Chapter 4

Antiproteases as Therapeutics to Target Inflammation in Chronic Obstructive Pulmonary Disease

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Additional information is available at the end of the chapter

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive inflammation of the airways and is one of the major causes of death in the elderly. COPD leads to development of airflow limitation as evident by decreased forced expiratory volume in one second (FEV_1) and reduction in the percentage of FEV_1/ vital capacity. Standardised spirometric tests showing the presence of airflow obstruction are used in clinical diagnosis and more recently the use of high resolution CT scanning has been employed to detect early emphysematous changes which may be present prior to severe airflow obstruction (Figure 1).

In the lungs of patients with COPD elevated levels of pro-inflammatory cytokines such as interleukin (IL)-8, leukotriene-B_4 (LTB_4) and tumour necrosis factor-α (TNFα) have been recorded which can act as neutrophil chemoattractants. Once recruited to the airways, neutrophils are activated and release various compounds including reactive oxygen species, proteases and cationic proteins in order to clear infections. When released in excessive amounts however, these molecules can cause extensive damage to the respiratory epithelium resulting in yet more pro-inflammatory cytokine release and further neutrophil influx, thereby creating a cycle of inflammation.

It is now clear that neutrophil serine proteases, including neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G (CathG) are major pathogenic determinants in chronic airway inflammatory disorders. Moreover, accumulating evidence indicates that the expression of matrix metalloproteases (MMPs) is dysregulated in COPD and these proteins are involved in small airway remodelling. Increased levels of both MMPs and serine

proteases can participate in proteolytic attack on the alveolar wall matrix and, as a consequence the lung extracellular matrix is damaged, resulting in obstruction of small airways and development of emphysema.

Due to their implication in the pathology of COPD airways disease both MMPs and serine proteases have been in focus for drug-development efforts over the last two decades. Such concepts are further reinforced by scientific findings indicating that a variety of broad spectrum serine protease and MMP inhibitors significantly ameliorate emphysema in experimental animal models of COPD. To this end, in recent years continued efforts to identify and optimize novel mechanism-based inhibitors have led to a number of new inhibitors being reported. For example three natural protease inhibitors, secretory leucocyte protease inhibitor (SLPI), elafin and alpha-1 antitrypsin (AAT), have therapeutic potential for reducing the protease-induced inflammatory response and show promising potency and selectivity profiles. Other therapy options for the modulation of inflammation associated with excessive protease activity is the use of recombinant, synthetic or semi synthetic protease inhibitors. In this chapter we aim to describe the clinical and scientific evidence for the involvement of proteases and their proposed mode of action in COPD development and progression. Potential intervention with natural or synthetic inhibitors in the management of emphysematous symptoms associated with COPD will be reviewed in this chapter.

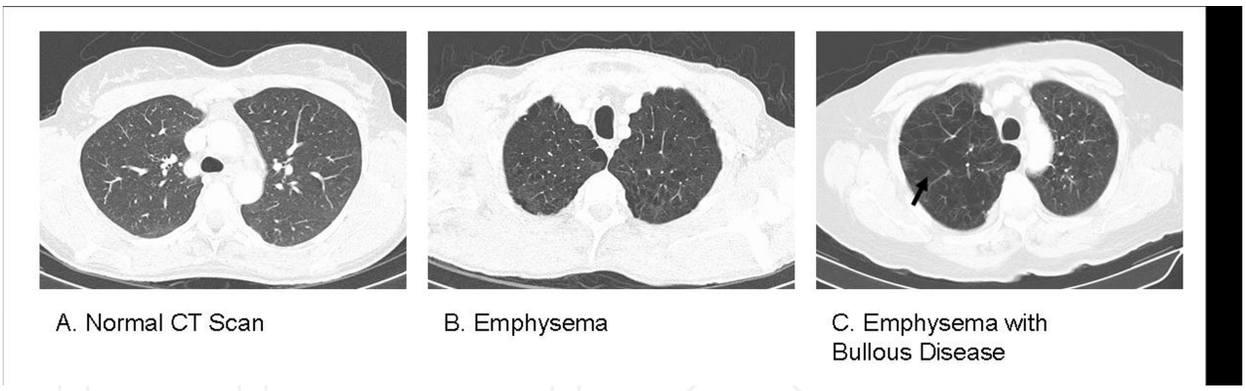

Figure 1. Chest high resolution CT scan of COPD. A) A single slice from a high resolution CT (HRCT) scan of thorax demonstrating normal healthy lung parenchyma. B) A single HRCT thorax image of the upper lobes demonstrating loss of lung density in keeping with emphysematous change. C) Severe emphysema of the upper lobes showing markedly reduced lung density and bullous disease present (indicated by the arrow).

2. The role of proteases in COPD

There are several classes of proteases and these include serine-, cysteine-, aspartate-, threonine-proteases and metalloproteases. For the purpose of this chapter we will focus on proteases which have been implicated in the pathogenesis of COPD and discuss particularly serine proteases and metalloproteases including NE, PR3, cathepsins and matrix metalloproteases.

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2.1. Serine proteases

Serine proteases are a class of proteases which are involved in various physiological and pathological processes throughout the body and universally contain a serine as the amino acid at the active site of the enzyme [1]. Serine proteases include NE, CathG and PR3. They are primarily inactivated by serine protease inhibitors, the archetypal member of this family being AAT [2]. The main model for the protease-antiprotease imbalance is AAT deficiency, thus providing us with an insight into the importance of proteases in the development of emphysema [3, 4].

NE, PR3 and CathG are produced during neutrophil development in the bone marrow and are stored in the azurophilic (primary) granules of neutrophils [5]. These serine proteases are found in high concentrations in airway secretions of patients with inflammatory lung conditions [6, 7] and are seen in the circulating plasma of patients with severe sepsis and acute respiratory distress syndrome [8]. They have effects upon a broad range of extracellular matrix proteins including elastin, collagens, fibronectin, proteoglycans and laminin [9, 10]. Apart from AAT deficiency a large number of experimental animal studies also support the role of the protease/antiprotease imbalance in the pathogenesis of COPD, including the involvement of NE and PR3 in the development of emphysema [11, 12].

2.1.1. The serine protease neutrophil elastase

NE is a single polypeptide glycoprotein comprising 218 amino acids and is quite homologous with other serine proteases including PR3 and CathG [13]. NE is stored in the primary granules of neutrophils and when neutrophils are activated or primed by cytokines, it is released from the cell to the extracellular environment and may also rebind and become expressed on the cell surface [14]. The activity of NE is primarily inhibited and regulated by AAT [2, 15] but other inhibitors of NE have been reported including monocyte neutrophil elastase inhibitor (MNEI/Serpin B1) [16], elafin [17, 18] and SLPI [19, 20]. Of interest, the converse has also been shown whereby NE has been shown to inactivate and cleave SLPI. [21]

Smoking may lead to an imbalance between proteases and antiproteases through the reduction of the functional activity of AAT in the lung and also as described earlier by increasing the number of proteases produced hence increasing the proportion of elastolysis in the lung. Moreover, cigarette smoke has been reported to inhibit the anti-NE activity of AAT in bronchoalveolar lavage (BAL) fluid of smokers compared to healthy non-smoking control subjects [22, 23], however some studies have argued against this point [24, 25]. Cigarette smoke has also been shown to induce the release of NE in BAL fluid [26] and also to increase the circulating NE levels seen in plasma [27]. This implicates NE in the pathogenesis of emphysema and studies in BAL from COPD patients demonstrated a direct correlation between the NE burden in BAL and the degree of emphysema seen on CT scans. Further to this, results showed an inverse relationship between the anti-elastase activity in the BAL of COPD patients and the degree of emphysema and diffusing capacity, supporting the protease-antiprotease imbalance theory of emphysema [28].

Further studies on the presence of NE in the airways have shown that NE can cleave a number of epithelial cell surface receptors [29], cell activators and signalling cytokines [30] thereby potentially orchestrating the airway inflammatory milieu. Moreover, data have shown that alveolar macrophages may bind and internalise NE [31] and a later study has reported increased levels of NE in alveolar macrophages of patients with emphysema, providing further evidence of the increased protease burden [32]. NE has also been demonstrated using immunohistochemistry to be localised to the elastin fibres in the lung parenchyma of patients with emphysema [33]. The theory of NE induced emphysema has been supported not only from human studies but excellent animal models. Indeed, tracheal instillation of NE has been shown to induce the infiltration of neutrophils into the lung and cause emphysema in experimental models [11, 34].

Apart from the well-known role of NE as a protease in the protease/antiprotease imbalance in emphysema it exerts additional effects which contribute to the pathogenesis of COPD. For example NE exposure has been shown to significantly increase macrophage production of cathepsin B and latent and active MMP-2 [35], whilst the addition of AAT to BAL fluid greatly reduced NE-induced cathepsin B and MMP-2 expression in macrophages *in vitro* [36]. Additionally, NE is involved in the hypersecretion of mucus seen in COPD and is also a secretagogue, inducing the submucosal gland cells and goblet cells to secrete mucus [37-39]. NE has been shown to induce the expression of MUC5AC at the gene level in epithelial cells and hence increase the production of mucin in the airway. This mechanism has been shown to be dependent on the presence of reaction oxygen species, linking the role of NE inducing mucus hypersecretion to smoking [40, 41]. Moreover, not only does NE increase the amount of mucus produced in the lung but it also interrupts the mucociliary clearance mechanism by decreasing the ciliary beat frequency of bronchial epithelial cells [42].

The importance of the immune system and the inflammatory process in the pathogenesis of COPD is becoming more apparent and NE is involved in several of these immune mechanisms. NE can induce the expression of IL-8 in bronchial epithelial cells via TLR-4, subsequently leading to neutrophil chemotaxis and increasing the inflammatory burden in the lung [43, 44]. NE also plays an important role in neutrophil migration both through the cleavage of fibrin [45] and through the chemo-attractant properties of NE complexed with AAT [46]. The immune function can be further affected by NE through the cleavage of CD4 and CD8 glycoproteins from the surface of T-cells, thus impairing the function of T-cells and contributing to the inflammation seen in COPD [47]. Moreover, NE up-regulates the expression of TGF-β1 in smooth airway muscle via the NFκB pathway and this may further contribute to the airflow limitation seen in COPD [48]. Additionally, NE plays a role in the apoptosis seen in the pathogenesis of emphysematous lesions. Accordingly NE has been demonstrated to induce endothelial cell apoptosis [49], cytolysis of epithelial cells [42, 50] and cleaves the hydrophobic phospholipid substrate phophatidylserine receptor on macrophages, which subsequently impairs the ability of macrophages to clear apoptotic cells [51]. Collectively, these documented reports demonstrate that NE is an integral enzyme involved in the pathogenesis of COPD and emphysema and is a key target for therapeutic intervention.

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2.1.2. Neutrophil derived proteinase 3

PR3 is the most abundant serine protease found in the neutrophil [52] and consequently more PR3 than NE is released by the neutrophil during the phagocytosis process [53]. Similar to NE, following activation of neutrophils by inflammatory cytokines, PR3 can be expressed on the surface of neutrophils [52] and AAT potently inhibits PR3 in the circulation [2, 54]. In contrast to AAT, SLPI has no effect upon PR3 and in fact PR3 is capable of degrading SLPI, thereby enhancing the activities of NE and other serine proteases [55].

Animal studies have demonstrated that PR3 plays a role in the development of emphysema, with studies showing that instillation of PR3 intra-tracheally leads to emphysematous lung disease in hamsters [12]. PR3 is found in the sputum of both stable COPD patients and AAT deficient individuals and increases at times of pulmonary exacerbations. Elevated PR3 levels correlate with an increase in neutrophilic burden [56]. However, its elastolytic rate is relatively low compared to NE, suggesting possibly a more minor role in the pathogenesis of emphysema [57]. Studies have also shown that PR3 maintains pro-inflammatory properties, possessing the ability to activate TNFα and IL-1β [58]. PR3 also has a strong effect on promoting the secretion of mucus in the airways [59] and can lead to cell apoptosis in the lung [49, 60].

2.1.3. Cathepsin G and other cathepsins

Not all cathepsins are serine proteases and in fact include aspartate and cysteine proteases. In this section we will discuss CathG, a serine protease related to NE and PR3 and briefly discuss other cathepsins and their role in COPD. Of major importance, increased levels of cathepsins have been demonstrated in BAL fluid of emphysema patients and therefore may play a role in the pathogenesis of the condition [61].

CathG is a serine protease and is stored in the primary granules of neutrophils. Upon release, activity of this protease is under the regulation of AAT and SLPI [19]. Increased levels of cathepsins can be seen in the BAL fluid of patients with emphysema [61] but unlike NE and PR3, *in vivo* animal studies have not demonstrated the same effect of CathG in inducing emphysema [62]. Similar to the other serine proteases however, CathG can induce secretion of mucus, hence adding to the airflow limitation and symptoms found in COPD [37]. Moreover, CathG can impair T-cell function by cleaving trans-membrane glycoprotein co-receptors on the cell surface in a similar fashion to NE [47].

Increased levels and activity of cathepsin L and cathepsin S have been shown in alveolar macrophages of smokers [63, 64]. The expression of cathepsin S is induced by INF-γ and this occurs in multiple cell types including smooth muscle cells. Subsequently, it has been shown that over-expression of IFN-γ can increase the expression of cathepsin B, D, L and S [65]. Moreover, in murine investigational studies cysteine-protease inhibitors markedly reduced the amount of airway inflammation and emphysema associated with over expression of cathepsins B, H, K, L and S [66]. In addition, bacterial killing by the antimicrobial peptide LL-37 and by human beta-defensins is inhibited due to proteolytic degradation by cathepsin D [67] and inactivation by the cysteine proteases cathepsins B, L, and S, respectively [68]. Although there is far less evidence and reported studies on cathepsins they most likely play a contribu-

tory role to the pathogenesis of emphysema and should also be considered as possible therapeutic targets in the future.

3. Matrix metalloproteases in COPD

MMPs are proteolytic enzymes which degrade components of the lung matrix, including collagens and elastins. This occurs both under normal physiological conditions and during abnormal pathological processes. MMPs are secreted as pro-enzymes and are activated by proteolytic conversion and have a close relationship with cytokines and growth factors [69, 70]. MMPs have both collagenase and elastase activity and account for 50% of the elastolytic activity of BAL fluid in smokers [71]. Indeed, MMPs account for the majority of the elastolytic activity of macrophages in COPD patients [72] and are counteracted by tissue inhibitors of matrix metalloproteases known as TIMPs. Additionally, MMPs do not only demonstrate degrading properties but also are pro-inflammatory in nature, with liberated matrix fragments possessing pro-inflammatory and monocytic chemotactic properties [73, 74].

3.1. Macrophage derived matrix metalloprotease-12

MMP-12, also known as macrophage-metalloelastase, is perhaps the most studied and best understood MMP in emphysema and COPD and numerous animal models have demonstrated its role in lung disease. It is involved directly in matrix degradation and is also a pro-inflammatory peptide. A recombinant form of human MMP-12 has been used to demonstrate the direct role that MMP-12 plays in the inflammatory process in the airways of mice [75]. This recombinant form of MMP-12 caused an increase in neutrophils and in macrophages accompanied by increased levels of pro-inflammatory cytokines and MMPs. Human studies have reported that the number of alveolar macrophages in BAL fluid expressing MMP-12 was higher in COPD patients than in controls [76]. MMP-12 can also be produced by bronchial epithelial cells [77] and is expressed by airway smooth muscle [78]. Cigarette smoke has been repeatedly shown to up-regulate both the release and production of MMP-12 [79]. How this occurs is complex in nature and likely through several mechanisms. For example, upon exposure to smoke, proteins including plasminogen and prothrombin are released into the alveolar space where they are converted to plasmin and thrombin, both of which are serine proteases. Their action upon proteinase activated receptor-1 (PAR-1) subsequently leads to the secretion and activation of MMP-12 [80, 81]. A second mechanism by which smoke may activate MMP-12 has been shown in mice where it was shown to up-regulate GM-CSF production which in turn controls MMP-12 release [82]. Moreover, the gene expression of MMP-12 in epithelial cells is affected by reactive oxygen species [83] and in murine studies a direct link between MMP-12 and cigarette smoke induced airway inflammation has been demonstrated [84-86]. Moreover, in MMP-12 knockout mice a marked reduction in monocyte recruitment and in IL-13 and IFN-γ induced emphysema has been demonstrated, implicating the importance of MMP-12 in the inflammatory response seen in COPD [65, 66]. How MMP-12 exerts this inflammatory effect is multifactorial. One reported mechanism involves the pro-

inflammatory action of MMP-12 through the release and activation of TNFα [87]. Secondly, MMP-12 has also been shown to possess the ability to induce the production and release of IL-8 via the EGFR pathway in epithelial cells [88]. The importance of MMP-12 in inducing airway inflammation is further supported by the use of MMP-12 inhibitors which were shown to ameliorate emphysema in experimental animals [89] and to lower both the concentration of immune cells in BAL fluid, and to affect inflammation [90]. MMP-12 also impacts upon the activity of other proteases, especially neutrophil derived enzymes including NE, PR3 and cathepsins, and can degrade AAT hence up-regulating the activity of these serine proteases [91].

Despite convincing evidence provided by experimental models and compelling data showing an association between MMP-12 and emphysema, the data from human studies is conflicting and not as clear cut. A number of studies have suggested increased expression of MMP-12 in alveolar macrophages and increased MMP-12 protein in sputum of COPD individuals compared to healthy controls [76, 92, 93] while others have shown no difference [94-96]. Thus further studies on MMP-12 are essential to confirm the role of this matrix metalloprotease in inflammation and tissues destruction associated with COPD.

3.2. Neutrophil released matrix metalloprotease-9

The next most studied and understood MMP in COPD is MMP-9. Also known as gelatinase-B and similarly to other matrix metalloproteases MMP-9 has multiple substrates including collagens IV, V, VII, X and XI as well as elastin, gelatin, pro-MMP9 and pro-MMP13. MMP-9 is secreted by bronchial epithelial cells, mast cells, neutrophils, eosinophils and alveolar macrophages in response to TGFβ, IL-13 and IL-8 [79].

Increased levels of MMP-9 have been demonstrated in smokers with and without airflow obstruction [97, 98] and a correlation between the levels of MMP-9 in sputum and the extent of airflow obstruction and symptoms in COPD has been reported [99]. There are also increased levels of MMP-9 in AAT deficient individuals, where levels correlated with clinical parameters including FEV1, DLCO, lung density and exacerbation frequency [98, 100]. Elevated levels of MMP-9 in COPD individuals have been demonstrated to correlate to the number of neutrophils, implicating MMP-9 in the inflammatory burden in COPD [98, 101]. Moreover, polymorphism in the promoter region of the human MMP-9 gene has been shown associated with emphysema in 2 separate Japanese cohorts [102, 103] and with COPD in a Chinese population [104].

In support of a role for the involvement of MMP-9 in COPD, *ex vivo* studies have shown that alveolar macrophages from BAL fluid in emphysema patients demonstrate increased expression of MMP-9 and MMP-1 compared to control subjects [95]. This is also true for smokers with and without COPD where there is increased expression of MMP-9 on alveolar macrophages compared to healthy controls [105, 106]. In a study comparing COPD patients to smokers with no evidence of airflow obstruction, alveolar macrophages from COPD individuals released increased levels of MMP-9 and *in vitro* the stimulation of these cells by IL-1β, lipopolysaccharides (LPS) and cigarette smoke increased the secretion of MMP-9 [106].

Consequently, emphysematous lung tissue has been shown to contain higher levels of MMP-9 compared to disease free tissue [107, 108].

Other studies of interest that provide evidence for the involvement of MMP-9 in the inflammatory process associated with COPD include data that demonstrate that MMP-9 possesses levels of TACE (TNFα converting enzyme) like activity and mediates acute cigarette smoke-induced inflammation via TNFα release [87]. MMP-9 may also be involved in the link between destruction of alveolar tissue and fibrotic proliferation observed in emphysema, potentially mediated by proteolytic cleavage of latent TGF-binding-protein-1 with subsequent release of TGFβ-1, possibly linking elastolysis and fibrosis [109, 110]. Interestingly, MMP-9 may be activated by NE via cleavage of pro-MMP-9 to active MMP-9 [111]. Moreover, NE has been shown to degrade TIMP-1 thereby leading to enhanced MMP-9 activity [112], implicating both NE and MMP-9 in the pathogenesis of COPD. Finally, a role for MMP-9 in mucus hypersecretion in COPD patients has been suggested, as MMP-9 activates epidermal growth factor which in turn increases the expression of MUC5AC production hence leading to increased mucus secretion [113, 114].

3.3. Matrix metalloprotease-1 and other matrix-metalloproteases

MMP-1, also known as interstitial collagenase-1, is active against multiple collagens including collagen I, II, III and VII, gelatin and pro-MMP9 and over-expression in mice has been associated with development of emphysema [115]. Loss of collagen type II leads to an increase in lung compliance and hence the development of emphysema [116]. In humans, BAL fluid from emphysema patients has increased MMP-1 expression compared to healthy controls [117] and increased expression of MMP1 was reported in the lungs of patients with emphysema [94]. This expression was localised to type II epithelial cells but not alveolar macrophages. MMP-1 is also expressed by airway smooth muscle [118] and the production of MMP-1 is driven by the MAPK pathway and also by hydrogen peroxide, an important pathogenic component of cigarette smoke [119]. In mouse models, MMP-1 has been shown to contribute to alveolar destruction leading to emphysema development [120, 121]. Similar to MMP-9, MMP-1 also possesses levels of TACE like activity which may contribute to the inflammation seen in COPD [87].

MMP-2 which is also known as gelatinase-A has activity against collagens I-V, VII, X, XI and XIV as well as elastin, fibronectin and gelatin. Increased levels of MMP-2 have been demonstrated in COPD lungs [107, 108] and also in experimental mice exposed to cigarette smoke [122]. Interestingly, in guinea pigs exposed to wood-smoke increased expression of MMP2 was recorded, possibly providing an insight into the pathogenesis of emphysema in individuals not exposed to cigarette smoke [123]. Moreover, increased levels of both MMP-8 and MMP-10 are seen in sputum of COPD patients and have been shown to correlate with airflow obstruction [97, 124], with increased levels of MMP-8 recorded at the time of acute pulmonary exacerbation [125]. Finally, MMP-12 is up-regulated in mice exposed to cigarette smoke [126] and elevated levels of MMP-13 are seen in individuals with emphysema [127].

Collectively, this information on MMPs contributes to the protease/antiprotease theory in COPD pathogenesis and provides targets that may be investigated when developing future

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therapeutic strategies. MMPs play a mixed role in COPD and are involved in the proteolytic destruction of lung tissue and possess a close relationship to neutrophil derived serine proteases. These latter processes are intertwined and hence targeting certain elements of protease induced inflammatory processes may provide encouraging and exciting therapeutic options.

4. Approaches to treatment

Current therapies for the treatment of COPD are similar between the non-genetically inherited form of the lung disease and COPD as a result of AAT deficiency, and are in line with the American Thoracic Society/European Respiratory Society published guidelines [128]. Of particular importance is the cessation of smoking for individuals diagnosed with the disorder, as smoking aggravates the condition and is a predominant prognostic factor for the outcome of COPD patients. Therapeutic strategies aimed at the protease-antiprotease imbalance can be expected to have the greatest effect on lung disease associated with COPD and AAT deficiency. Ensuing sections will focus on AAT augmentation therapy in both intravenous and aerosolized forms, transgenic and recombinant forms of AAT and administration of either natural (elafin and SLPI) or synthetic (Sivelestat) antiproteases for treatment of COPD.

4.1. AAT replacement therapy

The importance of the protease: antiprotease balance is evident by the development of emphysema in AAT deficient individuals due to unopposed NE activity, of which AAT is the major inhibitor. Indeed the most logical approach to treatment is the reestablishment of physiological levels of AAT. The minimum level of AAT required to protect the lung from protease mediated damage was set as 11μmol/L (or 80mg/dL), and this has been referred to as the "protective" threshold level [129]. This value is based on the levels of AAT recorded in non-smoking individuals with the PiSZ phenotype, which is not thought to confer an increased risk of developing lung disease. Intravenous infusion of human plasma derived AAT, also referred to as AAT augmentation therapy, provides a method of maintaining this protective threshold and thus delays the progression of emphysema (Table 1). In 1981, researchers developed a method for partially purifying AAT from pooled human plasma and with the knowledge that the serum half-life of AAT is 5.4 days [130], devised an infusion schedule that allowed once weekly administration of purified AAT to AAT deficient individuals. Following such infusions the results of this study demonstrated that significant amounts of AAT, with full anti-NE capacity, diffused to the lower respiratory tract. Consequently, infusion of purified human plasma AAT at a dose of 60mg per kilogram of body weight per week, a dose that was sufficient to maintain serum levels ≥ 11μmol/ L, was FDA-approved and is now widely used in Europe and North America in treatment of AAT deficient individuals [131]. The first available preparations of AAT included pooled human plasma AAT prepared by pasteurisation (Prolastin, Bayer, West Haven, CT, USA). In turn, more recent studies examined the biochemical effectiveness of AAT purified by solvent detergent and nano-filtration methodology (Aralast, Baxter, Westlake Village, CA, USA) [132] and a third preparation (Zemaira,

CSL Behring, PA USA) has received US FDA approval [133]. Currently there are six FDA approved AAT preparations available in the USA. The clinical effects of infused AAT therapy documented to date consist of patient outcome measures including the rate of FEV_1 decline [134, 135], the level of desmosine as a biomarker of elastin degradation [136, 137], the incidence of acute exacerbations [138], frequency of lung infections [139] and the change in lung density [140] calculated by CT scanning [141]. Nevertheless, though studies have demonstrated that infusion of AAT is safe and well tolerated [142], there remains considerable debate over the clinical benefits and the cost-effectiveness of the treatment [134, 143, 144].

Study Type	AAT Infusion	Reference	Patient outcome
Observational	Weekly	(Ma et al. 2013)	Reduced elastin degradation in treated group
Observational	Weekly	(Tonelli et al. 2009)	Slower rate of decline in FEV1% predicted in augmentation group
Randomized	Weekly	(Dirksen et al. 2009)	Attenuated loss of lung tissue in treated cohort measured by CT lung density
Observational	Weekly	(Wencker et al. 2001)	Reduced rate of FEV1 decline post augmentation therapy
Descriptive	Weekly	(Gottlieb et al. 2000)	Elastin degradation was not reduced
Observational	56% weekly & 26% biweekly	(Lieberman 2000)	Decline in number of infections per year
Randomized controlled	4-weekly	(Dirksen et al. 2009)	Reduced loss of lung tissue by CT densitometry with augmentation
Observational parallel controls	51% weekly & 25% biweekly	(group 1998)	Slowed decline in FEV1 of 27ml/year in treated group
Observational parallel controls	Weekly	(Seersholm et al. 1997)	Reduced rate of decline of FEV1 in treated group of 21ml/year.

Table 1. Treatment of patients with AAT deficiency with augmentation therapy

The biochemical efficacy of AAT augmentation therapy has been examined in a number of studies and in 1987 Wewers and colleagues demonstrated that a weekly dosage of 60mg/kg not only restored AAT levels in serum and BAL fluid but also increased anti-NE capacity from a baseline value of 5.4 ± 0.1μM in the serum to 13.3 ± 0.1μM [142]. Indeed, additional studies have focused on the impact of augmentation therapy on the inflammatory environment of the lung. For example, AAT deficiency is characterised by increased neutrophil numbers and thus increased sputum and BAL fluid levels of NE [145]. This burden of neutrophils in the lung as a result of excessive trafficking is attributed to chemotactic agents such as IL-8 and LTB_4 and significantly higher levels of these chemo-attractants have been recorded in sputum from individuals with AAT deficiency compared to COPD patients. To understand this further, an

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in vitro study discovered increased release of LTB_4 from AAT deficient alveolar macrophages as a result of unopposed NE activity. Of relevance, the addition of exogenous AAT reduced the amount of LTB_4 being released by macrophages in response to NE [146]. In confirmation of this latter study, Stockley *et al* demonstrated that sputum samples from AAT deficient individuals on day one post infusion of AAT augmentation therapy possessed reduced NE activity and a dramatic attenuation of LTB_4 concentration from a level of 13.46nM to 8.62nM [147]. Moreover, the anti-inflammatory effects of AAT augmentation therapy were confirmed as decreased concentrations of IL-8, and the chemotactic activity mediated by this chemokine, were reduced to normal levels in AAT deficient individuals post treatment. [148, 149]

One of the first studies to examine the effect of AAT augmentation therapy compared the ΔFEV_1 of 97 ex-smokers from a Danish AAT deficient registry to a German group of 198 patients treated with weekly infusions of AAT (60mg/kg) for at least one year. Overall, the ΔFEV_1 in the treated group was significantly lower than in the untreated group, with annual declines of 53 mL yr^{-1} and 75 mL yr^{-1}, respectively (p=0.02). However, there was no beneficial effect of augmentation therapy in 103 patients with an initial $FEV_1 \leq 30$ or in the 25 patients with an $FEV_1 > 65\%$ [134]. In 1998, one of the largest observational studies (n=1129) centred on the NHLBI Registry for individuals with severe AAT deficiency [150] was carried out. Although no overall difference in FEV_1 decline was recorded between the augmentation-therapy treated and untreated groups, in a subgroup with FEV_1 between 39-45% predicted there was a significant difference of -27ml/year between treated and untreated. This study also suggested that survival was enhanced in individuals receiving augmentation therapy. The risk ratio for death in augmentation therapy recipients was 0.64, significantly lower than non-recipients (P=0.02) and the risk ratio for individuals receiving augmentation therapy with stage II COPD was 0.21 (P<0.001). The possibility that these differences may have been due to other factors, such as the socioeconomic status of enrolled patients could not be ruled out and this latter point is a potential limitation of this study. In a subsequent randomised study of 164 AAT deficient individuals enrolled in the Alpha-1 foundation DNA and tissue bank, when adjusted by age at baseline, sex, smoking status, baseline FEV_1 % of predicted, a slower rate of decline in FEV_1% predicted was observed in the augmentation group (10.6±21.4 ml/year) in comparison to the non-augmented group (36.96±12.1ml/year). The authors of this study concluded that augmentation therapy was effective in subjects with AAT deficiency, favouring ex-smoker subjects with an FEV_1 below 50% of predicted [151].

Results from studies measuring changes in urine desmosine levels, a marker of elastin breakdown, in response to AAT augmentation therapy have been mixed. One study of two AAT deficient patients observed a 35% reduction in urine desmosine levels after monthly doses of 260mg/kg AAT [152]. In contrast, a larger study by Gottelieb *et al* showed no change in the level of elastin breakdown after eight weeks of augmentation therapy (P=0.85). Although this latter study recruited only twelve AAT deficient individuals, the authors suggested that elastin degradation at this point in the disease was possibly NE independent [137]. In a recent study desmosine levels were assessed in plasma, BAL fluid and urine in a cohort of AAT deficient patients receiving AAT augmentation therapy. A 13.9% reduction in plasma and 37% reduction in BAL fluid desmosine levels were observed 12 weeks after receiving IV augmentation

therapy compared to baseline levels prior to treatment [153]. The study showed that augmentation therapy maintained the ability to inhibit NE and to reduce degradation of elastin both systemically and in the lungs, however the findings suggested that the dose of AAT was not sufficient to reduce elastin degradation to that of control levels of healthy individuals. There is a relatively limited body of clinical evidence supporting the benefits of 60mg/kg dosage, with some even questioning the recommendation of this dose of AAT to AAT deficient patients [154]. The concentration of 60mg/kg aims to increase AAT levels to values above that of the protective threshold (11μM) yet levels are still below that of serum AAT concentration observed in non-AAT deficient individuals (20-53μM) [155]. To address this issue Campos *et al* assessed the safety and efficacy of an increased 120mg/kg weekly dose of AAT in 30 adults with AAT deficiency in a multicentre, randomised, double-blind crossover study. A weekly dose of 120mg/kg resulted in a serum concentration of 27.7μM four weeks after treatment, well within the healthy control range, compared to 17.3μM at the same time period for the group receiving 60mg/kg. Concurrently, this increased dosage was considered to be safe and well-tolerated, however further investigation is warranted due to the relatively small cohort size and the lack of analysis of clinical parameters such as FEV_1 and CT densitometry [156].

Currently desmosine is the only clinical biomarker for assessment of lung tissue destruction, however it is limited by lack of specificity and may be influenced by diet and renal function which may in part provide an explanation for the lack of association between desmosine levels in the urine and FEV_1 [157-159]. Current research is aimed at identifying cost effective yet stable novel biomarkers that are central to the pathophysiological process, which can act as a predictor of disease progression and are sensitive to therapeutic intervention [160, 161]. In this regard, a novel potential biomarker has been recently reported specific to cleavage of fibrinogen and related to NE in the lungs [136]. The fibrinogen cleavage product Aα-Val^{360} was measured in plasma from subjects recruited in the EXACTLE trial and the levels of this product were decreased in subjects receiving AAT replacement therapy while remaining unchanged in the placebo group.

Determining exacerbation rates in patients receiving therapy is an additional parameter that is employed to determine the efficacy of AAT replacement therapy. In a web based questionnaire prepared by Lieberman and colleagues, key questions addressed issues such as frequency of respiratory infections pre- and post-AAT augmentation therapy [139]. Out of eighty nine individuals who had received augmentation therapy for more than one year, seventy four felt that the treatment was of benefit, with almost half accrediting the benefits to a reduction in the number of infectious exacerbations from approximately five down to one per year ($P<0.001$). CT lung density measurements are currently considered to be a more sensitive outcome to measure the impact of AAT replacement therapy. The first of these studies was a double blind trial with 26 Danish and 30 Dutch ex-smokers, randomized to either AAT (250 mg/kg) or albumin (625 mg/kg) infusions at 4 week intervals for at least 3 years [140]. Using self-administered spirometry, no difference was found in FEV_1 decline between treatment and placebo. Conversely, upon analysis of CT scans (slices 5 cm below carina), a trend towards a slower rate in loss of lung density was observed in augmentation receiving patients with measurements of 2.6 ± 0.41 g/L/yr for placebo as compared with 1.5 ± 0.41 g/L/yr for AAT

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infusion, however the reported differences were shown to be non-significant (P=0.07), possibly due to the inadequate study size. Following this, in 2009, Dirksen *et al*. carried out a randomised, double-blind, placebo-controlled, parallel-group study conducted at three European centres (Copenhagen (Denmark), Birmingham (UK), and Malmö (Sweden)) known as the EXACTLE trial. CT scans were performed at baseline and at 12 and 24 months and the difference in the decline of lung density between treated and placebo groups suggested a trend towards a beneficial treatment effect, with P values for treatment difference ranging from 0.049 to a non-significant value of 0.084 depending on the outcome algorithm chosen. In 2010, Stockley and colleagues analysed the combined outcome measures of the EXACTLE and Danish-Dutch trial and concluded that augmentation therapy conferred a significant decline in the rate of CT densitometric loss but not in FEV_1 decline [162].

A number of studies have addressed the potential clinical use of AAT augmentation therapy in treatment of disorders outside the context of AAT deficiency, including transplant rejection, type1 diabetes mellitus, inflammatory bowel disease, rheumatoid arthritis, viral infection and cystic fibrosis [163]. In light of this, there is a need that goes beyond that afforded by purified human plasma and thus transgenic and recombinant AAT have been considered.

4.2. Recombinant and transgenic AAT

Despite the accumulation of encouraging data supporting the use of AAT augmentation therapy, significant questions remain regarding the cost-effectiveness of this intravenous strategy for treatment of AAT deficient related COPD, which is estimated to cost the US $100,000 per patient per year worldwide [143]. Such deliberation has prompted the development of new strategies, including the use of recombinant and transgenic protein to boost the natural antiprotease screen. A variety of systems have been employed to produce humanized recombinant AAT including plants, yeast fungi, animals, insect cells, bacteria and mammalian cells. One of the approaches involved expression of recombinant AAT in *E. coli*, but AAT produced via this system lacked glycosylation and consequently exhibited a short half-life within the circulation. To address this issue the presence of a single thiol residue at the surface of AAT was exploited by researchers, who conjugated maleimido-polyethylene glycol to the thiol group. Results revealed that site-specific conjugation with polyethylene glycol at Cys^{232} of nonglycosylated recombinant human AAT gave rise to active inhibitor with prolonged *in vivo* stability [164]. An alternative approach included production of a secreted fully functional AAT by the yeast *Saccharomyces cerevisiae* [165]. This secreted protein had high mannose-type glycosylation [166], which was thought to give rise to an immune response in humans because of non-human glycan residues, an adverse effect shared by active recombinant AAT protein expressed in the insect cell baculovirus expression vector system [167]. In an attempt to overcome the issue of glycosylation, a method of hyper glycosylation of the protein was introduced. This was achieved by adding terminal sialic acids to the existing glycans on the human AAT molecule. Such modification conferred increased protein half-life while maintaining the anti-NE capacity of the protein when injected into a mouse model [168]. In a further attempt to prepare fully glycosylated human AAT, Blanchard *et al* employed a human neuronal cell line (AGE1.HN®) to produce a recombinant AAT protein which retained biological activity and

displayed a similar glycosylation patterns to native AAT [169]. Moreover, the recombinant AAT protein was biologically active and maintained anti-elastase activity and anti-inflammatory capacity, including modulation of TNFα production in neutrophils and monocytes [169]. *In vitro* testing of this protein revealed that the inhibitory effect of the recombinant AAT was comparable to that of Prolastin, however this has yet to be confirmed *in vivo*. More recently, AAT has been successfully produced and purified from the human PER.C6 cell line. The resultant protein shares the same primary, secondary and tertiary structures and *N*-linked glycosylation sites as AAT derived from human plasma and exhibits equivalent anti-NE capacity [170, 171]. While these studies strengthen the argument for the therapeutic use of recombinantly produced AAT, issues still remain with regards to the safety and efficacy of protein produced in this manner.

Subsequently, it was envisaged that sources of recombinant AAT from transgenic animals might overcome this posttranslational glycosylation challenge and fulfil the demand for mass production of AAT. To this end rabbits [172], mice [173] and sheep transgenic for a fusion of the ovine beta-lactoglobulin gene promoter to the human AAT genomic sequences were generated [174]. Human AAT purified from the milk of these animals appeared to be fully *N*-glycosylated and biologically active. While the use of transgenic sheep certainly tackled the issue of cost effectiveness, 5,000kg of AAT could be produced in one year, unfortunately however, a trial of inhaled AAT derived from sheep milk proved disappointing and observations of a systemic antibody response in recipients to residual native sheep AAT and alpha-1 antichymotrypsin were observed [175]. Thus it is clear that transgenic preparations of AAT will only be of therapeutic value if they are of high purity in order to avoid immunogenicity.

While enhancing production of AAT seems promising from a cost and mass production point of view, significant challenges remain for the development of recombinant and transgenic AAT and they cannot compete with the current source of AAT from pooled human plasma for safety and efficacy in the treatment of AAT deficiency.

4.3. Aerosolized AAT augmentation therapy

Interest in the administration of AAT in aerosolized form has increased in recent years due to the advantageous properties provided such as a direct route to the lungs, avoidance of systemic deliver and a reduction in costs. Aerosolization of AAT has been shown to increase AAT levels and to restore anti-NE capacity in lung epithelium lining fluid of both COPD and cystic fibrosis individuals [176]. AAT administered in aerosolized form was found to positively impact upon neutrophil mediated killing of *Pseudomonas* [177], possibly by preventing cleavage of neutrophil complement receptors by serine proteases, a previously reported adverse effect of NE [178] or by preventing cleavage of CXC chemokine receptor 1 (CXCR1) [179]. In addition, the presence of increased AAT in serum post aerosolization supports the hypothesis that augmented AAT is capable of diffusing across the pulmonary interstitium after administration [180] and affords anti-NE protection to the interstitial compartment [176]. Measurements of the rate of transfer of AAT from the lung and of the rate of appearance of AAT in plasma resulted in a calculated permeability of the alveolar-capillary membrane to AAT of 3.49-6.39 X 10^{-10} cm/s. In a canine model, levels of AAT rose to a maximum value at 48 hours after

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administration, remained elevated from 48-72 hours, and then declined slowly by 144 hours after administration [180]. Comparable results were reported by Hubbard *et al.* who evaluated aerosol administration of recombinant AAT to COPD individuals with AAT deficiency. Post aerosolization of single doses of 10-200mg of AAT, AAT anti- NE defences in ELF were augmented and aerosolized AAT was detectable in serum indicating that recombinant AAT was capable of gaining access to lung interstitium [176].

Further support for the use of aerosolized AAT in treatment of COPD was provided by Griese and colleagues who examined the effect of four weeks of plasma purified AAT inhalation on lung function, protease–antiprotease balance and airway inflammation in patients with cystic fibrosis [181]. The authors reported an increased concentration of aerosolized AAT accumulation in the airways. Post treatment, levels of NE activity, numbers of infiltrating neutrophils, pro-inflammatory cytokines levels and the numbers of bacteria (*P. aeruginosa*) were reduced culminating in a marked reduction in airway inflammation. In a second study by this research group, the authors documented that the inability of neutrophils to eradicate *Pseudomonas* infection despite the abundance of neutrophils in the cystic fibrosis lung was due to cleavage of CXCR1 from the neutrophil. *In vivo* administration of aerosolized AAT was shown to restore CXCR1 expression and improve the killing capacity of neutrophils as assessed by sputum levels of *P. aeruginosa*, thus supporting the beneficial effects of aerosolized AAT therapy [179]. In a further study employing a mouse model of smoke induced emphysema, inhaled AAT resulted in reductions in airspace enlargement of up to 73%. The results of this investigation indicated that delivery of AAT directly to the lung can prevent NE mediated tissue damage. Moreover, further evidence of the ability of aerosolised AAT to directly protect the lung tissue from proteolytic breakdown was demonstrated as aerosolised AAT abrogated NE-induced expression of cathepsin B and MMP-2 in BAL fluid, thus indirectly protecting key anti-inflammatory and antimicrobial peptides including SLPI and lactoferrin from cathepsin mediated proteolysis within the milieu of the COPD lung [36]. In terms of clinical efficacy of aerosolised AAT, a phase III trial of inhaled Kamada AAT is currently on-going with results on outcome measures including safety and efficacy, exacerbation frequency and progression of emphysema to be released presently. The outcome of this study will be pivotal to shaping the future development of aerosolised AAT augmentation therapy (NCT01217671).

4.4. Naturally occurring antiproteases elafin and SLPI as therapies for COPD

A number of studies have generated data to support the use of naturally occurring antiproteases, other than AAT, as potential anti-inflammatory therapies to modulate the high level of inflammatory within the lungs of COPD patients. Within the literature reports suggest that serine protease inhibitors may potentially be used as a treatment option for COPD. One such antiprotease is elafin, a small cationic protein (6kDa) which is a member of the chelonianin family. Throughout the literature it is often referred to as skin derived anti-leukoprotease (SKALP) due to its first identification in psoriatic epidermis [182]. More recently however, it has been shown to be produced and released by epidermal keratinocytes [183], lung derived epithelia [184], T-cells [185], macrophages [186] and also neutrophils [187].

Elafin is a subunit derived from the precursor molecule trappin-2 which has a molecular weight of 12.3 kDa [188]. The conversion of trappin-2 into elafin can occur by cleavage by several proteases including cathepsin L, cathepsin K, plasmin, trypsin but the most efficient protease at releasing elafin is tryptase [189]. The mature form of trappin-2 consists of two domains; the first domain is unique to only trappin-2, the *N*-terminal cementoin domain. This domain aids as a transglutaminase substrate which allows trappin-2 to interact and bind to extracellular matrix proteins such as fibronectin, collagen IV, fibrinogen, laminin V, vitronectin, thus prolonging its presence within in the lung [188]. The second domain, which is present in both trappin-2 and elafin, is the whey acidic protein (WAP) domain. The WAP domain provides antiprotease activity with specificity against NE [18] and PR3 [17], two key proteases that play a role in the lung pathophysiology associated with COPD [56, 190].

Several studies have demonstrated that elafin is produced under inflammatory conditions suggesting it to be an acute phase reactant [191]. Interestingly, NE can impact on elafin expression and it has been demonstrated that exposing human alveolar epithelial cells to NE can result in increased protein expression and secretion of elafin [192]. Additional studies have demonstrated that pro-inflammatory mediators such as TNFα and IL-1β increase elafin expression in lung derived cell lines, indicating that in response to cytokine induced inflammation, epithelial cells can increase their antiprotease shield [191]. This induced increase in elafin expression is not limited to only host derived inflammatory mediators as it has been shown that bacterial LPS can increase elafin expression in murine airways [193] and in macrophages from transgenic mice expressing human elafin [194].

The immuno-modulatory impact of elafin on inflammation has been established in a number of studies and animal models. For example, Vachon and colleagues utilised a murine model and following intranasal administration of LPS, pre-treatment with recombinant human elafin was shown to diminish neutrophil infiltration into the airways [195]. In the same study elafin was shown to reduce gelatinase activity, macrophage inflammatory protein-2 (MIP-2), keratinocyte chemoattractant and significantly reduced mRNA levels of three members of the IL-1 ligand family [195]. Conversely, in an earlier study involving a transgenic murine model expressing human trappin-2, an increase in neutrophil recruitment to the lungs of LPS challenged mice was observed [193]. The discrepancy between these two latter studies could in part be due to differences in the mode of elafin administration and the murine models employed. Of interest, the positive impact of elafin on COPD related complications such as pulmonary hypertension has been reported [196]. Moreover, in a hypoxia associated pulmonary hypertension murine model the protease activity, muscularization of pulmonary arteries and right ventricular pressure were reduced in transgenic mice that over expressed elafin [197]. Indeed, both elafin and its precursor molecule trappin-2 have anti-inflammatory properties, as evident by the inactivation of key inflammatory signalling pathways and immune cell activity associated with COPD pathogenesis. In a study using a human myelomonocytic cell line, elafin was shown to exert its anti-inflammatory effect intracellularly [198]. The mode of action involved direct impact on the ubiquitin-proteasome pathway, delaying turnover of polyubiquitinated proteins thus affecting NFκB activation and the AP-1 pathway leading to reduced MCP-1 expression in response to LPS [198]. Moreover, in transfection

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studies it was demonstrated that the impact of elafin on chemokine production was cell specific [199]. Results demonstrated that elafin reduced TNFα secretion by human macrophages and IL-8 release by human umbilical cord endothelial cells (HUVECs) in response to TNFα, LPS and oxidized LDL, through modulation of NFκB signalling. In contrast, in the same study transfecting an alveolar epithelial cell line with elafin had no significant effect on LPS induced IL-8 production [199].

In addition to possessing antiprotease and anti-inflammatory properties, elafin also demonstrates anti-microbial properties which may prove beneficial in the treatment of infective exacerbations in COPD patients. In a study carried out by Simpson *et al*., the anti-bacterial capability of elafin was evaluated and the results demonstrated that elafin maintained bactericidal effect against both *P. aeruginosa* and *S. aureus* [200]. This effect could in part be due to the cationic charge of elafin which may destabilize bacterial membranes similar to classical antimicrobial proteins [201]. Moreover, a study by Baranger *et al*., demonstrated that trappin-2 had antibacterial properties against other clinically important bacteria including *Klebsiella pneumoniae, Haemophilus influenzae, Streptococcus pneumoniae* and *Branhamella catarrhalis* [202]. Furthermore, this study highlighted the novel anti-fungal effect of trappin-2 against *Aspergillus fumigatus*, with the outcome of this study demonstrating the anti-microbial properties of trappin-2 to be independent of its antiprotease property. Aside from direct activity against microbes, trappin-2 in turn has been shown to enhance the clearance of *P. aeruginosa* from the lungs of infected mice [203], an effect mediated via opsonisation of bacteria with trappin-2 for more efficient CD14-dependent clearance by macrophages [197]. In additional studies it has been shown that elafin can modulate inflammation associated with LPS, through inhibition of AP-1 and NFκB activation [198], possibly by direct interaction with this microbial inflammatory mediatory [204].

These innate attributes of elafin make it an ideal candidate for a replacement therapy in treatment of inflammatory lung diseases such as COPD which is associated with excess protease burden and/or infection. However caution must be exercised, as like many other endogenous protease inhibitors, elafin is susceptible to inactivation by neutrophil-derived oxidants resulting in loss of its antiprotease activity [205]. Moreover, elafin can be cleaved by NE [206], which is present in high concentrations in the COPD lung [207] and cleavage can result in diminished ability of elafin to bind LPS and its capacity to be immobilized by transglutamination [206]. A similar observation was made in regard to the *P. aeruginosa* derived proteases pseudolysin and aeruginolysin which can cleave elafin thereby negatively impacting on its biological functions [208]. Collectively, these findings suggest that within an environment of high protease burden, as found in the COPD lung, this could have a negative impact upon the clinical efficacy of elafin.

An additional native antiprotease that has been well documented for its potential as a therapy for COPD is human SLPI, which was originally identified in parotid secretions [20]. Being a member of the antileukoprotease family, SLPI shares 40% homology with elafin. A number of cell types have been documented to produce SLPI including macrophages [186], monocytes, neutrophils [187] and lung epithelial cells [209]. It is well accepted that SLPI is a potent inhibitor of an array of serine proteases including NE, cathepsin G, trypsin and chymotrypsin. Similar

to elafin, SLPI expression levels are increased post inflammatory mediator exposure; for example TNFα and IL-1β treatment of lung epithelial cells [191]. Other host derived inflammatory proteins including proteases from neutrophils (e.g. NE and defensins [184]) can increase and induce SLPI expression in bronchial epithelial cells. In more recent studies, aside from host derived inflammatory mediators, bacteria have also been shown to induced SLPI expression in macrophages by a TLR2-dependent but MyD88-independent signalling pathway [210]. Additionally, bacterial products such as LPS can increase SLPI expression in macrophages and neutrophils [211], suggesting that SLPI functions as an acute phase reactant.

SLPI exhibits an array of anti-inflammatory properties including inhibition of monocyte production of MMPs [212, 213] which play an important pathophysiological role in tissue remodelling and are observed to be in high abundance in COPD [95, 107, 214]. The range of anti-inflammatory properties of SLPI includes inhibition of nitric oxide and TNFα production in macrophages in response to LPS [215] and inhibition of IFN-γ induced cathepsin S expression in macrophages [216]. In monocytes it has been demonstrated that SLPI can cross the plasma membrane and enter the cell cytoplasm and nucleus [217]. Localization to these cell compartments facilitates the ability of SLPI to inhibit degradation of key proteins that activate NFκB and to competitively compete with p65 binding to NFκB consensus sequences thereby preventing NFκB binding to promoter regions of inflammatory genes [217]. In a more recent study involving neutrophils from COPD patients, the intracellular inhibitory activity of SLPI was further expanded as it was shown that SLPI modulated calcium flux and inositol 1,4,5-triphosphate generation thereby reducing cell migration [218] Additionally, SLPI possesses anti-microbial capabilities against *P. aeruginosa, S. aureus, S. epidermidis, E. coli and S. aureus* [219, 220]. SLPI's antifungal properties have been observed against *A. fumigatus* and *Candida albicans* [221] and in this latter study SLPIs fungicidal and fungistatic activity were shown comparable to that of human defensins and lysozyme.

Despite the potential advantages, delivery of SLPI to the lungs has proven unimpressive [222]. Aerosolisation of recombinant SLPI (100mg) twice daily to individuals with cystic fibrosis was associated with reduced NE activity on the respiratory epithelial surface, as well a reduction in the level of IL-8 and neutrophil numbers [223]. However, a greater concentration of recombinant SLPI compared to AAT is required to suppress NE activity in individuals with lung disease. Furthermore accumulation of recombinant SLPI on respiratory epithelial surfaces does not occur and SLPI does not penetrate significantly into the interstitium following aerosolization. Further drawbacks include the fact that most of the anti-NE effects are gone within 12 hours of administration. Reasons for this latter phenomenon have been proposed and may be due to uptake of SLPI by epithelial cells and macrophages, and/or binding of recombinant SLPI to molecules in the interstitium after passing through the epithelium [222, 224].

More recently, it has also been shown that SLPI is vulnerable to degradation and inactivation by cysteinyl cathepins [225] and NE [21], and thus the utilization of SLPI as effective therapy for COPD presents some challenges. One approach to this challenge is delivery of recombinant SLPI via a liposomal carrier which protects the activity of SLPI against cathepsin L mediated degradation, whilst having no adverse effect on SLPI access to intracellular sites of action *in*

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vitro [226]. Alternatively, co-treatment of SLPI with surfactant protein A has been proposed, which aids in the preservation of SLPI and protects it from cleavage by matrix metalloproteases [227].

4.5. Synthetic and semi-synthetic engineered protease inhibitors as alternative therapies for COPD

In a COPD animal model, ADAM-17 (a membrane bound MMP) has been shown to contribute to progression of COPD lung disease through activation of TNFα [228]. Recent studies have demonstrated that AAT, a natural serine protease inhibitor, also acts as an ADAM-17 inhibitor thereby regulating soluble immune complex induced neutrophil chemotaxis [148] and TNFα production in lung endothelial cells [229]. However, other therapeutic options for the modulation of inflammation associated with excessive protease activity is the use of synthetic or semi synthetic protease inhibitors. These molecules, for example the non-peptide inhibitor ONO-5046 or sivelestat, offer better accessibility into the lung milieu due to their reduced size. Sivelestat is a specific inhibitor of NE and in LPS animal studies was shown to significantly reduce the number of infiltrating neutrophils and elastase activity levels, thereby decreasing lung tissue damage [230-232]. Moreover, sivelestat has been reported to reduce neutrophil-mediated endothelial cell injury by inactivating extracellular elastase and by suppressing release of this serine protease by neutrophils [231]. A recent study demonstrated the ability of sivelestat to inhibit bleomycin induced pulmonary fibrosis and apoptosis in human epithelial cells [233]. The authors of this study also verified that sivelestat reduced pulmonary neutrophil counts by decreasing BAL fluid levels of cytokine-induced neutrophil chemoattractant (CINC)-1. So far there have been no *in vitro* or *in vivo* investigations on the potential use of sivelestat in COPD, but studies involving other pulmonary conditions including post-cardiopulmonary bypass lung injury [234], acute lung injury with sepsis [235] and adult respiratory distress syndrome [236] have shown some therapeutic success with this synthetic inhibitor. To date, Japan has been the only country to approve sivelestat for the above conditions [237].

An alternative synthetic inhibitor that has been utilized in several studies is the cyclic thiol compound MR889. This synthetic inhibitor demonstrated inhibition against several serine proteases *in vitro* and in sputum from patients with chronic bronchitis [238]. When used in clinical studies however, although MR889 was considered safe for COPD patients, the results demonstrated that there was no significant reduction in the level of biomarkers of lung disease except for a small subset of individuals who had a short disease duration [239]. Other synthetic elastase inhibitors such as ZD0892 and FR134043 have been shown to reduce lung inflammation associated with cigarette smoke [240, 241] or NE [242] in animal models and in cultured cells [243]. ZD0892 was shown not to exert an adverse effect on neutrophil function including uptake and killing of *S. aureus*, but use of this compound in clinical studies in humans has not been documented.

More recently, the synthetic selective inhibitor of elastase, AZD9668, has attracted a lot of attention and was shown to have significant impact on NE activity *in vitro* [244]. In rodent models, AZD9668 was shown to prevent NE-induced lung injury, cigarette smoke induced

inflammatory responses, airspace enlargement and small airways remodelling [244]. Despite these positive findings however, results of a recent clinical trial involving AZD9668, queried the efficiency of this inhibitor in COPD patients as no significant change in lung function and clinical measurements was observed [245]. Although these later results suggest that targeting NE activity alone may not be an effective treatment option in COPD, a subsequent smaller study indicated the potential clinical efficacy of AZD9668 in the treatment of bronchiectasis [246].

Aside from serine proteases, and as already mentioned, MMPs also play a key role in the development of COPD. TIMPs are endogenous MMP inhibitors and in COPD, MMP activity levels associated with lung disease are not counteracted by TIMPs [247]. To date no native TIMPs have been used as potential therapeutics, but synthetic and selective MMP inhibits have been well documented. The orally administered MMP-9 and MMP-12 inhibitor AZD1236 was well tolerated by patients in two separate clinical trials but overall it was deemed to have no effect on clinical parameters [248] or disease related biomarkers in COPD patients [249]. In a recent study by Wu *et al*., several potential MMP-12 inhibitors were analysed *in vivo*, with one specific inhibitor, Compound 26, modulating cell infiltration into the lungs of mice with MMP-12 induced inflammation [250]. Similar findings were observed for a second MMP-12 inhibitor, Compound 14 [251]. An additional *in vivo* study involved the use of the broad spectrum MMP inhibitor RS113456, which possessed comparable anti-inflammatory properties to AAT [252]. In the latter study the authors noted multi-facetted attributes of RS113456, whereby this MMP inhibitor reduced neutrophil influx, modulated NFκB activity and reduced MCP-1 and MIP-2 expression in the short term [252].

Other synthetic MMP inhibitors evaluated in cigarette smoke induced models of COPD have shown significant reductions in lung macrophage numbers [126], neutrophilia [253, 254], elastase/ MMP levels [254], air space enlargement [126, 254] and inflammatory markers [90, 126]. To date the benefits of MMP inhibitors has only been assessed in animal models and their successful use as therapeutics for treatment of COPD in humans has not been reported. Thus caution needs to be exercised due to the potential for tumour growth promotion as a result of inhibition of angiogenesis factors [255].

An alternative to synthetic inhibitors is the production of semi-synthetic protease inhibitors. These agents are created through the chemical modification of naturally occurring protease inhibitors. One such semi-synthetic inhibitor is the engineered protein inhibitor of human NE (EPI-HNE-4) which is derived from the Kunitz type domain from the naturally occurring inter α inhibitor. EPI-HNE-4 has been shown resistant to degradation by both human and bacterial MMPs and its antiprotease activity was reported impervious to oxidative inhibition [256]. Further studies involving the use of a rat model and sputum from cystic fibrosis patients demonstrated the anti-NE capacity of EPI-HNE-4 along with its ability to decrease neutrophil migration towards the bacterial peptide *N*-formyl-L-methionyl-L-leucyl-phenylalanine [257]. Subsequently however, contrary data on the immuno-regulatory effects of this inhibitor have been documented as Honoré *et al*., demonstrated that EPI-HNE-4 had little effect on neutrophil migration or impact on bacterial clearance in a *P. aeruginosa* model of pneumonia [258].

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Within this expanding field of potential antiprotease therapies lies the development of novel chimeras that are active against NE, PR3 and CathG. A study by Zani *et al*., reported on the development of a chimera consisting of domains from both elafin and SLPI and a trappin-2 variant (A62L) [259]. The authors demonstrated that both A62L and the elafin-SLPI chimera retained their polypotent inhibition of protease activity while covalently cross-linked to fibronectin or elastin by a tissue transglutaminase, in a similar fashion to wild-type elafin and trappin-2 [188]. A recent extensive study on A62L in an epithelial cell line demonstrated that this engineered trappin-2 molecule prevented neutrophil and protease induced epithelial cell injury by inhibiting cell detachment, tight junction disruption and ultimately reducing apoptosis [260]. Although the preliminary data has been generated for these engineered antiproteases, overall their effect on the inflammatory status and clinical parameters in COPD patients remains to be investigated.

5. Conclusion

Extensive studies have been performed to extend our knowledge of the involvement of proteases in respiratory manifestations of COPD, the outcome of which has served to illustrate the multifactorial complexity of the disease (Figure 2). Nevertheless, this clinical and scientific knowledge has advanced the design of both fundamental and novel therapeutic strategies, directed at relieving the protease burden associated with this disorder (Table 2). There have been major advances in the evaluation of AAT augmentation therapy in the treatment of the lung disease associated with AAT deficiency with newer focus on aerosolization approaches. These studies will inevitably show within the next number of years whether this approach works clinically. No single treatment that effectively prevents activity of the broad spectrum of proteases present in the airways is available as yet. Such pharmacological approaches including the development of antiprotease chimeras would essentially correct lung disease processes and conceivably exert anti-inflammatory effects. However, this would require a more complete understanding of the mechanism of disease and of the action of presently accessible and newly discovered drugs.

Protease	Role in COPD pathogenesis	Inhibitors and inactivators	Clinical trials and evidence for antiproteases as therapeutics
Neutrophil Elastase	Elastolysis/destruction of extracellular matrix proteins including elastin, collagens, fibronectin, proteoglycans & laminin Cleaves epithelial cell surface receptors Cleaves signalling cytokines	Alpha-1 antitrypsin (AAT) Monocyte neutrophil elastase inhibitor (MNEI/ Serpin B1) Elafin Secretory leucocyte protease inhibitor (SLPI)	Intravenous augmentation therapy of AAT.(Multiple Clinical Trials in Humans, see Table 1) Clinical effects of IV AAT in AATD: Slows rate of FEV1 decline Reduces elastin degradation Reduces exacerbation frequency

Protease	Role in COPD pathogenesis	Inhibitors and inactivators	Clinical trials and evidence for antiproteases as therapeutics
	Induces IL-8 expression in bronchial epithelial cells Upregulates TGF-β in airway smooth muscle Impairs T-cell function Induces macrophage production of cathepsin B and MMP-2 Induces expression of MUC5AC leading to hypersecretion of mucus. Decreases ciliary beat frequency of bronchial epithelial cells	Sivelestat (synthetic non-peptide inhibitor) MR889 (synthetic cyclic thiol compound) ZD0892 (synthetic elastase inhibitor) FR134043 (synthetic elastase inhibitor) AZD9668 (synthetic selective elastase inhibitor)	Attenuates loss of lung density Clinical effects of aerosolized AAT: Restores anti-NE capacity (176, 177) Improved neutrophil killing of *P. aeruginosa* (179) Outcome of phase III clinical trial pending (NCT 0217671) SLPI – Reduced NE activity and reduced IL-8 levels in CF patients (223) Sivelestat – Reduced number of neutrophils in BAL. Has shown therapeutic benefit in acute lung injury and ARDS, but no trials in COPD to date (230-232, 235, 236) MR889 – Safe but no significant reduction in the level of biomarkers of lung disease in COPD patients (239) ZD0892 & FR134043 – Reduce cigarette induced lung inflammation, but no human trials to date (240,2410) AZD9668 – Decreased NE activity *in vitro* (244) No change in clinical parameters in COPD (245)
Proteinase-3 (PR3)	Elastolysis/destruction of extracellular matrix proteins including elastin, collagens, fibronectin, proteoglycans & laminin. Less destructive than NE Pro-inflammatory, activates TNFα and IL-1β Hypersecretion of mucus Cellular apoptosis	Alpha-1 antitrypsin (AAT)	See above for clinical effects of AAT therapy in AATD and COPD

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Protease	Role in COPD pathogenesis	Inhibitors and inactivators	Clinical trials and evidence for antiproteases as therapeutics
Cathepsin G	Proteolysis and destruction of extracellular matrix of the lung Induces mucus secretion Impairs T-cell function	Alpha-1 antitrypsin (AAT) Secretory leucocyte protease inhibitor (SLPI)	See above for clinical effects of AAT therapy in AATD and COPD
Other Cathepsins (Cathepsin B, D, H, K, L and S)	Parenchymal destruction and emphysema development Proteolytic degradation of beta-defensins thus impairing bacterial killing Induce airway inflammation	Cystatins are a group of endogenous reversible, tight-binding competitive cysteine protease inhibitors for cathepsins B, H, and L. SLPI inhibits cathepsin S expression in macrophages	
Matrix Metalloprotease-12 (MMP-12)	Degradation of collagen and elastin Monocyte recruitment Activates TNFα Induces IL-8 production Can degrade AAT	Tissue inhibitors of metalloproteases (TIMP) Secretory leucocyte protease inhibitor (SLPI) AZD1236 (synthetic inhibitor of MMP-12 and MMP-9) Compound 26 (synthetic compound) Compound 14 (synthetic compound) RS113456 (synthetic MMP-12 inhibitor)	SLPI – Anti-inflammatory properties and specifically inhibits monocyte production of MMPs (212, 213) AZD1236 – Well tolerated in COPD but not effect on clinical parameters (248,249) Compound 26, Compound 14, RS113456 – Reduced neutrophilic inflammation but no human trial data (250-252)
Matrix Metalloprotease-1 (MMP-1)	Degradation of collagens, elastin, gelatin, pro-MMP9, pro-MMP13 TACE (TNFα converting enzyme) like activity Induces TGFβ-1 – leading to fibrotic changes Induces mucus hypersecretion	Tissue inhibitors of metalloproteases (TIMP) Secretory leucocyte protease inhibitor (SLPI) AZD1236 (synthetic inhibitor of MMP-12 and MMP-9)	SLPI – Anti-inflammatory properties and specifically inhibits monocyte production of MMPs (212, 213) AZD1236 – Well tolerated in COPD but no effect on clinical parameters (248,249)
Other Matrix Metalloproteases (MMP-2, MMP-8, MMP 10, MMP-13)	Degradation of collagens, gelatin, pro-MMP9, pro-MMP13 TACE (TNFα converting enzyme) like activity	Tissue inhibitors of metalloproteases (TIMP)	

Protease	Role in COPD pathogenesis	Inhibitors and inactivators	Clinical trials and evidence for antiproteases as therapeutics
	Degradation of collagens, elastin, gelatin and fibronectin	Tissue inhibitors of metalloproteases (TIMP)	

Table 2. Summary table listing the various proteases, their targets, their inhibitor/inactivators, and clinical trials.

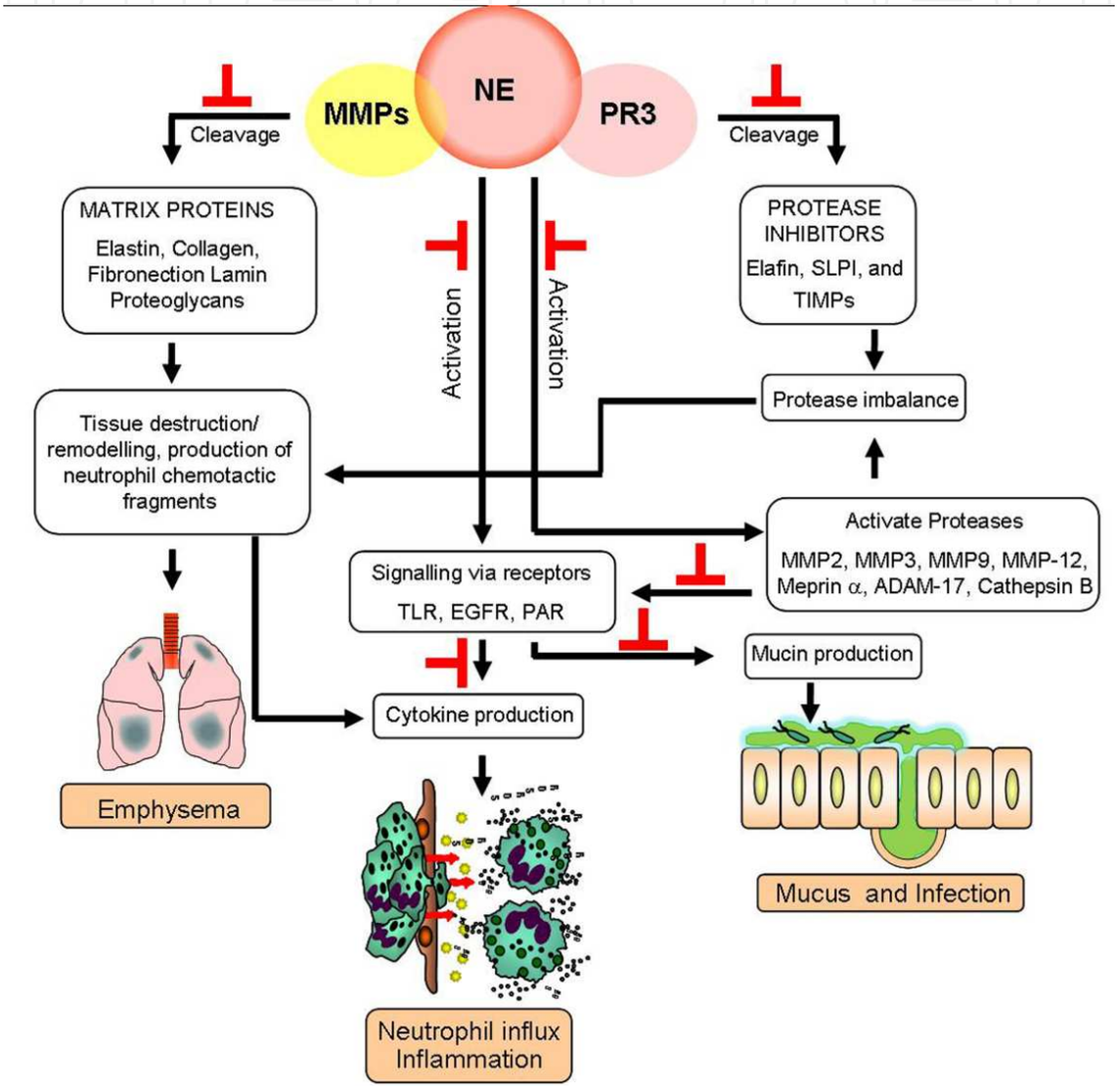

Figure 2. The effect of protease activity on the pathophysiology of COPD. A diagramatic illustration of the primary effects of excessive protease activity on inflammation in COPD. The effects include activation of signalling pathways and proteases, to cleavage and degradation of matrix proteins and antiproteases. The secondary effect of the excessive protease burden in the COPD lung can result in tissue destruction/ remodelling and excessive cytokine and mucin production. Consequenrly proteases play a key role in emphysema, neutrophil influx, inflammation, mucus production and infection. As shown by the red lines, antiprotease therapy (AAT, SLPI and elafin) can impact on several aspects of this complex and intertwined pathway and potentially modulate disease progression.

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