



# Endoplasmic reticulum stress and glutathione therapeutics in chronic lung diseases



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## 1. Introduction

The lung is a unique organ, as it is constantly exposed to air and airborne pollutants including oxidant gases such as highly reactive nitrogen dioxide and ozone, particulates, viruses, other microbes, immune activators and irritants. Defense from these pollutants is provided by a number of cell types that include epithelial cells and macrophages. Differentiated ciliated airway epithelial cells contain cilia, specialized organelles that beat in waves to propel pathogens and inhaled particles trapped in the mucous layer out of the airways [1]. Secretory epithelial cells produce mucins, and an array of anti-microbial products [2]. Airway epithelial cells also express hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-producing dual oxidases (DUOX) providing defense from microbes and other irritants [3]. Located in the distal alveolar lung regions, type II epithelial cells produce surfactant proteins with anti-microbial or surfactant lowering activities [4]. Type II epithelial cells have been estimated to constitute 60% of alveolar epithelial cells and 10–15% of all lung cells. These cells are highly bioenergetically active due to their role in surfactant production, barrier protection, metabolism of xenobiotics, and as progenitor cells to replace damaged alveolar epithelial cells. The functions of distal epithelial cells and other cell types allow the lung to

function to exchange oxygen and carbon dioxide to allow for normal aerobic function [5].

The high metabolic demand on airway and alveolar type II epithelial cells which secrete mucins, surfactant proteins, cytokines, etc., can create stress in the endoplasmic reticulum (ER). A tightly regulated, evolutionary conserved stress response termed the unfolded protein response (UPR), is triggered to rectify the stress, thereby allowing adaptation. However, when the stress is excessive, apoptosis occurs [6]. ER stress has become a well-recognized feature of chronic lung diseases. Less well recognized is the fact that protein folding within the ER creates oxidants, and that the ER environment is oxidizing to allow disulfide bond formation in proteins to occur. Therefore, altered redox-based processes that accompany ER stress have the potential to contribute to chronic lung diseases.

Changes in the redox environment have long been implicated in the pathophysiology of a myriad of lung diseases that include cancer, asthma, acute respiratory distress syndrome, chronic obstructive pulmonary disease and pulmonary fibrosis. The low MW thiol molecule, glutathione (GSH) has been at the forefront of investigation in these chronic diseases, and oxidation of glutathione to its disulfide, (glutathione disulfide, GSSG) is a frequently used oxidative stress marker.

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While an increased oxidative burden originally was believed to contribute to lung injury, for instance by damaging epithelial cells, changes in the redox environment are now believed to promote lung disease via the oxidation of proteins, notably via the oxidation of reactive cysteines that typically occur in highly conserved regions. S-glutathionylation, the covalent attachment of glutathione to protein cysteines, is one of the numerous protein cysteine oxidations that has received considerable attention recently, given that S-glutathionylation affects numerous regulatory proteins and other redox systems. Dysregulation of S-glutathionylation is speculated to contribute to the pathogenesis of chronic lung diseases by affecting cellular pathways that govern death, proliferation, migration, plasticity, inflammation and production and profibrogenic factors [7–9]. Herein we will describe glutathione biochemistry, processes that regulate S-glutathionylation, and provide evidence that dysregulation of S-glutathionylation is associated with ER stress in settings of chronic lung diseases. Lastly, we provide a rationale for developing small molecules or biologics that harness S-glutathionylation chemistry for the treatment of chronic lung disease. Parts of this review were recently published as a book chapter entitled: “Redox Mechanisms in pulmonary disease: Emphasis on pulmonary fibrosis”, in “Oxidative eustress”, edited by Helmut Sies (Elsevier), and is expanded here with additional focus on ER stress, chronic obstructive pulmonary disease and asthma.

## 2. Chronic lung diseases: Pulmonary fibrosis, asthma and chronic obstructive pulmonary disease

### 2.1. Pulmonary fibrosis

Pulmonary fibrosis is an unrelenting progressive disease, characterized by a loss of normal alveolar architecture, repopulation of alveolar spaces with extracellular matrix, an overpopulation of activated myofibroblasts, and loss of alveolar epithelia [10]. While the causes of pulmonary fibrosis might be suspected in some cases, for instance as a result of exposure to asbestos or silica, in other cases the causes remain unknown. The diagnosis idiopathic pulmonary fibrosis (IPF) is made when other potential etiological factors have been excluded, and is based upon strict radiological criteria, sometimes also requiring histopathological confirmation of a pattern of usual interstitial pneumonia. Pulmonary fibrosis presents in patients in their 50–70's, and affects more men than women [10]. Each year, pulmonary fibrosis kills 40,000 people in the USA alone, and it affects over 5 million people worldwide [11]. The age-associated enhanced susceptibility to fibrosis is believed to reflect the lack of proper repair in an aging individual following repeated cumulative stresses [10]. Defective autophagy, telomere attrition, altered proteostasis, and cell senescence have been observed in epithelial cells and fibroblasts from IPF lungs, as compared to age-matched controls [12]. IPF is a dismal diagnosis, and current FDA-approved therapies, pirfenidone and nintedanib (marketed as Esbriet and OFEV, respectively), have limited effectiveness to halt progression of idiopathic pulmonary fibrosis (IPF), which typically leads to death of patients with IPF within 3–5 years from the time of diagnosis [10]. A number of environmental insults have been shown or are speculated to cause pulmonary fibrosis, that include inhalation of the aforementioned particulates, asbestos or silica, smoking, viral infections, radiation, etc. The chemotherapeutic drug, bleomycin, causes pulmonary fibrosis as a side effect, which limits its use as a chemotherapeutic modality. Radiotherapy for the treatment of lung cancer can also induce pulmonary fibrosis, one of its most common side effects [13]. Pulmonary fibrosis also can have a genetic component, and in cases of familial IPF, patients are known to have germline mutations in certain genes, including surfactant protein C (SFTPC) [14,15]. These genes encode proteins that are highly expressed in epithelia, and mutations in these genes result in defects in protein folding, leading to ER stress and mitochondrial dysfunction as will be further discussed below [16]. Besides IPF, a number of other disorders can result in lethal fibrosis, including

systemic sclerosis (SS) and Hermansky-Pudlak syndrome (HPS).

### 3. Asthma

Asthma is a complex spectrum of diseases that affects approximately 7% of the US population [17], and is characterized by symptoms such as wheezing, frequent cough, or shortness of breath, and is typically diagnosed by spirometry and methacholine challenge tests as well as allergen skin tests. Asthma is a chronic inflammatory disease characterized by increased numbers of eosinophils, T-Helper 2 (T<sub>H</sub>2) lymphocytes and activated mast cells and by structural changes in the airways, including epithelial cell mucus metaplasia, smooth muscle hypertrophy and hyperplasia, subepithelial fibrosis (with increased deposition of collagens, fibronectin, and tenascin), changes in submucosal gland cells, and increased blood vessel formation [18,19]. The subepithelial fibrosis in the airways of asthmatics differs anatomically from the parenchymal fibrosis seen in IPF, but the molecular pathways that underlie fibrotic remodeling may be quite similar [20]. The majority of asthmatics are hyperresponsive to common allergens such as house dust mite, cat dander, pollen, fungal allergens, etc. but a large subgroup of asthmatic patients displays a different phenotype not characterized by allergy. Asthma severity varies greatly and can be grouped into 4–5 phenotype clusters based on systematic analysis of age, gender, atopy, lung function, or obesity. These include large subgroups of early-onset asthma typically characterized by allergic sensitization and exacerbations (T<sub>2</sub> High Asthma), and distinct groups of adult-onset asthma which are often independent of allergy and atopy (T<sub>2</sub> Low Asthma) and are associated with obesity, smoking, and other factors [18,19] in addition to frequently being characterized by severe airway obstruction and exacerbations. Efforts to define asthma based on specific functional or pathobiological mechanisms also revealed different asthma endotypes, which may benefit from treatments specifically targeted at these causative molecular mechanisms. Treatment and management of asthma typically involves bronchodilators (e.g. long-acting beta agonists) and/or anti-inflammatory treatments that target eosinophilic inflammation (corticosteroids or biologics that directly target eosinophils or T<sub>H</sub>2 lymphocytes). However, certain subgroups of asthma do not benefit from such treatments, probably due to alternative underlying mechanisms such as neutrophilic inflammation and exacerbations due environmental pollutants or infections [21,22].

### 4. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is one of the most common lung diseases in the world, currently affecting over 251 million people. In 2015, it caused 5% of all deaths and the World Health Organization estimates that COPD will become the third leading cause of death by 2030. Symptoms include breathlessness, chronic cough and sputum production, which hinder patients in their daily activities as the disease worsens and greatly impact their quality of life. COPD is defined as a common, preventable and treatable disease characterized by persistent airflow limitation, due to airway and/or alveolar abnormalities caused by significant exposure to noxious particles or gasses [23]. The lack of reversal of airflow limitation after the administration of a short-acting bronchodilator distinguishes COPD from asthma. Current treatments are aimed at relieving symptoms and can slow down the progressive loss of lung function, but they fail to halt disease progression, and do not recover lung capacity that is lost. The loss of lung function in COPD is attributed to airflow obstruction, which is caused by chronic bronchitis, small airway remodeling and emphysema in varying combinations and severities. It is therefore not a single disease, but rather a set of complex conditions, each consisting of various components and clinical presentations [24]. Chronic bronchitis is an inflammatory state of the airways associated with bronchial wall thickening and excess mucus production arising from mucus hyperplasia. The small airways (< 2 mm) are affected by mucus plugging and inflammation, and the

mucosal thickening and increased deposition of extracellular matrix (ECM), a hallmark of fibrosis [25]. This sub-epithelial fibrosis is again different from IPF, but also in COPD molecular pathways that underlie fibrotic remodeling might be very similar [20]. Emphysema on the other hand, is defined by a loss of alveolar septae, which reduces the surface area available for gas exchange and can lead to hypoxia. In addition, it makes small airways more likely to collapse upon exhalation, resulting in further air trapping. Cellular damage to pneumocytes and degradation of alveolo-capillary membranes by an excess of proteases, are thought to underlie emphysema development. Importantly, the remaining alveolar walls in emphysema have been shown to display matrix alterations that are similar to the ones described in small airways. Increases in collagens, glycoproteins and glycosaminoglycans, and loss of elastin have been shown in both compartments [25–28]. The presence of chronic pulmonary inflammation, which includes neutrophils, macrophages, B-cells, lymphoid aggregates and CD8<sup>+</sup> T-cells, and which persists after smoking cessation is the driving force for the pathological alterations in the airways and alveoli [24]. Although they represent very different pathological entities, chronic bronchitis, small airways remodeling and lung emphysema can each be considered the result of defective tissue repair ensued by the inhalation of noxious particles or gasses, in which chronic inflammation plays a major role. Unfortunately, the inflammatory reaction in COPD is unresponsive to corticosteroid treatment, in which mechanisms similar as in the unresponsive subgroups of asthmatics mentioned earlier likely play a role [29]. The disease is considered preventable, because the main risk factor for COPD is (second-hand) exposure to tobacco smoke. Additional risk factors which are not all avoidable, include exposures to other environmental pollutants, and dusts and chemicals in an occupational setting [24]. Many genetic factors have been shown to contribute to the risk of a smoker to develop COPD. Only a genetic  $\alpha$ 1-antitrypsin deficiency has been causally linked to early onset emphysema. Because the normal decline in lung function with advancing age is accelerated in COPD patients, and because of the similarities between normal structural alterations occurring in lung tissue upon aging which lead to the development of senile emphysema and smoke-induced emphysema, COPD has been considered a disease of accelerated lung aging. At a molecular level many hallmarks of aging have been shown to be involved in the pathogenesis of COPD [30,31]. These include, among others, telomere attrition, a loss of proteostasis and cellular senescence. These aging hallmarks represent a marked overlap between COPD and IPF [31].

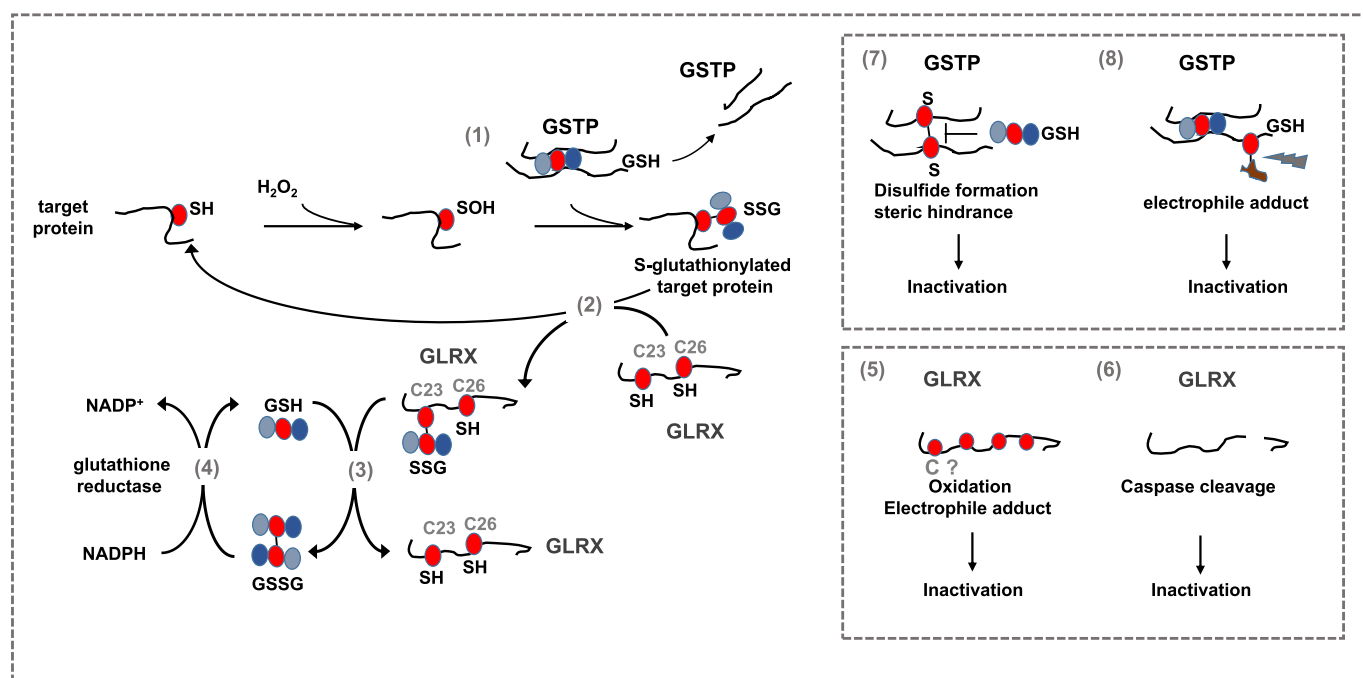
## 5. Glutathione and glutathione-mediated protein oxidation

Glutathione is considered a major redox buffer that exists in the reduced (GSH) and oxidized (GSSG, glutathione disulfide) forms [32], in addition to the chemical forms described below. Glutathione consists of the three amino acids, glutamate, cysteine and glycine, with a unique gamma glutamyl linkage of glutamate and cysteine. Cysteine, glycine and glutamate are derived from dietary sources or can be synthesized via the conversion of methionine, through the transsulfuration pathway [33]. Intriguingly, cysteine can have additional sulfur atoms, with cysteine persulfide (CysSSH) representing a cysteine derivative having one additional sulfur atom bound to a cysteinyl thiol group. Consequently, GSH can also exist as glutathione persulfide (GSSH), which has been reported to exist at concentrations as high as 100  $\mu$ M in vivo [34]. GSSH has higher nucleophilicity than parental glutathione, and therefore GSSH exhibits strong scavenging activities against oxidants [34,35]. GSH can also exist in conjunction with various lipid-derived mediators, such as leukotriene C4 (LTC4), a leukotriene isoform that is generated by enzymatic coupling of GSH to LTA4 [36], and has important biological implications as a vaso- and bronchoconstrictor [37]. Another modified version of glutathione, S-geranylgeranyl-L-glutathione (GGG) was recently identified and shown to be a ligand for the P2RY8 receptor critical to the biology of germinal centers and B-cells,

and GGG was shown to be metabolized by gamma-glutamyltransferase-5 [38]. Lastly, methylglyoxal is reactive metabolite formed as a by-product of glycolysis. Conjugation of glutathione with methylglyoxal results in the formation of lactoyl-glutathione (LGS) in a reaction catalyzed by glyoxalase, important in the detoxification of methylglyoxal. This metabolic pathway was recently shown to be important in the survival of non small cell lung cancer cells and tumor growth in mice [39]. Interestingly, glyoxylase II also has been implicated in promoting S-glutathionylation reactions [40]. The extent to which some of these modified versions of glutathione contribute to lung physiology and disease represents an unknown domain that warrants further exploration. The enzymes in the  $\gamma$ -glutamyl cycle that regulate GSH synthesis and degradation include glutamate cysteine ligase (GCL, also known as  $\gamma$ -glutamylcysteine synthetase); glutathione synthase (GS);  $\gamma$ -glutamyltranspeptidase (GGT);  $\gamma$ -glutamylcyclotransferase (GGCT); 5-oxoprolinase (OPLAH); and dipeptidase (DP). The recently identified cytosolic glutathione-specific gamma-glutamylcyclotransferase (ChaC) 1 and ChaC 2, can metabolize GSH directly into 5-oxoproline and Cys-Gly [41]. Thus, the existence of multiple and redundant enzyme systems that precisely maintain GSH and its precursor amino acids point to exquisite regulation of GSH.

The oxidized disulfide form of glutathione, GSSG can be reduced to 2 molecules of GSH by glutathione reductase (GR) oxidizing NADPH to NADP<sup>+</sup>. The pentose phosphate pathway is a major pathway that reduces NADP<sup>+</sup> to NADPH, thereby maintaining the glutathione redox buffer [32,42] (Fig. 1). GSH concentrations in cells range from ~ 1 mM–10 mM [42] with cytosolic GSH:GSSG ratios ranging from 100:1 to > 500:1 [42]. GSH levels in extracellular fluids such as blood plasma are much lower (only several  $\mu$ M) but extracellular levels ranging from 100  $\mu$ M to 400  $\mu$ M have been measured in the lung lining fluid [43,44], demonstrating the unique regulation of GSH in lung tissue. In contrast to the cytosol, the endoplasmic reticulum is far more oxidizing (see below) and the reported GSH:GSSG ratio is notably lower and ranges from 1:1 to 7:1. Reported variations in GSH:GSSG ratios likely stem from variations in sample preparation, and methods used to block the thiol in reduced GSH. Lack of adequate blocking, in addition to auto-oxidation, may result in an overestimation of GSSG values, and these concerns continue to plague the field [45]. GSH is utilized in multiple processes. It is consumed by glutathione peroxidases to reduce oxidized lipids [46]. GSH contributes to the excretion of xenobiotics wherein glutathione S-transferases (GSTs) conjugate GSH to electrophiles or other toxic compounds leading to their excretion via mercapturic acids [47]. Lastly, and the focus of this review, GSH can be conjugated to proteins in a process called protein S-glutathionylation (PSSG). PSSG protects protein cysteines from being overoxidized to sulfinic and sulfonic species that are not readily regenerated. S-glutathionylation also changes the protein structure and function as will be further described next.

Cysteines in proteins have emerged as important biological sensors for redox perturbations that in turn regulate biological responses. Reactive, low pKa cysteines can be oxidized in specific, reversible, and regulated manners, in order to permit cells to sense and respond to redox changes. The specificity of redox modifications of target proteins is controlled via compartmentalization of oxidant formation, and the presence of enzymes that regulate protein cysteine redox status in subcellular domains [8,48,49]. As stated earlier, protein S-glutathionylation (PSSG) represents a cysteine oxidation wherein GSH is conjugated covalently to the protein cysteine. PSSG has emerged as a critical regulatory mechanism that regulates protein structure and function. The conjugation of glutathione to protein cysteines changes protein conformation (addition of three amino acids) as well as charge (glutamic acid), thereby affecting function [8,50–52]. The precise pathways that lead to PSSG in biological settings are not completely known, but it is thought that an oxidant, such as H<sub>2</sub>O<sub>2</sub>, can first lead to a sulfenic acid intermediate (-SOH) (or sulfenylamide (-SN)), a gateway oxidation, that can subsequently be S-glutathionylated. While such GSH



**Fig. 1.** Steps in the catalytic cycle of protein S-glutathionylation (PSSG) and de-glutathionylation believed to be relevant in the pathogenesis of chronic lung diseases. A number of biochemical events can induce PSSG. The mode of PSSG is likely to be target and context specific, dependent upon the proximity of oxidant producing enzymes, redox relays, the oxidation state of the GSH/GSSG redox couple etc. In epithelial cells in settings of a pro-fibrotic environment, oxidants originating from multiple sources lead to a sulfenic acid intermediate (SOH) which can be a platform for subsequent PSSG, which can happen spontaneously or catalyzed by glutathione S-transferases (GST), notably GSTP [1]. Conversely GLRX acts to de-glutathionylate proteins [2], restoring the original sulfhydryl group. GLRX induces de-glutathionylation via the monothiol mechanism requiring only the N-terminal cysteine in the thioredoxin (TXN) domain [2], which can thereafter be reduced by reduced glutathione (GSH) [3] re-establishing the reduced thiol group of the N-terminal cysteine in GLRX. Glutathione disulfide (GSSG), formed in this process can be reduced back to GSH through the action of glutathione reductase (GR) consuming reducing equivalents from NADPH [4]. GLRX can be inactivated via oxidations of one or more cysteines outside of the TXN domain [5] including electrophiles from cigarette smoke. GLRX can also directly be cleaved by caspases 8 and 3 [6]. Similarly, GSTP also is subject to oxidant-mediated inactivation, potentially involving a disulfide which creates steric hindrance that interferes with GSH binding [7], or electrophile-induced modification [8]. We refer the reader to the body of text for detailed information. Red circle: protein cysteine or cysteine in glutathione. SH: reduced cysteine, SOH: Sulfenic acid, SSG: S-glutathionylated protein, GSH: Reduced glutathione, GSSG: glutathione disulfide, comprised of two glutathione molecules with a disulfide bond between the cysteines of each glutathione molecule, S-S: disulfide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conjugation can occur spontaneously, it can also be catalyzed by glutathione-S-transferases (GSTs) (Fig. 1). The majority of studies conducted thus far on GST-catalyzed PSSG have centered on glutathione S-transferase  $\pi$  (GSTP) [53,54], and therefore we will focus on GSTP herein. GSTP is a dual function enzyme that has both chaperone activity and can catalyze GSH conjugation to biological and xenobiotic electrophiles as well as S-glutathionylation reactions. Separate domains within GSTP regulate these functions. The N-terminal thioredoxin-like G-site binds GSH and activates the cysteinyl moiety of GSH via tyrosine 7 whereas the C-terminal alpha-helical domain shapes the co-substrate binding site, termed the H-site, based upon the hydrophobic (H) character of co-substrates [55,56]. Although the role of GSTP in protein S-glutathionylation is well recognized, the exact mechanisms whereby GSTP induces PSSG still remain unclear. Studies of the 1 cysteine-containing PRDX6 demonstrated that oxidation of PRDX6 to a sulfenic acid intermediate is associated with formation of a heterodimer with GSH-containing GSTP, which in turn leads to S-glutathionylation of PRDX6, followed by GSH-mediated reduction of the disulfide to regenerate the active form of PRDX6 [57,58]. An array of proteins are now known to be S-glutathionylated by GSTP. However, S-glutathionylation of these proteins in some cases has been demonstrated in cell lines under conditions of overt stress. The repertoire of proteins S-glutathionylated by GSTP and other GSTs in settings of lung (patho)physiology remains poorly understood. Interestingly, GSTP also can be oxidatively inactivated, and it has been suggested that cysteines-47 and -101 regulate the binding of GSH, and that disulfide bond formation between these cysteines results in steric hindrance and resultant

inactivation of GSTP [59] (Fig. 1).

The family of glutaredoxins (GLRX) under physiological conditions acts to specifically de-glutathionylate proteins restoring the reduced sulfhydryl group [60] (Fig. 1). It is worthy of mention that under conditions where GSSG levels are high, GLRX can catalyze S-glutathionylation. Four mammalian GLRX have been described, the dithiol GLRXs, GLRX (active site; CPYC) and GLRX2 (active site; CSYC) and monothiol GLRX3 (also known as PICOT, active site; CGFS) and GLRX5 (active site; CGFS). GLRX exert specificity toward the  $\gamma$ -linkage-glutamyl moiety of glutathionylated proteins (or free GSH) [61,62]. Mammalian GLRX catalyzes de-glutathionylation in a two-step mechanism in which the first step involves the nucleophilic displacement of the GSH moiety in the S-glutathionylated protein target by the N-terminal active site cysteine, resulting in S-glutathionylation of GLRX itself. In the second step, the thiolate ion of the N-terminal active site cysteine is regenerated by consuming one molecule of GSH, forming GSSG [63] (Fig. 1). Mammalian GLRX has 5 reactive cysteines. Only the active site N-terminal cysteine is required for the de-glutathionylation reaction, as the mutation of individual or all four cysteines other than active site cysteine 23 does not affect its activity [64–66]. However, oxidation, including S-glutathionylation, of the cysteines outside of the catalytic site results in inactivation of GLRX [67,68] (Fig. 1), and suggests that GLRX is subject to negative feed-back regulation by its substrate, GSH. In summary, regulatory pathways comprised of GSTs and GLRX, among others, that collectively control protein S-glutathionylation and de-glutathionylation operate to precisely control the oxidation state of target cysteines through utilization of glutathione. These



observations point to the importance of GSH as a critical regulator of biological pathways, beyond the traditional view that GSH is a mere quencher of oxidants.

## 6. ER stress

The endoplasmic reticulum (ER) is an organelle essential to normal cellular function that plays key roles in the regulation of proteostasis, calcium storage, lipid synthesis, mitochondrial function, among others. Protein misfolding and ensuing ER stress triggers the activation of a signaling network known as the unfolded protein response (UPR), mentioned above, through distinct effector pathways that involve activation of PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ). These pathways are controlled by a chaperone protein, immunoglobulin heavy-chain-binding protein (Bip, also known as glucose regulated protein 78 (GRP78) or heat shock 70 kDa protein 5, (HSPA5)), and their activation is restricted by binding of Bip to these ER sensors (PERK, ATF6, and IRE1 $\alpha$ ). Bip also binds to misfolded proteins in the ER. As misfolded proteins accumulate, Bip binding to the three UPR sensors is reduced and resultant UPR signaling in turn modulates new protein synthesis, increases production of ER chaperones, and induces components of the ER-associated degradation (ERAD) system [14,69]. The UPR response is triggered to rectify the stress, but severe stress can result in cell death. In epithelial cells, ER stress can lead to apoptosis, pro-inflammatory signaling, and epithelial-mesenchymal transition (EMT), features that have all been linked to chronic lung diseases, reviewed in Refs. [14,70,71]. During the UPR, various chaperones including oxidoreductases such as protein disulfide isomerases (PDIs) are upregulated in order to restore ER homeostasis [72,73]. Twenty members exist in the PDI family of proteins. PDIs exhibit varying numbers of thioredoxin-like domains (CXXC) which catalyze oxidative folding and disulfide bond isomerization of proteins in the ER lumen [74]. It has been speculated that a large amount of redundancy in client proteins of the various PDIs exists. However, recent studies using mutants to trap PDI-associated proteins indicate more nuanced roles for the individual PDIs toward regulating specific client proteins [75]. The environment of the endoplasmic reticulum (ER) is more oxidizing than the cytosol, allowing disulfide bridge formation [76]. As mentioned earlier, the GSH/GSSG ratio is substantially lower in the ER than the cytosol, and a majority of glutathione in the ER is present as mixed disulfides with proteins (S-glutathionylated proteins) [77]. Not surprisingly, it has therefore been suggested that GSH regulates oxidative folding processes [78].

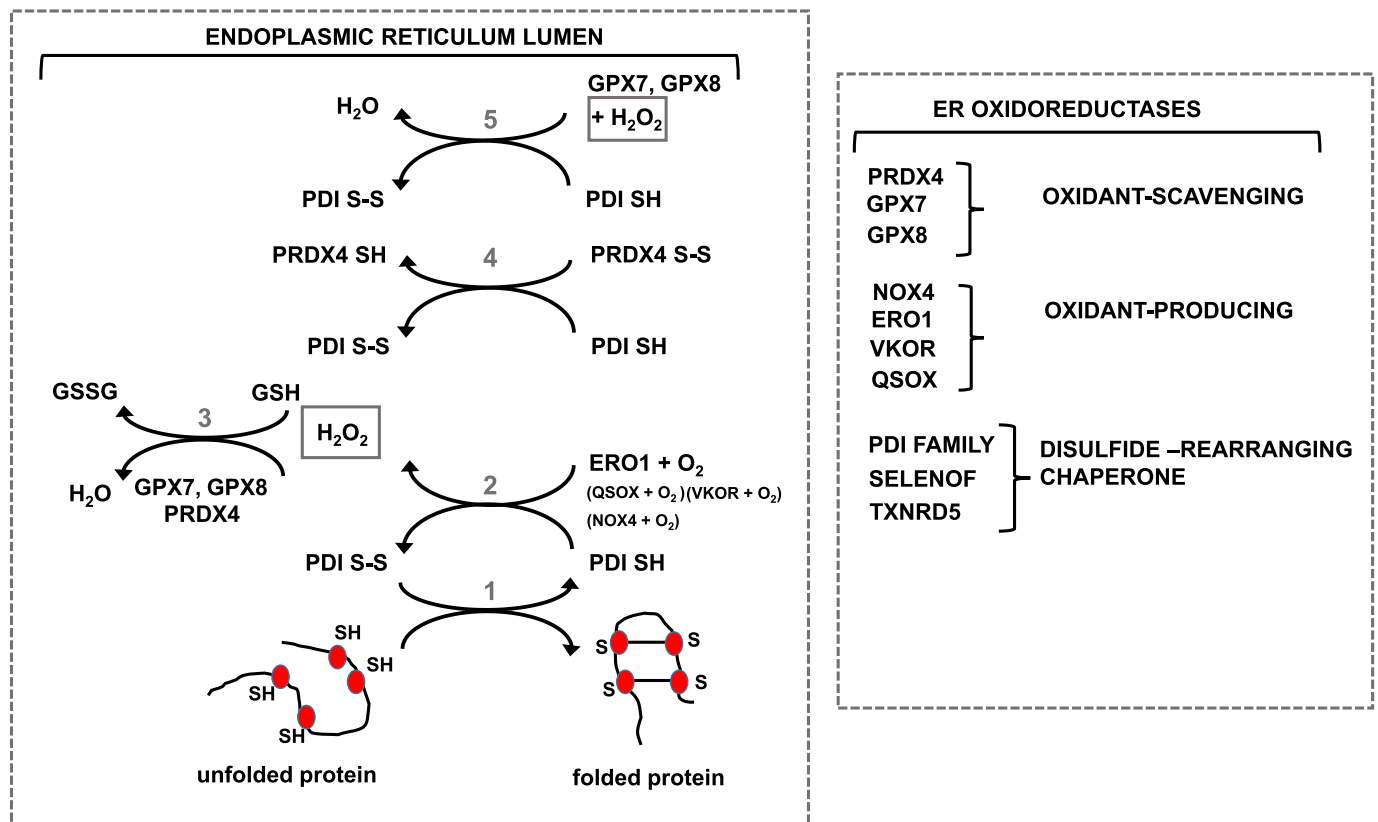
Many proteins that enter the secretory pathway, including the aforementioned surfactant proteins, mucins (79) etc. contain cysteines that are oxidatively processed into disulfide bonds in the ER in order to stabilize the protein. Disulfide bridge formation in proteins is regulated by the PDI family. Oxidized PDI (S-S) will covalently bind its reduced client protein (SH) to catalyze disulfide bridge formation, via a sulfhydryl-disulfide exchange reaction in which the client protein will be oxidized (S-S), while PDI will be reduced (SH). In order to regenerate catalytically active PDI, it has to be re-oxidized by ER oxidoreductin-1 (ERO1). During this regeneration cycle of PDI by ERO1, H<sub>2</sub>O<sub>2</sub> is formed [80] (Fig. 2). Thus, oxidative processing of proteins in the ER leads to production of H<sub>2</sub>O<sub>2</sub>. Steady state levels of H<sub>2</sub>O<sub>2</sub> are precisely controlled in part via oxidation of GSH to GSSG, and H<sub>2</sub>O<sub>2</sub> metabolism by ER-resident peroxiredoxin-4 (PRDX4). Besides ERO1, other mechanisms have emerged to regenerate oxidized PDI, which are speculated to involve H<sub>2</sub>O<sub>2</sub> produced by the NADPH oxidase, NOX4, acceptance of electrons by oxidized PRDX4, reduction of vitamin K with concomitant oxidation of PDI by Vitamin K epoxide hydrolase (VKOR), quiescinsulfhydryl oxidase (QSOX), and use of H<sub>2</sub>O<sub>2</sub> generated by ERO1 by glutathione peroxidases (GPX) 7 and 8 [80–83] (Fig. 2). Similarly, additional quality control systems exist in the ER. For example, Selenof is an ER-resident thioredoxin-like oxidoreductase that acts as a gatekeeper for immunoglobulins and likely other disulfide-rich glycoproteins,

thereby providing redox quality control of secreted glycoproteins [84]. The import of cystine via system Xc has emerged as a pathway that controls ER homeostasis. System Xc imports cystine, while exporting glutamate, increasing intracellular levels of cystine (the oxidized version of cysteine), which is rapidly reduced to cysteine, the rate limiting amino acid required for GSH biosynthesis. Inhibition of system Xc results in decreases in GSH, and induces ER stress as manifested by activation of the eIF2 $\alpha$ -ATF4 branch of the ER stress response pathway, and increased expression of notably of ATF4, ATF3 and CHOP [85]. These observations collectively suggest important links between glutathione homeostasis and ER stress.

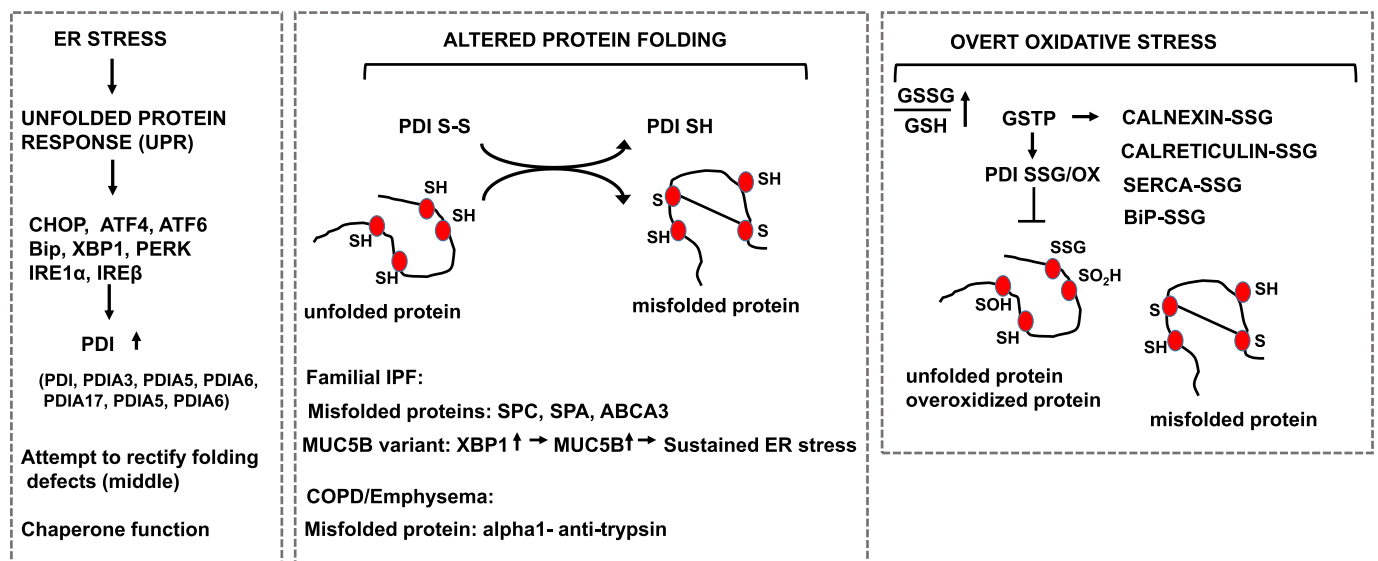
Relevant to this review are findings that, among other cellular compartments, GSTP is found in the ER [86,87], where it promotes S-glutathionylation of a number of ER resident proteins, including calnexin, calreticulin, endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA), PDI and Bip in settings of overt oxidative stress [86] (Fig. 3). Genetic ablation of *Gstp1/2* increased sensitivity of mouse embryonic fibroblasts to death induced by the UPR inducers tunicamycin or thapsigargin, in association with increased expression of UPR proteins [86], suggesting that GSTP is critical in the regulation of ER stress responses. It remains unclear to date whether the chaperone and/or S-glutathionylation functions of GSTP regulate ER stress responses and whether GSTP contributes to enhanced ER stress in patients with chronic lung diseases.

## 7. ER stress and lung fibrosis

Chronic ER stress is associated with the development of fibrotic disorders in the lung, liver and kidney. ER stress within epithelial cells has been strongly implicated in the pathogenesis of lung fibrosis, based upon discoveries of germline mutations in genes expressed exclusively in epithelial cells that result in defects in folding and/or processing of a nascent peptide, causing prolonged ER stress and subsequent fibrosis in patients with familial IPF [14,88]. Evidence that ER stress occurs in patients with IPF was first reported by the laboratory of Dr. Timothy Blackwell in 2008, documenting that expression of various markers of ER stress was increased in airway epithelial cells of patients with IPF, in association with the presence of herpes virus [15]. These markers include ATF4, ATF6, and CCAAT-enhancer-binding protein homologous protein (CHOP), Bip, and X-box binding protein 1 (XBP-1) [14]. Some patients with familial IPF have germline mutations in surfactant protein C (*SFTPC*), surfactant protein A2 (*SFTPA2*), or the lipid transporter, ATP binding cassette class A3 (*ABCA3*) genes. These genes encode proteins highly expressed in epithelia, and mutations in these genes result in defects in protein folding, leading to ER stress [14] (Fig. 3). Some affected individuals with familial IPF shared a rare missense variant (L188Q) containing a mutation adjacent to a cysteine residue in pro-SP-C that is required for proper folding [4]. Similarly, a mutation of cysteine 121 within the so-called BRICHOS domain of pro-SPC has been reported in a child with interstitial lung disease [89]. Misfolded pro-SP-C arising from these mutations aggregates in the ER and activates the UPR. The relevance of these mutations for the pathogenesis of lung fibrosis has been corroborated in mouse models where it was demonstrated that transgenic overexpression the L188Q mutant variant of *SFTPC* in type II alveolar lung epithelial cells increased the sensitivity to the development of fibrosis [90]. Knock-in mice expressing of an isoleucine-to-threonine substitution at codon 73 (I73T) of *SFTPC*, or a cysteine-to-glycine substitution at codon 121 (C121G), developed spontaneous fibrosis, [89,91]. The mucin, MUC5B, has been shown to play a key role on the obstruction of peripheral airspaces and honeycomb cysts in patients with IPF, and a MUC5B promoter polymorphism (rs35705950) has been identified as a strong risk factor for development of IPF. A recent study demonstrated that the UPR is critical in inducing expression of the *MUC5B* gene. Notably, the endoplasmic reticulum to nucleus signaling 2 protein (ERN2, also known as IRE1 $\beta$ ) and its downstream target, spliced XBP1, in cooperation with the mucus cell



**Fig. 2.** ER oxidoreductases and their roles in protein folding and maintenance of ER redox homeostasis. **Left:** Role of protein disulfide isomerase (PDI) in oxidative disulfide bond (S-S) formation in proteins [1]. Oxidized PDI (containing S-S disulfide) oxidizes its client protein by introducing disulfide bonds, while PDI itself as a result becomes reduced (SH) [2]. ER oxidoreductin (ERO1) plays a major role in re-oxidizing PDI, a reaction that generates hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [3]. H<sub>2</sub>O<sub>2</sub> can be detoxified by GSH, or the actions of peroxiredoxin 4 (PRDX4), or glutathione peroxidases (GPX) 7 or 8. Alternative pathways for the regeneration of oxidized PDI are the abstraction of electrons by oxidized PRDX4 [4], or a H<sub>2</sub>O<sub>2</sub>-dependent GPX7/8 catalyzed reaction. **Right:** Overview of the most studied ER-oxidoreductases and their potential roles in oxidant-production/scavenging or other functions. We refer the reader to the body of text for detailed descriptions.



**Fig. 3.** Disregulation of the Unfolded Protein Response (UPR) and protein disulfide isomerase in settings of chronic lung diseases. **Left:** Schematic overview of the link between ER stress, the resultant unfolded proteins response, and the effector pathways that are increased in chronic lung diseases. **Middle:** Illustrations of misfolded proteins relevant to IPF (SPC, SPA, ABCA3) or COPD (α1 anti-trypsin). Also illustrated is MUC5B which has been linked to ER stress in settings of familial IPF. Observed increases in PDI in chronic lung diseases may be to rectify the burden of ER stress and/or misfolded proteins. **Right:** In settings of overt oxidative stress, the function of PDI, and other ER proteins shown, may be compromised through oxidations and/or other modifications, allowing misfolded and/or overoxidized proteins to accumulate. Note that thus far, data to support this scenario was obtained in cell lines and/or settings of overt oxidative stress. Relevance of these putative events to chronic lung disease will require detailed analyses of human tissue specimens. We refer the reader to the body of text for detailed descriptions.

transcription factor, SAM pointed domain containing ETS transcription factor (SPDEF), bind the MUC5B promoter to induce its expression in a rs35705950-specific manner [92] (Fig. 3). PDIs also are emerging as potential contributors to pulmonary fibrosis. As will be described below, PDIA3 promotes disulfide formation of FAS, leading to epithelial cell death [87]. PDIA3 also has been implicated in the trans-differentiation of murine type II alveolar epithelial cells into type I cells, in association with increased Wnt/ $\beta$ -catenin signaling [93]. Furthermore, recent studies have highlighted that PDIs interact with and potentially regulate disulfide bonds in numerous pro-apoptotic and extracellular matrix regulating proteins including BAK [94], FAS [87], collagen 1a1 (95), transglutaminase 2 [96] matrix metalloproteinase 9 [97], and the collagen crosslinking enzyme lysyl oxidase like 2 (LOXL2) [75]. PDI also plays a role in integrin-mediated cell adhesion [98]. Intriguingly, fibronectin itself contains protein-disulfide isomerase activity thought to be relevant to the disulfide-mediated cross linking of fibronectin in the extracellular matrix [99]. Relevant to fibrosis, it is worthy to mention that PDI also acts as a non-catalytic component of the enzyme prolyl 4-hydroxylase, important in the hydroxylation of collagen [100,101].

Links between ER stress, loss of proteostasis and mitochondrial dysfunction in settings of IPF also have become apparent [12]. The ER tightly controls the calcium pool available for mitochondrial uptake through a number of proteins that include the mitochondrial calcium uniporter via sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  ATPase and the inositol triphosphate receptor, providing a mechanism whereby the ER regulates cellular bioenergetics (reviewed in Refs. [14]) and cell death [102]. Specialized regions of interaction and communication between ER and mitochondria have been demonstrated and represent an area of active investigation [103]. Notably, aging and enhanced ER stress during aging have been shown to lead to mitochondrial dysfunction in type II alveolar epithelial cells [12]. A higher frequency of enlarged mitochondria with a bias toward mitochondrial fusion and an increased mitochondrial area occurred in aging type II alveolar epithelial cells [104]. Additional findings of mitochondrial abnormalities in the aging lung include increases in mitochondrial reactive oxygen species, decreases in mitophagy, impaired respiration, mitochondrial DNA deletions, and decreased expression of sirtuin 3 [12,105]. ER stress in epithelial cells has been linked to mitochondrial perturbations via decreases in levels of PTEN-induced putative kinase 1 (PINK1), a regulator of mitochondrial homeostasis. A role for PINK1 in lung fibrosis was corroborated based upon findings demonstrating that *PINK1*-deficient mice exhibited increased susceptibility to apoptosis, and spontaneous transforming growth factor beta (TGFB)-dependent fibrosis, in association with abnormal mitochondria in type II alveolar epithelial cells [104]. Epithelial cells expressing the I73T-mutant variant of human SPC showed defects in autophagy in association with increases in mitochondria biomass and decreases in mitochondrial membrane potential [11]. Mitochondrial dysfunction and ER stress also have been linked to the senescent fibroblast phenotype present in the IPF lung [106]. Fibroblasts from IPF patients have shorter telomers, express a number of senescence-associated proteins and produce cytokines related to the senescence-associated secretory phenotype. Upon TGFB stimulation, markers of ER stress increased to greater extents in IPF fibroblasts compared to age-matched cells from control lungs [107]. TGFB was furthermore shown to stimulate increases in mitochondrial mass in human lung fibroblasts via the SMAD2/3 (SMAD represents an acronym from the fusion of *Caenorhabditis elegans* Sma genes and the *Drosophila* Mad, Mothers against decapentaplegic) and CCAAT/enhancer binding protein  $\beta$  (CEBPP) pathways [108]. Interestingly a role of oxidants has been suggested in promoting the senescence phenotype in fibroblasts and this had been linked to the activation of signal transducer and activator of transcription (STAT)-3, a transcription factor implicated in premature senescence [109,110]. Human lung fibroblasts exposed to  $\text{H}_2\text{O}_2$  displayed increases in expression of senescence markers and expression of interleukin-6 (IL6), in association with

translocation of STAT3 to the nucleus and mitochondria. These observations were accompanied by increased basal respiration, proton leak and an associated increase in oxidant production in senescent fibroblasts. Using a small molecule inhibitor of STAT3, the authors demonstrated attenuated IL6 production, senescence markers and restoration of normal mitochondrial function [109]. Collectively, these findings strongly support the concept that ER dysfunction/loss of proteostasis in alveolar type II cells play key roles in driving the pathogenesis of IPF. ER stress is not limited to those individuals with familial IPF, and ER stress is now recognized as a common feature of sporadic IPF. Disruptions in proteostasis are also linked to mitochondrial dysfunction and the senescence phenotype of fibroblasts, relevant to pulmonary fibrosis.

A few studies have been conducted to suggest a link between dysregulated oxidative processing in the ER and altered extracellular matrix biology. Mice with combined loss-of-function mutations in genes encoding ERO1 $\alpha$ , ERO1 $\beta$ , and PRDX4 display a compromised extracellular matrix due to defects in the intracellular maturation of procollagen. Combined absence of these enzymes resulted in increased cysteinyl sulfenic acid modification of ER proteins. Intriguingly, the increased cysteinyl sulfenic acids led to oxidation of ascorbate to dihydroascorbate. Consistent with the requirement of ascorbate in maintaining the function of ER-localized prolyl-4 hydroxylases, the decreases in ascorbate in the ER of mouse embryonic fibroblasts lacking ERO1 $\alpha$ , ERO1 $\beta$ , and PRDX4 resulted in a strong decrease in procollagen 4-hydroxyproline content [111]. Thioredoxin domain containing 5 (TXNDC5), an ER-localized protein disulfide isomerase, was shown to facilitate ECM protein folding, and to contribute to the stability of ECM proteins. Enhanced expression of TXNDC5 promoted redox-sensitive activation of cardiac fibroblasts and augmentation of cardiac fibrosis [112]. Quiescin sulfhydryl oxidase 1 (QSOX1) is an atypical disulfide catalyst, as it is localized to the Golgi and can be secreted from cells. QSOX1 activity was shown to be essential for the incorporation of laminin into the extracellular matrix synthesized by fibroblasts [113]. A role of VKOR in organ fibrosis has is emerging based upon a study showing that inhibition of VKOR with 3-acetyl-5-methyltetronic acid (AMT) attenuated the progression of cisplatin-induced renal fibrosis in rats [114]. Although GPX7 has not been linked to fibrosis, GPX7 expression was decreased in senescent cells, and knock down of GPX7 promoted premature senescence. Moreover, the ability of metformin to extend life span in worms has been linked to the upregulation of GPX7 [115]. GPX7 was also shown to be essential to promote Bip's chaperone function [116]. As fibrosis is associated with aging, it will be interesting to explore the function of GPX7 in settings of age-associated fibrosis. Lastly, overexpression of PRDX4 elicited protective effects against nonalcoholic steatohepatitis and type 2 diabetes in a mouse model [117]. Based upon these observations, it is highly plausible that dysregulation of proteostasis in the lung, linked to altered functions in ER-redox processes, contribute to the pathogenesis of lung fibrosis (Table 1).

## 8. Glutathione biochemistry, S-glutathionylation and lung fibrosis

Numerous studies in patients with IPF and rodent models have demonstrated that perturbations of the redox status occur and are linked to disease pathogenesis [118–120]. Disruptions in mitochondria and, as mentioned earlier, the ER have been implicated in the pathogenesis of IPF [12,14,121], although the extent to which redox perturbations originating from dysfunctional mitochondria or ER, respectively, or other sources contribute to IPF remain unclear. In rodent models of fibrosis, genetic ablation of the antioxidant enzyme extracellular superoxide dismutase (SOD3) increased the susceptibility to fibrogenesis [122], and similar findings were observed in mice lacking the transcription factor, nuclear factor erythroid 2-related factor (NRF2) [123], which regulates numerous antioxidant and cytoprotective responses including expression of enzymes important in GSH synthesis.

**Table 1**  
Implications of ER oxidoreductases and/or ER stress in lung fibrosis or extracellular matrix biology.

ER oxidoreductase	Putative function	Model	References
PDIA3	Fas-SS formation, Epithelial cell apoptosis AEC2- AEC1 differentiation, regulation of Wnt/ $\beta$ -catenin signaling	Epithelial cells, Mouse models, IPF	[87] [93]
PDI	Disulfide bond formation in LOXL2, collagen 1a1, transglutaminase 2, matrix metalloproteinase 9, Non-catalytic component of prolyl-4 hydroxylase Integrin mediated adhesion	Primary lung epithelial cells Cell lines, mouse models Human renal fibrosis Rat liver, chick embryos, C. Elegans	[75,95–97] [100,101] [98]
ERO1 $\alpha$ , ERO1 $\beta$ PRDX4	Prolyl-4 hydroxylation of collagen	Platelets Triple knock out mice Mouse embryonic fibroblasts	[111]
PRDX4	Protection of NASH and diabetes	Mouse model	[117]
TXNDC5	Stabilization of ECM proteins, Augmentation of cardiac fibrosis	Cardiac fibroblasts, mice	[112]
QSOX1	Laminin incorporation in the ECM	mice, fibroblasts	[113]
GPX7	Regulation for longevity, protection from senescence	Mouse models Fibroblasts, mesenchymal stem cells C.Elegans	[116]
Fibronectin	Intrinsic PDI activity	In vitro	[99]
VKOR	Promotes cisplatin-induced hepatic fibrosis	rats	[114]

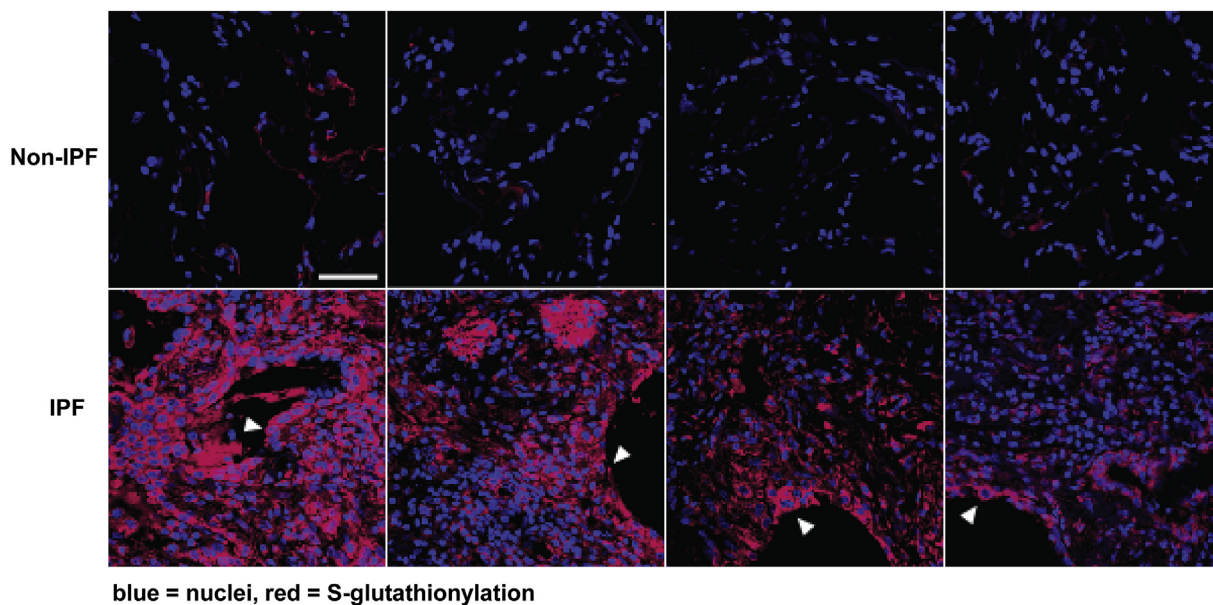
Conversely, administration of various antioxidants has been suggested to be protective against development of fibrosis (for review [124,125]). NOX4, which produces H<sub>2</sub>O<sub>2</sub>, has been shown to play a causal role in myofibroblast activation and resultant fibrosis in the bleomycin and fluorescein isothiocyanate models [126]. NOX4 dampens mitochondrial bioenergetics and biogenesis via an NRF2-dependent pathway. Interestingly, NOX4 silencing increased the levels of NRF2 mRNA, while NRF2 silencing led to a decrease in NOX4 mRNA expression, showing counter-regulation between NOX4 and NRF4. A NOX4/NRF2 redox imbalance also was demonstrated to contribute pulmonary fibrosis in aged mice where persistent fibrosis was linked to upregulation of NOX4 and failure to induce expression of NRF2 [127]. Levels of GSH were reported to be markedly decreased in lung lining fluids, induced sputum and plasma of patients with IPF, in association with increases in GSSG in plasma [118,128,129]. Increased levels of H<sub>2</sub>O<sub>2</sub> occur in exhaled breath condensates from patients with IPF, compared to control groups [130]. To address the potential impact of GSH on the biology of lung fibroblasts, studies as early as 1990 found that extracellular GSH in the 500  $\mu$ M range, similar to the concentration of GSH in the lung lining fluid, decreased lung fibroblast proliferation, suggesting that lowered levels of reduced GSH might promote fibroblast activation. The effect of GSH was mimicked by other sulfhydryl-containing molecules including cysteine and N-acetylcysteine (NAC), precursors of GSH, 2-mercaptoethanol, and low concentrations of dithiothreitol, but not by GSSG [131]. Transforming growth factor beta-1 (TGFB1) is growth factor critical to organ fibrogenesis, and overexpression of active TGFB1 is sufficient to induce lung fibrosis in rodent models [132]. TGFB1 alters the GSH status in the lung. Intranasal instillation of AdTGFB1(223/225), an adenovirus expressing constitutively active transforming growth factor beta 1 (hereafter referred to as AdTGFB1) suppressed the expression of catalytic and modifier subunits of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, resulting in decreases of GSH. This resulted in increases in epithelial apoptosis, which occurred prior to the development of lung fibrosis [133]. The microRNA, miR-433, has been shown to directly target GCL. In vivo models of renal and hepatic fibrosis were associated with a TGFB1-mediated reduction of GCL levels that were miR-433 dependent, resulting in decreases in GSH levels, increases in S-glutathionylation and enhanced fibrogenesis [134]. It remains unclear whether miR-433 has a similar impact on lung fibrosis. TGFB1 is one of the major repressors of GCL expression leading to GSH depletion [133,135], suggesting that in a pro-fibrogenic environment, depletion and/or oxidation of GSH occurs through multiple mechanisms, which in turn promote fibrosis. Using the bleomycin model of fibrosis, the redox potential (E(h)) of plasma GSH and Cys redox systems was shown to change uniquely as a

function of progression of bleomycin-induced lung injury. Plasma E(h) GSH/GSSG was shown to be selectively oxidized during the proinflammatory phase, whereas oxidation of E(h) Cys/CySS occurred at the later fibrotic phase. Increased oxidation of E(h) Cys/CySS in the lung lining fluid was also detected and was linked due to decreased food intake in mice exposed to bleomycin [136].

To address the role of glutathione disruption in pulmonary fibrosis, a number of studies involving the GSH precursor, N-acetyl-L-cysteine (NAC), were conducted with the goal to augment pulmonary GSH levels and to alleviate oxidative stress. NAC was shown to dampen bleomycin-induced inflammation and subsequent fibrosis in mice in association with decreases in lysyl oxidase [137,138]. A number of clinical trials have been also been conducted to determine whether increases in GSH in the lung would attenuate the progression of disease in patients with IPF and maintain lung function. One clinical trial that included the GSH precursor, NAC, along with another drug, initially showed therapeutic effects associated with NAC [139,140]. However, re-evaluation of NAC in a clinical trial with over 130 IPF patients in the NAC or placebo groups failed to show a clinical benefit of NAC on disease progression or lung function decline [141]. However, a pharmacogenomic interaction involving toll interacting protein (*TOLLIP*) and *MUC5B* single nucleotide polymorphisms (SNPs) and a response of IPF patients to NAC has become apparent [142].

Although changes in glutathione homeostasis were first demonstrated in lungs from IPF patients over 30 years ago, the extent to which changes in S-glutathionylation occurred in fibrotic tissues had remained unclear. In order to assess the potential implications of PSSG for the pathophysiology of lung fibrosis, the highly specific catalytic activity of mammalian GLRX toward reduction of PSSG was used to visualize PSSG in formalin-fixed, paraffin-embedded (FFPE) tissues in situ using microscopy approaches [143]. Notable PSSG immunoreactivity was apparent in bronchiolar epithelial cells and alveolar macrophages under physiological conditions. In mice with bleomycin-induced fibrosis increases in PSSG occurred in bronchiolar epithelial cells, as well as parenchymal regions [143]. Increases in PSSG also occurred in lungs from patients with IPF (Fig. 4), and increases in PSSG inversely correlated with lung function in these patients [68]. Airway epithelial cells showed noticeable PSSG. Interestingly, GSTP was shown to be highly expressed in bronchiolar epithelial cells and type II alveolar epithelial cells [144], consistent with a role of these cells in protection against environmental insults and metabolism of xenobiotics. In lungs from patients with IPF, GSTP was prominently increased in bronchiolar epithelia as was as in the distal lung epithelial cells, in areas of re-bronchiolarization, and in reactive type II epithelial cells, including those type II pneumocytes at the leading edge of disease progression





**Fig. 4.** Increases in S-glutathionylation in lung tissues from patients with IPF. Lung sections were deparaffinized, rehydrated, permeabilized and reduced protein thiols blocked with N-ethyl maleimide (NEM). Sections were then subjected to GLRX-catalyzed protein cysteine labeling in order to detect regions of PSSG, as described in the text. Red = PSSG, Blue = DAPI counterstain. Note the increases in PSSG in lungs from IPF patients ( $n = 4$ ), compared to non-IPF controls ( $n = 4$ ). This image was first published in Nature Medicine. 2018 Aug; 24 (8):1128–1135. <https://doi.org/10.1038/s41591-018-0090-y>. Epub 2018 Jul 9. by Anathy V et al. and was reproduced with permission from Nature Medicine [68]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

[144]. In contrast to increases in GSTP immunoreactivity in lungs from patients with IPF, the enzymatic activity of GLRX was decreased in IPF lungs, in a partial dithiothreitol-specific manner, indicative of oxidative inactivation of GLRX. As mentioned above, increases in GLRX S-glutathionylation were observed in lungs from patients with IPF, and in mice with bleomycin-induced fibrosis [68], suggestive of negative feedback inhibition of GLRX via S-glutathionylation. Earlier studies reported decreases in GLRX content in lungs from patients with IPF and COPD [145,146] and downregulation of GLRX by TGFB1 [147], consistent with a putative role of enhanced PSSG in promoting lung fibrosis.

## 9. S-glutathionylation, the death receptor FAS, and lung fibrosis

A critical role of epithelial cell death in the pathogenesis of pulmonary fibrosis has clearly emerged. In fibrotic lung disease, including idiopathic pulmonary fibrosis (IPF), loss of or damage to distal conducting airway or alveolar epithelial cells represent common histopathological features that are believed to contribute to diminished lung function. Chronic injury leads to death of epithelial cells, and lack of normal epithelial restitution is a cardinal driving process for fibrosis, with concomitant activation and proliferation of myofibroblasts [148,149]. Numerous preclinical studies strongly support the importance of epithelial cell death per se in subsequent fibrogenesis [150]. For example, upon administration of diphtheria toxin to transgenic mice expressing the diphtheria toxin receptor in type II epithelial cells or Club cells (epithelial cells with secretory functions that exist in the proximal airways of the lung where gas exchange does not occur), marked death of these cells occurred with resultant prolonged fibrosis [151,152]. The functional role of the death receptor, FAS (also known as CD95) in the development of pulmonary fibrosis is evident from studies showing that agonistic FAS antibody (which mimics the cross-linking between FAS ligand (FASL) and FAS) induces apoptosis of bronchial and alveolar epithelial cells leading to fibrosis. Conversely, bleomycin-induced fibrosis could be prevented using soluble anti-FAS, or anti-FASL antibodies, and did not occur in mice that lack functional FAS or FASL [148,153,154]. Apoptosis of epithelial cells has been

shown following co-culture with myofibroblasts isolated from patients with IPF which express FASL, while these myofibroblasts themselves are resistant to FASL-induced apoptosis [154,155]. Epithelial cell death by IPF-derived fibroblasts has been linked to  $H_2O_2$  produced by myofibroblasts [156]. Low immunoreactivity of FAS was found in fibroblasts within fibroblastic foci, and upregulation of surface FAS in fibroblasts sensitized them to FASL-induced apoptosis [157]. A recent study showed that expression of protein tyrosine phosphatase-N13 mediated the resistance of human lung (myo)fibroblasts to FAS-induced apoptosis and promoted pulmonary fibrosis in mice [158]. Collectively, these findings demonstrate the importance of FAS and epithelial cell apoptosis in the pathogenesis of lung fibrosis, and suggest that avenues that dampen the extent of epithelial cell death and target the biology of the FAS receptor may attenuate fibrotic remodeling.

Work from our laboratories has demonstrated that FAS is subject to S-glutathionylation which amplifies its apoptosis-inducing function. Specifically, S-glutathionylation of FAS at cysteine 294 (murine FAS) augments the ability of FASL to induce epithelial cell death. S-glutathionylation of FAS coincided with a loss of GLRX enzymatic activity. Inhibition of caspases 8 and 3, or knock-down of caspase 8 prevented the loss of GLRX activity, FAS-SSG, and abolished cell death. Consistent with a role of S-glutathionylation of FAS in promoting apoptosis, absence of GLRX enhanced FASL-induced apoptosis, in association with more FAS accumulating in lipid rafts and FASL binding to FAS, while overexpression of GLRX protected against epithelial cell apoptosis, and diminished accumulation of FAS onto lipid rafts [159]. The demonstration that caspases 8 or 3 can directly cleave GLRX [159] suggests a protease-dependent mechanism towards inactivation of GLRX, in addition to aforementioned mechanism of oxidative inactivation of GLRX (Fig. 1).

A functional link between FAS, ER redox stress, epithelial cell death and lung fibrosis is emerging. In this regard, we have demonstrated that the molecular events that culminate in FAS-SSG originate within the ER [87]. FAS contains 24 cysteines, 20 of which occur as disulfide bridges, formed in the ER, that stabilize the receptor and enable binding of FASL. Epithelial cells were shown to contain a pool of latent FAS, not in the disulfide-bonded state. Administration of FASL triggers a calcium-

dependent signal that promotes disulfide bridge formation of FAS within the ER. The protein disulfide isomerase, PDIA3 was shown to be responsible for disulfide bridge formation in FAS (FAS S-S). Absence of PDIA3, or its pharmacological inhibition, maintained FAS cysteines in a sulfhydryl state, and resulted in attenuated FAS-SSG and attenuated epithelial cell death. Incubation of cells with the sulfenic acid trapping agent, dimedone, also prevented FAS-SSG, suggesting that formation of a sulfenic acid intermediate preceded the formation of FAS-SSG. In epithelial cells stimulated with FASL, GSTP was found to bind to FAS, and interaction between FAS and GSTP was first observed in the ER-enriched fraction [87].

A role of FAS-SSG in lung fibrosis also has emerged based upon the use of lung tissues from patients with IPF. In these tissues, an interaction between FAS and GSTP was also observed [144], along with increases in FAS-SSG [68]. siRNA-mediated ablation of GSTP or its pharmacological inhibition attenuated FAS-SSG and decreased cell death. The active GSTP inhibitor, TLK117, is an analog of GSH and the active metabolite of TLK199 has been used clinically [160,161]. TLK117 is a highly specific inhibitor of GSTP, having binding affinity greater than GSH itself and a selectivity for GSTP over 50-fold greater than other GSTs (inhibition constant  $[K_i] = 0.4 \mu\text{M}$ ) [162,163]. TLK117 was shown to attenuate both Fas-SSG as well as cell death [87,144].

To address the role of PSSG chemistry in lung fibrosis, our laboratories conducted a number of studies to modulate GSTP and GLRX. Mice that lacked functional *Gstp1* and *Gstp2* were subjected to bleomycin- or AdTGFB1-induced fibrosis, and were shown to have attenuations in FAS-SSG, decreased caspases 8 and 3 activity and diminished fibrosis, compared to WT controls. Direct administration of TLK117 into airways of mice with existing bleomycin- or AdTGFB1-induced fibrosis blocked the progression of fibrosis, in association with decreases in overall PSSG, decreases in S-glutathionylation of FAS, and decreased activities of caspases 3 and 8, compared to mice receiving vehicle control [144]. The importance of PSSG in lung fibrosis was further corroborated by studies that addressed the role of GLRX, using transgenic mice overexpressing *Glrx* in lung epithelia, as well as mice that globally lack *Glrx*. Mice that lack *Glrx* were more sensitive to AdTGFB1- or bleomycin-induced fibrosis, whereas mice that overexpress *Glrx* in lung epithelial cells showed increased resistance to fibrosis. Absence of *Glrx* augmented FAS-SSG in mice with bleomycin- or AdTGFB1-induced fibrosis, compared to WT mice, in association with enhanced caspase 3 activities, consistent with increases in cell death. Overexpression of *Glrx* in epithelial cells dampened FAS-SSG and caspase 3 activation. Furthermore, ablation of caspase-8 which is activated following activation of the FAS pathway, also attenuated FAS-SSG, caspase-3 activation and fibrosis [68]. Collectively, these data demonstrate that attenuation of epithelial cell death confers protection from bleomycin- or AdTGFB1-induced fibrosis, and that S-glutathionylation of FAS in epithelial cells is an important death-inducing signal that promotes fibrogenesis. In addition to the aforementioned findings, direct administration of recombinant GLRX into airways of mice augmented GLRX activity in the lung tissue, dampened PSSG, and reversed increases in collagen content, while inducing collagenolytic activity within the lung. Similar protective effects of GLRX were observed in lungs from aging mice, which were more prone to bleomycin-induced fibrosis. A mutant of GLRX lacking cysteine 23, which is critical in the deglutathionylation reaction (Fig. 1), failed to elicit protective responses, demonstrating that the catalytic activity of GLRX is important in the anti-fibrotic responses [68]. These collective observations point to the importance not of GSH per se, but a unique facet of GSH chemistry, which involves its covalent incorporation into proteins (i.e. PSSG), catalyzed by GSTP, and reversed by GLRX, a biochemical pathway regulated by enzymes that had not previously been recognized in settings of pulmonary fibrosis (Fig. 1). Thus, the consideration of clinical studies utilizing the clinically relevant GSTP inhibitor, TLK199, or a variant thereof, in conjunction with active GLRX appears well warranted and may yield new

insights into the functional importance of S-glutathionylation chemistry in the pathogenesis and progression of this deadly disease.

## 10. ER stress in COPD

Like IPF, activation of the UPR also has been detected in lung tissue of patients with COPD and has predominantly been associated with the emphysema phenotype [164–166]. Chronic ER stress was shown to promote alveolar epithelial cell death, although ER stress has also been demonstrated in airway epithelial cells, lung endothelial cells [167] and fibroblasts [168] as well. Moreover, ER stress in COPD is not limited to lung tissue, but was also shown to be involved in COPD-related comorbidities. In skeletal and respiratory muscles for instance, increased ER stress was associated with muscle dysfunction [169]. In this review only the evidence for ER stress in the lungs will be further elaborated on. The induction of the UPR has been linked with the exposure to cigarette smoke per se, as many harmful components in cigarette smoke lead to oxidative damage of proteins, which tend to misfold, aggregate and accumulate. In fact, the evidence for the presence of ER stress in COPD itself is rather limited, as more studies have examined ER stress in smokers compared to non-smokers, or in *in vitro* or *in vivo* models of smoke-exposure. Since the animal models of smoke exposure use emphysema development as their main endpoint, ER stress has been solely linked to this disease phenotype. The few studies that used patient-derived material have confirmed the link between ER stress and emphysema.

*In vitro* exposure of lung epithelial cells to smoke rapidly and dose-dependently activated the PERK and ATF6 arms of the UPR, whereas inconsistent induction of the IRE1 $\alpha$  arm occurred [170–172]. The effects of smoke on the UPR could be largely inhibited by compounds with antioxidant-like activities suggesting the involvement of smoke-induced oxidation of target proteins [172,173]. Importantly, the vapor phase appeared to be a more potent activator of the UPR as compared to the particulate phase of cigarette smoke [171]. Acrolein, a particular component of the vapor phase, was shown to cause UPR activation and ER stress *in vitro*, as well as in acute and chronic exposure models in rats [174,175]. Mechanistically, other thiol-reactive compounds present in smoke, such as acrolein and hydroxyquinones were shown to oxidize and inhibit PDI located in the ER, thereby limiting protein folding [176]. When comparing the induction of the UPR by smoke in air liquid interface cultures of primary bronchial epithelial cells isolated from COPD patients compared to cells isolated from controls, it appeared that an induction of all three branches of the UPR could only be observed in cells isolated from COPD patients. Glutathione peroxidase 1 (GPX1), which is known to be deficient in COPD patients, was down-regulated in the COPD-derived cultures in response to smoke [166], and a role for the tyrosine kinase SRC in promoting the accelerated decay of *GPX1* mRNA in airway epithelial cells from COPD patients was shown. Chemical inhibitors of SRC prevented the loss of lung GPX-1 expression in COPD-derived epithelial cells and in response to chronic smoke exposure *in vivo* [177], although in this study, the impact of SRC inhibition on the UPR was not evaluated. However, the reintroduction of GPX1 in the COPD-derived cultures was sufficient to reduce the UPR. Furthermore, ER stress markers were more prominently induced by chronic smoke exposure in *GPX1* deficient mice, in association with enhanced cell death, inflammation and emphysema development. These findings further confirm that an altered redox environment, possibly linked to SRC activation and loss of GPX1 is involved in the induction of the UPR in response to smoke [166]. In a variety of murine smoke-exposure models of COPD, ER stress has been documented. In mice, exposure to a single cigarette was sufficient to induce changes to the organization of the ER network, to induce phosphorylation of eIF2 $\alpha$ , and to increase nuclear ATF6 levels [178]. ER stress was still evident when extending the exposure to 3 months or even a year [166,179]. No time-course studies have been performed to evaluate whether ER stress progressively increases with the duration of the

exposure, when a maximal level is reached, or when the adaptive UPR fails and/or becomes pathological. As in *in vitro* experiments, curcumin, a compound with an electrophilic character present in the diet which has been attributed antioxidant-like properties in part through activation of NRF2 [180], reversed the increased expression of ER stress markers, as well as apoptosis in smoke models [181].

In addition to cigarette smoke itself, other smoke- or disease-related factors are implicated in the activation of ER stress in COPD. The depletion of calcium from the ER triggered by smoke exposure for instance possibly also contributes to UPR activation [182]. In air liquid interface cultures of epithelial cells from COPD patients, calcium release from the ER and calcium signaling were found to be disrupted, but this has not yet been linked to ER stress [183]. Factors related to mitochondrial dysfunction, of which the contribution to COPD pathogenesis and the dysregulation of epithelial cell function was recently reviewed [184], include low energy levels and mitochondrial-derived ROS. Hypoxia is another important factor in COPD that could trigger the UPR [185], but for which there is no direct evidence yet. Plasma heme, which is released from damaged red blood cells and which is a potent trigger of oxidative stress, was found to be increased in patients with very severe COPD. In a ferret model of fibrosis and emphysema induced by the inhalation of Br<sub>2</sub>, heme scavenging inhibited ER stress and the development of both pathological features of COPD, fibrosis and emphysema. Inhibition of ER stress-mediated apoptosis using salubrinal or genetic deficiency of ATF4, similarly prevented the development of fibrosis and emphysema in this injury model. Reduced numbers of macrophages and neutrophils and elastase activity were implicated in these protective effects. Collectively findings from this study shows that ER stress induced by free heme could play a role in the development of airway fibrosis and pulmonary emphysema as observed in COPD [186].

The only monogenetic cause underlying COPD is  $\alpha$ 1-antitrypsin deficiency, which leads to early onset emphysema. The liver is the main source of circulating  $\alpha$ 1-antitrypsin, a secretory glycoprotein that counterbalances trypsin activity. Interestingly, the deficiency which is caused by a lysine to glutamate 342 substitution (E342K), leads to the misfolding of  $\alpha$ -antitrypsin and subsequently to its polymerization and hyper-aggregation. The protein can no longer be secreted and accumulates in all components of the secretory pathway, but mostly in the early part. In the liver, the proteotoxic effects of misfolded protein accumulation causes an ER storage disease. Accumulation of mutated  $\alpha$ 1-antitrypsin activates NF- $\kappa$ B and autophagy, but modest UPR activation [187]. In the lungs on the other hand, emphysema results mainly from the lack of trypsin inhibition and consequent excessive breakdown of connective tissue. However, small quantities of  $\alpha$ 1-antitrypsin are also produced by epithelial cells. In a mouse model of the  $\alpha$ 1-antitrypsin E342K mutation, accumulation of the  $\alpha$ 1-antitrypsin Z variant occurred also in pneumocytes, through a process similar as in the liver. In these mice the UPR was induced, and promoted inflammation [188]. Transgenic mice expressing the  $\alpha$ 1-antitrypsin Z variant also spontaneously developed pulmonary fibrosis as a consequence of the ineffectiveness of proteostasis mechanisms, which include autophagy, to counteract the proteinopathy [189]. It was furthermore shown that  $\alpha$ 1-antitrypsin mutation carriers displayed a dysregulated expression of various miRNAs that target ER protein folding responses, which was associated with enhanced expression of UPR activation markers. miR-199a-5p in particular was upregulated in asymptomatic mutation carriers, likely in response to ER stress. A downregulation was on the other hand found in symptomatic mutation carriers, which appeared to be driven by hypermethylation of the miR-199a-5p promoter. Importantly, overexpression of pre-miRNA-199a-5p significantly reduced Bip, ATF6, CHOP, GADD34 and XBP1 levels in monocytes. RELA expression was also attenuated, in concert with lower LPS-induced production of various inflammatory cytokines [190]. In COPD, as in IPF however, miR-199a-5p was upregulated [191], but to date its association with ER stress has not been examined.

Triggers of COPD exacerbations, including viral and bacterial infections, are also known inducers of ER stress and might further amplify existing stress [166,192,193]. A normal ER stress response is required for an effective innate immune response to infection, but in COPD the increased susceptibility to infection and defective clearance mechanisms might be related to the pre-existing aberrant stress responses [194,195]. As was described above, dissociation of the chaperone Bip from the ER luminal surface triggers the dimerization and autophosphorylation of the triad of UPR sensors PERK, ATF6 and IRE1 $\alpha$ . In small airway epithelial and type II alveolar epithelial cells of lung tissue of smokers, in rats, as well as in epithelial cells exposed to smoke *in vitro*, Bip expression was found to be increased in association with ER stress [179,196,197]. In lung tissue, Bip was present in higher levels in alveolar epithelial cells and macrophages from emphysema patients compared to controls [164]. Bip could furthermore be detected in BALF, and levels were shown to be elevated in smokers. The secretion of Bip occurs through a non-classical mechanism, and could be induced from human airway epithelial cells by smoke exposure, by pharmacologically-induced ER stress, as well as inhibition of histone deacetylases (HDAC) [198]. Increased levels of Bip have also been reported in plasma and serum of subjects with COPD, and are considered a potential biomarker for the disease, correlating with disease severity and the degree of emphysema [186,199]. Whereas Bip located in the ER acts as the main trigger that activates the three UPR sensors, extracellular Bip exerts anti-inflammatory effects by augmenting the release of anti-inflammatory molecules, such as IL1RA and soluble TNF $\alpha$  receptor II [200]. In addition to increased levels of Bip, plasma of COPD patients was found to contain anti-Bip IgG autoantibodies. Bip is an antigen that has been associated with autoimmune diseases [201]. A specific association with the presence and severity of emphysema was noted for these auto-antibodies in COPD, as for the auto-antigen itself. The auto-Bip antibody increased baseline production of various inflammatory mediators by macrophages, and treatment of PBMCs from patients in whom auto-antibodies were measured, with recombinant Bip induced CD4<sup>+</sup> T-cell proliferation [164]. This finding potentially connects ER stress to the paradigm of an autoimmune component of COPD development. It is furthermore of interest that the HLA-DRB1\*15 haplotype appears to protect smokers from auto-Bip antibody production, whereas the haplotype is overrepresented in IPF patients with anti-HSP70 autoantibodies [202]. Disease-specific reactions seem thus to underlie these auto-immune responses.

In lung tissue of smokers, increased protein expression of PDI was observed compared to non-smokers, likely as an adaptive response to smoke exposure. It is not known if this adaptive response fails in smokers who develop COPD. In mice, exposure to a single cigarette did not affect PDI expression, whereas protein expression was increased in airway lining cells when exposure was extended to 6 weeks. This was not found to be related to enhanced PDI mRNA levels. Moreover, a reduced turnover rate of PDI protein is unlikely as PDI is a long-lived protein. It was speculated to occur rather through preferential loading of PDI mRNA into ribosomes, as was reported for other effectors of the ER stress response [203]. On the other hand, the increased protein level of PDI in a smoke-exposure model of COPD was associated with high levels of oxidized, including sulfenic acid-containing forms of the protein. These observations are in line with the reported alkylation of PDI by acrolein and hydroxyquinones described above. PDI oxidation was furthermore found to progressively increase with age. The consequent inhibition of its reductase and isomerase activity would limit its capacity to restore ER homeostasis, and potentially contribute to fueling chronic ER stress responses [176,178] (Table 2).

Because of the chronic nature of the insult, and the self-perpetuating mechanisms of injury that occur in COPD, the UPR is unable to repair or dispose of damaged proteins through the ERAD. Likewise, other proteostasis processes such as autophagy fail. The processes of ER stress and autophagy have both been found to contribute to COPD and are interrelated. For instance, inhibiting autophagy in a murine smoke



**Table 2**  
Implications of ER stress in COPD.

ER stress features	Putative function	Model	References
PDI	Inhibition and oxidation of PDI by cigarette smoke, diminished protein folding Increased expression reflecting potential adaptive response	Mouse models In vitro Humans	[176,178]
Bip	Increased in smokers and COPD; Extracellular BiP: Anti-inflammatory effect Anti-BiP antibodies: Linked with enhanced inflammation, emphysema and auto-immunity	Humans Human airway epithelial cells, lung lavages Humans Mouse model	[164,186,197–199,201]
PERK/ATF6	Induced by cigarette smoke vapor phase, acrolein, hydroxyquinones Impact: adaptive response or cell death	Cell lines Human airway epithelial cells	[170–172,175]
UPR	Induced by cigarette smoke in epithelial cells from COPD patients Impact: cell death, regulated by GPX1	Patient-derived cells	[166]
ATF4/ER stress	Induced by free heme following Br2 inhalation Impact: Emphysema and fibrosis	Mouse and ferret models	[186]
ATF4/PERK	mutant alpha 1 anti-trypsin > Inflammation	Mouse model Human lung explants from COPD patients	[188]
Bip/ATF6	Controlled by miRNA-199a-5p protease activation > inflammation, fibrosis and emphysema	Blood monocytes from patients with $\alpha$ anti-trypsin mutation	[190]

model was shown to limit apoptotic cell death and attenuated the expression of ER stress proteins PERK and CHOP. In concert, this led to limited emphysema development as well as attenuated bronchial wall thickening [181,204]. Failure of these adaptive mechanisms results in aberrant cell functioning or excessive cell death. ER stress in response to smoke indeed contributes to epithelial cell death and this has been put forward as a mechanism underlying emphysema development. In primary alveolar epithelial cells smoke-induced apoptotic cell death was associated with caspase 12 activity, CHOP expression, and phosphorylation of PERK, eIF2 $\alpha$  and IRE1. These effects could be attenuated by overexpression of HERD1, a specific ubiquitin E3 ligase located in the ER and part of ERAD [205]. Other reports also demonstrated that a dysfunction of the proteasomal system in smoke-exposed conditions contributed to ER stress and excessive epithelial cell death [206]. Inhibition of ER stress in smoke-treated epithelial cells and a mouse model using the chemical chaperone, 4-phenylbutyric acid (4-PBA), reduced the amount of epithelial cell death and inflammation, and could partially suppress emphysema development [179]. Protection against ER stress and consequent cell death therefore provides a plausible strategy to prevent the development of emphysema (Table 2).

In addition to inducing cell death, ER stress results in pro-inflammatory signaling which is demonstrated in many of the COPD-related studies described above. Activation of antioxidant defense via concerted actions of NRF2 and ATF4, fails in COPD because of defects in NRF2 signaling, further discussed below. A role for ER stress in the induction of airway fibrosis in COPD is inferred from only a limited number of studies conducted so far. Likewise, it has not been examined whether ER stress in airway epithelial cells could contribute to airway features of COPD, such as mucus metaplasia and ciliary dysfunction. In this regard, a recent study demonstrated that cigarette smoke exposure caused internalization of cystic fibrosis transmembrane conductance regulator (CFTR) and promoted its retrograde trafficking to the ER. Inhibition of CFTR has been linked to decreases in airway hydration and mucus stasis in COPD patients [207].

## 11. COPD and S-glutathionylation

Oxidative stress is considered a major driver of the development of COPD [208]. Cigarette smoke itself of course contains a myriad of oxidants and the state of chronic inflammation further challenges the pulmonary anti-oxidant defenses. In recent years, the contribution of NADPH oxidases in epithelial cells to the local redox imbalances have furthermore become known. The downregulation of DUOX1 in patients with COPD [209], for instance could contribute to diminished epithelial responses to injury [210]. Furthermore, increased NOX4 levels co-localized with  $\alpha$ SMA and TGF $\beta$  in airway smooth muscle cells, and

correlated with small airway remodeling and COPD disease severity [211,212]. As part of the adaptive response to these oxidant exposures, GSH levels increase in smokers and in early stages of COPD. Increased GSH levels were shown in epithelial lining fluid, sputum, as well as plasma of COPD patients compared to controls [129]. GSSG was elevated as well in sputum, indicating a more oxidizing environment of the lungs [213]. The excess oxidant levels are indeed insufficiently counteracted by the pulmonary defense systems. The antioxidant defenses naturally decline with aging [214], and GSH is not an exception to this phenomenon [215,216]. In COPD, NRF2 constitutes an important mechanism underlying the failing antioxidant defense. Indeed, lower levels of NRF2 were present in lung tissue of emphysema patients, in association with a disequilibrium between NRF2 and its inhibitors KEAP1 and BACH [217]. Moreover, genetic ablation of *NRF2* enhanced the susceptibility to smoke-induced emphysema in mice [218]. Pharmacological activation of NRF2 on the other hand attenuated emphysema development in mice [219]. Short duration clinical trials have however not been successful at even restoring NRF2-target gene expression or inflammation [220]. Prior to targeting NRF2, clinical trials have examined the therapeutic potential of increasing GSH levels in COPD. The GSH precursor, NAC, the main compound investigated in most studies failed to demonstrate clinical improvement [221–225]. However, a meta-analysis showed that NAC, and related compounds erdosteine and carbocysteine, lowered the number of disease exacerbations, their duration and severity [226]. These effects are however likely to be effectuated through NAC's mucolytic activity, which involves reducing cysteine oxidations in mucin polymers increasing their solubility, rather than its GSH precursor function per se [227].

In contrast to the large number of studies that have examined oxidative stress and GSH responses in general in COPD, the potential importance of PSSG and its regulatory enzymes is only recently emerging. GLRX protein levels were decreased in lung tissue of patients with COPD, paralleling decreases in the number of GLRX-positive macrophages. Macrophages were shown to be the main cell type expressing GLRX and the number of GLRX-positive macrophages positively correlated with lung function. Furthermore, during disease exacerbations, significantly increased GLRX protein could be detected in sputum [146]. Our unpublished observations confirm decreases in protein level of GLRX in lung tissue of COPD patients, which was also found to correlate with lung function (N.L. Reynaert). Moreover, the lower protein level of GLRX was associated with attenuated GLRX activity and overall increased PSSG in lung tissue of COPD patients compared to smoking controls. Other unpublished observations confirm the increased level of GLRX in sputum during a disease exacerbation in COPD patients, which was also associated with a higher GLRX activity level and an attenuated level of sputum PSSG. Although we were unable to



determine the cellular source of GLRX in sputum supernatant, there was a positive correlation with the total number of viable cells present in the sputum (N.L. Reynaert, unpublished). A non-classical export mechanism was proposed for GLRX [228], which remains to be further elucidated, as is the cellular source, as well as its particular functions.

Contrasting aforementioned findings with GLRX, GSTP1 levels were found to be increased in lung tissue lysates of patients with mild COPD. However, using immunohistochemistry methods, no differences were observed when examining GSTP1 positive cell populations between controls and COPD patients. Like GLRX, GSTP1 could be detected in sputum, but here the levels were not different between patients and controls [229]. The causal role of GSTP1 in COPD pathogenesis has been investigated. Deletion of *GSTP1* in fibroblasts induced apoptosis [230], whereas overexpression protected against smoke-induced cell death [230], findings that contrast the aforementioned death promoting effects of GSTP1 in FASL-exposed lung epithelial cells [87]. At a genetic level, *GSTP1* has been studied in COPD more frequently, because it is thought that variations in the gene could contribute to COPD susceptibility by attenuated detoxification of, and antioxidant protection against cigarette smoke and associated products. The GSTP1 I105V polymorphism in particular has been studied frequently in COPD, but a recent meta-analysis concluded that there is no significant association between this polymorphism and disease risk [231]. No studies to date have examined the putative protective role of GSTP1 to smoke-induced emphysema, nor has GSTP1 been related to PSSG in this context.

Exposure of lung epithelial cells to cigarette smoke extract reduced GLRX protein levels and activity, and increased PSSG [232]. In mice, exposure to cigarette smoke also reduced GLRX expression and activity, but here PSSG was decreased, as were free protein thiol levels, with a concomitant increase in protein carbonylation [233]. In bronchoalveolar lavage fluid, as well as in macrophages isolated from smoke-exposed mice, an increase in PSSG was however observed [232]. PSSG seems thus to be distinctly regulated in different regions and cellular compartments of the lungs in response to cigarette smoke. Importantly, recombinant GLRX was found to be irreversibly oxidized and inhibited by the electrophilic compound acrolein present in smoke [232]. Extracellular GLRX could be more prone to such modification by smoke. Overall, the attenuated activity of GLRX in lung tissue of COPD patients, and in lungs and cells treated with cigarette smoke is likely the result of decreased mRNA expression in combination with smoke-induced post-translational modifications of the protein.

GLRX and PSSG play a role in regulating epithelial cell death in response to cigarette smoke. Overexpression of GLRX protected epithelial cells from smoke-induced cell death, which was associated with attenuated PSSG, while the converse was observed in the absence of GLRX [232]. The mode of cell death triggered by smoke was not specifically examined and could involve ER stress. In mice, the absence of *Glrx* decreased neutrophil influx into lung tissue in response to 4 weeks of cigarette smoke exposure, whereas the number of macrophages was increased. Bronchoalveolar lavage fluid of these mice contained lower KC levels and epithelial cells from *Glrx*<sup>-/-</sup> mice produced less KC in response to smoke as well [232]. Macrophages from *GLRX*<sup>-/-</sup> mice were however smaller, expressed lower levels of PU.1 and showed a diminished phagocytic capacity, and lower inflammatory responses to LPS [234]. Another study on the other hand found that 3 days after smoke exposure the neutrophil influx was increased in *Glrx*<sup>-/-</sup> mice, with a concordant increase in pro-inflammatory cytokines [235]. These contrasting results are likely related to the different durations of the exposure to cigarette smoke. Interestingly, the protective effects of the absence of *Glrx* on smoke-induced inflammation we showed using a 4 week exposure regimen is in line with our previous study in which the absence of *Glrx* in epithelial cells, through enhanced protein S-glutathionylation of IKK $\beta$  was shown to limit the activation of NF- $\kappa$ B in response to both TNF $\alpha$  or LPS [236]. Conversely, in the 3 day exposure regimen NF- $\kappa$ B activity was enhanced in *Glrx*-deficient mice, in

association with S-glutathionylation of IKK $\alpha$  as well as IKK $\beta$  [235]. The duration of the exposure to smoke thus also seems to divergently affect the regulation of NF- $\kappa$ B by the GLRX/PSSG axis, which should be examined in more detail. It has not been examined whether *Glrx*<sup>-/-</sup> mice are more or less susceptible to smoke-induced emphysema. The positive correlation between GLRX and lung function reported in the clinical studies points to a putative protective role of GLRX towards COPD, a scenario supported by the demonstrated protection that GLRX confers against smoke-induced cell death, which is an important driver of emphysema development.

When considering additional pathways that could be involved in COPD pathogenesis through differential S-glutathionylation, neutrophil migration and phagocytic capacity are prominent candidates. Negative influences of PSSG were shown on actin polymerization in neutrophils, which limited migration and phagocytic capacity [237,238], effects which were exacerbated in *Glrx*-deficient neutrophils [238]. Conversely, S-glutathionylation of  $\alpha$ 4 integrin positively affected the migration of neutrophils by increasing the binding to VCAM1 on endothelial cells, thereby increasing the egression of neutrophils from the bone-marrow [239]. *Glrx*-deficient neutrophils in addition showed defects in formation of neutrophil extracellular traps (NET). In COPD, NET formation was increased and implicated in the enhanced susceptibility of patients to develop disease exacerbations [240]. Pathogens that colonize the airways of COPD patients on the other hand can evade trapping and killing in NETs. A bacterial peroxiredoxin-glutaratedoxin bifunctional enzyme in nontypeable *Haemophilus influenzae* has been shown to promote such resistance and to be involved in the colonization and recurrence of infections by this pathogen in COPD [241]. It remains to be determined to what extent PSSG and the regulation by GLRX is involved in the dysregulation of neutrophil trafficking and functioning in COPD. Therefore, the GLRX/PSSG axis in pathogens should not be overlooked as a potential contributor to COPD pathogenesis and progression.

Lastly, S-glutathionylation of  $\alpha$ 1-antitrypsin was shown to attenuate its anti-protease function, whereas S-glutathionylation of MMP-1,-8 and -9 was associated with enhanced activity [242]. If  $\alpha$ 1-antitrypsin and MMPs were found to be more S-glutathionylated in lung tissue of COPD patients, this scenario would have the potential to contribute to enhanced tissue destruction by disrupting both sides of the protease-antiprotease imbalance.

## 12. The unfolded protein response and protein disulfide isomerases in allergic asthma

Like IPF and COPD, activation of the UPR also has been observed in the airway epithelium of patients with asthma and indicates that ER stress also accompanies asthma. Activation of the UPR transducer, IRE1 $\beta$ , subsequent XBP-1 splicing and transcriptional increases of chaperone anterior gradient homolog 2 (AGR2, also known as PDIA17) were shown to be important for mucin production in human primary bronchial epithelial cells [79,243] in addition to the aforementioned role of XBP-1 in the transcriptional upregulation of MUC5B relevant to IPF [92]. The increases in expression of IRE1 $\beta$  in human asthmatic epithelium as compared to the non-asthmatic epithelium were also correlated with increased mucus production and goblet cell metaplasia [79]. Consequently, depletion in IRE1 $\beta$  or deletion of AGR2 significantly attenuated mucus metaplasia in a mouse model [79,243] and suggests that the IRE1 $\beta$  axis is important in allergen-induced mucus metaplasia in the lung epithelial cells.

Genetic association studies have linked polymorphisms of Orosomucoid-like (ORMDL)3 with asthma [244–246]. ORMDL3 is an ER-localized protein with homologs in yeast that decreases the activity of serine palmitoyltransferase thereby inhibiting de novo sphingolipid biosynthesis [246]. Expression of ORMDL3 was increased in mouse models of allergic asthma [247] and ORMDL3 over-expressing mice showed selective activation of ATF6 in association with increases in

**Table 3**  
Implications of ER oxidoreductases and/or ER stress in asthma.

ER oxidoreductase	Putative function	Model	References
PDIA3	Apoptosis, chemokine/growth factor folding	Cell culture, mouse models, human epithelial cells	[254,256]
AGR2 (PDIA17)	Mucus metaplasia	Cell culture, mouse models, human epithelial cells	[243]
IRE $\beta$	Mucus metaplasia linked to upregulation of XBP1 and AGR2	Cell culture, mouse models human samples	[79]
XBP1	Mucus, Eosinophil differentiation, type II inflammation and IFN-related gene expression	Cell culture, mouse models, human samples	[243,250,304]
ATF6 $\alpha$	Linked to OMRDL3 and SERCA2b, macrophage activation, epithelial cell death airway hyperresponsiveness, smooth muscle hypertrophy	Cell culture, mouse, human samples	[247,248,256,305]
CHOP	IL-4/macrophage signaling, ATF6 expression	Cell culture, mouse, human samples	[249]

airway remodeling, including increased airway smooth muscle, sub-epithelial fibrosis, and mucus metaplasia [247]. Furthermore, ATF6 was found to regulate airway hyperresponsiveness, smooth muscle proliferation and contractility in a mouse model of asthma [248]. In addition to ATF6, allergic asthmatics as well as mouse models of asthma exhibited increases in expression of CHOP [249]. CHOP in association with ATF6 exacerbated allergic airway inflammation by enhancing M2 programming in macrophages in a mouse model of asthma [249]. These reports collectively highlight that the canonical UPR transducer pathways impact various facets of allergic asthma [250] and have led to the hypothesis heterogeneous severe asthma could be potentially be endotyped based upon the UPR, moving beyond type 2 high/low paradigm [251–253] (Table 3).

PDIs are upregulated in asthma, however their function in disease pathophysiology is not well understood [254,255]. We have identified that increases in expression of various PDIs correlated with a higher bronchodilator response or blood eosinophilic counts in allergic asthmatics, indicative of worse disease [254,255]. The protein disulfide isomerase A3 (PDIA3) can associate with Bak and promote its oligomerization thereby triggering intrinsic apoptosis under conditions of ER stress. In a house dust mite model of allergic inflammation, genetic ablation of PDIA3 specifically in lung epithelial cells decreased proapoptotic Bak oligomerization, and caspase-3 activity in mice, in association with decreases in airway fibrosis [254], and similar results were obtained following siRNA mediated ablation of ATF6- $\alpha$  [256]. Ablation of PDIA3 resulted in more exposed sulfhydryls (-SH) in the glycoproteins periostin and epidermal growth factor (EGF), both of which are involved in airway remodeling, suggesting a potential mechanism whereby PDIA3 promotes airways remodeling. PDIA1, PDIA5, PDIA6, and ERp18 are known to modulate the activity of PERK, ATF6 and IRE1 through oxidoreductase activities in various biological settings [257–260]. In addition to PDIA3, we found that both PDIA5 and PDIA6 were also upregulated in epithelial cells from allergic asthmatics and airway epithelial cells using mouse models of asthma [255]. Furthermore, in mouse models of asthma, therapeutic administration of the UPR modulators, tauroursodeoxycholic acid (TUDCA) or Alanine  $\beta$ -Muricholic acid (A $\beta$ M), decreased the UPR and subsequently decreased PDIA5 and PDIA6 expression [255]. Furthermore, these decreases were associated with an attenuation of various parameters of allergic airway disease in mice. Retrospective analysis of patient data revealed that increases in PDIA5 significantly correlated with increased eosinophilic and diminished bronchodilator response in allergic asthmatics, again being indicative of worse disease [255].

To date, no FDA-approved therapeutics are available to inhibit PDIs; however, decades of research from various laboratories have identified numerous inhibitors that have shown *in vivo* and *in vitro* efficacy in inhibiting PDIs. Interestingly, rutinoides (plant flavonoids) that are known to inhibit PDIs are now being used in different clinical studies [261]. It also is interesting to note that Kaplan et al. have identified LOC14 as a specific inhibitor of PDIA1 and PDIA3 [262]. These observations suggest that the UPR and subsequent induction of PDIs

regulate the pathophysiology of asthma, among other diseases, and that inhibition of an oxidoreductases such as PDIs may be a potential therapeutic approach that would benefit patients with chronic lung diseases (Table 3). One of the modulators of UPR, TUDCA is being increasingly used in clinical studies (Clinicaltrials.gov) for various indications. A few studies show that TUDCA administration is safe in humans and potentially effective in alleviating symptoms in patients with amyotrophic lateral sclerosis [263](NCT00877604), and in increasing insulin sensitivity in obese men and women [264](NCT00771901). These results suggest that modulating the UPR may have potential beneficial effects in neuro-degenerative and metabolic diseases. Therefore, future studies using TUDCA in patients with chronic lung diseases appear well warranted.

### 13. Glutathione biochemistry in asthma

Many studies have addressed potential alterations in GSH redox homeostasis in patients with asthma, and these have been summarized in several recent reviews [33,265,266]. Not surprisingly, given the high diversity in asthma subtypes and severity, different methodologies to measure GSH and/or GSSG, and variable impact of corticosteroids or other medications, reported values vary dramatically [265]. Some reports indicate that total levels of GSH are increased in asthmatics, which has been attributed to increased oxidative stress and resultant adaptive responses in asthmatic patients [266], for example by activation of NRF2 and induction of genes involved in GSH biosynthesis [219,267]. However, increased activation of NRF2 pathways in severe asthmatics was not associated with increased GSH or cysteine levels in either BAL or plasma, and GSH redox status was in fact more oxidizing, suggesting dysregulation of NRF2 signaling [268]. The importance of cellular GSH status in asthma pathology has been supported by animal studies showing that disruption of cellular cysteine uptake by deletion or inhibition of GGT [269,270], or inhibition of GSH synthesis using buthionine sulfoximine [271], worsened allergen-induced airway reactivity and/or inflammation, and have promoted studies with GSH administration or precursors such as NAC to alleviate disease phenotypes, with variable success [271]. GSH may also be protective in asthma based on its involvement in formation of nitrosoglutathione (GSNO), which acts as endogenous bronchodilator [272]. In fact, GSNO levels were found to be decreased asthmatic airways compared to airways of healthy subjects, which was related to increased expression of GSNOR reductase (GSNOR) [273]. Mice lacking the *Gsnor* gene showed increases in S-nitrosothiols and were protected from airways hyperresponsiveness in models of allergic asthma [274], prompting development of GSNOR inhibitors as novel therapeutic agents in therapeutic management of asthma [275]. Other biochemical forms of GSH, such as LTC4 and related cysteinyl leukotrienes have been found to be elevated in asthma, and are thought to contribute to asthma pathology due to their broncho-constrictive properties [276]. Redox alterations in asthma are also manifested in the form of enhanced disulfide cross-linking in mucus proteins, by oxidative pathways likely involving

eosinophil activation, thereby contributing to formation of mucus plugs and airflow obstruction [277]. The beneficial effects of thiol-based antioxidants such as N-acetylcysteine, often thought to act by fueling GSH synthesis, likely involve its ability to break such disulfide bonds and act as a mucolytic, and novel strategies are being developed to promote such a mucolytic action [227].

Alterations in some GSH-dependent enzymes have also been documented in asthma. For example, various isoforms of glutathione peroxidase, critical in GSH-mediated detoxification of H<sub>2</sub>O<sub>2</sub> or related peroxides, are upregulated rodent models of allergic airway disease or in patients with asthma [278,279]. Glutathione S-transferases have also been implicated in the pathogenesis of asthma, and common genetic variants of a number of GSTs have been linked asthma, with variable correlations to disease severity [280,281]. This variability likely relates to the diverse functions of GSTP1, as well as the varying asthma endotypes. GSTP1 may be protective against environmentally-induced or occupational asthma by contributing to metabolism of environmental toxicants, but may also enhance features of asthma through its role in protein S-glutathionylation (see below). Interestingly, GSTP1 has also been reported to enhance the proteolytic activity of the HDM allergen Der p1, and may thereby enhance allergen-induced immune responses [282]. *Gstp1* was found to be downregulated in mouse models of allergic inflammation and in children with asthma [283], and studies in mice showed that *Gstp1* deletion enhanced OVA-induced allergic airways inflammation and hyperresponsiveness in mice, and also enhanced indices of collagen deposition and smooth muscle proliferation [284]. However, it is unclear to what extent these latter findings are relevant for alternative models of asthma and exacerbations, or whether these effects of GSTP1 are related to S-glutathionylation.

Relevant to the main topic of this review, emerging evidence also suggests changes in protein S-glutathionylation in asthma. Analysis of sputum samples from asthmatics revealed a decrease in PSSG compared to controls, which was attributed to increased levels of extracellular GLRX and increased *GLRX* mRNA in bronchial epithelial cells [285]. However, studies of nasal brushings from asthmatic patients indicated a decrease in *GLRX* mRNA expression [286], possibly reflecting the different sites of sampling. Studies in mouse models of allergic airway disease indicate increased *GlrX* mRNA during allergic inflammation induced by ovalbumin (OVA) or house dust mite (HDM), whereas *GlrX2* mRNA was unchanged. In spite of these increases in GLRX, overall lung tissue levels of PSSG were also increased in models of allergic asthma [147,287,288]. Evaluation of temporal changes of GLRX, GSH and PSSG in airways and lung tissues from mice subjected to the OVA model showed transient increases in BAL GSH and GLRX protein after OVA challenge, and more persistent increases in lung tissue PSSG and GLRX. Observed increases in GLRX levels in the BAL were found to positively correlate with increases in cytokines [288], suggesting a link between GLRX and airway inflammation [288,289]. Studies with *GlrX* deletion showed somewhat disparate findings, depending on the mouse strain and allergen model used. OVA-induced allergic airway inflammation and remodeling in BalB/C mice developed similarly in *GlrX*<sup>-/-</sup> mice but appeared to resolve faster [288], suggesting that PSSG may actually contribute to resolution of allergic airways disease. In a HDM-induced model of allergic airways disease, different patterns of inflammatory profiles and cytokine secretion were observed in BalB/C *GlrX*<sup>-/-</sup> mice, with a decrease in Th2 cytokines and airway eosinophils, but an increase in airway macrophages and neutrophils, which was associated with increased production of the Th17 cytokine, IL17. *GlrX*<sup>-/-</sup> mice displayed altered profiles of airway hyperresponsiveness in this model, with increases in central airway resistance but with dampened peripheral elastance in response to HDM, indicating variable impact of GLRX and PSSG on airway and parenchymal lung function [287,288].

While the critical protein targets for S-glutathionylation in the context of allergic airway inflammation have not been identified, several proteins relevant for asthma pathobiology are known to be regulated by S-glutathionylation. Among these are proteins involved in the

NF- $\kappa$ B signaling pathway, which plays a central role in immune responses and inflammation [290] and in asthma pathophysiology [291,292]. As was described in the previous section, S-glutathionylation can regulate the NF- $\kappa$ B pathway at multiple levels, and S-glutathionylation of IKK $\beta$ , IKK $\alpha$  and RelA have been reported, in association with decreases in or alterations of expression various epithelial cell-derived pro-inflammatory mediators in epithelial cells exposed to LPS, TNF $\alpha$  or IL17A [236,293,294]. The adaptor protein, MyD88 adaptor-like (MAL or TIRAP), critical in signaling induced by various Toll-like receptors, can also be targeted by S-glutathionylation of Cys91, which appears to enhance pro-inflammatory signaling [295]. A recently identified redox-based mechanism relevant to asthma pathology involves epithelial secretion of the alarmin IL33, as an innate response mechanism to allergens in activation of type 2 cytokines such as IL-13, and subsequent mucus metaplasia and fibrotic remodeling. Allergen-induced secretion of epithelial IL33 was found to require activation of the H<sub>2</sub>O<sub>2</sub> producing NADPH oxidase homolog dual oxidase 1 (DUOX) [296]. Epithelial DUOX1 expression and activation is enhanced in the airways of asthmatic subjects, in associated with increased cysteine oxidation in tyrosine kinases such as SRC and EGFR [296,297], which have been previously found to be involved in asthma pathology. Cell-based studies indicate that DUOX1 activation can promote S-glutathionylation of several target proteins [298], including SRC and EGFR [299], but their activation is enhanced by oxidation of conserved cysteines within their kinase domains to a sulfenic acid, whereas the importance of S-glutathionylation for activation of these kinases is less clear [300,301]. Induction of IL33 in macrophages is also subject to regulation by S-glutathionylation, based on observations that LPS-induced increases in *IL33* mRNA is diminished in macrophages lacking *GlrX*, in association with enhanced S-glutathionylation of TRAF6 resulting in attenuated NF- $\kappa$ B activation. Conversely, IL33 was found to enhance *GlrX* induction in response to cockroach, based upon observations demonstrating that increases in *GlrX* were attenuated in lungs from mice lacking *IL33* [302]. Collectively, these findings indicate reciprocal actions of GLRX and IL33 in settings of allergen exposure and suggest a role of GLRX and PSSG in the regulation of type 2 responses in asthma in part via the regulation of IL33. Lastly, in addition to regulating inflammatory signaling pathways in diverse ways, S-glutathionylation can also directly regulate the biological activity of several asthma-relevant cytokines, such as IL1 $\beta$ , IL6, or IL17 [303]. For example, the activity of IL1 $\beta$  can be inhibited by overoxidation of its Cys188, and S-glutathionylation of Cys188 protects against such inhibition and helps sustain IL1 $\beta$  activity [303].

Collectively, these various findings illustrate diverse mechanisms by which S-glutathionylation can impact inflammatory signaling and other signaling pathways that contribute to asthma pathobiology, which likely depends on cell-specific actions of different oxidant sources (e.g. NOX enzymes, mitochondria, ER stress).

#### 14. Concluding comments and challenges for future research and development

Although the etiology of IPF, COPD and asthma highly complex, in the present review we highlighted the importance of altered GSH redox homeostasis and ER stress, particularly in the development of fibrotic remodeling that is common to all three diseases, despite differences in the anatomical location of fibrosis in asthma/COPD (airways) and IPF (parenchyma). We presented arguments that avenues to normalize redox homeostasis warrant targeted approaches to alleviate ER stress and/or activation of PDIs, dampen oxidant production from mitochondria, or NOX enzymes such as NOX4 or DUOX1, as these diverse processes provide important sources of oxidants in settings of chronic lung diseases (Table 4). The demonstration of increased protein S-glutathionylation, related to altered expression and/or enzymatic function of GSTP or GLRX, points to the importance not of GSH per se, but a unique facet of GSH chemistry, protein S-glutathionylation, which



**Table 4**

Proposed development of GSH-based/redox therapeutics for chronic lung diseases.

- Develop a variant of GLRX for the treatment of IPF and COPD
- Repurpose TLK199 for the treatment of IPF
- PDI inhibitors for the treatment of asthma, COPD and IPF
- NOX4 inhibitors for the treatment of IPF
- DUOX1 inhibitors for the treatment of asthma

**Note:** TLK199 and TUCDA have already been used clinically, but the undesirable intellectual property domain along with concerns for future profit margins makes it unattractive to invest in costly clinical trials and re-development of these compounds.

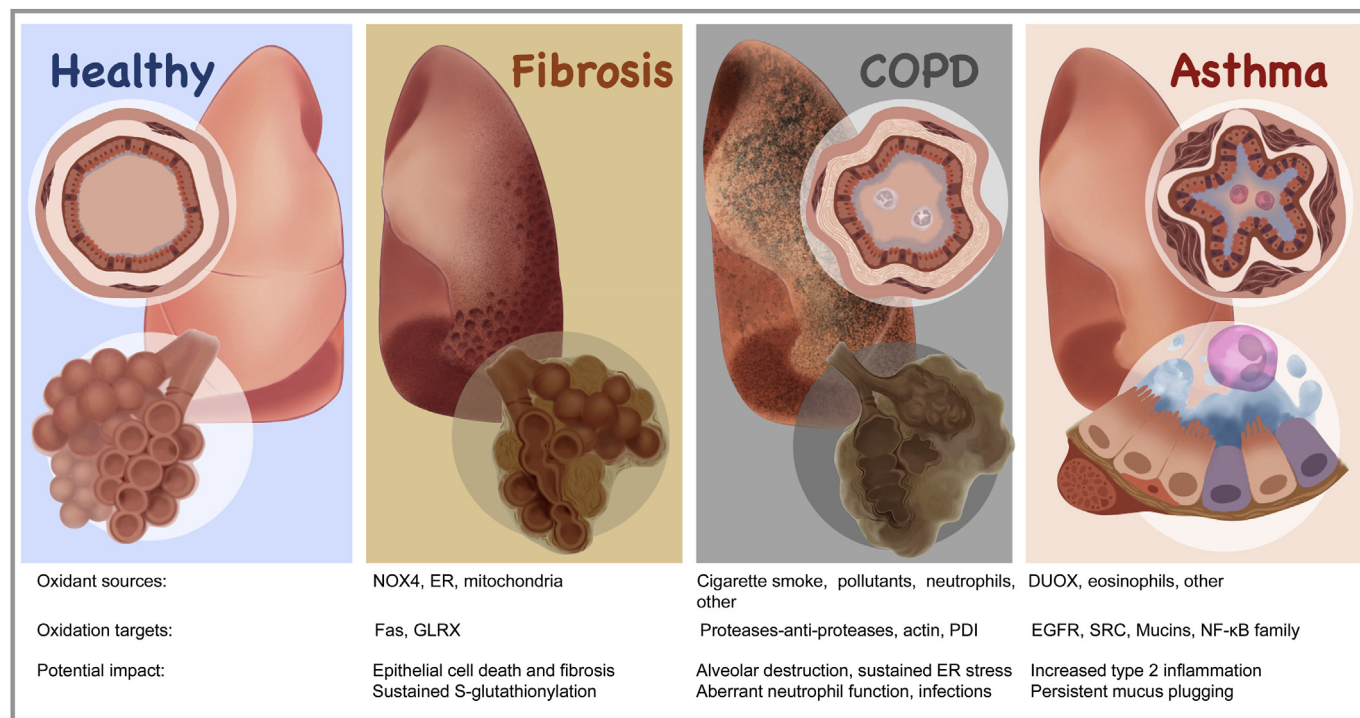
involves its covalent incorporation into proteins, catalyzed by GSTP, and reversed by GLRX. The present review highlights the importance of S-glutathionylation as a critical enzymatically-controlled biochemical pathway that had not previously been recognized in settings of chronic lung diseases, and that is amenable to unique therapeutic intervention, although alternative redox-based thiol modifications may also be important in these pathologies.

A number of signaling molecules, structural proteins, ER resident proteins, mitochondrial proteins, the death receptor Fas, proteases, the protease inhibitor A1AT, and cytokines are targets for S-glutathionylation, and therefore have the potential to affect cell death, migration, mitochondrial function, release of alarmins, innate and adaptive immune responses, epithelial and immune cell activation, and protease

activities. Many of these aforementioned processes are involved in or altered in IPF, severe asthma and COPD (Fig. 5). Therefore, additional translational studies to elucidate the exact PSSG targets in these diseases will be critical. Notably redox proteomics studies to unravel alterations in the oxidized cysteine proteome including the S-glutathionylated proteome remain to be performed in lung tissue specimens or epithelial and other cells directly isolated from patients. Our hope is that the recent discoveries pertaining to glutathion(e)ylation chemistry provide a rapid path to drug development. The consideration of clinical studies utilizing the clinically relevant GSTP inhibitor, TLK199, or a variant thereof, potentially in conjunction with local administration of recombinant active GLRX, appears well warranted and may yield new insights into the functional importance of S-glutathionylation chemistry in the pathogenesis and progression of chronic lung diseases. Similarly, approaches to inhibit PDI, NOX4 and DUOX1 also come to the forefront as new avenues to treat chronic lung diseases, based upon the compelling observations made in pre-clinical models (Table 4). We are hopeful that such strategies, perhaps in conjunction with standard of care treatment, have the potential to increase the survival and quality of life of the millions of patients living with chronic lung diseases.

#### Declaration of competing interest

Yvonne Janssen-Heininger, Niki L. Reynaert, and Vikas Anathy hold patents: United States Patent No. 8,679,811, "Treatments Involving Glutaredoxins and Similar Agents" (YJ-H, VA), United States Patent No.



**Fig. 5.** Illustration highlighting the defining histopathological features of fibrosis, COPD and asthma, and redox-controlled events that may control these disease manifestations. **Healthy:** Normal lung tissue depicted with healthy bronchiole, alveolar ducts and alveoli. **Fibrosis:** Shown is the honey-comb appearance visualized by CT cans. Also shown are the disruption of alveolar structures. The death receptor Fas, a critical player in epithelial cell death as well as lung fibrosis is S-glutathionylated in the ER. NOX4 derived from fibroblasts is one of the oxidant sources important in fibrosis. **COPD:** COPD classically has been linked to inhalation of cigarette smoke or other environmental pollutants, although it also is linked to  $\alpha 1$  antitrypsin deficiency. The bronchioles of COPD patients are inflamed and neutrophils are depicted. Mucus plugging and subepithelial collagen deposition also are features of COPD. Narrowing of small airways and loss of alveoli lead to gas trapping and poor oxygen exchange. An imbalance in protease-antiprotease activity is believed to contribute to alveolar destruction. Pollutants in cigarette smoke induce ER stress, linked to alkylation of PDI. The UPR reflects an adaptive response that in some cases contributes to epithelial cell death. Mutant  $\alpha 1$  antitrypsin also induces the UPR. Oxidation of the proteases, antiproteinases contribute to their imbalance and resultant tissue destruction. **Asthma:** Depicted is an inflamed airway, characterized by increases in eosinophils, and mucus plugging in type-2 high asthmatics. An expansion of smooth muscle cell mass contributes to constriction of the airways. Severe asthma also is characterized by sub-epithelial fibrosis. ER-redox processes are implicated in mucus metaplasia, cytokine disulfide content, and type-2 responses. Oxidation of mucins contributes to persistent mucus plugging. Although numerous oxidation targets have been identified in cell cultures and mouse models, for the most part these remain to be verified in human lung tissues. We refer the reader to the text for more detailed information.



8,877,447, “Detection of Glutathionylated Proteins” (YJ-H, NLR), United States Patent, 9,907,828, “Treatments of oxidative stress conditions” (YJ-H, VA) Yvonne Janssen-Heininger and Vikas Anathy have received consulting fees from Celdara Medical LLC for their contributions with the commercialization of glutaredoxin for the treatment of pulmonary fibrosis.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101516>.

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