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Mechanisms of emphysema in α_1-antitrypsin deficiency: molecular and cellular insights

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ABSTRACT: The severe, early onset emphysema that occurs in patients with circulating deficiency of α_1-antitrypsin (α_1-AT) attests to the importance of this protease inhibitor in maintaining lung parenchymal integrity. It has led to the powerful concept of protease:antiprotease balance being crucial to alveolar homeostasis.

Pathogenic mutations cause α_1-AT to self-associate into polymer chains that accumulate intracellularly rather than proceeding along the secretory pathway. Polymerisation of α_1-AT abolishes antiprotease activity and confers toxic gain-of-function effects. Since α_1-AT is predominantly synthesised in the liver, where it does not play a major homeostatic role, the directly toxic effects of polymerisation are clearest here. However, data from molecular, cellular, animal and *ex vivo* studies indicate that intrapulmonary polymerisation of α_1-AT and inflammatory positive feedback loops may augment the destructive effects of decreased antiprotease levels in the lung.

This review integrates the findings from these different approaches and highlights how multiple pathways may converge to give the severe, panacinar emphysema phenotype seen in α_1-AT deficiency.

KEYWORDS: α_1-Antitrypsin deficiency, elastase, interstitial inflammation, lung, mechanism of emphysema, serpin polymer

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The syndrome of circulating deficiency of the plasma protein α_1-antitrypsin (α_1-AT) was first identified in 1963 by LAURELL and ERIKSSON [1] who characterised its association with pulmonary emphysema. Subsequently, major associations were identified with neonatal hepatitis, hepatic cirrhosis and hepatocellular carcinoma [2, 3]. The condition has also been associated with asthma [4], bronchiectasis [5], panniculitis [6] and the antineutrophil cytoplasmic antibody (ANCA)-positive vasculitides [7].

α_1-antitrypsin (also known as α_1-proteinase inhibitor) is a 52-kDa secreted glycoprotein of the serpin (serine protease inhibitor) protein superfamily [8–10]. The circulating protein is synthesised within hepatocytes, although other cells also express it at lower levels. Many of these cells are intrapulmonary, notably bronchial epithelial cells [11], type II pneumocytes [12], neutrophils [13] and alveolar macrophages [14]. Its major function is the inhibition of human neutrophil elastase [15]. Normal α_1-AT and most pathogenic variants are named by letters of the alphabet according to the relative electrophoretic mobilities of their mature glycosylated forms. The wildtype protein is therefore also known as M α_1-AT, and the most common variant to cause severe clinical disease in populations of North European descent is the Z variant, in which a glutamate at residue 342 is replaced by a lysine residue (Glu342Lys). The characteristics of the common variants are summarised in table 1. The

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α_1-AT genotype (SERPINA1 gene [10], located on chromosome 14 [16]) can therefore be expressed by Pi (protease inhibitor) notation, in which PiM (or PiMM) represents an M homozygote state, PiZ (or PiZZ), a Z homozygote, and PiMZm, heterozygosity for both alleles.

Pathogenic mutations block secretion of synthesised α_1-AT to cause the observed deficiency of circulating protein [17]. The observation that deficiency of a major circulating proteinase inhibitor predisposes to early onset emphysema led directly to the protease:antiprotease imbalance hypothesis of lung disease [18]. In this model, alveolar and interstitial tissue destruction is driven by excessive proteolysis. It was supported experimentally by animal models in which airspace enlargement was induced by intratracheal instillation of elastolytic enzymes [19–22]. Understanding the pathogenic mechanisms set in train by protease:antiprotease imbalance has provided many useful insights into disturbances of alveolar and interstitial homeostasis that are likely to be relevant to human disease. In the last two decades, great advances have been made in understanding the molecular and cellular basis of α_1-AT deficiency.

In this review we describe the pathways that have been linked to the pathogenesis of emphysema in α_1-AT deficiency by molecular, cell, animal and clinical studies. These relate both to the original concept of protease:antiprotease imbalance and also to functions of α_1-AT in its native and disease-associated polymeric states that may also play an important role in this condition. Incorporation of these data into the design of future therapies may lead to improved patient outcomes.

GENETIC AND ENVIRONMENTAL FACTORS IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

While α_1-AT deficiency is likely to represent a specific subphenotype of chronic obstructive pulmonary disease (COPD), it shares general pathogenic mechanisms with the development of COPD in individuals with normal levels of α_1-AT. Cigarette smoke and, in the developing world, pollutants from biomass fuel sources [23] trigger oxidative stress and an inflammatory response within the lung that causes parenchymal destruction. The magnitude of this effect varies between individuals; thus, additional genetic and environmental factors must modify the response at the tissue level. This was highlighted by the finding that the airway and emphysema components of COPD cluster within families and show different relationships with smoking history [24]. These data strongly support the presence of distinct genetic factors for COPD in addition to α_1-AT genotype. Indeed, polymorphisms in genes implicated in oxidative stress, the proteinase:antiproteinase balance, and elastin and matrix homeostasis have been linked to the pathogenesis of COPD [25].

The linking of environmental factors to chronic disease is particularly powerful when it involves the elucidation of a mechanism. This was recently exemplified by a study that demonstrated an immune pathway within the lung by which acute viral infection in childhood can predispose to obstructive airways pathology in later life [26]. Infection has also been proposed to accelerate respiratory decline in COPD more acutely. This is an intuitively appealing concept since clinically severe or rapidly deteriorating individuals with COPD are often frequent infective exacerbators; however, proving causality has not been straightforward [27].

POLYMERISATION: A CENTRAL EVENT IN α_1-AT DEFICIENCY

While homozygosity for null alleles of the α_1-AT gene is responsible for a few cases of α_1-AT deficiency [28],the vast majority of pathogenic genotypes are associated with no reduction in translation of the full-length polypeptide sequence. However, pathogenic mutations affect post-translational folding of α_1-AT, favouring the adoption of an intermediate conformational state [29]. These species self-associate *via* intermolecular

TABLE 1 Common pathogenic variants of α_1-antitrypsin (α_1-AT)

Variant, mutation, polymerisation tendency	Circulating deficiency in homozygotes	Association with clinically significant liver disease	Epidemiology
Z, Glu342Lys, +++	Severe (10–15% of normal levels)	Yes in homozygotes. Lower burden of hepatocyte inclusion bodies seen in heterozygotes, not associated with clinical disease	1 out of 27 of North European populations' heterozygotes. Most common severe deficiency variant. Allele frequency decreases from North-West to South-East Europe
Siiyama, Ser53Phe, +++	Severe	Yes	Most common severe deficiency variant in Japanese populations
Mmalton Δ52Phe, +++	Severe	Yes	Most common severe deficiency variant in Sardinian populations
S, Glu264Val, +	Moderate (60% of normal levels in homozygotes, equivalent to MZ α_1-AT heterozygotes)	Reported in SZ α_1-AT compound heterozygotes	Most common deficiency allele; 1 out of 5 Europeans are heterozygotes. Frequency decreases from South-West to North-East Europe
I, Arg39Cys, +	Mild (extrapolation from levels in heterozygote)	Case report in IZ α_1-AT heterozygote	Only reported in compound heterozygotes

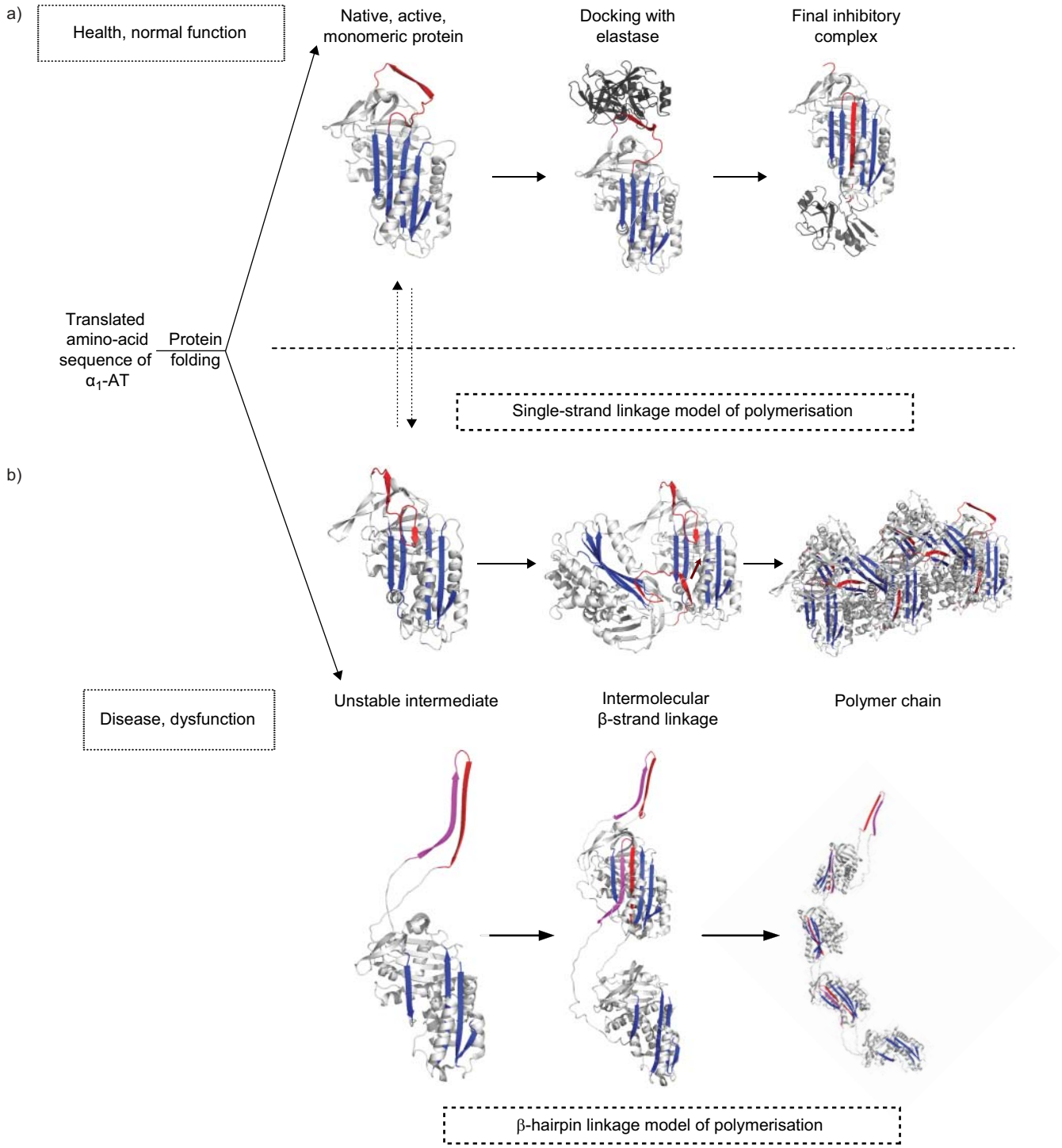

FIGURE 1. Folding and function, misfolding and dysfunction in α_1-antitrypsin (α_1-AT) deficiency. a) In the absence of pathogenic mutations, α_1-AT is normally expressed and folded to its native, active state. The native protein has the potential to undergo dramatic conformational change and a series of high-resolution crystal structures shows how this is utilised in its mechanism of inhibition of neutrophil elastase. The reactive site loop (red) of α_1-AT represents the ideal substrate ''bait'' for the enzyme (dark grey). The interaction results in cleavage of the reactive site loop, allowing its insertion as a central strand within the underlying β-sheet A (blue). Strand insertion is coordinated with expansion of β-sheet A, and results in hyperstabilisation of the α_1-AT molecule. Some of the energy released by this is used to distort the active site of the enzyme, which therefore remains bound in a physiologically irreversible complex. b) Two proposed molecular mechanisms of polymerisation, both based upon crystallographic, biochemical and biophysical data. Both mechanisms propose that pathogenic mutations favour the population of an unstable intermediate state during folding and/or in equilibrium with the native state following folding. In both schemes, the intermediate has an expanded β-sheet A, allowing formation of intermolecular β-structural linkages. However, in the upper scheme, the linkage is *via* a single β-strand formed from the reactive loop, while in the lower scheme, the linkage is *via* a β-hairpin formed from the reactive loop and the contiguous β-strand. Polymerisation abolishes the inhibitory activity of α_1-AT.

β-strand linkage to form polymeric chains of α_1-AT molecules with no enzyme inhibitory activity (fig. 1) [17, 30].

Since the liver is the major source of circulating α_1-AT, polymerisation is particularly prominent within the endoplasmic reticulum (ER) of hepatocytes where α_1-AT becomes sequestered, forming inclusion bodies. α_1-AT inclusion bodies are characterised by periodic acid-Schiff stain positivity and resistance to digestion by diastase. The hepatic manifestations of α_1-AT deficiency indicate that the intracellular polymerisation of α_1-AT is associated with gain-of-function toxicity. Moreover, the propensity for polymerisation is not restricted to the endoplasmic reticulum of the hepatocyte. α_1-AT synthesised in cells outside the liver, notably bronchial and type II alveolar epithelial cells, retains the potential to form polymers. These will also predictably form within the endoplasmic reticulum at the time of folding. However, polymers are also found in extracellular environments in the circulation [31], lung lavage [32, 33] and interstitium of the lung [34] in individuals homozygous for polymerogenic variants of α_1-AT. Moreover, when such variants of α_1-AT are purified from the plasma in the correctly folded, monomeric, functional conformation, this native material readily converts into polymers under physiological conditions [35, 36]. This strongly suggests that polymerisation can occur outside the cellular folding environment and that polymers found extracellularly are likely to be formed from circulating native monomers.

However, we cannot discount an alternative possibility, that extracellular polymers are formed within the endoplasmic reticulum and trafficked *via* the secretory pathway. This matters for two reasons. First, if all pathologically relevant polymerisation occurs within the ER then therapies targeted to block polymerisation can only be effective in this environment. This represents a greater challenge for drug delivery than targeting the extracellular milieux. Secondly, a recent crystal structure of a dimer of the related protein [37], the anticoagulant serpin antithrombin, suggests a model for serpin polymerisation in which the intermolecular linkage involves β-hairpin exchange (fig. 1b). This is coupled with loss of secondary structural features and implies an intermediate state with a substantial degree of exposure of hydrophobic core residues. This is more readily understandable as an intermediate seen during initial folding from a relatively unstructured polypeptide chain than as a species formed by facile interconversion from native protein after correct folding. Thus, if polymers are formed extracellularly this step must be somehow facilitated, or else such polymers are linked through a different mechanism and are therefore distinct from those formed in the endoplasmic reticulum. The β-hairpin linkage model is hard to reconcile with previous biochemical studies of polymerisation involving blocking peptides [17, 38–40]. These often use native material as the starting point and the discrepancy may therefore reflect different mechanisms of polymerisation. It is clearly a priority to confirm whether polymers extracted from the intracellular environment show features consistent with the β-hairpin model of linkage.

INTRACELLULAR CONSEQUENCES OF THE ACCUMULATION OF POLYMERS OF α_1-AT

Proteins are non-branching polypeptide chains that contain thousands of atoms. The folding of proteins in three dimensions is a process of such complexity that prediction of tertiary structure from amino acid sequence alone remains difficult, despite great efforts and major advances in computational power [41]. Although proteins are typically able to fold spontaneously to their most stable state in cell-free systems *in vitro*, protein folding in the intracellular milieu must be tightly controlled. Protein synthesis is energetically expensive for the cell, while the potential for misfolding and nonspecific aggregation of polypeptide chains is very high at the protein concentrations found within the cell. Moreover, since molecular structure determines function, misfolded proteins that evade cellular control mechanisms are likely to be deleterious to the cellular machinery. Chaperone molecules optimise the folding environment for nascent polypeptide chains after they emerge from the ribosome and shield them from nonspecific interactions with other molecules [42, 43]. Cells may also dispose of misfolded proteins by degradative pathways, such as the ubiquitin-proteasome system [44] and the autophagy-lysosome system [45]. Within the ER, misfolded proteins are targeted for retrograde transport into the cytosol where they are ubiquitinated before degradation by the proteasome (ER-associated degradation, or ERAD) [46]. Excessive protein misfolding typically triggers the unfolded protein response (UPR) as a reaction to ER stress [47]. The UPR upregulates synthesis of both chaperones and the molecules involved in ERAD; ultimately, if these are insufficient to relieve the load of misfolded protein, this leads to apoptosis. *In vivo* accumulation of α_1-AT polymers is so marked that it greatly distorts the normal ER architecture [17]. It is therefore remarkable that overexpression of Z α_1-AT in eukaryotic cells leads to massive polymer accumulation yet does not trigger the UPR in the absence of a second hit, such as heat or induced accumulation of other misfolded protein [48, 49]. This implies that polymers are not initially recognised as misfolded due to their essentially ordered structure. Instead, the presence of α_1-AT polymers within the ER is associated with a distinct response, termed ''ER overload''.

The hallmark of ER overload is chronic activation of the transcription factor nuclear factor-κB (NF-κB). The effects of NF-κB are complex, with either pro-survival or pro-apoptotic functions dependent on varying inflammatory gene expression profiles. In general terms, either outcome may be pathological. Widespread apoptosis can lead to compromised organ function and maladapted repair responses. However, inappropriate stimulation of pro-survival pathways favours the accumulation of mutated protein in individual cells that may lead to dysfunction or malignancy. Precise characterisation of pathways linking the activation of NF-κB to cellular toxicity in α_1-AT deficiency and those involved in the second hit mechanism will provide important insights into the associated liver disease. Furthermore, such progress will also imply ways in which pulmonary cells expressing mutant α_1-AT may be prone to dysfunction and cell death to precipitate or exacerbate the development of emphysema.

While α_1-AT is primarily recognised as a protein that functions extracellularly, animal models of emphysema provide evidence to support further, intracellular roles. Vascular endothelial growth factor (VEGF) prevents alveolar cell apoptosis, and so maintains alveolar integrity [50, 51]. Blockade of VEGF in mouse and rat models resulted in apoptotic emphysema in the absence of neutrophilic inflammation [52]. However, this

phenotype was rescued by α_1-AT overexpressed locally or systemically (tested in the mouse model) or given intravenously (rat model). The α_1-AT localised within type II pneumocytes and alveolar endothelial cells, and suppressed oxidative stress and caspase-3 activation [53]. Whilst active, monomeric α_1-AT can bind and inhibit intracellular caspase-3, polymeric α_1-AT is inactive as an enzyme inhibitor. Analogously, PiZZ individuals who have high levels of polymeric α_1-AT but low levels of the native, active form may therefore be at increased risk of alveolar cell apoptosis and hence emphysema *via* the action of VEGF and caspase-3.

EFFECTS OF α_1-AT DEFICIENCY ON THE INTERSTITIUM

The dominant model for the pathogenesis of emphysema in α_1-AT deficiency is that of increased tissue turnover as a result of reduced pulmonary protection against proteolysis, most specifically *via* the activity of human neutrophil elastase. An animal knockout model of α_1-AT deficiency has not been developed to test this, as mice express five paralogues of human α_1-AT with overlapping functions. While the pallid mouse model is sometimes referred to as a model of α_1-AT deficiency [54], it is in fact a model of wide-ranging secretion defects with a complex phenotype. *In vivo* data on the effects of loss of α_1-AT function are therefore extrapolated from animal models with excessive elastase activity. Elastase digests a wide range of substrates of relevance to the development of emphysema. These include components of the extracellular matrix (elastin, collagen, fibronectin and fibrin), molecules attached to the cell surface (cadherins), and plasma proteins (immunoglobulins, complement, proteinase inhibitors) [55]. While apoptotic mechanisms can produce models of emphysema, the phenotypes may be reversed on removal of the apoptotic stimulus [56]. Models involving destruction of the lung extracellular matrix, however, are not reversed by removal of the initial insult alone but may be reversed by treatment with all-trans retinoic acid [57].

There is strong evidence that excessive elastase-mediated degradation of elastin plays a major role in the pathogenesis of emphysema. Elastin, a core component of lung elastic fibres that is cross-linked to other key extracellular matrix components, is responsible for reversible pulmonary expansion during the respiratory cycle. Loss of elastic tissue therefore plays a major role in fixed obstructive airways diseases [58]. Elastin expression is noted to be maximal during alveologenesis in mice and then declines to very low levels in adulthood [59]. Its assembly is a complex multi-step process requiring coordinated interactions with multiple co-expressed components; the subsequent development of elastic fibres is similarly elaborate [60]. It seems likely that in adulthood there is limited capacity to restore functional elastin. Mice expressing ~50% of normal elastin levels ($Eln^{+/-}$) have normal lung architecture at maturity. However, they develop more severe emphysema in response to cigarette smoke exposure compared to wildtype mice [61]. Mice expressing lower levels of elastin (0 or 37% compared to normal levels *i.e.* $Eln^{-/-}$, with or without partial transgenic rescue) than this have abnormal alveolar development consistent with a congenital, non-inflammatory emphysema. Moreover, low elastin levels correlate with increased mechanical strain within lung tissue in response to stresses such as those occurring during the respiratory cycle. Similar phenotypes are seen in association with knockout models of other elastic fibre components [62–64], while missense mutations in elastin underlie familial syndromes characterised by COPD and emphysema [65, 66]. Thus, excessive elastase-mediated degradation of elastin will affect both the integrity of the interstitium and its ability to respond to other insults. The resultant proteolytically cleaved elastase fragments are strongly chemotactic, driving neutrophilic inflammation and further tissue damage [67].

Dysregulated elastase activity in α_1-AT deficiency will additionally impact upon growth factor signalling. Elastic fibres are a major component of the large reservoir of transforming growth factor (TGF)-β within the interstitium [68]. Elastase cleavage of elastin and the latent TGF-β-binding protein mobilises activated TGF-β [69, 70]. This results in increased tissue inflammation and delayed wound healing in the context of excessive elastase activity in a knockout model of the small leukocyte proteinase inhibitor [71]. A similar effect may therefore contribute to a maladapted inflammatory response in α_1-AT deficiency.

A number of cellular and *in vitro* studies support a range of effects of elastase activity in the direction and facilitation of neutrophil migration in response to inflammatory stimuli [55, 72–74]. Defective regulation of elastase activity would enhance any such effects in individuals with α_1-AT deficiency. This is consistent with the observed increase in bronchoalveolar lavage (BAL) neutrophil counts [72, 75] and the increased inflammatory response to infections and bacterial colonisations [76, 77] seen in PiZZ individuals. Neutrophil elastase also induces expression of the proteases cathesin B and matrix metalloprotease-2 *in vitro* and in a mouse model [78]. Treatment of PiZZ patients with aerosolised α_1-AT has been shown to reduce the activity of these two proteases in BAL samples, supporting the relevance of this cascade *in vivo* [79]. Moreover, elastase is potentially cytotoxic and, while epithelial cells are resistant to this effect [80], endothelial cells may be more susceptible [81].

Excessive airway elastase is also implicated in impairing pulmonary bacterial killing by neutrophils in a range of lung diseases *via* cleavage of the cell surface chemokine receptor CXCR1. In a fascinating study by HARTL *et al.* [82], cells were obtained from BAL and induced sputum samples of healthy controls, patients with COPD, patients with bronchiectasis and patients with cystic fibrosis (CF). Levels of airway inflammatory mediators were also measured. The receptors CXCR1 and CXCR2 mediated the effects of interleukin (IL)-8 upon neutrophils [83]. CXCR2 was shown to mediate respiratory burst and α-defensin release responses but not bacterial killing, which was mediated *via* CXCR1 [82]. Airway neutrophils from all patients showed reduced surface expression of CXCR1 (but not CXCR2) and a directly correlated reduction in bacterial killing capacity relative to controls. The reduction in CXCR1 levels correlated directly with levels of airway free elastase, which were elevated above control levels to the greatest extent in the CF patients and to a lesser extent in the COPD subjects. Treating neutrophils derived from peripheral blood samples with patients' airway fluids caused similar reductions in these measures, but this effect was not seen when the airway fluids were pre-treated with elastase inhibitors. In addition, proteolytically cleaved fragments of CXCR1 were themselves able to

stimulate IL-8 release *via* a mechanism involving Toll-like receptor (TLR)2 from bronchial epithelial cells *in vitro*. Taken together, these effects would exacerbate a situation in which bacterial infection stimulated pulmonary neutrophilia and oxidative burst responses but in which bacterial killing was disabled. Amongst the patient groups studied by HARTL *et al.* [82], the effects were greatest in CF patients and smallest (though still present) in those with usual COPD. α_1-AT-deficient patients were not represented in the study. However, given the tight inverse correlation between airway free elastase and airway neutrophil surface CXCR1 expression, the data strongly support an important role for this pathway in the lung disease of α_1-AT deficiency

A further effect of α_1-AT deficiency upon the lung interstitium, independent of its anti-elastase activity, was indicated by *in vitro* data demonstrating that α_1-AT can promote fibroblast proliferation and procollagen synthesis [84]. This effect appears to be mediated *via* classic mitogen-type interactions with cell-surface receptors and activation of tyrosine kinase and mitogen-activated protein kinase pathways. Therefore, in α_1-AT deficiency, excessive proteolysis of the interstitium may be coupled with loss of pro-repair functions to worsen tissue damage. A number of pulmonary effects of reduced circulating and lung levels of α_1-AT may also be deduced from observations of the effects of exogenous α_1-AT. These are discussed further later, in the context of α_1-AT replacement therapy.

α_1-AT REPLACEMENT THERAPY: LESSONS AND QUESTIONS

Given the clear importance of the protease:antiprotease imbalance in causing emphysema in α_1-AT deficiency, augmentation of circulating α_1-AT to achieve physiological levels is a logical treatment [85, 86]. Accordingly, intravenous administration of exogenous α_1-AT to achieve serum levels $\geqslant$11 μM (replacement therapy) is widely used in Europe and North America [87, 88]. Systemic augmentation is reflected by increases in lung concentrations of α_1-AT [86, 89]. Inhaled α_1-AT supplementation therapies capable of similar augmentation of pulmonary levels have also been developed [90] and these have been shown to be safe and efficacious in CF lung disease [91]. In addition to directly protecting elastic tissue from the effects of neutrophil elastase, laboratory studies support anti-inflammatory roles for exogenous α_1-AT. *In vitro* α_1-AT abrogates bacterial endotoxin (lipopolysaccharide (LPS))-induced chemokine release from monocytes (tumour necrosis factor (TNF)-α, IL-1β) and neutrophils (IL-8). These effects are concentration dependent.

Nasal administration of α_1-AT also inhibits IL-8 release in response to LPS challenge [92]. A well-conducted prospective clinical trial assessed the effects of short-term replacement therapy on markers of neutrophil chemotactic stimuli and neutrophilic inflammation [93]. The study observed 12 PiZZ patients with moderate-to-severe airways obstruction. Replacement therapy was given over a period of 3 weeks and sputum levels and/or activity of α_1-AT, elastase, myeloperoxidase, IL-8 and leukotriene (LT)B_4 were measured before, during and 3 weeks after the last infusion. Replacement therapy resulted in a rise in levels and a smaller rise in activity of α_1-AT, reduced elastase activity and a reduction in LTB_4. Studies support a central role for LTB_4 in mediating pulmonary inflammation in patients with α_1-AT deficiency [72, 76, 94]. These include an association between exacerbations and LTB_4 levels [76] and a retrospective analysis indicating that exacerbations are reduced by augmentation therapy [94]. It is therefore particularly noteworthy that the reduction in sputum LTB_4 levels persisted 3 weeks after the final infusion even though levels of α_1-AT and elastase activity had returned to baseline [93]. Levels of IL-8 were not significantly affected in this study and neither, interestingly, were levels of myeloperoxidase, a marker of neutrophil activity that would not be directly inhibited by the supplementary α_1-AT. The pathways by which these anti-inflammatory effects of α_1-AT are mediated merit further investigation.

Support for beneficial effects of replacement therapy was also found in the previously discussed study of elastase effects on CXCR1 [82]. The authors assessed the effects of administration of 4 weeks of inhaled α_1-AT therapy to counteract the effects of free airway elastase on bacterial killing. They did so in the CF patient group as these were the worst affected. Neutrophil cell surface CXCR1 levels and bacterial killing capacity increased in line with a drop in free elastase levels and, impressively, bacterial lung colonisation also fell, as assessed by sputum levels of *Pseudomonas aeruginosa*.

Despite the accumulation of encouraging data, there are so far no robust clinical trials that prove benefits in respiratory function in patients receiving replacement therapy [95]. This may be a reflection of insufficiently powered studies and a failure to identify subgroups of α_1-AT-deficient patients who are most likely to derive benefit. *Post hoc* subgroup analysis of data from augmentation trials suggests this may be the case in patients with established but not end-stage lung disease, such as those with a forced expiratory volume in 1 s level of 30–65% predicted [96–98]. Computed tomography-based assessment methods may be more sensitive than spirometry in detecting benefit, but in the context of a small-scale trial, the difference between those who received active replacement therapy and those who received placebo failed to reach statistical significance [99]. However, the difficulty in detecting benefit may also indicate that the pulmonary disease seen in α_1-AT deficiency is not caused by a lack of functional α_1-AT alone.

INTRAPULMONARY POLYMERS OF α_1-AT, NEUTROPHIL INFLUX AND THE PANACINAR DISTRIBUTION OF TISSUE LOSS

Even when mutations result in a strong tendency for polymerisation, and hence retention within the ER, a proportion of the protein folds to a functional monomer and is secreted. Thus, homozygotes for the Z allele typically have circulating α_1-AT levels that are 10–15% of those seen in M homozygotes. As previously discussed, some of this secreted protein circulates in the form of polymers [33, 100] in patients with α_1-AT deficiency but not in healthy individuals nor in non-α_1-AT-deficient subjects with ''usual'' COPD. α_1-AT enters the lung from the circulation by passive diffusion [101]. It is supplemented by local synthesis, which may be stimulated by oncostatin M [102] and TGF-β [103] in pulmonary epithelial cells [11, 12]. This was initially supported by an animal model in which local synthesis induced in type II alveolar epithelial cells resulted in detectable levels of α_1-AT within the

interstitium [104]. It was elegantly proven by the demonstration of Z α_1-AT in BAL of a PiZZ individual who had previously undergone liver transplantation with an implanted organ from a PiMM donor [33]. Since all of that subject's circulating α_1-AT was of the M type, their intrapulmonary Z α_1-AT could only have derived from synthesis by local lung cells, which still carried the Z homozygote genotype [33]. High levels of α_1-AT polymers have been demonstrated in the lungs of Z homozygotes both in BAL [32, 33] and within the interstitium in a series of 10 explants from PiZZ individuals affected by end-stage COPD [34].

Extracellular polymers of α_1-AT are known to be chemotactic for human neutrophils *in vitro* [33, 105] to a similar degree to the powerful chemoattractant C5a. Chemotaxis occurs at physiologically relevant concentrations (EC50 ~1.5% of circulating α_1-AT levels of Z homozygotes). Polymers adhere to neutrophils, cause them to undergo the morphological changes induced by classical chemoattractants, and induce their degranulation to release degradative enzymes such as elastase and cathepsins, as evidenced by myeloperoxidase release [105]. Mouse neutrophils were shown to respond to α_1-AT polymers in the same way as neutrophils derived from humans. To investigate the significance of these data, α_1-AT was administered by intratracheal instillation to wildtype C57BL/6J mice, a strain widely used to model human disease, in polymeric or native form. Polymers, but not the native protein or control sample, provoked a vigorous neutrophilic response, as seen in BAL samples [34]. The major murine pulmonary inflammatory cytokines keratinocyte-derived chemokine and macrophage inflammatory protein-2 (paralogues of IL-8) were absent from BAL samples over 3 days. This indicated that the neutrophilia was mediated by a direct effect of α_1-AT polymers rather than *via* pro-inflammatory cytokines (although other murine cytokines were not measured). However, the lungs of PiZZ patients contain elevated levels of pro-inflammatory cytokines (IL-8, LTB_4) compared with matched controls [72, 106], which themselves are chemotactic for neutrophils. This elevation may in part result from α_1-AT polymerisation within cells. In a Chinese hamster ovary cell model, IL-6 and -8 were secreted as a response to the expression and intracellular polymerisation of Z α_1-AT [48]. This was not seen following expression of control M α_1-AT. Therefore, α_1-AT polymers may promote neutrophilic inflammation *via* both direct and chemokine-mediated actions, and the magnitude of these effects may depend on whether the polymers are present within cells or in the extracellular milieu.

The finding of interstitial polymers was given further interest by their co-localisation with interstitial neutrophils [34]. Lungs explanted for severe COPD in 10 PiMM and 10 PiZZ individuals were studied. Tissue sections were examined using immunohistochemistry to identify neutrophil elastase and α_1-AT polymers. The findings were independently reported by two pathologists blinded to the patients' underlying α_1-AT status. The data showed that α_1-AT polymers were found in the alveolar walls of PiZZ explants but not in those from PiMM patients, and were particularly focused around type II pneumocytes. This is consistent with the hypothesis that intrapulmonary polymers may be derived from locally synthesised material, concentrated within the interstitium. Whilst none of the explants showed evidence of bacterial infection, neutrophils were present in unusually high numbers within the interstitia of PiZZ explants (a more than four-fold increase over the numbers of alveolar neutrophils seen in PiMM explants). Two studies have shown both greater [75] and smaller [72] increases in BAL neutrophil counts in PiZZ emphysema. The first study [75] showed a 20-fold increase in BAL neutrophilia in five α_1-AT-deficient patients with chronic bronchitis compared to controls with chronic bronchitis but normal levels of α_1-AT. However, the groups were small and were not matched for age, sex or smoking history. Thus, a far higher proportion of the α_1-AT-deficient patients were current smokers. The second study [72] compared PiZZ individuals (all of whom had emphysema and half of whom had previously smoked) with healthy never-smoking controls in a similar/slightly younger age range, rather than with patients with usual COPD. This might be expected to exaggerate the differences attributable to α_1-AT deficiency, but in fact the increase was less than three-fold. Moreover, in usual COPD, the neutrophils are predominantly found within the conducting airways and relatively few are seen within the alveolar spaces [107]. These data therefore support the hypothesis that PiZZ individuals with emphysema are particularly predisposed to interstitial sequestration of neutrophils in the lung. The α_1-AT polymers with which the neutrophils co-localise are strong candidate agents for this, and for activation of neutrophils within the interstitium due to their effects on neutrophil chemotaxis and degranulation. The release of proteolytic enzymes would exacerbate elastin degradation and emphysema.

Taking these data together, we propose that in Z α_1-AT deficiency, an already pro-inflammatory, pro-degradative interstitial environment is exacerbated by interstitial polymerisation (figs 2–4). The normal response to an inflammatory stimulus within the airspace (such as cigarette smoke) is a massive migration of neutrophils from the vascular compartment along a chemotactic gradient into the alveoli, to accumulate at centriacinar sites (fig. 2). Neutrophil degranulation therefore leads to a centriacinar pattern of tissue damage and hence emphysema. Migration is usually so rapid that at any particular timepoint, relatively few neutrophils will be seen within the interstitium itself, even in severe, progressive COPD in PiMM individuals (where far more modest neutrophil accumulation is seen) [34]. In the lungs of PiZZ individuals, however, the local chemotactic effects of interstitial polymers would partially counterbalance the overall chemotactic gradient, introducing a lag into their transit across the interstium (fig. 3). As the numbers of neutrophils in transit are so great, any increase in the transit time will result in a prominent interstitial neutrophilia. Moreover, polymers will bind to these interstitial neutrophils and cause them to degranulate within the extracellular matrix itself, spreading the focus of tissue destruction to a panacinar distribution. Extracellular matrix loss would thus be maximised by the very proteolytic mechanism to which the patients are most vulnerable. This hypothesis may explain partly the finding that significantly reducing levels of sputum elastase and LTB_4 by α_1-AT augmentation did not result in a significant reduction in sputum myeloperoxidase (MPO) activity [93]. This was a surprising finding, since MPO activity is a marker of neutrophil activation; the finding is also surprising if we

consider the apparent importance of LTB_4 in mediating pulmonary inflammation in α_1-AT deficiency. However, these data are consistent with the presence of further factors, such as α_1-AT polymers that can maintain local neutrophil levels once recruitment has been initiated.

A study of all 12 known Dutch cases of α_1-AT null homozygotes provides a note of caution in assessing the potential contribution of any toxic gain-of-function effects of α_1-AT polymers [108]. This cohort had significantly lower spirometry than controls matched for age, sex, smoking history and potential ascertainment bias with α_1-AT deficiency caused by polymerisation (pooled data from 12 PiSZ and 12 PiZZ homozygotes). Since the PiNull cases by definition had neither circulating functional α_1-AT nor intrapulmonary α_1-AT polymers, these support a dominant role for loss-of-function effects in driving spirometric decline in α_1-AT deficiency. Interestingly, the data presented for the relationship between circulating α_1-AT levels and spirometric impairment appears to indicate a difference between PiSZ and PiZZ control groups. The latter group, which would predictably have higher polymer loads, appeared to show a steeper decline in spirometry with decreases in circulating levels, consistent with the presence of an exacerbating factor. However, interpretation is limited as the numbers of individuals studied was small and PiSZ individuals may be less affected by virtue of having circulating levels above a postulated ''protective threshold'' level.

BROADER IMPLICATIONS OF PRO-INFLAMMATORY GAIN-OF-FUNCTION EFFECTS OF EXTRACELLULAR POLYMERISATION

This gain-of-function model has also led to a proposed rationale for the high carrier frequency of the PiMZ allele type in populations of North European descent (~4%) [109]. Mutations that are deleterious for homozygotes are conserved

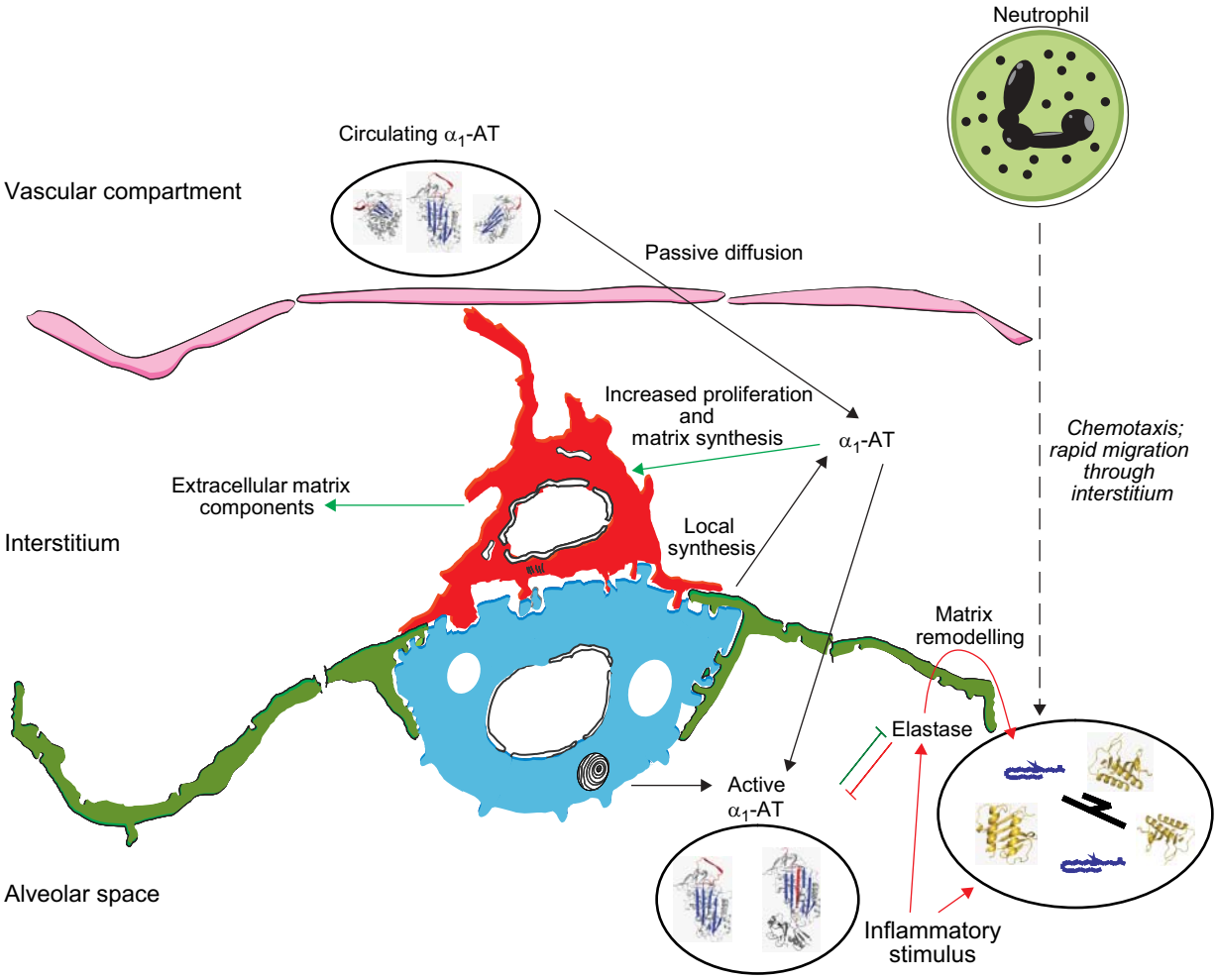

FIGURE 2. An integrated model of the molecular and cellular basis of emphysema in α_1-antitrypsin (α_1-AT) deficiency. Roles of α_1-AT in maintaining alveolar and interstitial integrity in health and in the normal response to an inflammatory stimulus such as cigarette smoke. The cells of the blood–gas barrier are represented by capillary endothelial cells (pink), a fibroblast (red), type I pneumocytes (dark green) and a type II pneumocyte (light blue); diffusion of molecules (solid black arrows) and neutrophil chemotaxis (dashed black arrow) are shown. Effects maintaining or degrading tissue integrity are indicated by green or red arrows, respectively. Molecules are depicted at higher scales within ellipses, with native and complexed α_1-AT depicted in the same colours as in figure 1. Inflammatory stimuli provoke elevated levels of chemoattractants interleukin-8 (gold), leukotriene B_4 (dark blue) and elastin degradation fragments (black). This creates a gradient along which neutrophils migrate rapidly from the circulation into airspaces.

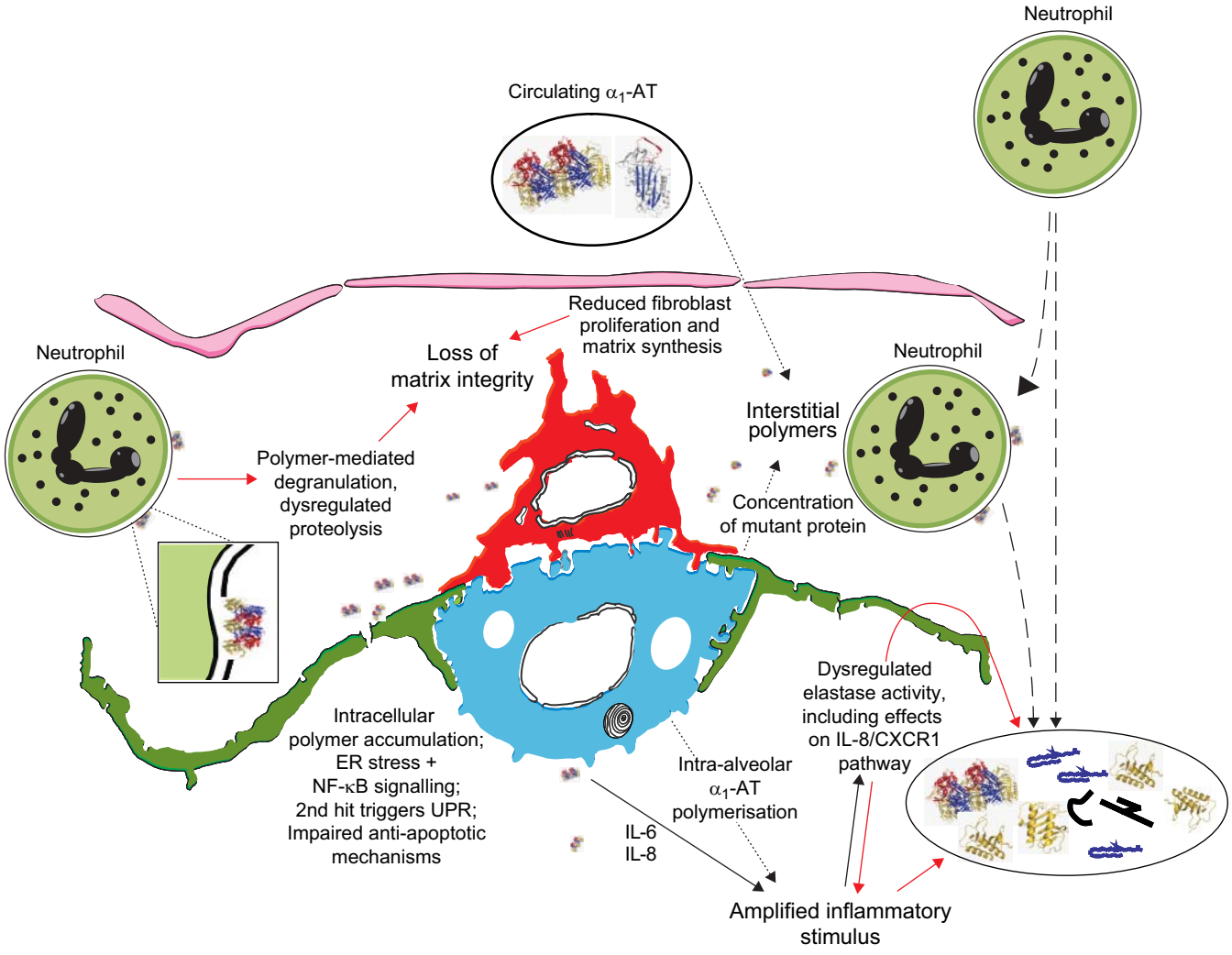

FIGURE 3. Compromise of alveolar and interstitial integrity in α_1-antitrypsin (α_1-AT) deficiency by multiple proposed pathways. Cells and molecules are represented in the same way as seen in figure 2, unless otherwise specified hereafter. The presence of pathogenic mutations in α_1-AT, results in secretion of reduced levels of the protein into the circulation by hepatocytes and into lung tissue by type II pneumocytes. The loss of adequate protection against elastase activity accelerates the pathogenesis of emphysema as does the loss of matrix-promoting effects of α_1-AT on fibroblasts. Recruitment of neutrophils may be stimulated, and their ability to kill bacteria disabled, by effects of excess elastase on the interleukin (IL)-8/CXCR1 pathway. Intracellular accumulation of polymerised α_1-AT within the endoplasmic reticulum (ER) of epithelial cells will, by analogy with hepatocytes and experimental models, damage the cell and surrounding tissue. It may result in chemokine secretion, nuclear factor (NF)-κB signalling and susceptibility to both the unfolded protein response (UPR) and apoptosis. α_1-AT, derived from a combination of local synthesis and passive diffusion from the systemic circulation, will be concentrated within the interstitium, resulting in extracellular polymerisation. Interstitial polymers will provide a local counterbalance to the chemotactic stimuli from the airspace, prolonging the interstitial transit time of neutrophils and hence causing interstitial neutrophilia. Within the interstitium, the effects of polymers (sets of three repeating molecular subunits shown in yellow, red and blue) will activate neutrophils and stimulate degranulation, focusing proteolysis in the midst of the extracellular matrix and spreading the focus of tissue destruction from a centriacinar to a panacinar distribution.

by evolutionary pressures if they confer a heterozygote advantage. This is classically exemplified by the sickle haemoglobin (S) allele in areas that are endemic for malaria, where heterozygotes are protected against disease. A similar heterozygote advantage may have applied in the pre-antibiotic era to a genotype that promoted a more vigorous antibacterial inflammatory response in the lungs. This would predictably be the case in PiMZ individuals in whom limited extracellular polymerisation would be expected. Such a response would reduce mortality from bacterial infections that cause pneumonia. Improved living conditions and the availability of antibiotic treatment have negated any heterozygote advantage in recent history. Conversely, increased longevity and the widespread adoption of cigarette smoking have unmasked a clearer homozygote disadvantage than was previously apparent.

The pro-inflammatory effects of extracellular polymers may further explain the association of α_1-AT deficiency with other inflammatory conditions. Within the lung, local polymerisation may predispose to asthma [4] and bronchiectasis [5] phenotypes in some individuals. α_1-AT deficiency is also associated with extrapulmonary inflammatory disorders: panniculitis [6] and the ANCA-positive vasculitides [7]. Circulating polymers of α_1-AT are potential triggers or exacerbators of these. However, the

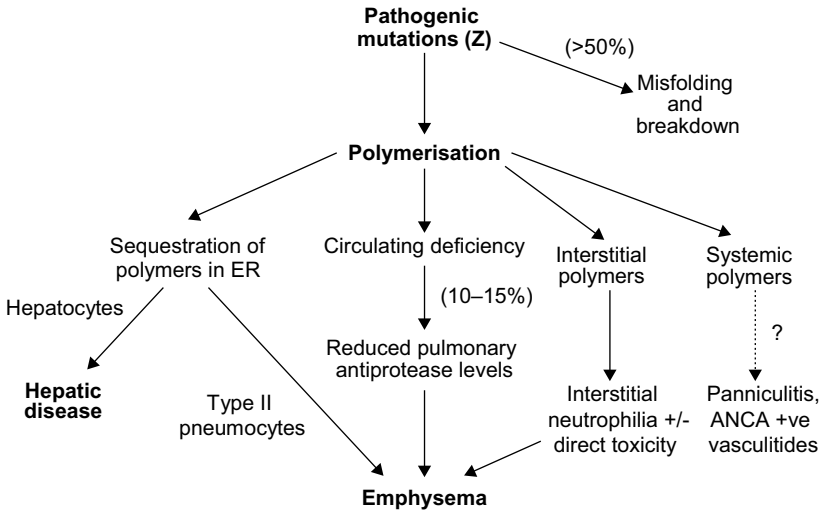

FIGURE 4. Integration of gain- and loss-of-function mechanisms (summarised in table 2) resulting from polymerisation of mutant α_1-antitrypsin (of which the Z variant is the archetype) within the clinical syndrome characterised for α_1-antitrypsin deficiency. ER: endoplasmic reticulum; ANCA: antineutrophil cytoplasmic antibody.

efficacy of α_1-AT replacement therapy in treating the panniculitis of α_1-AT deficiency supports a major role for loss-of-function effects in maintaining this condition [110]. Figure 4 integrates the various experimentally supported mechanisms of disease resulting from α_1-AT polymerisation within the clinically characterised syndrome of α_1-AT deficiency.

CONCLUSION: THERAPEUTIC STRATEGIES AND ONGOING CHALLENGES

Taken together, the data from molecular, cellular, animal and *ex vivo* studies support a model in which emphysema in α_1-AT deficiency results from the cumulative and interacting effects of multiple pathways (figs 2–4) [111]. α_1-AT polymerisation within hepatocytes results in a circulating deficiency and this together with intracellular and extracellular polymerisation in lung tissue renders it vulnerable to unchecked elastase activity. The inflammatory state that initiates this devastating loss of extracellular matrix is augmented not only by the effects of elastin degradation fragments but also by a range of loss- and gain-of-function effects of α_1-AT polymerisation. These are listed in table 2. It is important that future work characterises these further and assesses their relative contributions to the pathogenesis of emphysema in α_1-AT deficiency to guide design of more effective therapeutic options.

Only loss-of-function effects will be addressed by α_1-AT replacement therapy to maintain circulating levels within a physiological range. Currently, α_1-AT can only be augmented by exogenous purified protein. However, the same considerations will remain valid if current attempts to boost endogenous α_1-AT synthesis by transfecting cells with the gene for M α_1-AT [112] or stem cell therapy become clinically viable. Rigorous clinical studies are required to identify the factors that determine the magnitude of the benefit of replacement therapy in different individuals. Synthetic elastase inhibitors are a logical alternative to replacement therapy in addressing the excessive damage caused by this protease. Despite longstanding interest in their development [113] this has not yet resulted in agents for clinical use.

Conversely, gain-of-function effects will require the development of novel therapies which can address these specifically. Since there is a broad spectrum of disease even amongst those who share the PiZZ genotype, elucidation of naturally occurring disease-modifying factors may help identify such therapeutic possibilities. Current knowledge is already sufficient to propose three broad approaches. The first would be to target the downstream consquences of polymerisation, *e.g.* by blocking polymer–neutrophil interactions, modulating NF-κB activity or by improving anti-inflammatory treatments.

Secondly, understanding the molecular mechanism of polymerisation allows this central event in α_1-AT deficiency to be targeted directly. Chemical chaperones have been shown in

TABLE 2 Loss- and gain-of-function mechanisms proposed to play a role in α_1-antitrypsin deficiency

Loss-of-function	Gain-of-function
Excessive elastase activity, destruction of extracellular matrix, increased LTB_4, increase in neutrophil chemotaxis, reduced bacterial killing capacity	ER overload, NF-κB signalling
Reduced fibroblast stimulation	IL-6, IL-8 expression
Reduced defence against caspase-3-mediated apoptosis	Chemotaxis, neutrophil binding and activation
	Neutrophil degranulation

LTB_4: leukotriene B_4; ER: endoplasmic reticulum; NF-κB: nuclear factor-κB; IL: interleukin.

clinical trials to rescue misfolding mutants of the CF transmembrane conductance regulator protein from the ER in CF [114]. Initial *in vitro* data on the use of the chemical chaperone 4-phenylbutyric acid in a model of α_1-AT deficiency have been encouraging [115] but were not confirmed by a clinical trial [116]. Other chemical chaperone molecules may be worth investigating, although there is the theoretical risk that agents that promote protein folding will in fact encourage Z α_1-AT to the lowest energy state, *i.e.* polymers. Peptide analogues of the α_1-AT reactive site loop that forms the intermolecular link within polymer chains can compete with it to prevent polymerisation, and can even disassemble preformed polymers [17, 38]. While work continues to optimise this approach *in vitro* [117], successful delivery of sufficient quantities of a peptide drug to the ER is likely to be highly challenging. A separate structure-based drug design approach therefore targets non-peptide, drug-like small molecules against an allosteric target, a surface accessible cavity that will be sealed by polymerisation. Partially filling the cavity by mutagenesis (Thr114Phe) resulted in 10-fold reduction in α_1-AT polymerisation *in vitro* without affecting inhibitory function. This mutation also significantly rescued the Z α_1-AT secretion phenotype in a *Xenopus* oocyte model of disease [118]. Rational targeting of the same site has now identified a promising lead compound that causes a 70% reduction in the half-time of the intracellular retention of Z α_1-AT in a hepatic (Hepa1a) cell line [119].

The final strategy involves the use of small fragment homologous replacement to correct the gene defect of Z α_1-AT deficiency [120]. Here, cells are transfected with small fragments of DNA coding for the wildtype sequence in the region of the α_1-AT gene containing the mutation. Homologous recombination of these fragments with the complementary regions of genomic DNA allows correction of the mutation to wildtype sequence. A proof-of-principal study has demonstrated the effectiveness of this in correcting the gene defect in monocytes derived from PiZZ individuals.

In theory, a treatment that effectively prevented polymerisation, promoted secretion and preserved enzyme inhibitory activity would entirely correct the underlying disease process. However, the emerging evidence of the multiple pathways at play in α_1-AT deficiency makes it likely that the optimal treatment of individuals will involve the tailoring of a number of therapeutic strategies. These would include novel therapies for the treatment of ''usual'' COPD such as agents to encourage alveolar regeneration [121].

STATEMENT OF INTEREST

None declared.

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