Targeting Proteases in Cystic Fibrosis Lung Disease: Paradigms, Progress, and Potential

Targeting proteases in cystic fibrosis lung disease: paradigms, progress and potential

Michael C. McKelvey[1], Sinéad Weldon[1], Daniel F. McAuley[2], Marcus A. Mall[3,4,5*], Clifford C. Taggart[1*]

[1]Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, Northern Ireland.

[2]Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, Northern Ireland.

[3]Department of Pediatric Pulmonology, Immunology and Intensive Care Medicine, Charité - Universitätsmedizin Berlin, Berlin, Germany.

[4]Berlin Institute of Health (BIH), Berlin, Germany.

[5]German Center for Lung Research (DZL), Berlin, Germany.

[*]These authors contributed equally as senior authors

Corresponding author: Clifford C. Taggart, Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, Northern Ireland. E-mail: c.taggart@qub.ac.uk

Author contributions: Conception, design and drafting of the article or revising it critically for important intellectual content: MCM, SW, MAM, CCT.

Sources of support: This work was supported by the Northern Ireland Department for the Economy (studentship to MCM), Cystic Fibrosis Foundation (WELDON18G0), Medical Research Council (MR/P022847/1 CCT/SW), Rosetrees Trust (Grant A2450 CCT/SW), the German Federal Ministry of Education and Research (82DZL004A1, MAM), the German Research Foundation (SFB-TR84TP B08, MAM) and the Einstein Foundation Berlin (EP-2017-393, MAM)**.**

Running title: Proteases in cystic fibrosis lung disease

Subject descriptor: 9.16 Cystic Fibrosis: Basic Studies

Word count: 3376

Abstract

Cystic fibrosis (CF) is the most common life-limiting hereditary condition of Caucasian populations and is characterised by chronic airways inflammation driving progressive structural lung damage. Despite tremendous advances in the treatment of CF and concomitant increased life expectancy for patients, chronic lung disease remains the main cause of morbidity and mortality among CF patients. While universal restoration of cystic fibrosis transmembrane conductance regulator activity remains a future hope, novel therapies aimed at reducing or preventing chronic airways inflammation and progressive structural lung damage are required.

It is well-established that proteolytic enzymes are important in the CF lung beyond the basic turnover of proteins and intracellular degradation of pathogens. When secreted, these enzymes play key roles in extracellular substrate modification implicated in important biological processes such as matrix and airway remodelling, goblet cell metaplasia and mucus hypersecretion, immune cell recruitment and dysregulation of epithelial ion channels. Importantly, the burden of proteases in the CF lung is significantly elevated, overwhelming the endogenous antiprotease shield. Indeed, free protease activity has emerged as a major risk factor of the onset and progression of bronchiectasis and lung function decline in patients with CF. Recent research has highlighted the importance of new players such as cathepsin S and matrix metalloprotease-12, as well as the membrane-associated activity of key proteases such as neutrophil elastase on the surface of neutrophils.

Here, we review the current knowledge and emerging concepts of the role of host proteases in the pathogenesis of CF lung disease and their potential as therapeutic targets.

Abstract word count: 250

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic condition, predominantly of Caucasian populations, which impacts multiple organ systems. However, it is the chronic progressive lung disease of CF that causes the greatest morbidity and mortality. The disease is caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Consequently, the function of epithelial CFTR anion (chloride and bicarbonate) channels is compromised, leading to impaired anion and fluid secretion and airway surface dehydration, which in turn results in highly viscous airway mucus and impaired mucociliary clearance, setting the stage for mucus plugging, chronic inflammation and polymicrobial infection (1). Such a state causes progressive and irreversible damage of the airways and lung parenchyma, as recruited immune cells (predominantly neutrophils) release proteases, DNA and reactive oxygen species, and promote further immune cell recruitment by cytokine signalling.

The introduction of CFTR modulators (potentiators, correctors and amplifiers) in recent years has transformed the treatment of CF. Phase two trials of triple combination therapy suggest that a CFTR modulator therapy approach may be effective in up to 90% of CF patients (2, 3). While the emerging therapies show immense promise, there are still CF patients whose specific genotypes may not be amenable to these therapies. Furthermore, CFTR modulation alone may be insufficient to allow complete and lasting clearance of chronic airways infection and resolution of pulmonary inflammation, especially in the context of chronic CF lung disease with established structural lung damage (4). Importantly, it is unknown whether, or to what extent, these CFTR-directed therapies decrease protease activity. Until such a decrease has been demonstrated, novel antiprotease strategies are still highly relevant to limit tissue damage in CF lung disease.

The protease-antiprotease hypothesis is a simple paradigm that attempts to explain certain disease states as a product of an imbalance of proteases and cognate antiproteases, resulting in

elevated protease activity and damaging consequences for lung homeostasis (5). It is now well established that proteases play a significant role in the pathobiology of the CF lung (6), whether they are derived from immune cells or indeed the cells of the lung itself. The perception of these enzymes' roles has moved far beyond the terminal degradation of proteins; it is now recognised that proteases are key signalling molecules and that specific substrate cleavage can have myriad effects (7), beneficial or detrimental in the CF lung *(Figure 1)*. The use of protease inhibitor therapy may offer an alternative option in mitigating the disease state to regain homeostasis, which may go hand-in-hand with pharmacological rescue of mutant CFTR by emerging modulator therapies.

So far, the serine protease class has drawn the most attention in CF, in particular neutrophil elastase (NE), with its free extracellular form previously thought to be the major player in CF lung disease pathogenesis. Indeed, free NE in sputum has long been known to correlate with FEV_1 in children with CF (8) and elevated NE activity in bronchoalveolar lavage fluid at 3 months of age was found to be associated with persistent bronchiectasis by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) (9). However, several novel concepts are unfolding in pulmonary protease biology, which have led investigators to broaden their view beyond NE. These concepts include the redundancy of function between proteases *(Table 1)*, the *trans*-class activation of proteases, the discovery of highly active membrane-bound proteases and the emergence of new key players from the cysteine and matrix metalloprotease (MMP) classes. This review will summarise the current knowledge of host protease function in CF lung disease and how this may inform therapeutic intervention. The role of bacterial proteases in CF lies beyond the scope of this review but we direct readers to the following recent references (10, 11).

The actions of serine proteases in CF

The neutrophilic nature of CF airways inflammation gives intuitive significance to the group of proteases known collectively as the neutrophil serine proteases (NSPs). Its members are NE, proteinase-3 (PR-3), cathepsin G (CTSG) and the more recently discovered, lesser studied, NSP-4. The NSPs may be activated by the cysteine protease cathepsin C (CTSC) (12) and are involved in the intracellular degradation of neutrophil-phagocytosed microbes, a particularly important process in the chronically infected CF lung. The NSPs are harboured in primary neutrophil granules and their exocytosis is increased in CF, even during early CF disease prior to the onset of bronchiectasis (13). Extracellular NSPs have been shown to actively mediate the recruitment of immune cells to the site of inflammation by processing an array of cytokines including members of the IL-1 family (14), upregulating neutrophil chemoattractants such as IL-8 (15) and triggering the release of damage associated molecular patterns (DAMPs) such as High Mobility Group Box 1 (HMGB1), which can act as a biomarker for CF lung disease severity (16, 17). This modulation of neutrophil chemotaxis leads to enhanced inflammatory cell infiltration, continuing the vicious cycle of CF inflammation. Furthermore, highly chemotactic, truncated forms of various chemokines including IL-8 can be produced by NSP-mediated cleavage (18).

Neutrophil extracellular traps (NETs), the complex matrix of secreted DNA, proteases and other cellular contents released by neutrophils in CF airways (19) are important reservoirs of NSPs in CF. It has been demonstrated that this DNA-based matrix effectively maintains protease activity by preventing interactions with cognate endogenous or administered antiproteases (20). While intended as a protective mechanism, NSP activity can adversely affect the body's innate response mechanisms to infection including antimicrobial peptides (AMPs) and surfactant proteins. A number of proteases including NE can cleave AMPs such as lactoferrin and midkine (21, 22) and degrade surfactant proteins (23) thereby compromising

the host response and/or susceptibility to infection. NE also cleaves extracellular haem-containing proteins such as ferritin, liberating sequestered iron into the airway. Not only does this increase oxidative stress in the airway epithelium but it also promotes bacterial proliferation and biofilm formation as iron is made accessible for microbial nutrition (24, 25). NSPs may also play an important role as regulators of other proteases, particularly the MMPs. This role is especially relevant when considering the tissue-destructive nature of the proteases; NSP-activated MMP-9 and MMP-12, as well as the NSPs themselves, contribute directly to extracellular matrix (ECM) remodelling and bronchiectasis that is characteristic in CF (9, 26, 27). The protease-mediated loss of elastin limits elastic recoil, while the loss of collagen creates a structural deficit, leading to the emphysematous phenotype that can occur in adolescent and adult CF patients (28). The body's endogenous protection against aberrant NSP activity is also compromised, as NE inactivates tissue-protective antiproteases (some of which also possess antimicrobial properties) such as secretory leukocyte protease inhibitor (SLPI) (29).

The inability of such endogenous antiproteases, even when intact, to perform their inhibitory function has also been highlighted in recent years. This may be due, in part, to membrane-association of NSPs such as NE and CTSG (30). More recently, novel Förster resonance energy transfer (FRET)-based probes were used to analyse membrane-bound activity of proteases such as NE on the surface of neutrophils (31). In this surface-bound form, proteases are less accessible for their prospective inhibitors, which are unable to access the enzyme's active site. Indeed, surface-bound NE has been found to correlate with severity of lung disease and various inflammatory markers in CF (32).

As well as influencing both inflammatory cell recruitment and tissue destruction, NE contributes to increased mucus production in the CF lung by upregulating mucin expression and inducing goblet cell metaplasia, a process thought to be mediated through tumour necrosis factor-α converting enzyme (33, 34). In addition, NE induces secretion of mucins from airway

epithelial cells, augmenting mucus plugging in the CF lung (35). NE has also been shown to decrease the frequency of ciliary beat and damage the airway epithelium (36) which may contribute to impaired mucociliary clearance and hence mucus plugging. Further, NE may directly impact on airway ion transport by degrading CFTR (37) and activating epithelial sodium channel (ENaC) (38), thereby aggravating the basic ion transport defect and airway surface dehydration in CF airways. While CF is caused by mutations in *CFTR*, CF airways are characterized by increased ENaC-mediated sodium absorption in addition to deficient CFTR-mediated chloride secretion. Mimicking the hyperactivity of ENaC by airway-specific overexpression in mice can produce a phenotype that is strikingly similar to that found in CF patients, demonstrating that airway surface dehydration is a key disease mechanism in CF lung disease (1, 39) and that increased ENaC activity contributes critically to this abnormality. In this context, proteolytic activation of ENaC by NE and other proteases may be a key mechanism leading to increased ENaC activity that aggravates airway surface dehydration in CF airways.

Collectively, these studies show that NE is a key mediator in each of the major pathologies contributing to CF lung disease. However, the roles of the other NSPs have been less well studied and more research into these and their relative importance in CF is warranted.

The actions of cysteine proteases in CF

The predominant group of cysteine proteases in CF is the cysteine cathepsins. These papain-like proteases are lysosomally derived and hence display optimal activity in a reducing and acidic environment; only cathepsin S (CTSS) is thought to maintain its activity in the neutral-alkaline pH range (40). In the intracellular context, cathepsins are involved in the degradation of host and pathogen proteins as well as the processing and presentation of antigens. These

functions are crucial in homeostatic protein turnover, fighting infection and in the development of adaptive immune responses to infections. However, like the NSPs, certain members can be found in the extracellular milieu of the CF lung. Cathepsins are secreted by macrophages but may also be sourced from neutrophils, other antigen-presenting cells, lung epithelial and endothelial cells; this secretion may be associated with acidification of the pericellular space (41).

While only more recently recognised as major players in CF, the cysteine proteases mirror many the actions of the NSPs. Like all classes of proteases, the cysteine cathepsins are capable of degrading various ECM components, contributing to the tissue-destructive web of proteases involved in CF. A series of studies demonstrated the potential of cathepsins B (CTSB), L and S to compromise mucosal immunity in the CF lung via mechanisms similar to those mentioned already for the NSPs. They were shown to cleave AMPs including lactoferrin, LL-37, surfactant protein A and the human β-defensins (42–45). Thus, by the loss of active airway AMPs, the ability to maintain a pathogen-free airway may be undermined in CF. Several cathepsins have also demonstrated the ability to process CXCL chemokines *in vitro*, though it has yet to be determined whether these modifications occur or are highly relevant *in vivo* (46). The role of CTSC in the activation of NSPs makes it an interesting candidate for therapeutic intervention in the context of neutrophilic CF lung disease and although inhibitors are in early phase clinical trials, their efficacy and potential for CF remains to be determined (12).

CTSS is emerging as an important player in early CF lung disease with extracellular CTSS levels correlating significantly with lung function decline and neutrophil recruitment into the airways (47). A recent study, using the βENaC-overexpressing mouse with CF-like lung disease, elucidated roles for CTSS in the *in vivo* pathogenesis of several key CF pathologies (48). In this study, genetic and pharmacological knockdown of CTSS was associated with a reduction in neutrophil recruitment and amelioration of airway mucus obstruction and lung

tissue destruction. It also highlighted that CTSS may mediate inflammatory cell recruitment and mucin expression via protease-activated receptor 2. In relation to airway ion transport, both CTSS and CTSB have been reported to activate ENaC (49, 50). As such, in concert with NE-mediated CFTR degradation, the cysteine cathepsins may accentuate the mucus dehydration intrinsic to CF airways pathology.

The actions of matrix metalloproteases in CF

The members of the MMP class are not abundant in the healthy lung; however, they are produced by lung and inflammatory cells in response to inflammatory chemokines, noxious stimuli and free oxygen radicals (51). These zinc and calcium-dependent endopeptidases are loosely numbered in order of discovery up to MMP-28 and, as their name suggests, are potent ECM-degrading enzymes (52). While some MMPs are mainly tissue-derived, MMP-8 and MMP-9 are predominantly derived from neutrophils, making them proteases of particular interest in CF (53). The degradation of interstitial collagen is key to the development of bronchiectasis and other aberrant structural formations of the CF lung. In addition, this cleavage process generates matrix fragments, which can produce secondary downstream effects. During airway inflammation the proline-glycine-proline (PGP, a potent neutrophil chemoattractant) fragments produced by collagenase activity are not matched by a concomitant rise in PGP-degradation by leukotriene A_4 hydrolase, causing PGP accumulation which contributes to CF neutrophilia (54, 55). In addition, MMP-9 is capable of truncating IL-8 into a highly chemoattractive form (56).

While it is clear that the dominant immune cell population in the CF lung is the neutrophil, macrophage-derived proteases are gaining reputation in CF, particularly as regards their membrane-associated activity. A noteworthy example of this is macrophage elastase (also

known as MMP-12). Recent studies in βENaC-overexpressing mice with CF-like lung disease and paediatric CF patients suggest that mucostatic conditions in the CF airways may trigger elevated membrane-associated MMP-12 activity via macrophage activation (57). Interestingly, a functional polymorphism in the *MMP12* promoter (rs2276109) that decreases *MMP12* expression was positively associated with FEV_1 % predicted in patients with CF (57). This work opens interesting lines of inquiry: what are the specific signals which precipitate protease release from specific cell types? Are they to be found in CF mucus? Can they be targeted therapeutically? Can protease gene expression be targeted?

It is worth noting that there is evidence of positive roles for macrophage MMPs in the inflamed lung. MMP-10, for example, is highly expressed in CF patient macrophages and appears to have a protective role in acute bacterial infection by moderating macrophage inflammatory responses (58). MMPs continue to draw most attention for their contribution to lung tissue damage, though there is an emerging sense that this may not be the limit of their influence in CF.

Therapeutic strategies targeting the protease-antiprotease imbalance

The combined contribution of proteases to the pathology of CF makes them promising targets for novel therapeutics. Endogenous protease inhibitors are overpowered as a consequence of quantitatively elevated levels of secreted 'free' protease and have limited efficacy in the inhibition of surface-bound activity in CF lung disease. Importantly, current CF therapy relies heavily on mucolytic agents like dornase alfa, which are known to markedly increase NE activity in CF sputum (59). Therefore, antiproteases may constitute an important adjunct therapy to help limit further lung injury. Indeed, it has been shown that certain antiproteases are most effective when used along with mucolytics (59). Increased protease secretion and

membrane-associated activity are likely already initiated during infancy and early childhood, even in the absence of detectable bacterial infection (5, 9), strengthening the case for early antiprotease treatment.

To directly redress the protease-antiprotease imbalance, two principal strategies may be employed: antiprotease replacement/augmentation and pharmacological protease inhibition.

There is an attraction to using antiprotease-based therapies such as α_1-antitrypsin augmentation, especially considering the success of this strategy in α_1-antitrypsin deficiency. In 2015, a long-term, randomised control trial was reported with weekly α_1-antitrypsin administration for up to 48 months (60). RAPID (Randomized, Placebo-controlled Trial of Augmentation Therapy in Alpha-1 Proteinase Inhibitor Deficiency) demonstrated the slowing of lung parenchymal damage after redressing the protease-antiprotease imbalance and significantly, that this effect was most evident over the course of months and years, rather than short-term improvements over weeks (60). This was not a surprising finding, considering that antiprotease therapy is predicted to slow the progression of irreversible lung damage and bronchiectasis rather than producing short-term improvements in lung function; short trials are therefore unlikely to capture these therapeutic benefits. α1-antitrypsin augmentation has also been tested in CF, though this has been limited to short trials, predominantly powered to establish safety (61). Endogenous antiprotease augmentation is not without its pitfalls given their propensity to be degraded by proteases (host or pathogen). Recombinant variants of the endogenous antiproteases such as SLPI with reduced susceptibility to protease cleavage have shown efficacy in reducing inflammation (62). However, the size and complexity of these proteins, their generally broad antiprotease activity and the quantities required to address the substantial protease burden in CF are all factors to be overcome. These molecules have also yet to demonstrate efficacy against surface-bound proteases.

With these considerations in mind, perhaps it is the synthetic, low molecular weight, specific chemical inhibitors that hold the answer? As with all drugs, walking the tightrope between specificity and bio-reactivity has proven a challenge. For various reasons (including safety, a propensity for hapten formation and the struggle for target selectivity) it is no longer the consensus that rapid irreversible inhibition is necessarily the gold standard for these compounds (63). A new generation of highly specific, reversible inhibitors of NE or the emerging proteases (CTSC, CTSS and MMP-12) might help to shape the future of antiprotease therapy in CF. Many synthetic inhibitors have demonstrated potency *in vitro* and in the preclinical *in vivo* settings. However, NE inhibition, which has been the focus of clinical antiprotease work, has so far not proved overly effective in reducing key measures of disease in CF or other inflammatory lung diseases (64). Therefore, it may be that the inhibition of other proteases or a spectrum of proteases in combination with conventional therapies produces more promising results. Comprehensive studies will be required to ensure that patient susceptibility to infection is not increased by protease inhibition, though there is little preclinical evidence that this will be the case. Interestingly, the genetic ablation of NE in βENaC-overexpressing mice did not increase susceptibility to spontaneous airways infection in this model with CF-like lung disease (33). NSP-deficient mice have exhibited weakened host defence against certain respiratory pathogens (65, 66), though it should be noted that inhibitor-treated and full knockout mice are not direct corollaries and as such, further research is required to assess the effects of therapeutically relevant protease knockdown on host immunity.

An interesting alternative to the use of canonical antiproteases is inhaled heparin, which has been shown to improve pulmonary function in COPD (67). 2-O, 3-O-desulfated heparin, a modified polysulfated molecule, possesses both anti-NSP and anti-inflammatory properties with minimal anti-coagulant activity (16, 68). In some instances, repurposed drugs might offer a simpler and faster route to protease inhibition than the development of novel inhibitors,

especially regarding their safety profile. One drug which has emerged is the tetracycline antibiotic doxycycline. A 2017 study highlighted FEV_1 improvements following doxycycline treatment during acute pulmonary exacerbations in CF patients, seemingly independent of doxycycline's antibiotic properties, via MMP-9 neutralisation and TIMP-1 enhancement (69). Currently, there are no other licensed drugs that are known to fall into this category.

Summary and outlook

What is evident from the research to date is that proteases play a role in many of the most damaging facets of CF lung disease and as such could be targeted in combination with current antibiotic, mucolytic, bronchodilator and CFTR modulator therapies. A return to protease-antiprotease parity may indeed facilitate the breaking of the inflammatory cycle and slow the rate of structural and functional decline in CF. For proteases such as NE and CTSS, which are elevated from an early stage in the pathogenesis of CF lung disease, the age of CF patients at the start of protease inhibitor therapy and the frequency and duration of treatments may well be crucial factors to consider for the design of clinical trials. A significant challenge remains in developing protease inhibitors that retain specificity, stability and efficacy in the complex milieu of the CF lung and that are well tolerated over longer courses of treatment.

Our knowledge of the role and functions of proteases continues to evolve through the development and use of new experimental tools, reagents and pathobiological models. Because of their differential expression and activity profiles in CF lung disease, proteases (and their endogenous inhibitors) may serve as useful biomarkers for diagnostic and monitoring purposes to enable, for example, detection of lung disease severity and prediction of progression or response to treatment. Nonetheless, further work is needed to extensively characterise the lung

degradome, in addition to the status of endogenous antiproteases, activators, substrates and cleavage products in the CF lung.

References

1. Mall MA, Hartl D. CFTR: cystic fibrosis and beyond. *Eur Respir J* 2014;44:1042–1054.
2. Davies JC, Moskowitz SM, Brown C, Horsley A, Mall MA, McKone EF, Plant BJ, Prais D, Ramsey BW, Taylor-Cousar JL, Tullis E, Uluer A, McKee CM, Robertson S, Shilling RA, Simard C, Van Goor F, Waltz D, Xuan F, Young T, Rowe SM, VX16-659-101 Study Group. VX-659–Tezacaftor–Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *N Engl J Med* 2018;379:1599–1611.
3. Keating D, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, Ramsey BW, Rowe SM, Sass LA, Tullis E, McKee CM, Moskowitz SM, Robertson S, Savage J, Simard C, Van Goor F, Waltz D, Xuan F, Young T, Taylor-Cousar JL, VX16-445-001 Study Group. VX-445–Tezacaftor–Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *N Engl J Med* 2018;379:1612–1620.
4. Hisert KB, Heltshe SL, Pope C, Jorth P, Wu X, Edwards RM, Radey M, Accurso FJ, Wolter DJ, Cooke G, Adam RJ, Carter S, Grogan B, Launspach JL, Donnelly SC, Gallagher CG, Bruce JE, Stoltz DA, Welsh MJ, Hoffman LR, McKone EF, Singh PK. Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. *Am J Respir Crit Care Med* 2017;195:1617–1628.
5. Birrer P, McElvaney NG, Rüdeberg A, Sommer CW, Liechti-Gallati S, Kraemer R, Hubbard R, Crystal RG. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *Am J Respir Crit Care Med* 1994;150:207–13.
6. Voynow J, Fischer B, Zheng S. Proteases and cystic fibrosis. *Int J Biochem Cell Biol* 2008;40:1238–1245.
7. Turk B, Turk D, Turk V. Protease signalling: the cutting edge. *EMBO J*

2012;31:1630–43.

8. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. *J Pediatr* 2002;141:811–817.

9. Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, Murray CP, Stick SM. Risk factors for bronchiectasis in children with cystic fibrosis. *N Engl J Med* 2013;368:1963–70.

10. Reihill JA, Moreland M, Jarvis GE, McDowell A, Einarsson GG, Elborn JS, Martin SL. Bacterial proteases and haemostasis dysregulation in the CF lung. *J Cyst Fibros* 2017;16:49–57.

11. Molloy K, Smith SG, Cagney G, Dillon ET, Greene CM, McElvaney NG. Characterisation of the Major Extracellular Proteases of Stenotrophomoas maltophilia and Their Effects on Pulmonary Antiproteases. *Pathogens* 2019;8:92.

12. Korkmaz B, Caughey GH, Chapple I, Gauthier F, Hirschfeld J, Jenne DE, Kettritz R, Lalmanach G, Lamort A-S, Lauritzen C, Łęgowska M, Lesner A, Marchand-Adam S, McKaig SJ, Moss C, Pedersen J, Roberts H, Schreiber A, Seren S, Thakker NS. Therapeutic targeting of cathepsin C: from pathophysiology to treatment. *Pharmacol Ther* 2018;190:202–236.

13. Margaroli C, Garratt LW, Horati H, Dittrich AS, Rosenow T, Montgomery ST, Frey DL, Brown MR, Schultz C, Guglani L, Kicic A, Peng L, Scholte BJ, Mall MA, Janssens HM, Stick SM, Tirouvanziam R. Elastase Exocytosis by Airway Neutrophils Is Associated with Early Lung Damage in Children with Cystic Fibrosis. *Am J Respir Crit Care Med* 2019;199:873–881.

14. Clancy DM, Sullivan GP, Moran HBT, Henry CM, Reeves EP, McElvaney NG, Lavelle EC, Martin SJ. Extracellular Neutrophil Proteases Are Efficient Regulators of

IL-1, IL-33, and IL-36 Cytokine Activity but Poor Effectors of Microbial Killing. *Cell Rep* 2018;22:2937–2950.

15. Devaney JM, Greene CM, Taggart CC, Carroll TP, O'Neill SJ, McElvaney NG. Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. *FEBS Lett* 2003;544:129–132.

16. Griffin KL, Fischer BM, Kummarapurugu AB, Zheng S, Kennedy TP, Rao N V, Foster WM, Voynow JA. 2-O, 3-O-desulfated heparin inhibits neutrophil elastase-induced HMGB-1 secretion and airway inflammation. *Am J Respir Cell Mol Biol* 2014;50:684–9.

17. Liou TG, Adler FR, Keogh RH, Li Y, Jensen JL, Walsh W, Packer K, Clark T, Carveth H, Chen J, Rogers SL, Lane C, Moore J, Sturrock A, Paine R, Cox DR, Hoidal JR. Sputum Biomarkers and the Prediction of Clinical Outcomes in Patients with Cystic Fibrosis. In: Kreindler JL, editor. *PLoS One* 2012;7:e42748.

18. Padrines M, Wolf M, Walz A, Baggiolini M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett* 1994;352:231–235.

19. Gray RD, Hardisty G, Regan KH, Smith M, Robb CT, Duffin R, Mackellar A, Felton JM, Paemka L, McCullagh BN, Lucas CD, Dorward DA, McKone EF, Cooke G, Donnelly SC, Singh PK, Stoltz DA, Haslett C, McCray PB, Whyte MKB, Rossi AG, Davidson DJ. Delayed neutrophil apoptosis enhances NET formation in cystic fibrosis. *Thorax* 2018;73:134–144.

20. Dubois A V., Gauthier A, Bréa D, Varaigne F, Diot P, Gauthier F, Attucci S. Influence of DNA on the Activities and Inhibition of Neutrophil Serine Proteases in Cystic Fibrosis Sputum. *Am J Respir Cell Mol Biol* 2012;47:80–86.

21. Britigan BE, Hayek MB, Doebbeling BN, Fick RB. Transferrin and lactoferrin undergo proteolytic cleavage in the Pseudomonas aeruginosa-infected lungs of patients

with cystic fibrosis. *Infect Immun* 1993;61:5049–55.

22. Nordin SL, Jovic S, Kurut A, Andersson C, Gela A, Bjartell A, Mörgelin M, Olin AI, Lund M, Egesten A. High Expression of Midkine in the Airways of Patients with Cystic Fibrosis. *Am J Respir Cell Mol Biol* 2013;49:935–942.

23. Rubio F, Cooley J, Accurso FJ, Remold-O'Donnell E. Linkage of neutrophil serine proteases and decreased surfactant protein-A (SP-A) levels in inflammatory lung disease. *Thorax* 2004;59:318–23.

24. Fischer BM, Domowicz DAL, Zheng S, Carter JL, McElvaney NG, Taggart C, Lehmann JR, Voynow JA, Ghio AJ. Neutrophil elastase increases airway epithelial nonheme iron levels. *Clin Transl Sci* 2009;2:333–9.

25. Ghio AJ, Roggli VL, Soukup JM, Richards JH, Randell SH, Muhlebach MS. Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients. *J Cyst Fibros* 2013;12:390–398.

26. Garratt LW, Sutanto EN, Ling K-M, Looi K, Iosifidis T, Martinovich KM, Shaw NC, Kicic-Starcevich E, Knight DA, Ranganathan S, Stick SM, Kicic A, Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF). Matrix metalloproteinase activation by free neutrophil elastase contributes to bronchiectasis progression in early cystic fibrosis. *Eur Respir J* 2015;46:384–394.

27. Guyot N, Wartelle J, Malleret L, Todorov AA, Devouassoux G, Pacheco Y, Jenne DE, Belaaouaj A. Unopposed Cathepsin G, Neutrophil Elastase, and Proteinase 3 Cause Severe Lung Damage and Emphysema. *Am J Pathol* 2014;184:2197–2210.

28. Wielpütz M, Eichinger M, Biederer J, Wege S, Stahl M, Sommerburg O, Mall M, Kauczor H, Puderbach M. Imaging of Cystic Fibrosis Lung Disease and Clinical Interpretation. *RöFo - Fortschritte auf dem Gebiet der Röntgenstrahlen und der Bildgeb Verfahren* 2016;188:834–845.

29. Weldon S, McNally P, McElvaney NG, Elborn JS, McAuley DF, Wartelle J, Belaaouaj A, Levine RL, Taggart CC. Decreased levels of secretory leucoprotease inhibitor in the Pseudomonas-infected cystic fibrosis lung are due to neutrophil elastase degradation. *J Immunol* 2009;183:8148–56.

30. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J Cell Biol* 1995;131:775–789.

31. Gehrig S, Mall MA, Schultz C. Spatially Resolved Monitoring of Neutrophil Elastase Activity with Ratiometric Fluorescent Reporters. *Angew Chemie Int Ed* 2012;51:6258–6261.

32. Dittrich AS, Kühbandner I, Gehrig S, Rickert-Zacharias V, Twigg M, Wege S, Taggart CC, Herth F, Schultz C, Mall MA. Elastase activity on sputum neutrophils correlates with severity of lung disease in cystic fibrosis. *Eur Respir J* 2018;51:1701910.

33. Gehrig S, Duerr J, Weitnauer M, Wagner CJ, Graeber SY, Schatterny J, Hirtz S, Belaaouaj A, Dalpke AH, Schultz C, Mall MA. Lack of Neutrophil Elastase Reduces Inflammation, Mucus Hypersecretion, and Emphysema, but Not Mucus Obstruction, in Mice with Cystic Fibrosis–like Lung Disease. *Am J Respir Crit Care Med* 2014;189:1082–1092.

34. Park J-A, Sharif AS, Shiomi T, Kobzik L, Kasahara DI, Tschumperlin DJ, Voynow J, Drazen JM. Human neutrophil elastase-mediated goblet cell metaplasia is attenuated in TACE-deficient mice. *Am J Physiol Cell Mol Physiol* 2013;304:L701–L707.

35. Shao MXG, Nadel JA. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme. *J Immunol* 2005;175:4009–16.

462 36. Bingle L, Richards RJ, Fox B, Masek L, Guz A, Tetley TD. Susceptibility of lung
463 epithelium to neutrophil elastase: protection by native inhibitors. *Mediat Inn Amm*
464 1997;6:345–354.
465 37. Le Gars M, Descamps D, Roussel D, Saussereau E, Guillot L, Ruffin M, Tabary O,
466 Hong SS, Boulanger P, Paulais M, Malleret L, Belaaouaj A, Edelman A, Huerre M,
467 Chignard M, Sallenave JM. Neutrophil elastase degrades cystic fibrosis
468 transmembrane conductance regulator via calpains and disables channel function in
469 vitro and in vivo. *Am J Respir Crit Care Med* 2013;187:170–179.
470 38. Caldwell RA, Boucher RC, Stutts MJ. Neutrophil elastase activates near-silent
471 epithelial Na^+ channels and increases airway epithelial Na^+ transport. *Am J Physiol*
472 *Cell Mol Physiol* 2005;288:L813–L819.
473 39. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway
474 epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med*
475 2004;10:487–493.
476 40. Kirschke H, Wiederanders B, Brömme D, Rinne A. Cathepsin S from bovine spleen.
477 Purification, distribution, intracellular localization and action on proteins. *Biochem J*
478 1989;264:467–73.
479 41. Reddy VY, Zhang QY, Weiss SJ. Pericellular mobilization of the tissue-destructive
480 cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived
481 macrophages. *Proc Natl Acad Sci U S A* 1995;92:3849–53.
482 42. Rogan MP, Taggart CC, Greene CM, Murphy PG, O'Neill SJ, McElvaney NG. Loss
483 of Microbicidal Activity and Increased Formation of Biofilm Due to Decreased
484 Lactoferrin Activity in Patients with Cystic Fibrosis. *J Infect Dis* 2004;190:1245–1253.
485 43. Andrault P-M, Samsonov SA, Weber G, Coquet L, Nazmi K, Bolscher JGM,
486 Lalmanach A-C, Jouenne T, Brömme D, Pisabarro MT, Lalmanach G, Lecaille F.

Antimicrobial Peptide LL-37 Is Both a Substrate of Cathepsins S and K and a Selective Inhibitor of Cathepsin L. *Biochemistry* 2015;54:2785–2798.

44. Lecaille F, Naudin C, Sage J, Joulin-Giet A, Courty A, Andrault P-M, Veldhuizen RAW, Possmayer F, Lalmanach G. Specific cleavage of the lung surfactant protein A by human cathepsin S may impair its antibacterial properties. *Int J Biochem Cell Biol* 2013;45:1701–1709.

45. Taggart CC, Greene CM, Smith SG, Levine RL, McCray PB, O'Neill S, McElvaney NG. Inactivation of human beta-defensins 2 and 3 by elastolytic cathepsins. *J Immunol* 2003;171:931–7.

46. Repnik U, Starr AE, Overall CM, Turk B. Cysteine Cathepsins Activate ELR Chemokines and Inactivate Non-ELR Chemokines. *J Biol Chem* 2015;290:13800–11.

47. Weldon S, McNally P, McAuley DF, Oglesby IK, Wohlford-Lenane CL, Bartlett JA, Scott CJ, McElvaney NG, Greene CM, McCray PB, Taggart CC. miR-31 Dysregulation in Cystic Fibrosis Airways Contributes to Increased Pulmonary Cathepsin S Production. *Am J Respir Crit Care Med* 2014;190:165–174.

48. Small DM, Brown RR, Doherty DF, Abladey A, Zhou-Suckow Z, Delaney RJ, Kerrigan L, Dougan CM, Borensztajn KS, Holsinger L, Booth R, Scott CJ, López-Campos G, Elborn JS, Mall MA, Weldon S, Taggart CC. Targeting of cathepsin S reduces cystic fibrosis-like lung disease. *Eur Respir J* 2019;53:1801523.

49. Tan C Da, Hobbs C, Sameni M, Sloane BF, Stutts MJ, Tarran R. Cathepsin B contributes to Na+ hyperabsorption in cystic fibrosis airway epithelial cultures. *J Physiol* 2014;592:5251–5268.

50. Haerteis S, Krappitz M, Bertog M, Krappitz A, Baraznenok V, Henderson I, Lindström E, Murphy JE, Bunnett NW, Korbmacher C. Proteolytic activation of the epithelial sodium channel (ENaC) by the cysteine protease cathepsin-S. *Pflügers Arch - Eur J*

Physiol 2012;464:353–365.

51. Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. *Physiol Rev* 2007;87:69–98.

52. Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol* 2014;15:786–801.

53. Gaggar A, Hector A, Bratcher PE, Mall MA, Griese M, Hartl D. The role of matrix metalloproteinases in cystic fibrosis lung disease. *Eur Respir J* 2011;38:721–7.

54. Akthar S, Patel DF, Beale RC, Peiró T, Xu X, Gaggar A, Jackson PL, Blalock JE, Lloyd CM, Snelgrove RJ. Matrikines are key regulators in modulating the amplitude of lung inflammation in acute pulmonary infection. *Nat Commun* 2015;6:8423.

55. Turnbull AR, Pyle CJ, Patel DF, Jackson PL, Hilliard TN, Regamey N, Tan H-L, Brown S, Thursfield R, Short C, Mc Fie M, Alton EWFW, Gaggar A, Blalock JE, Lloyd CM, Bush A, Davies JC, Snelgrove RJ. Abnormal pro-gly-pro pathway and airway neutrophilia in pediatric cystic fibrosis. *J Cyst Fibros* 2019;doi:10.1016/J.JCF.2019.05.017.

56. Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 2000;96:2673–81.

57. Trojanek JB, Cobos-Correa A, Diemer S, Kormann M, Schubert SC, Zhou-Suckow Z, Agrawal R, Duerr J, Wagner CJ, Schatterny J, Hirtz S, Sommerburg O, Hartl D, Schultz C, Mall MA. Airway Mucus Obstruction Triggers Macrophage Activation and Matrix Metalloproteinase 12–Dependent Emphysema. *Am J Respir Cell Mol Biol* 2014;51:709–720.

58. McMahan RS, Birkland TP, Smigiel KS, Vandivort TC, Rohani MG, Manicone AM,

McGuire JK, Gharib SA, Parks WC. Stromelysin-2 (MMP10) Moderates Inflammation by Controlling Macrophage Activation. *J Immunol* 2016;197:899–909.

59. Kummarapurugu AB, Afosah DK, Sankaranarayanan NV, Navaz Gangji R, Zheng S, Kennedy T, Rubin BK, Voynow JA, Desai UR. Molecular principles for heparin oligosaccharide-based inhibition of neutrophil elastase in cystic fibrosis. *J Biol Chem* 2018;293:12480–12490.

60. Chapman KR, Burdon JGW, Piitulainen E, Sandhaus RA, Seersholm N, Stocks JM, Stoel BC, Huang L, Yao Z, Edelman JM, McElvaney NG, RAPID Trial Study Group. Intravenous augmentation treatment and lung density in severe α1 antitrypsin deficiency (RAPID): a randomised, double-blind, placebo-controlled trial. *Lancet* 2015;386:360–368.

61. Brennan S. Revisiting 1-antitrypsin therapy in cystic fibrosis: can it still offer promise? *Eur Respir J* 2006;29:229–230.

62. Camper N, Glasgow AMA, Osbourn M, Quinn DJ, Small DM, McLean DT, Lundy FT, Elborn JS, McNally P, Ingram RJ, Weldon S, Taggart CC. A secretory leukocyte protease inhibitor variant with improved activity against lung infection. *Mucosal Immunol* 2016;9:669–676.

63. Scott CJ, Taggart CC. Biologic protease inhibitors as novel therapeutic agents. *Biochimie* 2010;92:1681–1688.

64. Polverino E, Rosales-Mayor E, Dale GE, Dembowsky K, Torres A. The Role of Neutrophil Elastase Inhibitors in Lung Diseases. *Chest* 2017;152:249–262.

65. Hirche TO, Benabid R, Deslee G, Gangloff S, Achilefu S, Guenounou M, Lebargy F, Hancock RE, Belaaouaj A. Neutrophil elastase mediates innate host protection against Pseudomonas aeruginosa. *J Immunol* 2008;181:4945–54.

66. Hahn I, Klaus A, Janze A-K, Steinwede K, Ding N, Bohling J, Brumshagen C, Serrano

H, Gauthier F, Paton JC, Welte T, Maus UA. Cathepsin G and Neutrophil Elastase Play Critical and Nonredundant Roles in Lung-Protective Immunity against Streptococcus pneumoniae in Mice. In: Weiser JN, editor. *Infect Immun* 2011;79:4893–4901.

67. Shute JK, Calzetta L, Cardaci V, di Toro S, Page CP, Cazzola M. Inhaled nebulised unfractionated heparin improves lung function in moderate to very severe COPD: A pilot study. *Pulm Pharmacol Ther* 2018;48:88–96.

68. Rao N V., Argyle B, Xu X, Reynolds PR, Walenga JM, Prechel M, Prestwich GD, MacArthur RB, Walters BB, Hoidal JR, Kennedy TP. Low anticoagulant heparin targets multiple sites of inflammation, suppresses heparin-induced thrombocytopenia, and inhibits interaction of RAGE with its ligands. *Am J Physiol Physiol* 2010;299:C97–C110.

69. Xu X, Abdalla T, Bratcher PE, Jackson PL, Sabbatini G, Wells JM, Lou X-Y, Quinn R, Blalock JE, Clancy JP, Gaggar A. Doxycycline improves clinical outcomes during cystic fibrosis exacerbations. *Eur Respir J* 2017;49:1601102.

70. Fonović M, Turk B. Cysteine cathepsins and extracellular matrix degradation. *Biochim Biophys Acta - Gen Subj* 2014;1840:2560–2570.

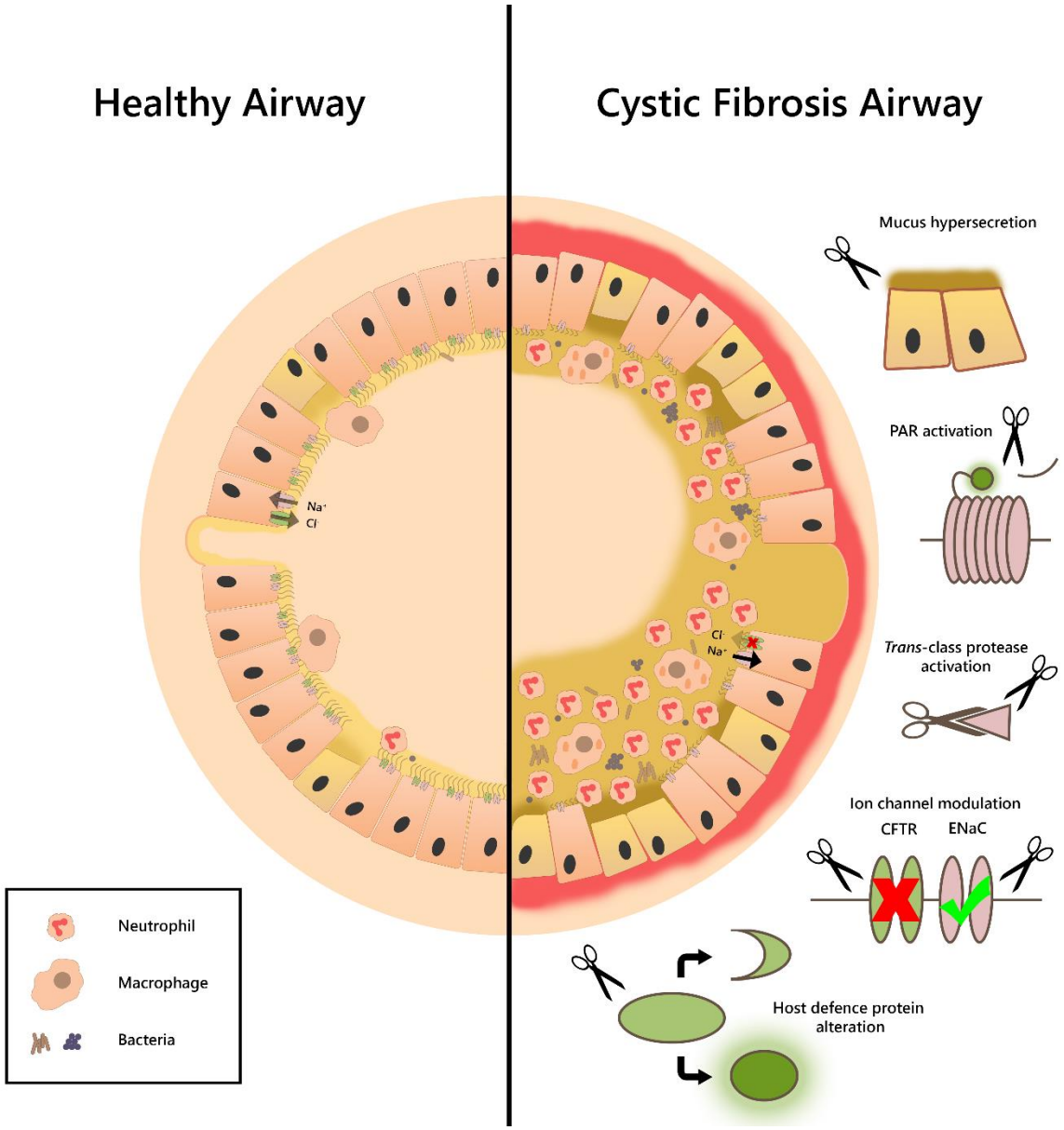
71. Heuberger DM, Schuepbach RA. Protease-activated receptors (PARs): mechanisms of action and potential therapeutic modulators in PAR-driven inflammatory diseases. *Thromb J* 2019;17:4.

Figure legends

Figure 1. A model of the cystic fibrosis (CF) airway and associated protease-mediated pathologies. A healthy airway maintains a thin layer of well-hydrated mucus covering the airway surface. Invading pathogens and particulates are trapped and subsequently removed from the airway by mucociliary clearance. In CF airways, malfunction of cystic fibrosis transmembrane conductance regulator (CFTR) anion channels and increased activity of epithelial sodium channels (ENaC) results in airway surface dehydration, altered viscoelastic properties of airway mucus and impaired mucociliary clearance, which makes the airway susceptible to chronic infection and inflammation. Neutrophils recruited to the airway, along with macrophages and epithelial cells, secrete proteases which aggravate key aspects of the pathophysiology of CF. Active proteases compromise the structural integrity of the airway through the degradation of elastin and collagen, leading to bronchiectasis. In addition, other protease roles in CF include *(top to bottom)*: the enhancement of mucin/mucus production and secretion; the activation of protease-activated receptors (PARs) leading to pro-inflammatory signalling; the *trans*-activation of other proteases by cleaving pro-domains and degrading cognate antiproteases; the aggravation of basic CF ion transport defects by the proteolytic degradation of CFTR and activation of ENaC and; the cleavage of various host protein substrates precipitating either activation (in the case of some pro-inflammatory cytokines) or inactivation (in the case of some antimicrobial peptides and surfactant proteins).

602 **Figures**

603 Figure 1.



604

Tables

Table 1. Protease functions and redundancy in CF lung disease

Function	Protease class involved	References
Matrix degradation	Serine, cysteine, MMP	(52, 54, 55, 70)
Cytokine processing	Serine, cysteine, MMP	(14, 18, 46, 56)
Cytokine upregulation	Serine, cysteine, MMP	(15, 48, 54, 55)
PAR activation	Serine, cysteine, MMP	(48, 71)
Trans-class protease activation	Serine, cysteine	(9, 12, 26, 27)
Host defence protein degradation (including antiproteases)	Serine, cysteine	(21–23, 29, 42–45)
ENaC activation	Serine, cysteine	(38, 49, 50)
CFTR degradation	Serine	(37)
Mucus modulation	Serine	(33–36, 48)
Iron liberation	Serine	(24, 25)

Definition of abbreviations: MMP = matrix metalloprotease; PAR = protease-activated receptor; ENaC = epithelial sodium channel; CFTR = cystic fibrosis transmembrance conductance regulator.