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

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38 **Abstract**

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

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

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

60

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62 **Introduction**

63 Cystic fibrosis (CF) is an autosomal recessive genetic condition, predominantly of Caucasian
64 populations, which impacts multiple organ systems. However, it is the chronic progressive lung
65 disease of CF that causes the greatest morbidity and mortality. The disease is caused by
66 mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.
67 Consequently, the function of epithelial CFTR anion (chloride and bicarbonate) channels is
68 compromised, leading to impaired anion and fluid secretion and airway surface dehydration,
69 which in turn results in highly viscous airway mucus and impaired mucociliary clearance,
70 setting the stage for mucus plugging, chronic inflammation and polymicrobial infection (1).
71 Such a state causes progressive and irreversible damage of the airways and lung parenchyma,
72 as recruited immune cells (predominantly neutrophils) release proteases, DNA and reactive
73 oxygen species, and promote further immune cell recruitment by cytokine signalling.
74 The introduction of CFTR modulators (potentiators, correctors and amplifiers) in recent years
75 has transformed the treatment of CF. Phase two trials of triple combination therapy suggest
76 that a CFTR modulator therapy approach may be effective in up to 90% of CF patients (2, 3).
77 While the emerging therapies show immense promise, there are still CF patients whose specific
78 genotypes may not be amenable to these therapies. Furthermore, CFTR modulation alone may
79 be insufficient to allow complete and lasting clearance of chronic airways infection and
80 resolution of pulmonary inflammation, especially in the context of chronic CF lung disease
81 with established structural lung damage (4). Importantly, it is unknown whether, or to what
82 extent, these CFTR-directed therapies decrease protease activity. Until such a decrease has
83 been demonstrated, novel antiprotease strategies are still highly relevant to limit tissue damage
84 in CF lung disease.

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

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

96 So far, the serine protease class has drawn the most attention in CF, in particular neutrophil
97 elastase (NE), with its free extracellular form previously thought to be the major player in CF
98 lung disease pathogenesis. Indeed, free NE in sputum has long been known to correlate with
99 FEV₁ in children with CF (8) and elevated NE activity in bronchoalveolar lavage fluid at 3
100 months of age was found to be associated with persistent bronchiectasis by the Australian
101 Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) (9). However, several
102 novel concepts are unfolding in pulmonary protease biology, which have led investigators to
103 broaden their view beyond NE. These concepts include the redundancy of function between
104 proteases (*Table 1*), the *trans*-class activation of proteases, the discovery of highly active
105 membrane-bound proteases and the emergence of new key players from the cysteine and matrix
106 metalloprotease (MMP) classes. This review will summarise the current knowledge of host
107 protease function in CF lung disease and how this may inform therapeutic intervention. The
108 role of bacterial proteases in CF lies beyond the scope of this review but we direct readers to
109 the following recent references (10, 11).

110

111

112 **The actions of serine proteases in CF**

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

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

137 the host response and/or susceptibility to infection. NE also cleaves extracellular haem-
138 containing proteins such as ferritin, liberating sequestered iron into the airway. Not only does
139 this increase oxidative stress in the airway epithelium but it also promotes bacterial
140 proliferation and biofilm formation as iron is made accessible for microbial nutrition (24, 25).
141 NSPs may also play an important role as regulators of other proteases, particularly the MMPs.
142 This role is especially relevant when considering the tissue-destructive nature of the proteases;
143 NSP-activated MMP-9 and MMP-12, as well as the NSPs themselves, contribute directly to
144 extracellular matrix (ECM) remodelling and bronchiectasis that is characteristic in CF (9, 26,
145 27). The protease-mediated loss of elastin limits elastic recoil, while the loss of collagen creates
146 a structural deficit, leading to the emphysematous phenotype that can occur in adolescent and
147 adult CF patients (28). The body's endogenous protection against aberrant NSP activity is also
148 compromised, as NE inactivates tissue-protective antiproteases (some of which also possess
149 antimicrobial properties) such as secretory leukocyte protease inhibitor (SLPI) (29).
150 The inability of such endogenous antiproteases, even when intact, to perform their inhibitory
151 function has also been highlighted in recent years. This may be due, in part, to membrane-
152 association of NSPs such as NE and CTSG (30). More recently, novel Förster resonance energy
153 transfer (FRET)-based probes were used to analyse membrane-bound activity of proteases such
154 as NE on the surface of neutrophils (31). In this surface-bound form, proteases are less
155 accessible for their prospective inhibitors, which are unable to access the enzyme's active site.
156 Indeed, surface-bound NE has been found to correlate with severity of lung disease and various
157 inflammatory markers in CF (32).
158 As well as influencing both inflammatory cell recruitment and tissue destruction, NE
159 contributes to increased mucus production in the CF lung by upregulating mucin expression
160 and inducing goblet cell metaplasia, a process thought to be mediated through tumour necrosis
161 factor- α converting enzyme (33, 34). In addition, NE induces secretion of mucins from airway

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

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

179

180

181 **The actions of cysteine proteases in CF**

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

187 functions are crucial in homeostatic protein turnover, fighting infection and in the development
188 of adaptive immune responses to infections. However, like the NSPs, certain members can be
189 found in the extracellular milieu of the CF lung. Cathepsins are secreted by macrophages but
190 may also be sourced from neutrophils, other antigen-presenting cells, lung epithelial and
191 endothelial cells; this secretion may be associated with acidification of the pericellular space
192 (41).

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

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

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

217

218

219 **The actions of matrix metalloproteases in CF**

220 The members of the MMP class are not abundant in the healthy lung; however, they are
221 produced by lung and inflammatory cells in response to inflammatory chemokines, noxious
222 stimuli and free oxygen radicals (51). These zinc and calcium-dependent endopeptidases are
223 loosely numbered in order of discovery up to MMP-28 and, as their name suggests, are potent
224 ECM-degrading enzymes (52). While some MMPs are mainly tissue-derived, MMP-8 and
225 MMP-9 are predominantly derived from neutrophils, making them proteases of particular
226 interest in CF (53). The degradation of interstitial collagen is key to the development of
227 bronchiectasis and other aberrant structural formations of the CF lung. In addition, this
228 cleavage process generates matrix fragments, which can produce secondary downstream
229 effects. During airway inflammation the proline-glycine-proline (PGP, a potent neutrophil
230 chemoattractant) fragments produced by collagenase activity are not matched by a concomitant
231 rise in PGP-degradation by leukotriene A₄ hydrolase, causing PGP accumulation which
232 contributes to CF neutrophilia (54, 55). In addition, MMP-9 is capable of truncating IL-8 into
233 a highly chemoattractive form (56).

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

237 known as MMP-12). Recent studies in β ENaC-overexpressing mice with CF-like lung disease
238 and paediatric CF patients suggest that mucostatic conditions in the CF airways may trigger
239 elevated membrane-associated MMP-12 activity via macrophage activation (57). Interestingly,
240 a functional polymorphism in the *MMP12* promoter (rs2276109) that decreases *MMP12*
241 expression was positively associated with FEV₁ % predicted in patients with CF (57). This
242 work opens interesting lines of inquiry: what are the specific signals which precipitate protease
243 release from specific cell types? Are they to be found in CF mucus? Can they be targeted
244 therapeutically? Can protease gene expression be targeted?

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

251

252

253 **Therapeutic strategies targeting the protease-antiprotease imbalance**

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

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

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

267 There is an attraction to using antiprotease-based therapies such as α_1 -antitrypsin augmentation,
268 especially considering the success of this strategy in α_1 -antitrypsin deficiency. In 2015, a long-
269 term, randomised control trial was reported with weekly α_1 -antitrypsin administration for up to
270 48 months (60). RAPID (Randomized, Placebo-controlled Trial of Augmentation Therapy in
271 Alpha-1 Proteinase Inhibitor Deficiency) demonstrated the slowing of lung parenchymal
272 damage after redressing the protease-antiprotease imbalance and significantly, that this effect
273 was most evident over the course of months and years, rather than short-term improvements
274 over weeks (60). This was not a surprising finding, considering that antiprotease therapy is
275 predicted to slow the progression of irreversible lung damage and bronchiectasis rather than
276 producing short-term improvements in lung function; short trials are therefore unlikely to
277 capture these therapeutic benefits. α_1 -antitrypsin augmentation has also been tested in CF,
278 though this has been limited to short trials, predominantly powered to establish safety (61).
279 Endogenous antiprotease augmentation is not without its pitfalls given their propensity to be
280 degraded by proteases (host or pathogen). Recombinant variants of the endogenous
281 antiproteases such as SLPI with reduced susceptibility to protease cleavage have shown
282 efficacy in reducing inflammation (62). However, the size and complexity of these proteins,
283 their generally broad antiprotease activity and the quantities required to address the substantial
284 protease burden in CF are all factors to be overcome. These molecules have also yet to
285 demonstrate efficacy against surface-bound proteases.

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

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

311 especially regarding their safety profile. One drug which has emerged is the tetracycline
312 antibiotic doxycycline. A 2017 study highlighted FEV₁ improvements following doxycycline
313 treatment during acute pulmonary exacerbations in CF patients, seemingly independent of
314 doxycycline's antibiotic properties, via MMP-9 neutralisation and TIMP-1 enhancement (69).
315 Currently, there are no other licensed drugs that are known to fall into this category.

316

317

318 **Summary and outlook**

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

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

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

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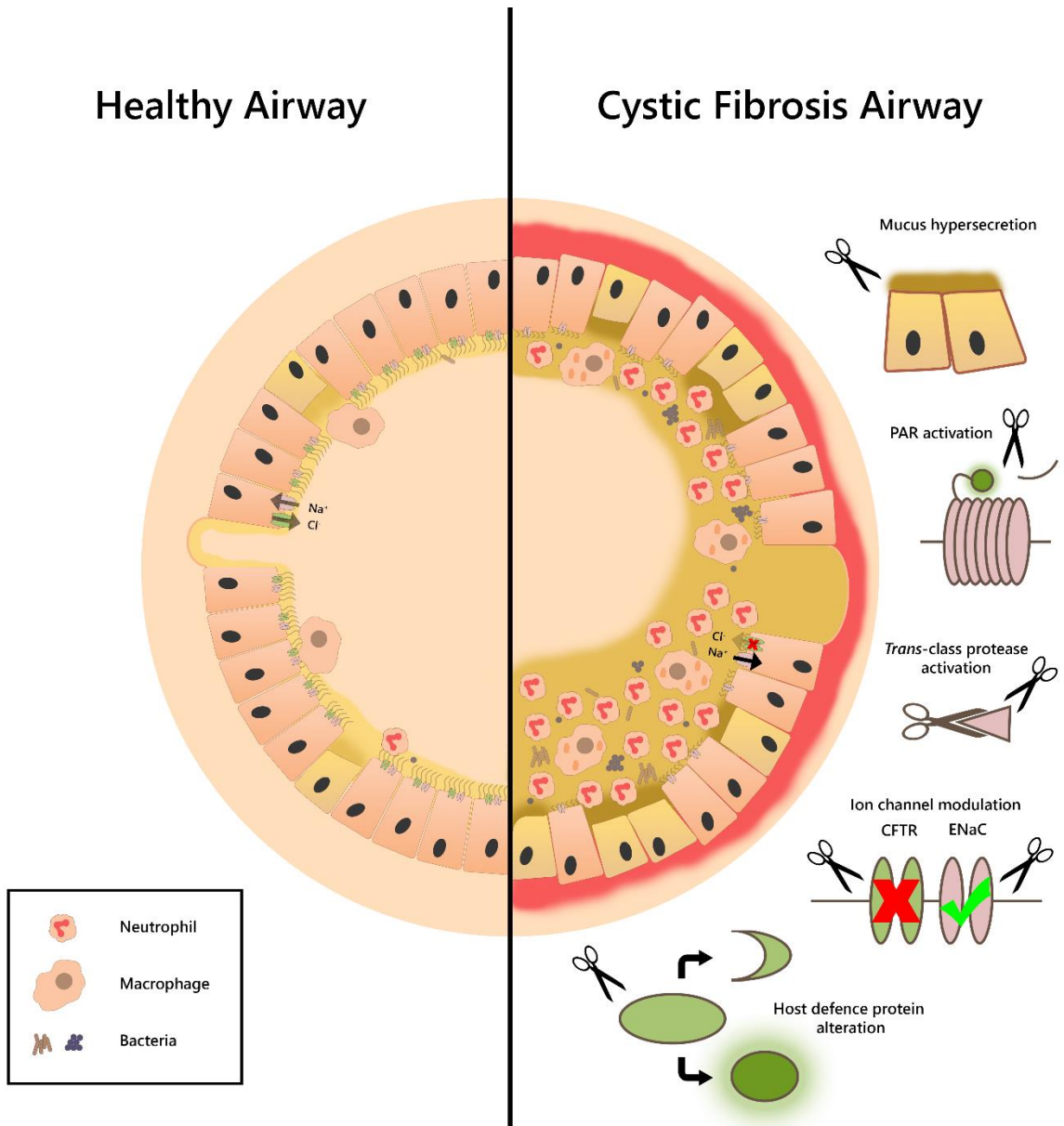
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582

583 **Figure legends**

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

602 **Figures**

603 Figure 1.



604

605 **Tables**

606 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)
<i>Trans</i> -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)

607

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