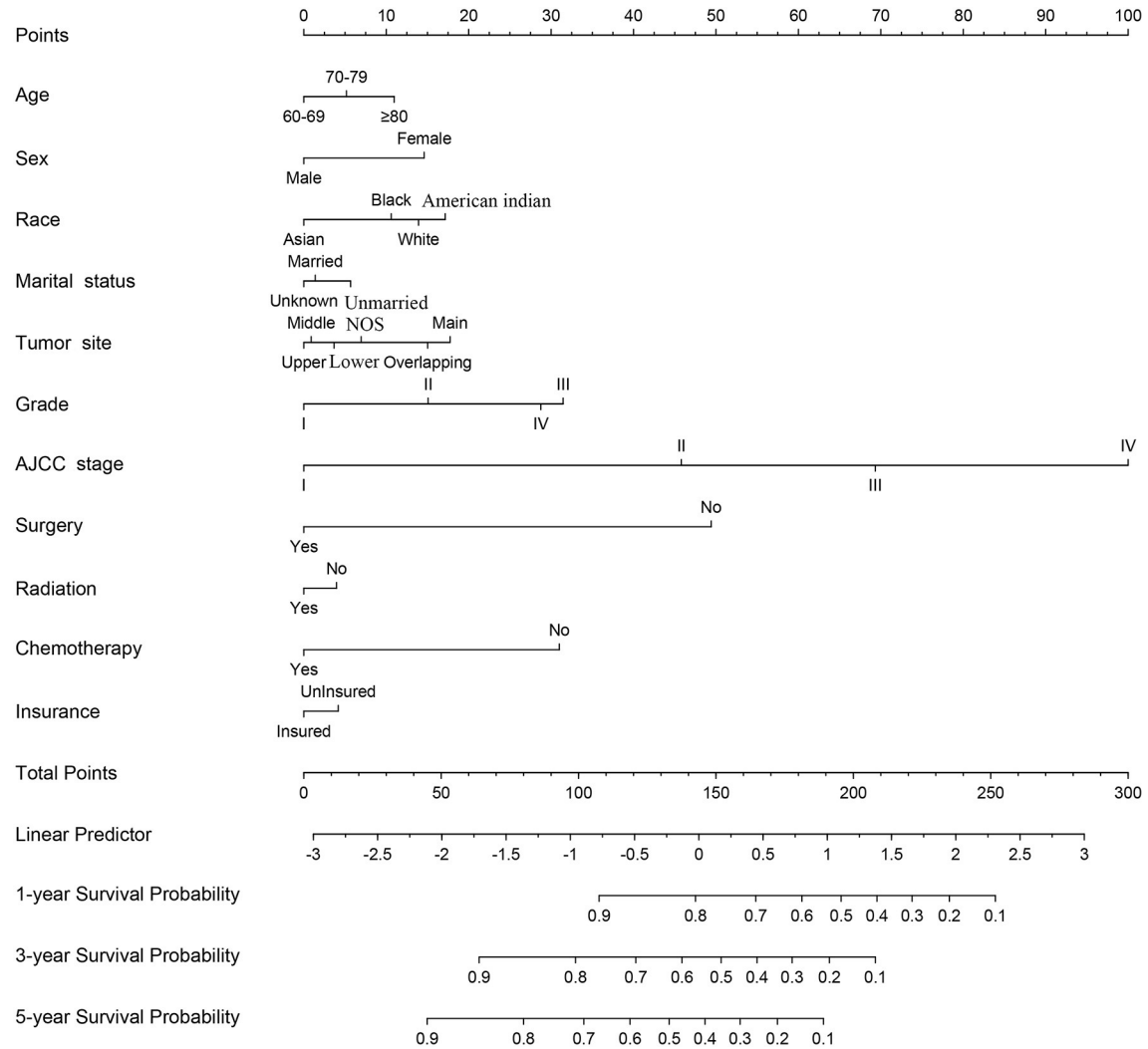

FIGURE 2 | Nomogram predicted 1-, 3-, and 5-year lung adenocarcinoma cancer-specific survival for patients with 11 available factors, including age, sex, race, marital status, tumor site, grade, AJCC stage, surgery, radiation, chemotherapy, and insurance. AJCC, the American Joint Committee for Cancer; NOS, not otherwise specified lung cancer.

improve clinical decision-making for patients (37). Histologic grade was a significant prognostic value for patient survival, and reflects the aggressiveness of lung tumors (38, 39). In particular, grade, surgery, and chemotherapy have greatly improved the performance of nomogram. Other factors also indicated as having prognostic value include patient age, sex, race, marital status, tumor site, radiotherapy status, and insurance type. These results were consistent with previous research (24). The prognostic factors of poorly differentiated tumor grade, male sex, increased age, late stage, and patient's performance status have been shown in multiple studies to have independent negative associations with long-term survival (40, 41).

Finally, the C-index, ROC curve, and calibration curve of our model were better in the validation cohort, indicating that it provides accuracy and reliable predictions (18, 20). The significantly higher C-index of the nomogram (in both cohorts) compared with the AJCC staging model indicates the good discrimination ability of the nomogram. This indicated that the model is very precise (42). In the current study, calibration plots of predictions corresponded well with actual observations indicated by the curve being close to the 45-degree line, verifying the repeatability and reliability of the established nomogram (20, 42). This is the first nomogram constructed to predict the survival of elderly AC patients that we know of. Both physicians and their patients can use the nomogram to individualize survival predictions. We believe that the nomogram is a more precise prognostic model than the AJCC staging model and other established prognostic models.

IDI and NRI were used to evaluate the performance and clinical application of the nomogram. Compared with the AJCC staging model, the nomogram has improved accuracy and discrimination of 1-, 3-, and 5-year survival predictions for

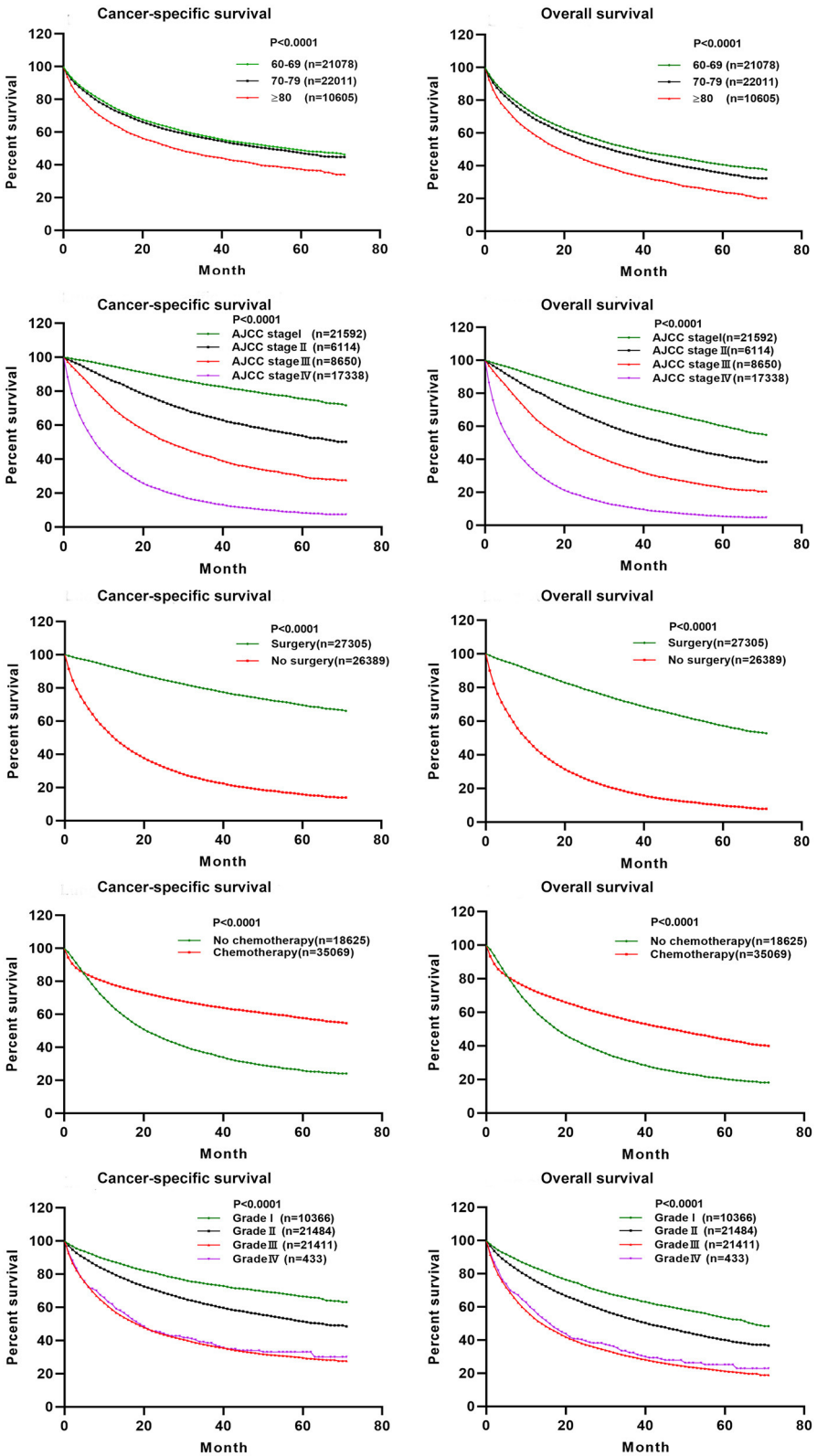

FIGURE 3 | The effect of AJCC staging, surgical treatment, chemotherapy treatment, histologic grade, and age at diagnosis on the cancer-specific survival and overall survival of elderly patients with lung adenocarcinoma. Kaplan–Meier curves for cancer-specific survival ($P < 0.001$) and overall survival ($P < 0.001$).

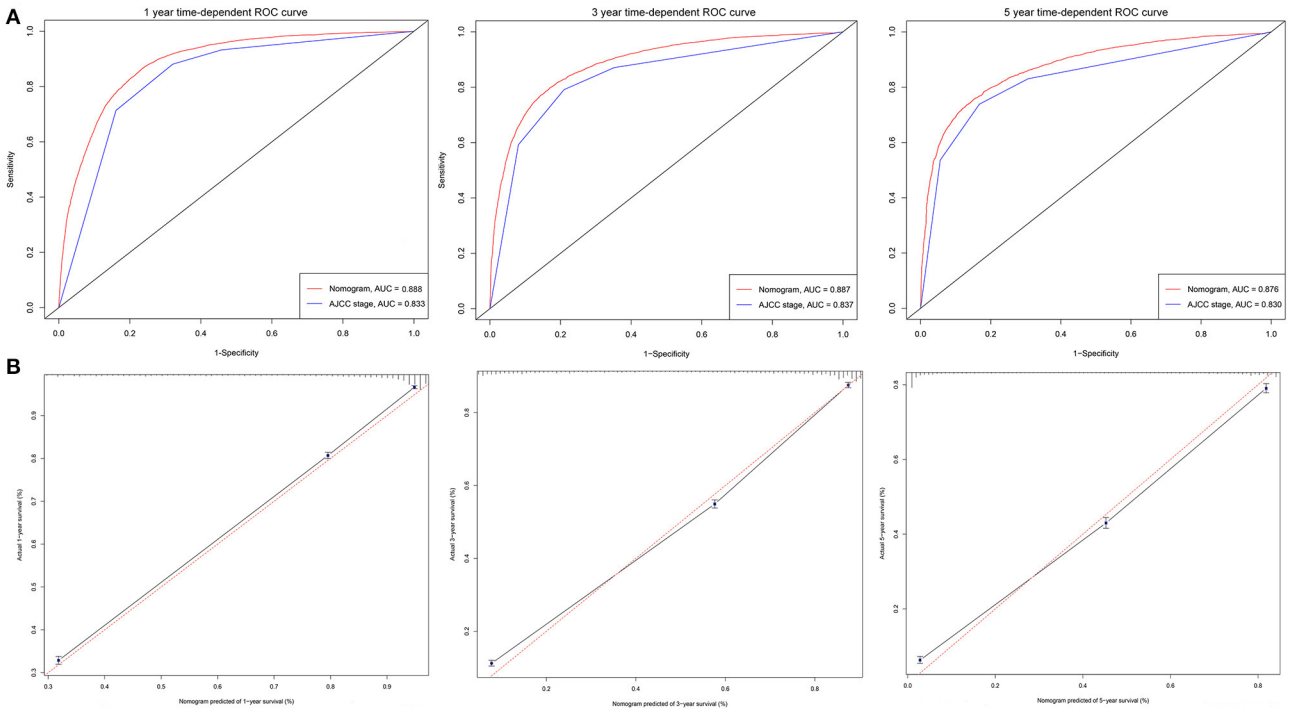

FIGURE 4 | ROC curves and calibration plots for predicting patients-specific survival at 1-, 3-, and 5-year in the training cohorts. **(A)** ROC curves of the Nomogram and AJCC stage in prediction of prognosis at 1-, 3-, and 5-year point in the training set. **(B)** The calibration plots for predicting patient survival at 1-, 3-, and 5-year point in the training set. ROC, receiver operating characteristic curve; AUC, areas under the ROC curve.

elderly AC patients. The nomogram had good discrimination and was well-calibrated, in which both IDI and NRI for 1, 3, and 5 years of follow-up examinations showed improvements in the C-index (20). DCA was also applied to compare the net benefits of the nomogram with those of the traditional AJCC staging model. Clinicians and patients can refer to the net benefit of our model according to their threshold probability during clinical decision-making. DCA values indicated that the newly established nomogram model had more practical and efficient survival predictions than the AJCC staging model (20). Our nomogram is an effective tool for predicting patient survival and optimizing treatment modalities in clinical practice.

This study was subject to several limitations. First, The SEER database does not include information on smoking history, radiotherapy doses, specific chemotherapy regimens, surgical methods, important molecular prognostic markers, comorbidity data, functional status, or other potentially important clinical information, which might reduce the predictive accuracy of the nomogram model. For example, targeted therapy and immunotherapy enhance response rates and prolong OS; The comorbidity data and functional status are most important part, closely related to the prognosis of elderly patients with lung AC. Karnofsky performance status for chemotherapy and anesthesia risk during the operation for elderly patients are important parameter in practice. Unfortunately, above information is not available in the SEER database. In the following research, these factors should be included in our model to achieve more comprehensive predictive ability for the prognosis of elderly AC patients. Second, this study was limited by collecting retrospective data from the SEER database, which may cause inherent and selection biases. The grade is also important factor of prognostic model. We screened patient data through strict inclusion and exclusion criteria. Consequently, a large number of patients without tumor grade information were excluded, which may affect the accuracy of model prediction. Finally, our nomogram is only constructed based on American patient data, and thus, may be underrepresented in the AC patients worldwide. In the following research, we would test the accuracy and generalizability of this model by external validation using Chinese patients or other populations with AC. Meanwhile, we will continue to optimize and improve this model by further clinical studies, hoping to finally have a better prognosis tool for patients with lung AC.

A nomogram for reliably determining the prognosis of elderly AC patients has been developed based on a large population sample. The nomogram includes 11 independent risk factors: AJCC stage, surgery status, chemotherapy status, histologic grade, radiotherapy status, age, sex, race, marital status, tumor site, and insurance type. Compared with the traditional AJCC staging model, the nomogram demonstrated excellent discrimination, and clinical usability, suggesting more accurate prognosis predictions for elderly AC patients. The nomogram may improve clinical decision-making as an auxiliary tool and provide accurate predictions of the prognosis of elderly AC patients.

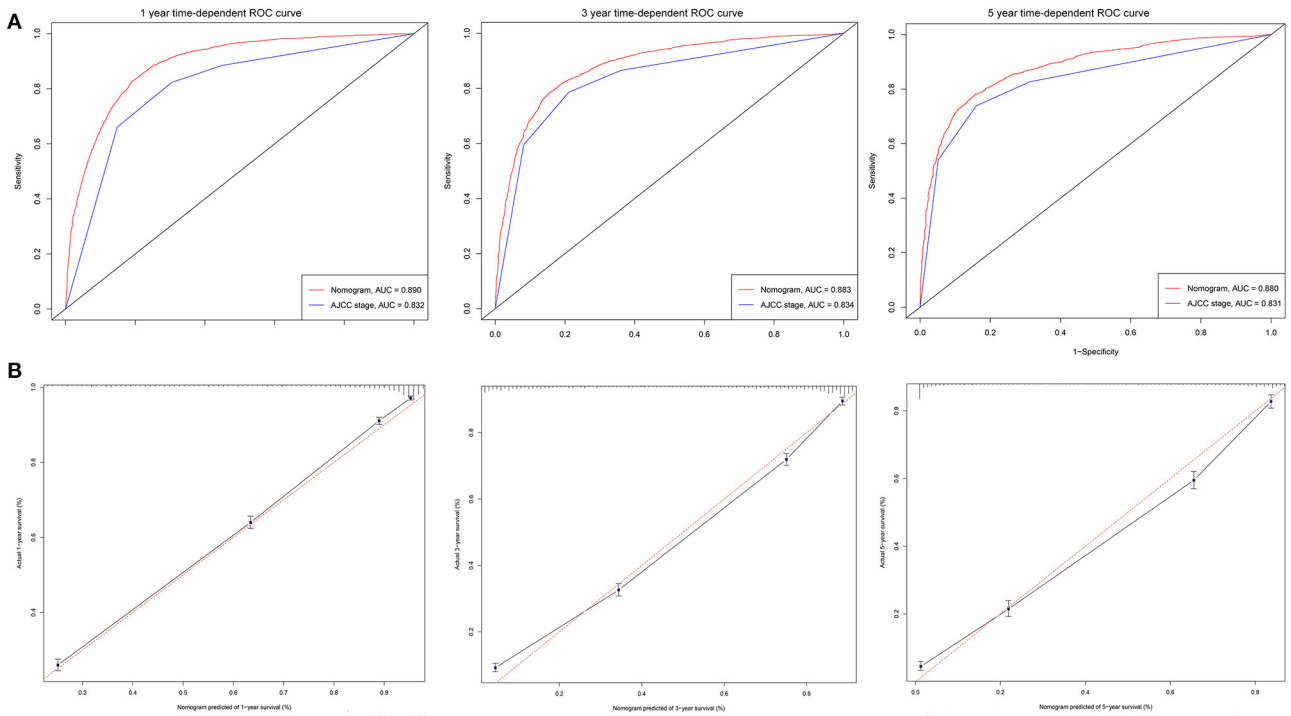

FIGURE 5 | ROC curves and calibration plots for predicting patients-specific survival at 1-, 3-, and 5-year in the validation cohorts. **(A)** ROC curves of the Nomogram and AJCC stage in prediction of prognosis at 1-, 3-, and 5-year point in the validation cohorts. **(B)** The calibration plots for predicting patient survival at 1-, 3-, and 5-year point in the validation cohorts. ROC, receiver operating characteristic curve; AUC, areas under the ROC curve.

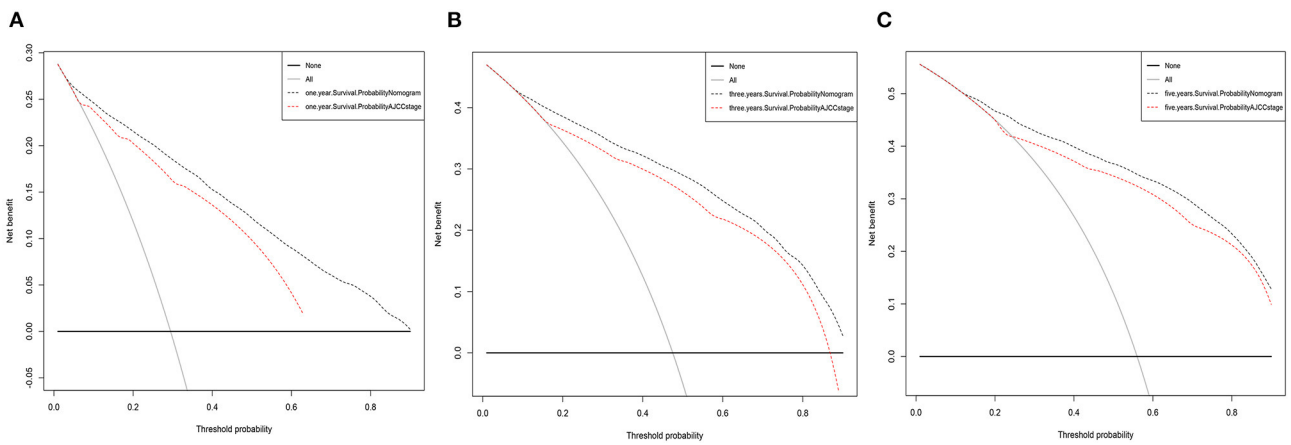

FIGURE 6 | Decision curve analysis for the Nomogram and AJCC stage in prediction of prognosis of elderly lung adenocarcinoma patients at 1-year **(A)**, 3-year **(B)**, and 5-year **(C)** CSS point in the validation cohorts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

All authors have signed the SEER Research Data Agreement to protect the privacy of patients, which is consistent with ethical principles.

AUTHOR CONTRIBUTIONS

HY, SC, and YD designed the experiments. MT, CG, BY, SH, and TW collected the data. HY and SC contributed to the statistical analysis of the data. HY wrote manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.680679/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Distribution of Pathogens in Elderly Chinese Patients With Pneumonia: A Systematic Review and Meta-Analysis

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Background: To summarize the distribution of pathogenic bacteria in elderly Chinese patients with pneumonia and provide guidance for the clinical application of antibiotics.

Methods: The electronic databases of PubMed, Embase, Cochrane library, and China National Knowledge Infrastructure were searched. The primary outcomes included the prevalence of gram-positive cocci, gram-negative bacilli, and fungus. The summary prevalence and 95% confidence interval (CI) were calculated using the random-effects model.

Results: A total of 17 retrospective studies reporting a total of 5,729 elderly patients with pneumonia were selected for final analysis. The summary prevalence of gram-positive cocci was 25% (95% CI: 20–30%; $p < 0.001$), whereas the prevalence of gram-negative bacilli was 56% (95% CI: 46–67%; $p < 0.001$). Moreover, the pooled prevalence of fungus in elderly patients with pneumonia was 11% (95% CI: 8–14%; $p < 0.001$). The most common gram-positive cocci were *Staphylococcus aureus* (ES: 8%; 95% CI: 6–11%; $p < 0.001$), *Streptococcus hemolyticus* (ES: 7%; 95% CI: 6–8%; $p < 0.001$), and *Streptococcus pneumoniae* (ES: 5%; 95% CI: 3–7%; $p < 0.001$). *Pseudomonas aeruginosa* (ES: 18%; 95% CI: 14–22%; $p < 0.001$) and *Klebsiella pneumoniae* (ES: 14%; 95% CI: 11–18%; $p < 0.001$) were most common gram-negative bacilli. Furthermore, the pooled prevalence of *Candida albicans* in elderly patients with pneumonia was 6% (95% CI: 5–8%; $p < 0.001$).

Conclusions: The findings demonstrated the comprehensive distribution of pathogenic bacteria in elderly Chinese patients with pneumonia, which could guide further antibiotic therapies.

Keywords: antibiotics, distribution of pathogens, elderly patients, pneumonia, clinical application

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INTRODUCTION

Pneumonia is the leading cause of infection-related deaths worldwide and the fourth-highest all-cause mortality in elderly patients (older than 65 years). It is characterized by cough, sputum production, dyspnea, and chest pain (1, 2). Underlying comorbid diseases, impaired mucociliary clearance, and waning immunity have been identified as risk factors for the incidence of pneumonia in elderly patients. The annual incidence of pneumonia in the elderly is nearly four times that of

younger populations (3). The number of elderly patients with pneumonia is rapidly increasing due to increasing sociodemographic aging, which has become a global problem. Moreover, the incidence of hospitalization due to pneumonia has significantly increased, and the burden of community-acquired pneumonia is more significant due to an expected 20% of the global population reaching elderly status by 2050 (4, 5).

Recently, the number of elderly patients with pneumonia has significantly increased in China due to the gradual increase in the aging population. Moreover, severe pneumonia was the main cause of death in elderly patients. Effective treatment strategies should be given to elderly patients with pneumonia to improve the prognosis through early diagnosis and treatment. Although there is the widespread use of vaccines and antibiotics, the prognosis for pneumonia in elderly individuals remains poor, and the pathogens were not systematically analyzed. Therefore, the current meta-analysis was conducted to illustrate the distribution of pathogenic bacteria in elderly Chinese patients with pneumonia, guiding the specific treatment strategies for such patients.

METHODS

Data Sources, Search Strategy, and Selection Criteria

This review was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Statement issued in 2009 (6). Any study investigating the distribution of pathogenic bacteria in elderly Chinese patients with pneumonia was eligible, and no restrictions were placed on publication status and language. Electronic searches of the PubMed, Embase, Cochrane library, and China National Knowledge Infrastructure databases were conducted for articles published in June 2019. The core search terms included "senile pneumonia" OR "elderly pneumonia" AND "pathogenic bacteria." The reference lists of retrieved studies were also reviewed to identify any new eligible studies.

Two authors independently evaluated and screened the potential studies. Any disagreement between these two authors was settled by group discussion or adjudicated by an additional author when necessary. The inclusion criteria for studies were as follows: (1) all participants diagnosed with pneumonia and aged $\geq$60 years; (2) patients received sputum culture analysis; (3) the study at least reported one of the prevalence of gram-positive cocci, gram-negative bacilli, and fungus. Moreover, the distribution of specific types of pathogenic bacteria was also summarized; and (4) prospective or retrospective study design.

Data Collection and Quality Assessment

Data from the included studies were independently abstracted and crosschecked by two authors using a standardized data extraction form, and any disagreement was settled by group discussion until a consensus was reached. The collected items included the first author's last name, publication year, study period, region, study design, sample size, age range, number of men and women, pneumonia subtypes, pathogen analysis, and the distribution of pathogenic bacteria. The quality of included studies was assessed by the Newcastle–Ottawa Scale, which is based on selection (four items: 4 stars), comparability (one item: 2 stars), and outcome (three items: 3 stars) (7). The "star system" for assessment of retrieved studies ranged from 0 to 9. Two authors independently evaluated the quality of included studies, and any disagreement was adjudicated by an additional author after referring to the original article.

Statistical Analysis

The prevalence (cases/patients) of gram-positive cocci, gram-negative bacilli, and fungus and the distribution of specific types of pathogenic bacteria were assigned as event and total sample size in each study. After that, the summary prevalence for investigated outcomes was calculated using the random-effects model (8, 9). The heterogeneity across included studies was assessed using I-square and Q statistic, and I-square $>$ 50.0% or $p < 0.10$ were considered as significant heterogeneity (10). Sensitivity analyses were conducted for gram-positive cocci, gram-negative bacilli, and fungus to assess the influence of every single study. Subgroup analyses for the prevalence of gram-positive cocci, gram-negative bacilli, and fungus were calculated based on mean age, percentage male, and study quality. Publication biases for investigated outcomes were evaluated using the funnel plots and Egger and Begg tests (11, 12). Moreover, the trim and fill method was used to adjust potential publication bias if significant publication bias was detected (13). All reported p-values are two-sided, and $p < 0.05$ was considered statistically significant for all included studies. Statistical analyses were performed using STATA software (version 10.0; Stata Corporation, College Station, TX, USA).

RESULTS

Literature Search

A total of 463 studies were identified in the initial search of the databases based on the search strategy mentioned earlier, of which 121 were excluded due to duplicate topics. An additional 317 studies were excluded because these were other types of articles (i.e., case reports, review articles, scientific abstracts) and studies not relevant to our study. The remaining 25 studies were retrieved for further evaluations, of which eight studies were excluded due to the following reasons: intervention study ($n = 4$), drug resistance study ($n = 3$), and review ($n = 1$). A total of 17 studies were selected for final analysis, and manual searching of the reference lists did not identify any new eligible study (14–30). The study selection process is presented in **Supplementary Figure 1**.

Study Characteristics

The 17 identified studies had a retrospective study design and included 5,729 elderly patients with pneumonia. The baseline characteristics of included studies and patients are summarized in **Supplementary Table 1**. The publication year ranged from 1996 to 2008, and 89–1,636 patients were included in each trial. The study period ranged from 1992 to 2016, and all patients received sputum culture analysis. Five studies included patients presented with community-acquired pneumonia and

hospital-acquired pneumonia, one study contained patients with community-acquired pneumonia, whereas the remaining 11 studies did not mention the pneumonia subtypes. All studies were published in Chinese, and the quality of included studies was low. The quality of included studies was assessed using the Newcastle–Ottawa Scale, and a study with 7–9 stars was regarded as high quality. Of the 17 included studies, six studies got 5 stars, nine studies with 4 stars, and the remaining two studies with 3 stars.

Gram-Positive Cocci

Data for the distribution of gram-positive cocci were available in 15 studies, and the summary prevalence of gram-positive cocci was 25% (95% CI: 20–30%; $p < 0.001$; **Figure 1A**). Moreover, substantial heterogeneity was detected among the included studies (I-square: 93.8%; $p < 0.001$). Sensitivity analysis indicated that the prevalence of gram-positive cocci ranged from 19 to 31% by sequentially excluding every individual study (**Supplementary Figure 2**). Moreover, potential significant publication bias for gram-positive cocci was detected (*p*-value for Egger: 0.030; *p*-value for Begg: 0.092; **Supplementary Figure 3**), and the prevalence of gram-positive cocci was 29% after adjustment using the trim and fill method (95% CI: 23–35%; $p < 0.001$; **Supplementary Figure 4**).

Gram-Negative Bacilli

Data for the distribution of gram-negative bacilli were available in 15 studies, and the pooled prevalence of gram-negative bacilli was 56% (95% CI: 46–67%; $p < 0.001$; **Figure 1B**). There was significant heterogeneity among the included studies (I-square: 98.5%; $p < 0.001$). Sensitivity analysis indicated that the prevalence of gram-negative bacilli ranged from 44 to 69% by sequentially excluding every individual study (**Supplementary Figure 5**). The Begg test indicated no significant publication bias for gram-negative bacilli ($p = 0.553$), whereas the Egger test indicated potential significant publication bias ($p = 0.011$) (**Supplementary Figure 6**). The prevalence of gram-negative bacilli was 67% after adjustment using the trim and fill method (95% CI: 42–90%; $p < 0.001$; **Supplementary Figure 7**).

Fungus

Data for the distribution of fungus were available in 14 studies, and the summary prevalence for fungus was 11% (95% CI: 8–14%; $p < 0.001$; **Figure 1C**). There was no significant heterogeneity among the included studies (I-square: 91.8%; $p < 0.001$). Sensitivity analyses indicated that the prevalence of fungus was 7–15% by sequentially excluding every individual study (**Supplementary Figure 8**). Moreover, there was significant publication bias for fungus (*p*-value for Egger: <0.001; *p*-value for Begg: 0.006; **Supplementary Figure 9**), and the prevalence of fungus was 9% after adjustment using the trim and fill method (95% CI: 6–12%; $p < 0.001$; **Supplementary Figure 10**).

Specific Type of Pathogenic Bacteria

The summarized results for the prevalence of specific type of pathogenic bacteria are presented in **Table 1**. The summary prevalence of *Staphylococcus aureus* (ES: 8%; 95% CI: 6–11%; $p < 0.001$), *Streptococcus hemolyticus* (ES: 7%; 95% CI: 6–8%; $p < 0.001$), and *Streptococcus pneumoniae* (ES: 5%; 95% CI: 3–7%; $p < 0.001$) indicated that they were the most common gram-positive cocci. Moreover, the pooled prevalence of *Staphylococcus epidermidis* and *coagulase-negative Staphylococcus* were 4% (95% CI: 3–6%; $p < 0.001$) and 3% (95% CI: 2–4%; $p < 0.001$), respectively. In addition, *Pseudomonas aeruginosa* (ES: 18%; 95% CI: 14–22%; $p < 0.001$) and *Klebsiella pneumoniae* (ES: 14%; 95% CI: 11–18%; $p < 0.001$) were the two most common gram-negative bacilli in elderly patients with pneumonia. Moreover, the prevalence of other specific types of gram-negative bacilli ranged from 1 to 8%. The pooled prevalence of *Candida albicans* in elderly patients with pneumonia was 6% (95% CI: 5–8%; $p < 0.001$), with no evidence of heterogeneity.

Subgroup Analyses

Subgroup analyses for the prevalence of gram-positive cocci, gram-negative bacilli, and fungus based on mean age, percentage male, and study quality were conducted (**Supplementary Table 2**). The prevalence of gram-positive cocci was high if the mean age was >75 years or the study was low quality. Moreover, patients aged ≥75 years, percentage male >70.0%, and study with high quality were associated with a high prevalence of gram-negative bacilli. The prevalence of fungus was high if the mean age of patients was >75 years, percentage male <70.0%, or study was low quality.

DISCUSSION

Pneumonia is the most common respiratory disease, and antibiotics are widely used for treating patients diagnosed with pneumonia. The incidence of pneumonia in the elderly is high due to organ function decline, cough reflex, and decrease in swallowing ability and bronchial mucociliary clearance. However, the data on the distribution of pathogenic bacteria in elderly Chinese patients with pneumonia are limited and inconclusive. The current quantitative meta-analysis recruited 5,729 elderly patients with pneumonia from 17 retrospective studies, with a wide range of patient characteristics. The findings of this study systematically reported the prevalence of gram-positive cocci, gram-negative bacilli, and fungus in elderly Chinese patients with pneumonia. Moreover, the prevalence of the specific type of pathogenic bacteria was illustrated. Furthermore, whether the prevalence of gram-positive cocci, gram-negative bacilli, and fungus are different according to mean age, percentage male, and study quality were assessed. The results of this study could guide the use of antimicrobial agents in elderly patients with pneumonia.

The current study indicated that the prevalence of gram-positive cocci in elderly patients with pneumonia was 25% (95% CI: 20–30%; $p < 0.001$), and the most common gram-positive cocci were *S. aureus*, *S. hemolyticus*, and *S. pneumoniae*. Xie et al. reported that the susceptibility rate of vancomycin was 100% for patients infected by gram-positive cocci, whereas the susceptibility to cefazolin sodium and ampicillin sodium was lower (15, 18). Moreover, Teng et al. suggested that gram-positive cocci are sensitive to vancomycin and teicoplanin

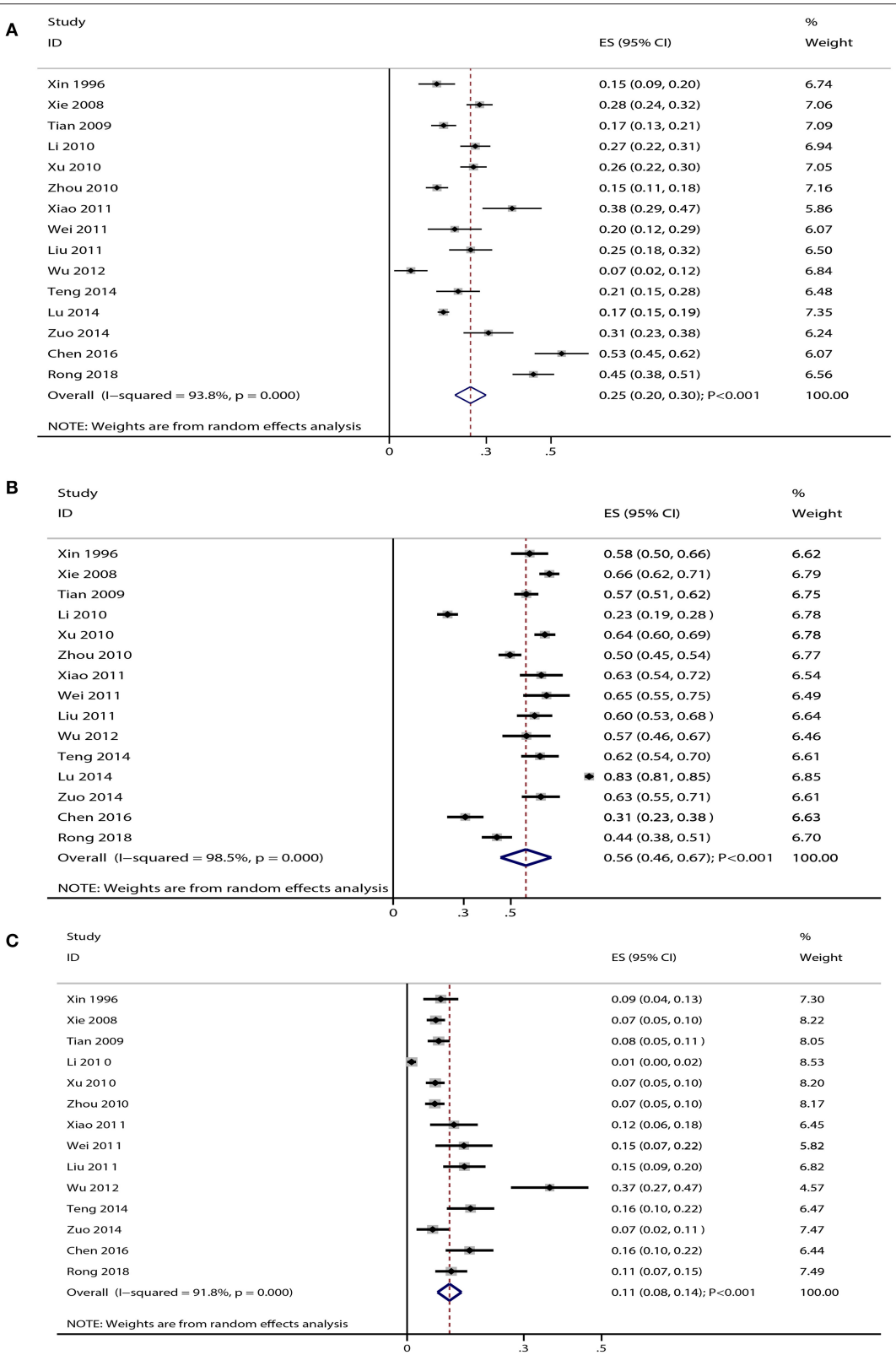

FIGURE 1 | (A) Summary prevalence for gram-positive cocci in elderly patients with pneumonia. **(B)** Summary prevalence for gram-negative bacilli in elderly patients with pneumonia. **(C)** Summary prevalence for fungus in elderly patients with pneumonia.

TABLE 1 | Summary results for specific pathogenic bacteria.

Pathogenic bacteria	Number of studies	Prevalence and 95% CI	*p*-value	Heterogeneity (%)	*p*-value for Heterogeneity	Egger test	Begg test
Staphylococcus aureus	16	0.08 (0.06–0.11)	<0.001	90.1	<0.001	0.017	0.034
Coagulase-negative staphylococcus	8	0.03 (0.02–0.04)	<0.001	65.4	0.005	0.005	0.009
Staphylococcus epidermidis	9	0.04 (0.03–0.06)	<0.001	79.2	<0.001	0.027	0.048
Streptococcus pneumoniae	12	0.05 (0.03–0.07)	<0.001	87.7	<0.001	0.004	0.003
Streptococcus hemolyticus	7	0.07 (0.06–0.08)	<0.001	0.0	0.965	0.352	0.368
Klebsiella pneumonia	17	0.14 (0.11–0.18)	<0.001	92.4	<0.001	0.200	0.127
Pseudomonas aeruginosa	16	0.18 (0.14–0.22)	<0.001	94.1	<0.001	0.026	0.444
Actinobacter baumannii	13	0.08 (0.06–0.11)	<0.001	92.1	<0.001	0.300	0.067
Escherichia coli	17	0.08 (0.07–0.09)	<0.001	65.7	<0.001	0.074	0.019
Enterobacter layer	4	0.07 (0.02–0.11)	0.002	92.3	0.001	0.010	0.308
Bacillus levans	13	0.03 (0.02–0.05)	<0.001	86.5	<0.001	0.007	0.200
Proteus vulgaris	5	0.02 (0.01–0.04)	0.010	87.6	<0.001	0.050	0.027
Stenotrophomonas maltophilia	13	0.04 (0.03–0.05)	<0.001	66.1	<0.001	0.013	0.033
Acinetobacter lwoffii	3	0.02 (0.01–0.02)	<0.001	0.0	0.872	0.163	0.296
Hemophilus parainfluenzae	6	0.03 (0.01–0.05)	0.001	88.5	<0.001	0.026	0.024
Citrobacter freundii	3	0.01 (0.00–0.02)	0.002	0.0	0.913	0.163	0.296
Pseudomonas alcaligenes	4	0.01 (0.00–0.02)	0.040	55.9	0.079	0.125	0.089
Candida albicans	8	0.06 (0.05–0.08)	<0.001	0.0	0.880	0.482	0.386

(26). Therefore, the sensitivity of gram-positive cocci to cephalosporins, penicillin, quinolones, and trimethoprim was low, whereas the sensitivity to vancomycin and teicoplanin was higher. The prevalence of gram-negative bacilli in elderly patients with pneumonia was 56% (95% CI: 46–67%; $p < 0.001$), and the most common gram-negative bacilli were *P. aeruginosa* and *K. pneumoniae*. Tian et al. reported that the resistance to ampicillin was highest, whereas resistance to imipenem was lowest, with the resistance rate from 0 to 14.3% for gram-negative bacilli (16). Xu et al. noted that the susceptibility rate of gram-negative bacilli to imipenem/cilastatin sodium reached 91% (18). The sensitivity of gram-negative bacilli to quinolones (ciprofloxacin, ofloxacin, ceftriaxone, and ceftazidime) and the third generation of cephalosporins was low, whereas the sensitivity to imipenem/cilastatin was high. The potential reasons for this could be: (1) quinolones are widely used as antimicrobial agents in China, and the pathogenic bacteria have high resistance to quinolones; (2) *P. aeruginosa*, *K. pneumoniae*, and other gram-negative bacilli could still induce gene mutation in beta-lactamase after treatment with the third generation of cephalosporins. Hence, it is necessary to formulate ultra-broad-spectrum beta-lactamase, which will be associated with a reduction in pneumonia pathogen susceptibility to antimicrobial agents. Therefore, the imipenem/cilastatin should be used for gram-negative bacilli owing to these antibiotics did not cross-resistance with other beta-lactamases (28). The prevalence of fungus in elderly patients with pneumonia was 11% (95% CI: 8–14%; $p < 0.001$), and the most common fungus was *C. albicans*. The potential reason for this could be because most elderly patients with lower respiratory tract infections have low resistance and comorbidity with other serious diseases. Moreover, the widespread use of broad-spectrum antibiotics and immunosuppressive agents causes susceptibility in patients. Furthermore, the *C. albicans* was contamination of upper airway secretion but not a pathogen for pneumonia. Patients presented positive for *C. albicans* could be caused by other pathogens. Therefore, an effective strategy should be used to prevent the spread of fungal infections.

Sensitivity analyses in the current study indicated the influence of a single study from the overall prevalence of gram-positive cocci, gram-negative bacilli, and fungus in elderly patients with pneumonia. The pooled prevalence for gram-positive cocci ranged from 20 to 30%, and the 95% CI for the prevalence of gram-positive cocci ranged from 19 to 31% by sequentially excluding every individual study, which indicated that the prevalence for gram-positive cocci was stable. Moreover, the summary prevalence for gram-negative bacilli ranged from 46 to 67%, and after sequentially excluding each study, the 95% CI for the prevalence of gram-negative bacilli ranged from 44 to 69%. The potential reason for this change could be the study conducted by Lu et al. (27), which specifically included elderly patients in an island area. The pooled prevalence for fungus in elderly patients with pneumonia ranged from 8 to 14%. The result of sensitivity analysis indicated that after sequentially excluding every individual study, the prevalence of fungus ranged from 7 to 15%, which indicated that the pooled prevalence of fungus in elderly patients with pneumonia had relatively high stability.

Subgroup analyses indicated that older patients could be easily infected with gram-positive cocci, gram-negative bacilli, and fungus, which might be the cause of the high risk of pneumonia in elderly patients. Moreover, percentage male >70.0% showed a relatively high prevalence of gram-negative bacilli, whereas the prevalence of fungus was relatively high when percentage male was <70.0%. These results suggested that males could be easily infected with gram-negative bacilli, whereas females had

a relatively high prevalence of fungal infection. The prevalence of gram-positive cocci, gram-negative bacilli, and fungus could be affected by the study quality, which is significantly associated with the reliability of abstracted data.

This study had several limitations. First, all the included studies had a retrospective design and uncontrolled selection. Hence, recall biases were inevitable. Second, all the included studies were of relatively low or moderate quality, and the summary results were restricted for clinical application. Third, the analysis of drug resistance was not available, which needs further study. Fourth, pathogen distribution might differ by region and pneumonia subtypes, whereas the stratified analyses based on these factors were not performed. Fifth, all of the included studies were performed in China, and the recommendation of results in our study to other countries was restricted. Finally, the analysis was based on published articles. Hence, publication bias was inevitable.

In summary, the findings of this study indicated that gram-negative bacilli were the most common bacterial infection in elderly patients with pneumonia, and the most common types of gram-negative bacilli were *P. aeruginosa* and *K. pneumoniae*. Moreover, *S. aureus*, *S. hemolyticus*, and *S. pneumoniae* were the most common gram-positive cocci in elderly patients with pneumonia. The most common fungus in elderly patients with pneumonia was *C. albicans*. Appropriate antibiotics should be applied based on the microbial surveillance data of each hospital.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LC substantially contributed to the conception, acquisition, analysis, and interpretation of data and drafted the manuscript for important content. HH contributed to design and critically revised the manuscript for important intellectual content. XC contributed to the acquisition of data and all authors gave final approval.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.584066/full#supplementary-material

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