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Adam Wanner
Robert A. Sandhaus *Editors*

Alpha-1 Antitrypsin

Role in Health and Disease

Humana Press

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Preface

Alpha-1 antitrypsin deficiency is an inherited condition that leads to lung disease in adults and liver disease in children and adults. The condition has a prevalence that varies from country to country, ranging from one in 2750 to one in 4500 live births, and currently is the only known genetic mutation firmly associated with chronic obstructive lung disease in adults. While the mechanisms underlying the clinical manifestations of alpha-1 antitrypsin deficiency have been largely clarified, specific treatment currently is only available for the lung disease. A growing interest of academic investigators and industry in finding new therapeutic solutions for lung and liver disease likely will lead to better clinical outcomes in the foreseeable future. This will necessitate a better effort to detect the condition, which is broadly underdiagnosed at present. The purpose of this book is to summarize what is known about the biology of alpha-1 antitrypsin and its deficiency, the clinical manifestations of alpha-1 antitrypsin deficiency, and the currently available therapeutic options.

The book begins with a chapter on the biologic role of serine protease inhibitors (SERPINS) including alpha-1 antitrypsin in general, followed by a chapter focusing on alpha-1 antitrypsin in particular. The next two chapters address the process of alpha-1 antitrypsin protein misfolding and polymerization, and their pathogenetic consequences in the liver and the lung, the principal sites of alpha-1 antitrypsin deficiency-related disease.

The condition is then characterized from a clinical perspective in the following three chapters that review the methods of and challenges to the detection of alpha-1 antitrypsin deficiency, and the manifestations and management of lung disease in adults, and liver disease in children and adults. With these three chapters we intend to provide clinicians with useful information on the diagnosis and treatment of alpha-1 antitrypsin deficiency.

The last two chapters cover topics that reach beyond alpha-1 antitrypsin deficiency per se. One chapter reviews what is currently known about the potential for using alpha-1 antitrypsin therapeutically in diseases not associated with alpha-1 antitrypsin deficiency. The final chapter addresses the important role of voluntary health organizations in the rare disease space, with a focus on alpha-1 antitrypsin deficiency. Voluntary health organizations raise awareness of the condition, support

research, promote new drug development, and bring the patient perspective to the table.

This book was conceived to provide the readership of health professionals and scientists with a comprehensive overview of alpha-1 antitrypsin deficiency. International authorities, who are widely recognized for their contributions to the basic and clinical science of alpha-1 antitrypsin deficiency, wrote the chapters. Certain difference of opinion may exist between authors. We have taken the position to allow for such differences and potential controversies and give the readers the opportunity to form their own conclusions.

Miami, FL — Adam Wanner, MD
Denver, CO — Robert A. Sandhaus, MD, PhD

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Chapter 1
Alpha-1-Antitrypsin and the Serpins

Robin Carrell

How α-1-Antitrypsin Became an Archetype

The identification in 1963 of the inherited deficiency of α-1-antitrypsin established its place as a model genetic disease [1]. This status as a model partly reflects the way in which this deficiency of a plasma protein results in both a loss of function, with the onset of emphysema [2], and a gain of function, in the form of progressive liver damage culminating in cirrhosis [3–5]. Interest in the deficiency was furthered by its common occurrence and from the realisation that the lung degeneration in α-1-antitrypsin deficiency resulted from a loss of lung elasticity exacerbated by tobacco smoking [6, 7]. This last focused attention on α-1-antitrypsin's action as a protease inhibitor and specifically as an antielastase [8–10]. Thus, early research addressed two prime questions. First and obviously, what was the molecular basis of this inherited deficiency? But it was a second subtler question that opened wider understandings. What was the special functional advantage of α-1-antitrypsin as compared to the apparently equal inhibitory efficiency of the much smaller protease inhibitors present in plants and other organisms?

An initial answer to these questions became apparent in the mid-1970s, with the amino acid sequencing of the terminal third of α-1-antitrypsin. This revealed not only the S and then the Z deficiency mutations [11, 12] but also identified the active centre of the molecule, the site of its cleavage by elastase and with that the clue to its inhibitory activity. The completion of this portion of sequence came with the bonus of the recognition in 1979 of its close homology with the sequence of another plasma protease inhibitor, the natural anticoagulant antithrombin [13, 14]. With that, α-1-antitrypsin took on a new and wider significance not just as a model for genetic disease but also as an archetype for a family of proteins controlling key functions of life [15].

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α-1-Antitrypsin and the New Superfamily

The alignment of the sequences of α-1-antitrypsin and antithrombin in 1979 indicated that we were looking at a new family of protease inhibitors, but the realisation that this was indeed a full-scale superfamily came from the subsequent alignment of a third family member, the egg-white protein ovalbumin [16]. The inclusion in the family of ovalbumin, a non-inhibitor, was an indication that this was indeed a protein superfamily with an ancient and diversified lineage—diversified in the functions of its members whilst still retaining a highly conserved framework structure. The recognition of this new superfamily at the commencement of the 1980s was timely, as it coincided with the introduction of cDNA sequencing. The availability of a simple and quick means of determining the amino acid sequence of previously unidentified proteins introduced a range of new members of the superfamily. These included the key functional proteins in human plasma, not only the protease inhibitors, antithrombin, C1 inhibitor, antichymotrypsin and the plasminogen activator inhibitor PAI-1, but also the non-inhibitory hormone carriers, thyroxine- and corticosteroid-binding globulins (TBG and CBG), and the source of the angiotensin vasopressors, angiotensinogen. Subsequently, manifold serpins have been identified as controlling vital intra- and extracellular functions in plants as well as in microbes and animals [17].

The breakthrough that tipped the balance in establishing α-1-antitrypsin as an archetype of the superfamily [18] was the solving in 1984 of its crystallographic structure [19], albeit in a modified physiologic form (Fig. 1.1). Much earlier experience with another protein family, the globins, had shown how the availability of the structure of just one member of a protein family allowed the accurate alignment of other members of the family, in a way that provides immediate insights into the specialist functions of each. Moreover, the study of clinical dysfunctions arising from genetic variations in any member of the family was seen to have implications for all [20]. Even more rewardingly, the lesson from the globins was that the identification of mutations in familial diseases can reveal previously unsuspected aspects, fundamental to the understanding of protein structure and function. The advance of science is overwhelmingly by vertical progression—the gradual building of one new increment in knowledge on another. But this stepwise approach narrows progress to preconceived concepts, which can become dogmas. The study of the consequences of mutations that result in the dysfunctions of disease opens lateral thought and provides insights to otherwise overlooked concepts. The lesson from the early work with haemoglobin is that individual genetic diseases are each unique experiments of nature, undertaken free of preconceptions. And similarly the story of the newly recognised α-1-antitrypsin superfamily is that of a series of advances in understanding, arising or authenticated by mutations that result in aberrant function and hence disease.

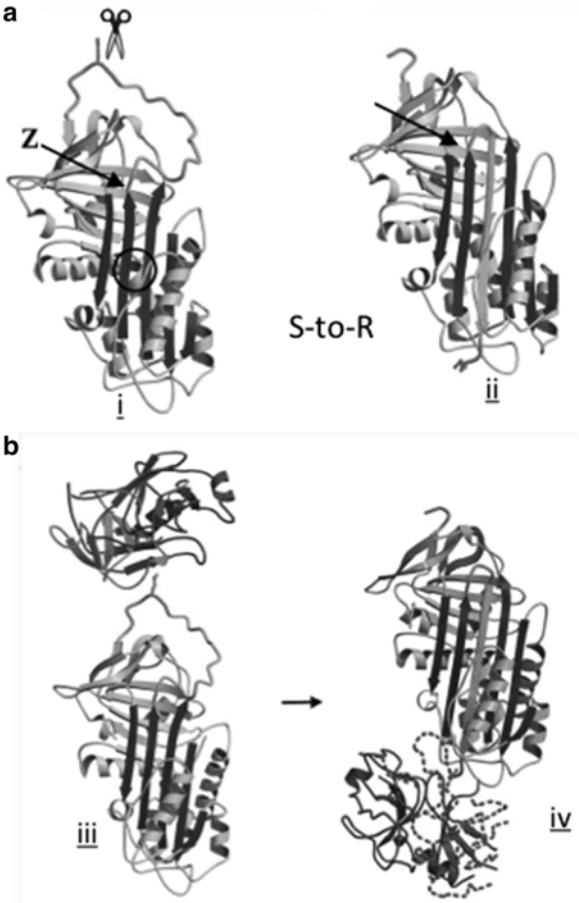

Fig 1.1 (**a**) The serpin mechanism. Cleavage of the reactive loop of α-1-antitrypsin triggers the archetypal serpin S-to-R transition: from a stressed active form (i) to a relaxed hyperstable form (ii) with inclusion of the cleaved loop as a middle strand in the main beta-sheet of the molecule. Mutations commonly causing intracellular aggregation in serpins occur at sites of hinge movements, as *arrowed* and *encircled*. (**b**) Serpins trap their target protease by exposing an ideal substrate (iii) with, on consequent cleavage, the spring-like S-to-R change flinging the entrapped protease to the other pole of the molecule (iv) with disruption of its activity

The Serpins as an Entity

By the mid-1980s, mutations in the plasma protein members of the family were known to result in multiple dysfunctions including thrombosis, haemorrhage and angioedema. Taken together, consideration of these mutations in terms of the template structure of α-1-antitrypsin promised new insights into the principles of protein structure and function. And so it turned out to be. There was first however a hurdle in encouraging the interest of the wider field of investigators needed to achieve the correlations of mutations and dysfunctions in terms of the family as a whole. A problem was an international confusion in nomenclature, between the medically based European researchers who had established the fundamentals of the inherited deficiency of *α-1-antitrypsin* [1, 15, 21] and the biochemically based researchers in the USA who were investigating the protease–antiprotease imbalances resulting from the inherited deficiency of the synonymous *α-1-proteinase*

inhibitor [8–10]. With time, the medical common-usage title of α-1-antitrypsin has become universally adopted, but a reminder of the controversy is still seen in the diplomatically chosen name of the major charity in the field—the Alpha-1 Foundation!

But there was also a real problem that hindered discussion and collective research on the family as a whole, due to its initial naming as the *ovalbumin–antithrombin-III–alpha-1-proteinase inhibitor superfamily of serine proteinase inhibitors* [16]. A title that if repeated twice was guaranteed to turn off not only audiences but also young investigators and funding bodies. Clearly, a simpler name was needed if the field was to progress. To meet this and with the precept of the globins in mind, the author proposed the name *serpins,* as an acronym for serine protease inhibitors. International acceptance of the new name was promptly assured in 1985 with the backing of the leading US investigator in the field, James Travis [22]. So, the stage was then set for what became a period of remarkable productivity in research. Now, 30 years later, some 60,000 serpin-based papers have been published with spin-off benefits to almost all branches of medicine and rewarding new insights in biology as a whole.

Z Mutation and Polymeric Aggregation

A satisfying feature of the studies of the serpin family is the way the findings in one member are frequently of direct relevance to all. This is especially true of the genetic dysfunctions in the protein family, as became apparent with the demonstration that the common Z mutation led to a misfolding and intracellular polymerisation of α-1-antitrypsin [23]. The consequent failure in secretion explained its plasma deficiency and the formation of large internal hepatocellular aggregates explained the accompanying liver degeneration. Several less common mutations of α-1-antitrypsin were also found to cause intracellular polymerisation and plasma deficiency. The significance of these mutations was demonstrated by alignments with other serpins [24]. These showed that the occurrence of identical mutations in other members of the family similarly resulted in intracellular aggregation, causing thrombosis when they occur in antithrombin, angioedema when they occur in C1 inhibitor and notably encephalopathy when they occur in the brain-specific serpin, neuroserpin.

The shared findings and pattern of dysfunctions, found in what came to be labelled together as the serpinopathies [25], strongly indicated that the causative mutations perturbed the conformational mechanism that is central to the serpin family. The realisation that this was so, motivated a switch in focus of the field in the 1990s, to the α-1-antitrypsin template and to the profound changes in conformation that enables the irreversible trapping of proteases. As illustrated in Fig. 1.1, it is the adaptation of this inherent ability of the template to spontaneously change its shape that explains not only the diversity of functions of individual serpins but also how their activity is modulated and the consequences of mutations affecting their structure.

How the Serpins Change Their Shape

α-1-Antitrypsin has a landmark place in molecular biology as the first protein to be shown to undergo a radical and functional change in shape. The serpins as a whole now demonstrate how this inherent change in conformation is not only central to the shared mechanism of the family but has also been adapted by individual serpins to meet their different roles. The first evidence of this conformational transformation came from the crystallographic structure solved by Huber and colleagues in Munich in 1984 [19]. Although they had commenced their study with intact α-1-antitrypsin (supplied by Laurell from Malmö), unbeknown to them, the protein had during the process of crystallisation been cleaved at its reactive site by a contaminating protease. The totally surprising feature of the solved structure was the finding that the reactive centre loop was not only cleaved but had become incorporated as a middle strand in the main beta-sheet of the molecule (Fig. 1.1a, ii). There was scepticism by many and even derision from some when it was proposed that this shift of some 70 Å resulted from a transition of a hyperstable stressed (S) native form, with an intact reactive loop, to a cleaved relaxed (R) form [26] and even more so with the suggestion that this spring-like S-to-R transition on cleavage of the reactive centre loop was central to the inhibitory mechanism of the serpins [27]. What was proposed was a molecular mousetrap. Nothing like it had been seen before. To settle the matter beyond all doubt required a series of crystallographic structures showing the different stages of the conformational change involved in the capture and trapping of a target protease. This daunting task took 15 years to complete, with the final frame of the complex, of α-1-antitrypsin with entrapped trypsin (Fig. 1.1b, iv), being solved in 2000 [28].

Inhibitory Mechanism The sequential changes involved in the transition in Fig. 1.1b provide a video depiction of the elegant though complex mechanism by which the serpins trap their target proteases. The exposed reactive centre loop contains a substrate sequence specific for the target protease, which forms a stabilised proteolytic intermediate with it. It is the subsequent changes that make the serpins unique amongst the many families of protease inhibitors. With the serpins, this initial binding with the protease moves beyond the reversible proteolytic intermediate to the formation of a covalent acyl-enzyme linkage. The consequent cleavage of the reactive site releases the energy of the stressed conformation, and the resulting spring-like S-to-R transition flings the bound protease to the other end of the serpin molecule, with effective irreversible destruction of the protease (Fig. 1.1b). This then is why the serpins have become the predominant protease inhibitors in higher organisms. The irreversibility of their action, as opposed to that of other inhibitors, is a vital advantage in tissue protection. But the video depiction of the S-to-R change reveals another factor that has ensured the success of the serpins. The S-to-R transition involves more than just the entry of the cleaved reactive loop into the main beta-sheet of the molecule; there is at the same time an overall conformational change as one pole of the molecule rotates about the other [29]. It is these concomitant changes in shape that have been adapted by evolution to give not only the specialist functions of the non-inhibitory serpin but also to allow the modulation of their activity by interactions with a range of ligands and receptors.

How α-1-Antitrypsin Became an Antithrombin

An intriguing report was published in 1978 of the finding in Pittsburgh, USA, of a severe bleeding disorder associated with the presence of a variant of α-1-antitrypsin [30]. Subsequent investigation [31] showed that the abnormality was due to the mutation of the methionine at the active centre of α-1-antitrypsin to an arginine, resulting in a complete change in its function, from being an inhibitor of elastase to that of a highly active inhibitor of thrombin (Fig. 1.2a).

This natural experiment in protein engineering, as well as showing the way in which a single mutation can completely change the function of a protein, also opened entirely new fields of understandings. The findings unequivocally settled controversies at the time as to the siting of the reactive centre of the serpins and elegantly illustrated how the inhibitory spectrum of the family could readily evolve from minor variations at the active site. Moreover, the demonstration that the variant Pittsburgh antitrypsin had an activity equivalent to that of heparin-activated

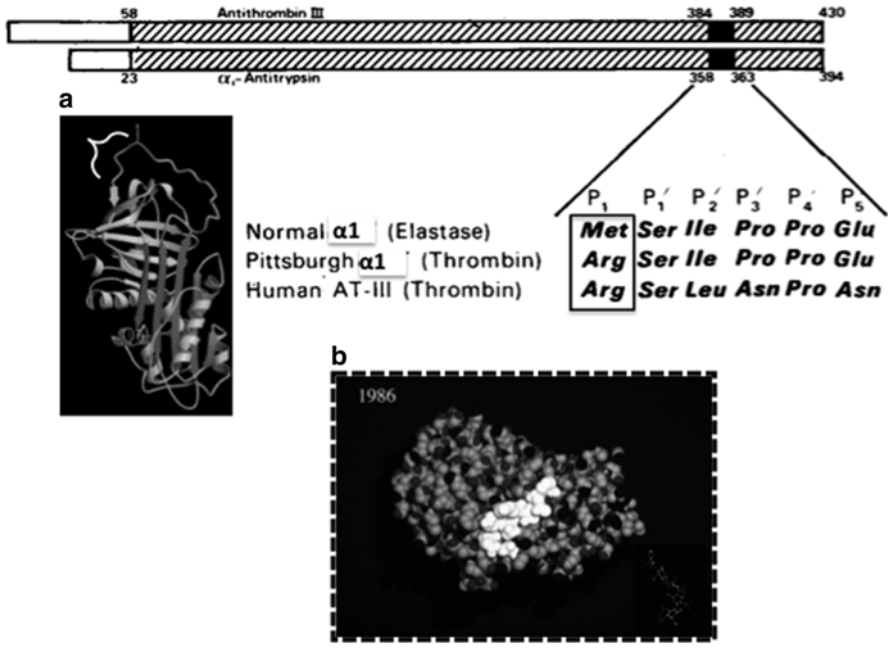

Fig 1.2 α-1-Antitrypsin as an archetype allowed the deduction of the mechanism of thrombin inhibition a decade prior to the solving of the structure of antithrombin. (**a**) At an early stage, when only the sequence but not the structure of α-1-antitrypsin was known, the finding of the mutation in the Pittsburgh variant (*boxed*) identified the active centre of both inhibitors and provided insights into the adaptive modulatory mechanism of antithrombin. (**b**) The heparin-binding site on antithrombin was unequivocally revealed a decade before the solving of the structure of antithrombin, by projection of conserved arginines and lysines onto the structure of antitrypsin. Heparin pentasaccharide, *shadowed bottom right*

antithrombin was the first evidence that heparin acts on antithrombin by releasing an otherwise suppressed inhibitory activity. This was a key to understanding how the anticoagulant function of blood is modulated by small movements of the reactive centre loop into and out of the main body of the antithrombin molecule, a mechanism that was found to be similarly utilised by other serpins to control a diversity of specialised functions. Yet another finding from this remarkable case opened a new field in biochemistry. The unexpected finding of proalbumin and other pro-proteins in the plasma of the Pittsburgh patient led to the first recognition of the nature of the propeptide-cleaving enzyme that has a key role in metabolism and endocrinology.

Ligand Activation of Serpins

Although not as yet known to be so with α-1-antitrypsin, the activities of many other serpins are affected by their interactions with ligands and receptors. Typically, the ligand binds to the serpin framework tightening its overall structure and hence affecting the movement of the intact reactive loop. In effect, the interaction with the ligand maintains the reactive centre 'protease bait' in an active exposed form.

PAI-1, Fibrinolysis and the Latent Transition The clearest example of this ligand-induced switching on-and-off activity is seen with the plasminogen activator inhibitor PAI-1, the serpin that regulates fibrinolysis by limiting the production of plasmin. The genetic deficiency of PAI-1 results in increased plasmin activity with consequent risk of haemorrhage, and its excess production results in decreased fibrinolysis with resulting thrombosis [32]. In normal function, circulating PAI-1 undergoes a ready transition to an inactive form, with the complete insertion of its intact reactive centre loop into the body of the molecule [33]. This conversion to an inactive latent conformation, which occurs spontaneously and reversibly in PAI-1, can also exceptionally occur, pathologically and irreversibly, in other serpins including α-1-antitrypsin. With PAI-1, however, the transition is reversible and so provides a physiological off-switch that maintains fibrinolytic activity in the circulation. When however the PAI-1 comes in contact with the extracellular matrix of the endothelium, it binds to vitronectin. The binding to this ligand expels the buried reactive loop and activates the plasmin-inhibitory activity of PAI-1 [34], with a consequent suppression of fibrinolysis.

Antithrombin Heparin and Thrombophilia A more subtle adjustment of activity occurs with antithrombin on its association with the complex polysaccharides that line the small vasculature and notably with their active component heparin. This interaction not only binds antithrombin to the endothelial surface but also activates it as an anticoagulant. Antithrombin differs from α-1-antitrypsin in that its reactive loop readily nudges in and out of the main beta-sheet of the molecule. In the circulating antithrombin, this equilibrated movement strongly favours partial insertion, with a consequently decreased inhibitory activity and hence an increased readiness

of coagulation. When however the antithrombin binds to the heparins lining the microvasculature, it changes to a full anticoagulant role, with the tightening of its structure and complete exposure of its reactive loop [35, 36]. The demonstration, now in video detail, of this activation by heparin fulfils the predictions decades before from the findings with the Pittsburgh variant of α-1-antitrypsin. In effect, heparin-activated antithrombin adopts the fully active conformation inherently present in α-1-antitrypsin and relevantly so in α-1-antitrypsin Pittsburgh.

Heparin is a heterogeneous mixture with its longer-chain forms containing sites that bind to and bridge both thrombin and antithrombin [36]. The shortest effective form however is a pentasaccharide. This binds to a highly specific site on antithrombin formed by the alignment on a surface helix of positively charged arginine and lysine side chains. Clinically, mutations at this site are a common cause of familial thrombophilia. The identification of this binding site on antithrombin further illustrates the critical part α-1-antitrypsin played in revealing features in other members of the serpin family. The binding of heparin occurs not only to antithrombin but also to three other heparin-binding serpins. Although in the 1980s the structure of antithrombin was unknown, the definitive recognition of the heparin-binding site was readily deducible from the precise alignment of the sequences of serpins, based on the template provided by the structure of α-1-antitrypsin. The alignment of serpin structures allowed the question: is there a conservation of arginine and lysines uniquely present in the four heparin-binding serpins? The result when projected on the framework structure of α-1-antitrypsin was stunning (Fig. 1.2b). It clearly defined the binding site of the heparin pentasaccharide, with unequivocal confirmation coming from mutations within the site identified in families with heparin-resistant thrombophilia [37].

Hormone Carriage: Serpins and the Modulation of Activity

When the initial listings of serpins in human plasma were made, three atypical members stood out. These were the non-inhibitory serpins: angiotensinogen and the two globulin carriers of thyroxine and corticosteroids, TBG and CBG. We now know that each of the three hormone carriers retains the serpin framework but, significantly, with inbuilt adaptations to allow the modulated control of hormone release. The findings raise the tantalising question, are there similar, as yet undetected, modulatory controls in α-1-antitrypsin?

TBG, CBG and the Mechanism of Modulation Thyroxine and corticosteroids bind to each of their carrier proteins in the circulation in a 1:1 ratio, and it was early realised that exposure to neutrophil proteases could trigger the S-to-R transition in both TBG and CBG, with the consequent massive release of the bound hormones [38]. The mechanism made sound physiological sense, as this would ensure hormone release at sites of greatest need, within the proteolytic milieu of loci of inflammation. And indeed this was demonstrably so. Yet this accelerated release in

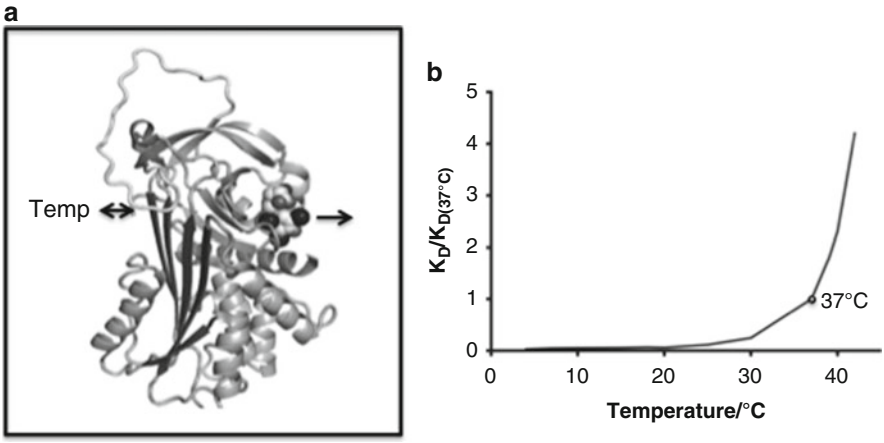

Fig 1.3 Hormone release from TBG and CBG, shown (**a**) in *side view. Upper left*, the exposed reactive loop nudging, as *arrowed*, in and out of the molecule and influencing the release of the bound hormone (space-filling depiction, *arrowed* on *right*). (**b**) The comparative decrease in binding affinity of CBG plotted against temperature shows the accelerated hormone release that will take place as the body temperature rises above 37 °C [42]

inflammation is an exception as for the most part the role of the circulating carriers is to supply hormones to healthy tissues. Until recently, it had been thought that such hormone release occurred passively, but the requirements of tissues inherently differ, between muscles and the brain, between being at exercise and rest and in hypothermia and fever.

An insight into the responsive way the hormone carriers meet these differential requirements came with the solving of the structures of TBG and CBG [39, 40]. The two structures are closely homologous, with identical external binding pockets on the exterior surface of each (Fig. 1.3a). The serpin framework in both TBG and CBG is strongly conserved and is identically superimposable on the framework of α-1-antitrypsin. Apart from the presence of the hormone-binding pocket, there is one other significant difference in the binding globulins as compared to α-1-antitrypsin, in that the main sheet of the molecule is partly opened. This is most markedly so in TBG, with the reactive loop being partially inserted into the sheet as is also seen in antithrombin. The striking similarity with antithrombin provided the clue to the mechanism influencing hormone binding and release in CBG as well as TBG, in that the mechanism is the converse of the interaction of heparin and antithrombin. With antithrombin, the binding of heparin tightens the molecule to favour the expulsion and hence activation of the reactive centre loop. TBG and CBG have a similarly conformed but inert loop, which, like that of antithrombin, can move in and out of the body of the molecule with an accompanying tightening and relaxing of the overall structure. But whereas in antithrombin this movement regulates inhibitory activity, in TBG and CBG, the accompanying tightening of structure modulates the release of the bound hormones. In this way, the flip-flop movement of the

loop in and out of TBG and CBG provides an equilibrated balance between higher and lower hormone-binding affinities. Such an elegant and balanced mechanism only makes sense in terms of a responsive modulation of hormone release to meet varying tissue or environmental needs.

Fever and a Serpin Thermocouple The tissue factors and receptors that influence differential hormone release from TBG and CBG are still unidentified. There is however one major mechanism that is well documented; this is the modulation in hormone-binding affinity that takes place with changes in body or tissue temperature. The affinity of binding of ligands to proteins in general decreases with increasing temperature, but this specifically occurs to a much greater extent with the hormone-binding globulins. In effect, TBG and CBG each act as protein thermocouples [39, 41–43]. The opening of the main sheet of the serpin molecule and the accompanying entry of the reactive loop is known to be temperature dependent. With TBG and CBG, this accelerated loop entry accompanying a rise in temperature will predictably result in a decreased affinity and hence an increased release of the bound hormone. Specifically, the structure of TBG shows how a triggering shift, as the loop enters the sheet, is transmitted directly to the binding site (Fig. 1.3a, b). Critically, a distinct drop in binding affinity will occur as the body temperature rises above 37 °C, such that a rise to 39 °C, as in fevers, will result with TBG in a 23 % increase in the free thyroxine concentration and with CBG in a doubling of the free cortisol concentration. This boosted release has clear physiological benefits, giving a raised release of cortisol in inflamed tissues and an increase in thyroxine delivery to meet the rise in metabolic activity that will accompany increases in body temperature.

Confirmation of the physiological significance of this thermally triggered increase in hormone release comes from two interactive mutations in TBG that have become established as a polymorphism [43]. The aboriginals of West Australia have lived for thousands of years in an environment where ambient temperatures consistently reach 40 °C and above. Under these conditions, the boost in thyroxine release as body temperatures reach above 39 °C, which in temperate climates is an advantageous response to inflammation, will historically for the aboriginal exacerbate the greater risk of heat exhaustion. The acquired polymorphisms in the aboriginal can be seen structurally to perturb the network of bonds that transmit the movement of the reactive loop to the thyroxine-binding site [39]. The effect of this is to recalibrate the protein thermocouple, with the boosted ‘fever’ release of thyroxine being cancelled, whilst the interactions of the two mutations otherwise maintain the physiological delivery of thyroxine.

Angiotensinogen and Redox Modulation Angiotensinogen, the source of the angiotensins that control blood pressure, is a plasma serpin that is not only a non-inhibitor but has also lost the ability to undergo the S-to-R conformational change. As with the other serpin hormone carriers, angiotensinogen also has an inbuilt mechanism to allow a responsive adjustment of hormone release. However, unlike the other carriers this modulation of activity is not dependent on reactive loop movements or other shifts in the serpin template but is a consequence of conformational adjustments in

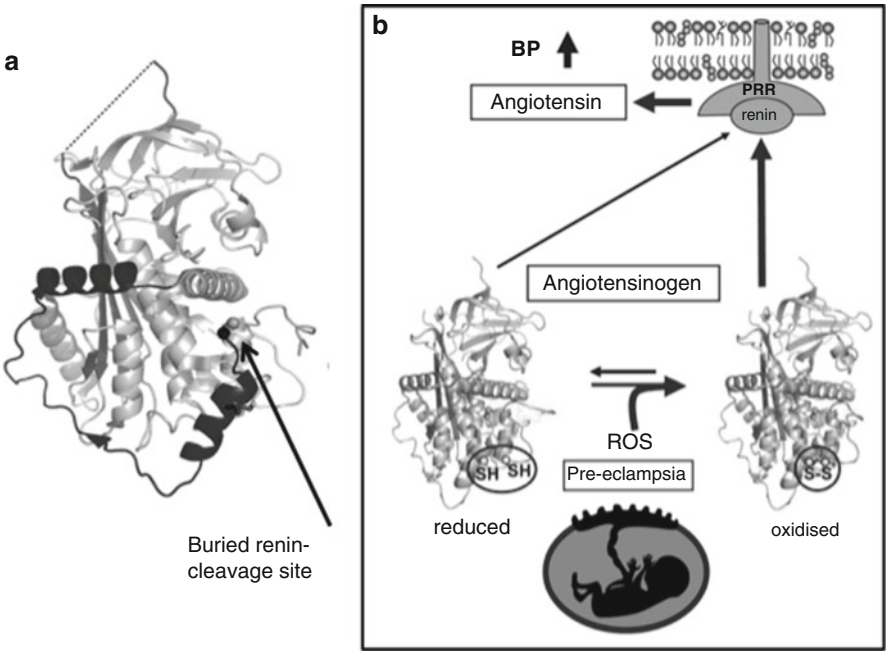

Fig 1.4 Angiotensinogen and the modulation of angiotensin release. (**a**) The amino-terminal tail of angiotensinogen, shown in *bold*, is firmly bound to the body of the molecule. The buried site that releases the terminal angiotensin I (*arrowed*) is revealed by a conformational change on binding to the enzyme renin. (**b**) Schematic, indicating how this renin-induced conformational change is affected by the oxidation of an S–S bridge, with a consequent effect on the efficiency of cleavage and hence the release of angiotensinogen. Although the modulating factor illustrated here is the release of reactive oxygen species ROS from the placenta, as in pre-eclampsia, other factors will influence the S–S bridging including nitrosylation by NO-releasing agents. *PRR* prorenin receptor

the amino-terminal tail of angiotensinogen [44]. This 61-residue extension contains the decapeptide angiotensin I, which is cleaved and released by a highly specific interaction with renin. Recent crystallographic structures show how the binding of the amino-terminal tail to the body of the circulating angiotensinogen inaccessibly obscures the angiotensin-cleavage site (Fig. 1.4). The interaction with renin, however, results in a major shift in the tail of angiotensinogen, to give the exposure of the otherwise buried site.

A key contribution to the coordination of this activating transition is provided by a disulphide bridge that links the amino-terminal tail of angiotensinogen to the body of the molecule. This disulphide bridge is labile, being readily broken by reduction, with some 60 % of the circulating angiotensinogen being in the bridged oxidised form and 40 % in the reduced unbridged form. The kinetics of cleavage of the two forms differs significantly, with the bridged oxidised form being a more effective substrate for cleavage and the release of angiotensin by renin. The proportions of

the bridged and unbridged forms vary however with tissue redox changes. In addition to this inbuilt ability of angiotensinogen to adjust its activity to oxidative changes, the susceptibility of the reduced bridge to nitrosylation will further allow the modulation of angiotensin release in individual tissues.

Evidence of the in vivo significance of such variations in activity comes from yet another experiment of nature, the finding of equivalent kinetic changes due to a mutation at the cleavage site in angiotensinogen [45]. The mutation was detected in five heterozygotes, each identified because of an association with hypertension and specifically with hypertension in pregnancy. Arising from this, the subsequent demonstration of markedly raised levels of oxidised angiotensinogen in pre-eclampsia now provides a persuasive explanation for the known association of hypertension in pregnancy with oxidative stress (schema: Fig. 1.4b).

Conclusion: Questions for α-1-Antitrypsin from the Serpins

Although the early studies of α-1-antitrypsin revealed its framework structure and unique inhibitory mechanism, subsequent studies of other serpins have shown the more subtle ways in which activity can be modified by tissue-specific receptors [46] and by changes in body temperature [42, 43]. Once again, key insights into these processes came from aberrations of function resulting in disease. The long list of disorders arising from mutations in the plasma serpins [24, 25] is now increasingly being added to by dysfunctions of the more recently characterised intracellular serpins, as with the collagen chaperone Hsp 47 and osteogenesis imperfecta and with the intracellular protease inhibitor SERPINB6A and familial hearing loss [47, 48].

Are there as yet undiscovered modulatory adaptations and dysfunctions in α-1-antitrypsin? Clues are likely to come from unexpected clinical leads. The non-committal label of the *α-1-antitrypsin syndrome* appropriately acknowledges that there is more to the ZZ clinical phenotype than just lung and liver degeneration [49]. The concentration of α-1-antitrypsin in the plasma at over a gram per litre is tenfold greater than that of any other plasma serpin and may rise several fold further in the acute phase of inflammation. What happens to the activity of α-1-antitrypsin as the body temperature increases in fever and pertinently does the acute phase response and a body temperature of 39 °C exacerbate the misfolding and intracellular aggregation of the variant Z that leads to liver cirrhosis? Another relatively unexplored part of the story of relevance to all the serpins is the catabolic pathway, with the turnover of α-1-antitrypsin notably being greater than a gram a day. What determines the senescence of the protein? What happens to it? Intriguing accounts in the earlier literature tell of high concentrations of the carboxy-terminal (leaving) peptide of α-1-antitrypsin in gallstones [50]. How could this happen? Examples of other challenges from the past that are yet to be followed through are the observations from Malmö of a 1:1 association of cholesterol with α-1-antitrypsin, putatively with the Z rather than M form [51], and of the studies in the USA of 30 years ago indicating the modulation of inhibitory activity by neutrophil oxidants [8, 10]. Overall, the lesson for α-1-antitrypsin from the serpins is that there is still much to be learnt.

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Chapter 2
Alpha-1 Antitrypsin: The Protein

Bethany Lussier and Andrew A. Wilson

Introduction

Alpha-1 antitrypsin (AAT), alternately referred to as alpha-1 proteinase inhibitor or alpha-1 protease inhibitor, is a member of the serine protease inhibitor (serpin) superfamily comprised of alpha-1 antichymotrypsin, C1 inhibitor, antithrombin, neuroserpin, and others [1, 2]. AAT is considered the major anti-elastase of the lower respiratory tract based on its unsurpassed ability to inhibit the serine protease, neutrophil elastase [3, 4]. In addition to its antiprotease activity, AAT has likewise been shown to have other biological effects, including the ability to modulate both inflammation and apoptosis [5, 6]. Mutant forms of AAT play a well-documented role in disease pathogenesis: misfolding of AAT protein, accumulation of misfolded protein polymers, and an associated decrease in secreted, functional monomers are known to cause clinical dysfunction and disease through both gain-of-function and loss-of-function mechanisms and are collectively known as "AAT deficiency." In this section, we review the protein's history, structural composition, regulation, and functional characteristics.

History and Classification

In 1963, the remarkable discovery was made by Laurell and Eriksson that serum protein electrophoreses of several individuals with severe obstructive lung disease of early onset lacked a band for alpha-1 globulin [1, 7, 8]. This missing electrophoretic band was later found to represent an inherited deficiency of a single protein with the

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capacity to inactivate serine proteases including trypsin, resulting in the designation "AAT" [8, 9]. Six years after its linkage to lung disease, Sharp and colleagues described an association between AAT deficiency and cirrhotic liver disease [10]. Since the mid-1970s, numerous AAT glycoforms have been documented by isoelectric focusing and used to identify deficiency states and phenotypes [11]. AAT glycoforms are classified based on differences in electrophoretic migration at acidic pH, with letters assigned to each variant in alphabetical order. Differences in this migration pattern depend primarily on the amino acid sequence, with heterogeneity of carbohydrate side chain modifications contributing to a lesser degree [9]. The most common normal AAT variant migrates a moderate distance and is designated "M," whereas the most common deficiency allele migrates further under the same conditions and is designated "Z" [9]. An individual inheriting one allele encoding each of these two variants would be referred to as "PiMZ" under the protease inhibitor ("PI") classification system. AAT is a pleiomorphic protein, with a large number of variants identified, including nine different glycoforms of the M-AAT protein (subtypes M0–M8) and at least six glycoforms of the Z-AAT mutant protein [3, 11–14].

Expression Pattern

AAT is a 52-kDa molecule produced primarily in liver hepatocytes and released directly into the bloodstream. The normal rate of synthesis is approximately 34 mg/kg/day leading to serum concentrations ranging from 150 to 350 mg/dL [3, 15]. While hepatocytes are the main source of circulating AAT, approximately 20 % of the total is produced by other AAT-producing cell types, a list that includes monocytes and macrophages, in addition to epithelial cells of the lung, kidney, intestine, pancreas, thymus, adrenal gland, ovary, testis, and corneum [16–24]. De novo synthesis has also been documented in some human cancers [25–28].

One-third of circulating AAT is degraded daily, and only a fraction of circulating AAT is transported into any given body compartment [3]. AAT is found in nearly all body fluids, including saliva, tears, breast milk, urine, and semen, with concentrations in bronchoalveolar lavage fluid reaching approximately 10 % of those in the circulation [15, 16, 23, 29–32]. AAT can likewise be found in feces with increased concentrations in the setting of inflammatory bowel disease [33, 34]. While local synthesis may contribute to body fluid or tissue AAT concentrations, it is generally believed that they are primarily determined by serum concentrations [15].

Transcription

The SERPINA1 gene encoding AAT is 12.2 kb in length and located on the long arm of human chromosome 14q31–32.3 [3, 35, 36]. The gene contains seven exons (designated Ia, Ib, Ic, II, III, IV, and V) and six introns [3, 37]. The 5′ untranslated

regions of AAT arise from the first three exons and a short 5′ segment of the fourth exon. The AAT protein sequence is encoded by 1434 base pairs from the fourth exon (exon II) and together with exons III, IV, and V [3, 9, 37]. The active site of the mature protein, encoded by the seventh exon (exon V), contributes specificity to the functional domain of the inhibitor [3, 9]. The transcriptional start site, found in exon Ic in hepatocytes, varies with cell type, yielding a longer mRNA transcript in monocytes where it is encoded by exon Ia [3, 9, 18].

Regulation of the AAT gene is thought to be predominantly at the transcriptional level in association with a hepatocyte-specific promoter, with posttranslational regulation playing a lesser role [21]. Expression is tissue specific and directed by structural elements within a 750-nucleotide region upstream of the hepatocyte transcriptional start site in exon Ic that contains hepatocyte-specific and nonspecific enhancer elements [3, 9, 38, 39]. Trans-acting factors, including HNF-1, HNF-3, and others, are able to bind these regions and thereby contribute to AAT expression [3, 9, 24, 38, 40].

Intracellular Trafficking and Posttranslational Modification

The processing involved in normal biosynthesis of AAT is classical, with transcription of the mRNA precursor from the AAT gene followed by splicing and translocation to the rough endoplasmic reticulum where translation occurs. The newly synthesized polypeptide chain is secreted into the cisternae of the rough ER and undergoes cleavage of its signal peptide. There, the AAT precursor undergoes dolichol phosphate-linked glycosylation at three distinct asparagine residues (numbers 46, 83, and 247) as the protein takes on a three-dimensional conformation [9]. These glycosylations have functional significance, as they help maintain solubility and allow attachment of protein-processing enzymes [41]. Glucosidase-based trimming of two glucose units produces a monoglycosylated oligosaccharide that is recognized by molecular chaperones [41–44]. Conformationally mature glycoproteins are then released from the protein-folding machinery while incorrectly folded forms undergo additional processing. Released glycoproteins are transported to the Golgi in coatomer protein complex II vesicles en route to the Golgi complex [41]. Terminal modification of the carbohydrate side chains within the Golgi complex produces a mature 52-kDa AAT protein, resistant to enzymatic cleavage by endoglycosidase H, which is then secreted [9].

Protein Structure

AAT shares both structural and functional characteristics with other members of the serpin family including alpha-1-antichymotrypsin, C1 esterase inhibitor, alpha-2 antiplasmin, protein C inhibitor, and antithrombin III [3, 9]. The fully processed,

mature AAT protein is a 394-amino acid peptide, 52 kDa in size, that includes three carbohydrate side chains [9]. These asparagine-linked side chains are composed of N-acetylglucosamine, mannose, galactose, and sialic acid arranged as a core with two to three branching "antennae" [9]. In addition to their contribution to differences in electrophoretic migration of AAT protein used in protein phenotyping, AAT glycosylations contribute to protein stability and half-life in the circulation [11, 13]. They have likewise recently been demonstrated to contribute to the anti-inflammatory capacity of AAT protein through negative regulation of IL-8-induced neutrophil chemotaxis [45].

The crystal structure analysis of AAT's three-dimensional conformation was described in 1984 by diffraction data and electron density mapping by Loebermann and colleagues [46]. Crystallographic analysis revealed a 6.7 nm × 3.2 nm globular protein with the three carbohydrate side chains on the outer surface, localized to one end. The AAT polypeptide chain is arranged into well-defined structural elements consisting of three beta-sheets (A–C) and nine alpha-helices (A–I), each formed by the first 150 residues. There are three internal salt bridges occurring within the molecule that have been implicated in folding and polymerization and subsequent deficiency states [9, 47, 48].

At the active site, a Met358–Ser359 bond is part of a highly stressed reactive center loop that if cleaved separates the two residues widely [9, 46] exposing the N-terminal region of the active site loop on two strands of beta-sheet A. It is this active site, designated "P1 residue", which is responsible for the functional capacity of the inhibitor as well as its specificity [49]. This contribution is dramatically illustrated by the so-called "Pittsburgh" AAT variant, in which a single amino acid substitution at this active site (358 Met → Arg) results in synthesis of a protein that is structurally similar to antithrombin III, another serpin family member, with significant thrombin inhibitory capacity manifested as a clinically significant bleeding disorder [50].

Circulating AAT

AAT is ultimately secreted as a 52-kDa single-chain glycoprotein; an average concentration of AAT is 150–300 mg/dL in circulating plasma [51, 52] and has a half-life of approximately 5 days [3, 51, 52]. About one-third of the intravascular AAT pool is degraded daily with some potential increase resulting in the setting of inflammation [3, 53]. Based on its tertiary structure, it was previously believed that AAT primarily trafficked across cell membranes and into most tissues via passive diffusion [4]. However, recent studies have demonstrated that AAT uptake occurs primarily via clathrin-mediated uptake resulting in transcytosis across the endothelial cell layer [54–56]. Protein trafficking has potential significance in disease pathogenesis as a mechanism by which cigarette smoking might alter local tissue AAT concentrations [55].

In the most common deficiency state, circulating AAT levels are reduced to 10–15 % of normal [52]. The strong correlation of bodily fluid AAT levels with circulating levels means that lung AAT concentrations decrease in parallel with

circulating levels in hereditary deficiency. While there is speculation that a gain-of-function mechanism, exerted either via extracellular, polymer-driven inflammation or direct cellular toxicity, could contribute to lung disease in AATD, alveolar destruction associated with disease has generally been considered to occur predominantly on a loss-of-function basis resulting from these low levels and their associated decrease in anti-elastase activity.

Protease Inactivation

AAT's most significant biological function is believed to be the inactivation of proteases, particularly in the lung. Although primarily recognized for its ability to neutralize neutrophil elastase (NE), AAT is also capable of inactivating other neutrophil proteases, including cathepsin G and proteinase 3 [57], as well as mast cell proteinase II [58]. It may also inhibit T-lymphocyte-derived serine esterases that are structurally similar to cathepsin G [59], though with lower binding efficacy [60].

NE is a highly active proteolytic enzyme formed in the promyelocytic phase of neutrophil maturation and stored in azurophilic granules for release along with other lysosomal oxidative enzymes upon neutrophil activation or death [61]. As depicted in Fig. 3.1, NE and other serine proteases recognize a 20-amino acid

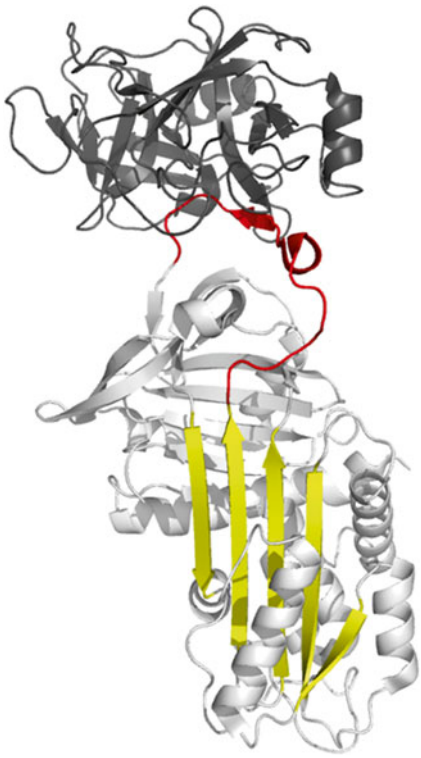

Fig. 3.1 Structure of the alpha-1 antitrypsin–trypsin complex. Ribbon diagram of alpha-1 antitrypsin (in *white*, *yellow*, and *red*) in non-covalent complex with trypsin (in *gray*). The active site of trypsin fits the reactive center loop of alpha-1 antitrypsin (*red*). Alpha-1 antitrypsin's beta-sheet A is designated in *yellow*. Figure courtesy of Bibek Gooptu, based on publications by Elliott PR et al. (*Nat Struct Biol* 1996;3:676–681) and Dementiev A et al. (*Journal of Biological Chemistry* 2003;278:37881–37887), as well as PDB ID 1OPH

"pseudo-substrate" within the AAT molecule that includes the reactive center loop [62]. Upon binding, AAT forms a rapid, strong association with NE with binding strength several orders of magnitude greater than other serine proteases and an association constant of $9.7 \pm 0.4 \times 10^6$ M^{-1} s^{-1} [63]. The binding of NE to AAT and its subsequent inactivation shares characteristic features with other serpin–enzyme pairs. Upon binding, NE cleaves AAT's reactive center loop, releasing stored potential energy and resulting in a conformational change in which NE is flipped to the opposite end of the AAT molecule. In the process, NE is distorted and catalytically inactivated [3, 48, 62, 64–66]. The resulting AAT–NE complex is then recognized by hepatic receptors and cleared from circulation [67].

The proteolytic capacity of normal AAT can be diminished in several circumstances. First, when exposed methionine residues are oxidized, the binding constant for serine proteases drops precipitously, significantly reducing functional protease activity [68–70]. Second, AAT can be cleaved and inactivated by the proteolytic activity of enzymes other than NE, including macrophage-derived metalloproteinase, neutrophil collagenases and gelatinases, and *Pseudomonas* elastase among others [60, 71].

Local and Systemic Fluctuations in AAT Secretion

Homeostatic mechanisms in response to tissue damage from infection or injury lead to characteristic changes in liver protein synthesis, known collectively as the acute phase response [72]. In this setting, IL-6-, TNFα-, and IL-1-driven STAT3 and NF-κB p65 (RelA) signaling result in increased hepatic production of a group of so-called "acute phase reactant" proteins. AAT, together with c-reactive protein, ferritin, complement factors, and others, is significantly upregulated, while that of negative acute phase proteins including cortisol-binding globulin, transferrin, and albumin are decreased [73, 74].

Circulating blood AAT levels in the acute phase have been found to increase three- to fourfold [75]. In addition to the acute phase response, other inflammatory settings including pregnancy, trauma, surgery, malignancy, and treatment with oral contraceptives can also increase tissue AAT levels [76–80]. Finally, there is some evidence that circulating granulocytes could contribute to local AAT levels through the transcription, storage, and release of AAT following migration into tissues [45, 81, 82]. While some studies have demonstrated that AAT is stored in secretory vesicles within neutrophils [81, 82], more recent data suggests that approximately 80 % of neutrophil-associated AAT is localized to the cell membrane within lipid rafts, where it colocalizes with FcγRIIIb [45].

Anti-inflammatory and Anti-apoptotic Effects of AAT

In addition to its primary role in protease inactivation, AAT has been demonstrated to exert both anti-inflammatory and immunomodulatory effects in a variety of conditions and cell types, in some cases independent of its antiprotease activity. Effects

on activation and trafficking of multiple immune cell types have been documented, including neutrophils [45], mast cells [83], macrophages [84], and T cells [85]. Of these, the interactions between AAT and neutrophils have been most thoroughly evaluated. AAT inhibits neutrophil chemotaxis through two independent mechanisms: first, it forms a complex with IL-8, blocking its ability to bind CXCR1 and induce downstream AKT phosphorylation and subsequent cytoskeletal rearrangement that is necessary for neutrophil chemotaxis [45]. In a second mechanism, AAT blocks the ADAM-17/TACE-mediated shedding of FcγRIIIb in response to stimulation by soluble immune complexes [45], a process integral to neutrophil chemotaxis.

Studies of organ ischemia or cigarette smoke exposure have demonstrated attenuation of neutrophilic infiltration of the kidney, liver, and lung [86–89] as well as a reduction in cell death due to simulated myocardial infarction [90] in the setting of supplementation with exogenous AAT. Collectively, these studies have likewise demonstrated direct anti-apoptotic effects of AAT, as well as inhibition of caspases and anti-inflammatory effects characterized by attenuation of increases in local or systemic TNFα. Similarly, in animal lung transplant models, treatment with AAT reduced ischemic-reperfusion injury by inhibiting neutrophilic infiltration of the lung together with reductions in cytokines including IL-1α, IL-4, IL-12p70, MCP-1, and TNF-α [91, 92].

Intriguingly, many of the anti-inflammatory properties of AAT appear to be independent of its antiprotease activity. Churg and colleagues demonstrated that oxidized AAT, lacking in antiproteolytic capacity, attenuated silica-induced increases in lung MCP-1 expression, MIP-2 α, activation of NF-κB, and associated neutrophilic migration into the lung [93, 94]. More recently, Jonigk and colleagues demonstrated that a recombinant form of AAT lacking elastase inhibitory function exhibited anti-inflammatory properties in LPS-challenged mice (decreased infiltration of neutrophils, decreased BAL TNF-α and KC, decreased lung tissue expression of DDIT3 and XBP-1) as well as in freshly isolated human blood neutrophils (decreased TNF-α and IL-8 secretion ex vivo) [93, 94]. While antiprotease activity may not be necessary for AAT-mediated effects on inflammation, proper protein glycosylation does seem to be significant: recombinant AAT lacking glycosylations is unable to bind and modulate IL-8 and may lack other associated anti-inflammatory capacity [11, 45, 95]. This finding has significance since recombinant AAT protein, currently under consideration for clinical use as an inhaled therapy, lacks these glycosylations and therefore may not fully recapitulate the functional capabilities of the native human protein [45].

In addition to its effects on inflammation, AAT has been demonstrated to inhibit apoptosis through blockade of caspase activation in a variety of settings. Exogenously administered AAT has been found to prolong pancreatic islet allograft survival in mice, while related in vitro studies demonstrated its inhibition of TNF-induced apoptosis in murine insulinoma cells and associated abrogation of caspase-3 activation [96, 97]. Petrache and colleagues demonstrated that human AAT transgene expression effectively protects against apoptosis-induced emphysema caused by VEGF receptor blockade in mice [98]. This effect was associated with a decrease in

caspase-3 activity, a decrease in apoptosis, and a decrease in markers of oxidative stress. Additional work by the same group demonstrated that AAT and caspase-3 colocalize intracellularly and that AAT-mediated abrogation of caspase-3 activity requires an intact reactive center loop [5], in contrast to its anti-inflammatory effects as outlined above.

Finally, AAT may have additional properties as an inhibitor of HIV-1 infection. In vitro studies have demonstrated that AAT reduces HIV-1 infectivity and blocks HIV-1 production, an effect associated with suppression of the transcription factor NF-κB. Additional work has subsequently demonstrated that this protective effect is exerted by the C-terminal 26-residue peptide fragment of AAT, also referred to as "VIRIP" or virus-inhibiting peptide, which blocks HIV-1 cell entry through binding and inhibition of the HIV gp41 fusion peptide [99]. Chemically synthesized variants of VIRIP have now been studied in patients and found to inhibit viral replication in HIV-infected individuals [100].

Together, these putative immunomodulatory and antimicrobial properties have driven research exploring potential novel therapeutic applications. Some examples include the use of AAT in the setting of inflammation and injury associated with diabetes mellitus, organ transplant rejection, reperfusion injury, asthma, and infectious diseases [77, 86, 91, 96, 100–106].

Summary

In summary, a great deal has been learned about this protein since its discovery 50 years ago, but many important questions remain. There is increasing awareness of a functional role for AAT that extends beyond its capacity to inactivate serine proteases, but the import of this role in persons with either normal or abnormal AAT genotypes/phenotypes is not yet well understood. Active research in this area, together with research examining protein trafficking within cells and across cell membranes, is likely to yield insights in the immediate future that will further our understanding of the role that this multifunctional protein plays in health and disease. Finally, it is anticipated that development of new, human cell-based models for studying AAT biology is likely to enhance the progress of research programs such as these in the years to come.

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Chapter 3
Misfolding and Polymerisation of Alpha$_1$-Antitrypsin: Conformational Pathology and Therapeutic Targeting

Bibek Gooptu and David A. Lomas

Introduction: Misfolding and Polymerisation Are Central Events in the Pathogenesis of α_1-Antitrypsin Deficiency

Abnormal behaviour of the α_1-antitrypsin polypeptide chain in pathophysiological conditions is at the root of the disease mechanism of α_1-antitrypsin deficiency [1, 2]. This is mediated by degradation of misfolded protein and by polymerisation [3]. In α_1-antitrypsin deficiency and other serpinopathies—conformational diseases due to mutations in members of the serpin (*ser*ine *p*rotease *in*hibitor) protein superfamily—pathology results from the combination of gain- and loss-of-function effects [4].

This understanding arises directly from the observations in patients with the condition. Initial characterisation related the severe deficiency of circulating α_1-antitrypsin, which results from misfolding and polymerisation, to emphysema [5, 6]. A powerful concept was inferred: loss of inhibitory function against neutrophil elastase disrupts a physiological protease–antiprotease balance that is responsible

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for lung tissue homeostasis. The association of α_1-antitrypsin deficiency with endoplasmic reticulum (ER) inclusion bodies and cirrhosis in the liver, an organ in which the protein had no major homeostatic function, further suggested the aggregated protein had deleterious effects [7]. Inclusion bodies result from the entanglement of highly flexible α_1-antitrypsin polymers, and so the toxic gain of function within liver cells is attributed to the polymerisation process [8].

In general, an excessive load of misfolding protein can overwhelm the local quality control mechanism of ER-associated degradation (ERAD) [9]. This leads to ER stress and unfolded protein response (UPR) repertoires that are implicated in multiple diseases [10, 11]. A rare variant of α_1-antitrypsin ($Null_{HongKong}$) is entirely unable to fold or polymerise due to a missense mutation that results in premature termination and a polypeptide lacking 60 C-terminal resides, and evokes exactly these responses [12, 13]. The ability of the more common Z (Glu342Lys) variant associated with severe disease to trigger ER stress and the UPR is somewhat contentious at present. It is reported differently by different groups and in different cell types [3, 14, 15]. The responses seen in PiZZ hepatocytes are likely the most relevant to liver disease [15]. Hepatocytes remain challenging to characterise ex vivo, and to culture in vitro. Cell lines that recapitulate the cellular and microscopic phenotypes of hepatocyte handling of M (high secretion), Z (inclusion bodies, low secretion), and $Null_{HongKong}$ (degradation, no inclusions, no secretion) α_1-antitrypsin expression have therefore been developed [16]. Data from hepatocytes and these models indicate that expression of Z α_1-antitrypsin primes cells for ER stress and the UPR such that they are sensitised to a "2nd hit", but does not itself trigger these repertoires [17, 18]. This is associated with sufficient misfolding of α_1-antitrypsin for ERAD to break down 70 % of the mutant polypeptide that is synthesised [3]. In other PiZZ cells, e.g. monocytes, direct activation of ER stress and the UPR is reported [14]. These responses could therefore be of pathological significance outside the liver, but this remains to be clarified.

The presence of extracellular polymers in patient plasma and media from cultured cells expressing mutant α_1-antitrypsin has been described previously [3, 19, 20]. However, the degree to which circulating Z α_1-antitrypsin is in a polymeric rather than a functional, native state has only recently been appreciated [21]. Z α_1-antitrypsin is present at only ~15 % of normal (PiMM) levels in PiZZ individuals, and the native monomer has reduced functional activity compared with the wild-type (M) protein [22]. The additional loss of function associated with substantial polymerisation is likely an important factor in the predisposition to emphysema. Indeed in PiZZ individuals, α_1-antitrypsin polymers have been found in both BAL and explanted lung interstitium [23]. They are also found in affected tissues in the rarer disease associations of panniculitis (subcutaneously) [24] and ANCA-associated vasculitis (renal glomeruli) [25]. These findings can be causally linked with pathogenesis as a result of local loss of function and hence excessive protease activity. In the case of panniculitis, this mechanism is further supported by published clinical experience indicating the efficacy of α_1-antitrypsin replacement therapy in what is otherwise a relatively severe, treatment-refractory condition [26–28]. Moreover, various lines of research support pro-inflammatory

gain-of-function effects of α_1-antitrypsin polymers [1, 23], in contrast to anti-inflammatory effects of the native protein [29–32], and these may further exacerbate disease progression.

Folding Interrupted: Metastability and Function

Hepatocytes face two seeming challenges in producing functional α_1-antitrypsin: quantity and quality. The task of generating 1–2 g/days of secreted protein is apparently compounded as the active, native conformation does not represent the most stable globular fold (latent conformation) of the polypeptide chain in physiological conditions (Fig. 3.1) [39, 46]. Instead, during folding it becomes kinetically trapped in a so-called metastable state. This can be considered as a local free energy minimum in the global folding landscape of the α_1-antitrypsin polypeptide chain. The potential represented by the free energy difference between the metastable state and fully stabilised conformers is used in the functional mechanism to trap its target serine protease, elastase (Fig. 3.2).

Functional conformational change can therefore be seen as a final step of an interrupted folding process. However, the metastability of native α_1-antitrypsin necessarily presents the possibility of alternative conformational transitions and folding steps that confer similar stabilisation on the protein. Despite its metastability, folding of the wild-type (M) polypeptide to the native state is a reasonably robust property of the polypeptide chain in cell-free conditions. A proportion of denatured protein refolds to the native state, although extensive aggregation is also observed [43, 44, 47]. Moreover, given a complementary pair of 2-chain constructs the "heavy chain" residues 1-334Cys can template folding of the "light chain" 335Cys-394 to the native conformation [48]. This relatively hardwired tendency to adopt the native state is conserved within the serpin superfamily of proteins. Plasminogen activator inhibitor (PAI)-1 spontaneously adopts the far more stable latent state; however, after denaturation it first repopulates the native conformation [49, 50]. A similar process is observed in a complementary-paired 2-chain construct of ovalbumin [51]. Ovalbumin is not ordinarily able to undergo intramolecular insertion of its reactive loop into β-sheet A and so self-stabilise. However it can do so if, as in this construct, the P14 residue is mutated to Thr.

In cells, the ability of native serpins to persist in a metastable native state is arguably more surprising still. Cells contain many chaperone molecules whose principal role is to shuffle proteins out of metastable conformations that are kinetically trapped in local minima within the free energy landscapes that determine folding pathways [52, 53]. Their action facilitates folding to stabilised states, and degradation is the general fate of polypeptides that do not achieve this. Based on current understanding, several mechanisms could plausibly favour population of the metastable native state over the highly stable latent conformation (Fig. 3.1). The relative kinetics of the secretory pathway and native-latent conformational change might favour export of native serpin from the ER folding compartment before the latent

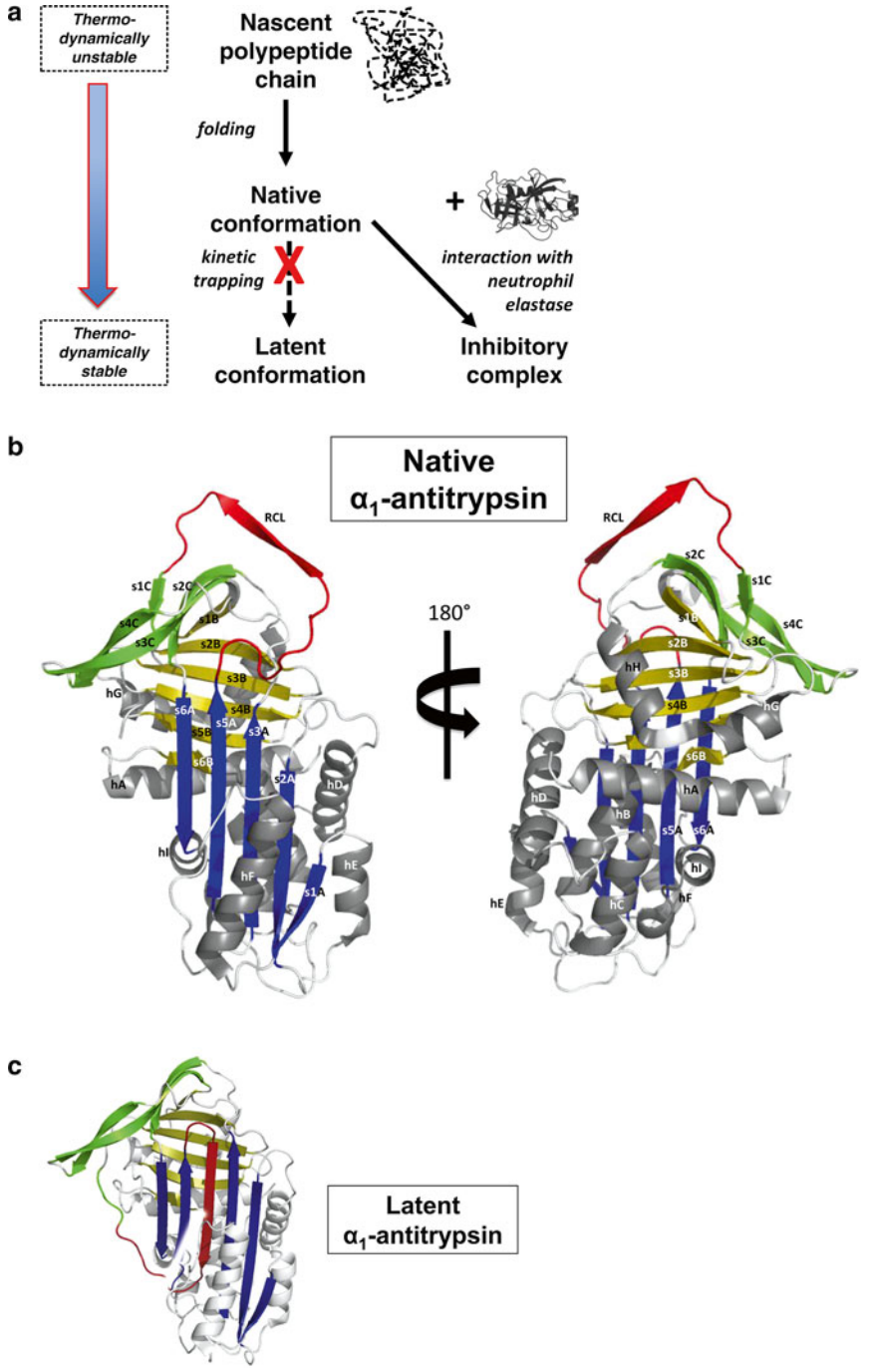

Fig. 3.1 Structures of native and latent α_1-antitrypsin. (**a**) Overall scheme of α_1-antitrypsin folding and function. The folding of the α_1-antitrypsin polypeptide chain (nascent state represented by *dashed* trace) is kinetically trapped in the monomeric, metastable, native state (**b**) rather than the

state was adopted. Alternatively, ER chaperones could mediate stabilisation of the native state. Despite the relative stability and foldedness of the latent state, it is also conceivable that it could be targeted for ERAD. Recent studies of α_1-antitrypsin folding in cells systematically blocked each of these mechanisms [35]. Surprisingly, however, neither preventing ER-to-Golgi transport, nor saturating ER chaperone systems, nor blocking degradation promoted formation of the latent conformer by M or Z α_1-antitrypsin. It therefore remains unclear how the folding pathways of α_1-antitrypsin and other serpins bypass general principles to avoid metastable states in vivo.

Aside from these studies, α_1-antitrypsin folding has been investigated predominantly by denaturant or refolding studies of polypeptide chain behaviour in cell-free conditions. Kinetic and equilibrium studies have been undertaken. Kinetic studies follow the process of unfolding or refolding and so provide information on rates of folding between unfolded, detectable intermediate, and folded states. Equilibrium studies establish ensemble characteristics of solution equilibria across a denaturant titration. In both cases, the interpretation of observations can be complicated by any tendency of the protein to aggregate in denaturing conditions. Both approaches have resulted in data consistent with the population of one or more intermediate states along unfolding or refolding pathways. Equilibrium studies support maximal population of intermediate ensembles at 4.0 M urea or 2.0 M GuHCl [41, 54, 55]. Kinetic studies have reported data consistent with an intermediate state along the unfolding pathway and two intermediates within refolding studies; of these the more folded

Fig. 3.1 (continued) more thermostable monomeric latent conformation. The latent state (**c**) can be induced by incubation with citrate at low protein concentration [33]. It can also occur more readily in the presence of multiple mutations that perturb the kinetic trapping of the native conformer [34]. However, in general, conditions within the ER and Golgi environments in which the protein folds and is trafficked in vivo appear to strongly resist latency [35]. Instead the potential for further stabilisation of the native conformer is utilised in its inhibitory interaction with its target protease (*black*). Formation of the final complex (Fig. 3.2) is associated with similar thermodynamic stabilisation to adoption of the latent conformation. (**b**) X-ray crystal structure of native α_1-antitrypsin at 1.8 Å resolution (PDB ID: 3NE4 [36]). The views are related by 180° rotation about the long axis of the protein. The reactive centre loop (RCL) is shown in *red*, β-sheet A is coloured *blue*, β-sheet B in *yellow*, β-sheet C in *green*, and α-helices (helix (h)A-hI) coloured *grey*. β-strands within β-sheets are labelled as per convention in the form: s(trand) followed by the number within, and then the name of, the β-sheet (e.g. s1A—strand 1 of β-sheet A). The high-resolution data that are contained within such crystallographic structures provide unparalleled detail, but they must be regarded as "snapshots" of the solution behaviour of native α_1-antitrypsin. Complementary methods, such as tryptophan fluorescence [41], native mass spectrometry (MS) [42], and NMR spectroscopy [43], demonstrate lability of various secondary structure elements (e.g. hF and β-sheet C) in native α_1-antitrypsin. Changes in such structural dynamics (increased lability or kinetic instability within the native and/or nascent chain conformational state ensembles) induced by disease mutations are likely key mediators of disease behaviour [44, 45]. (**c**) X-ray crystal structure of latent α_1-antitrypsin at 2.2 Å resolution (PDB ID: 1IZ2). In this conformation, the reactive loop inserts into β-sheet A in the absence of any interaction with a protease. To accommodate such reactive loop insertion, its C-terminal section and the contiguous sequence that forms s1C (*green*) in the native state are stretched between the lower pole of the molecule and the *N*-terminus of s4B

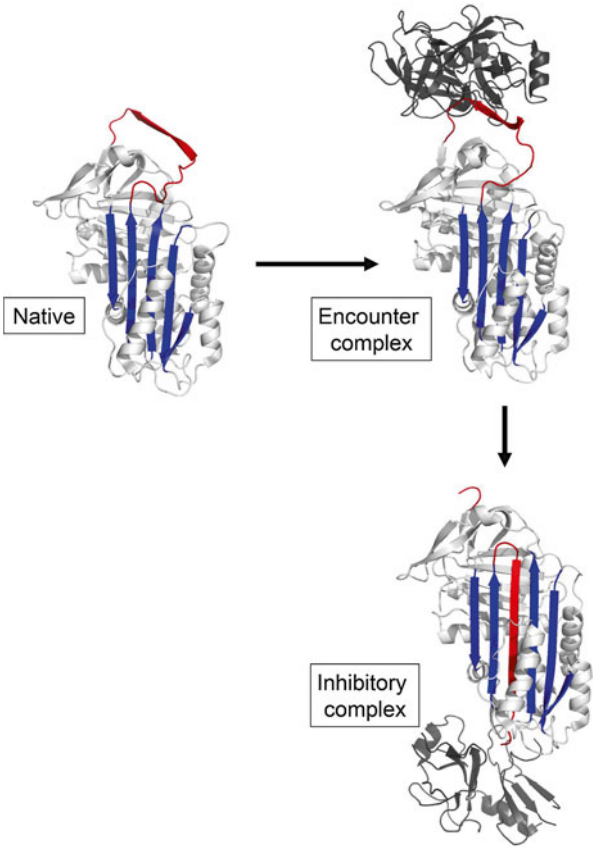

Fig. 3.2 Functional mechanism. *Upper panel*, *left*: *Native conformer* [36, 37]. The residues and conformation of the reactive centre bond (termed P1–P1′) of α_1-antitrypsin within its exposed reactive loop motif (*red*) represent an ideal substrate "bait" for the target serine protease, human neutrophil elastase. *Upper panel*, *right*: *Encounter complex* [38]. The target protease (*grey*) docks as shown in an initial non-covalent complex. The catalytic triad of residues in the elastase active site then cleaves between P1 and P1′ via nucleophilic attack, in association with the formation of an alternative covalent bond between P1 and the enzyme active site. The covalent enzyme–substrate complex is an obligate intermediate in the serine protease's catalytic mechanism that results in peptide bond hydrolysis. *Lower panel*: *Final covalent complex* [39, 40]. The reactive loop then inserts as a central 4th strand in the underlying β-pleated sheet A (*blue*). Loop insertion is strongly favoured in terms of thermodynamic stabilisation; some of the energy released is used to distort the catalytic triad of the serine protease such that it cannot break the covalent bond with α_1-antitrypsin to extricate itself

intermediate demonstrated some functional activity [56]. A number of studies have probed intermediate ensembles with the aid of assays that label or cleave intermediates with particular characteristics [44, 55, 57, 58]. Whilst some qualitative comparisons of observed behaviours between different states may be drawn, the propensity of such methods to stabilise the states they report upon will tend to

conformationally select for them within the ensembles [45]. There is therefore an inherent risk of skewing results to over-report some intermediate behaviours rather than others. A further concern arises from the tendency of partially folded α_1-antitrypsin to aggregate, since this may itself contribute an apparent signal. It is therefore ideal if observations interpreted in terms of folding or unfolding can be shown to be independent of α_1-antitrypsin concentration. This will support the lack of confounding effects from aggregation that will tend to increase at higher concentrations.

Studies involving denaturant conditions have raised interesting hypotheses about the normal folding of α_1-antitrypsin. However, a key message may be the apparent sensitivity of the polypeptide's solution behaviour to the experimental unfolding/refolding conditions that caveat the direct application of findings to the situation in cells [59]. It will therefore be important to develop improved methods for understanding α_1-antitrypsin folding in vivo, in the absence of chemical denaturant, and in the presence of ER protein folding quality control mechanisms. The recent development of in-cell NMR spectroscopy methods to study folding in cells may assist this goal [60].

Polymerisation: A Misfolding and/or a Post-folding Phenomenon?

Misfolding and polymerisation of α_1-antitrypsin both contribute to loss-of-function consequences of α_1-antitrypsin deficiency. Defining the structural mechanism of α_1-antitrypsin polymerisation in disease is therefore important as it appears to be a key target for novel therapeutic strategies that can address both loss- and gain-of-function mechanisms in disease [36, 59, 61, 62]. It is also a very logical goal given the powerful contribution that structural studies, in particular X-ray crystallography, have made to understanding serpin biology in unprecedented detail. However, crystallography is best suited to characterising relatively stabilised homogeneous states since these are required to form sufficiently ordered crystal lattices for X-ray diffraction. Pathological polymerisation is a dynamic process, occurring in solution and proceeding via an unstable intermediate state to give highly flexible polymers extended to a range of different lengths. Furthermore, in vitro the behaviour of α_1-antitrypsin is highly influenced by experimental conditions. Crystal structures therefore tend to report indirectly on polymerisation in disease. These factors likely explain the fact that multiple modes of polymerisation are described in the literature, supported by X-ray crystallographic, biophysical, and biochemical data [4] (Fig. 3.3). Linkage mechanisms observed to involve single β-strand linker motifs (Fig. 3.3a) include reactive loop insertion as an s7A strand [66], at the s1C position [65, 69], as well as at the s4A site of physiological loop insertion [8, 67]. Of these only insertion of a reactive loop at the s4A site appears to be a plausible candidate relevant to the α_1-antitrypsin polymers described in vivo in disease as the others dissociate to monomeric species in solution. However, α_1-antitrypsin polymers in disease are composed of intact rather than cleaved subunits.

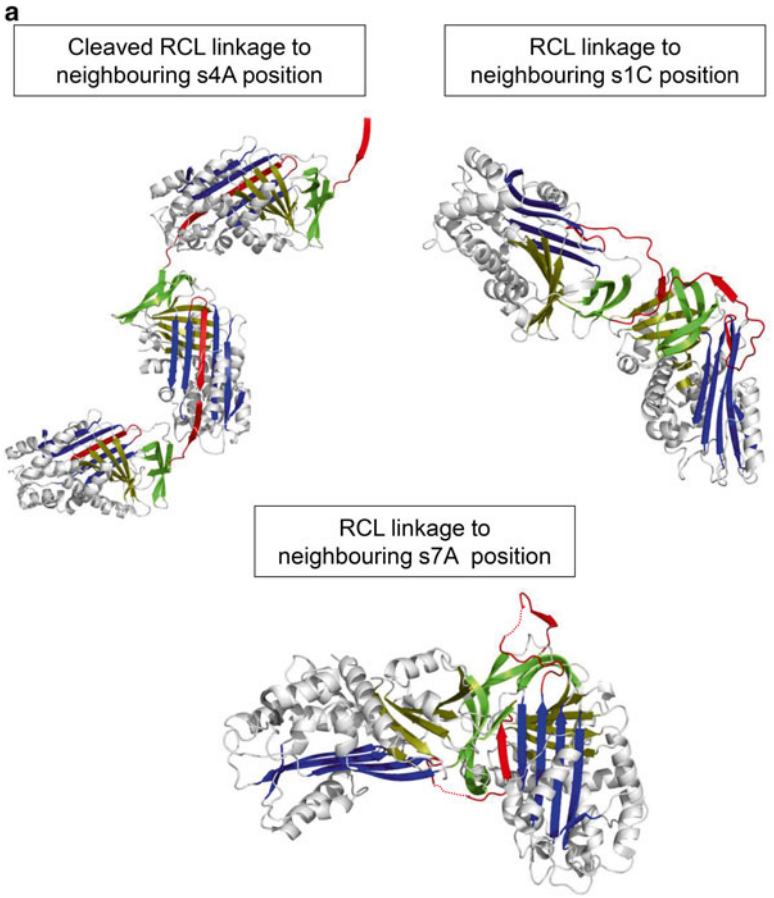

Fig. 3.3 Different models of α_1-antitrypsin polymerisation. (**a**) Different single-strand linkages observed crystallographically. *Upper left*, via insertion of reactive loop cleaved between P7 and P6 residues to s4A position. Intramolecular insertion occurs into P7; neighbouring subunits complement this to fill the lower s4A site by insertion from P6. Observed in α_1-antitrypsin [63, 64]. *Upper right*, via reactive loop insertion to replace an extruded s1C. Observed in antithrombin native-latent dimer [65], here modelled as for α_1-antitrypsin where partial loop insertion is sufficient to pull s1C away from the rest of β-sheet C. *Lower panel*, via reactive loop insertion as an extra strand in β-sheet A (s7A). Observed in native plasminogen activator inhibitor-1 (PAI-1) [66]. (**b**) Three alternative models of pathological polymerisation involving intact reactive loop-s4A insertion in association with different intermolecular β-strand complementation motifs. Different models consistent with structures of a deficiency variant of α_1-antichymotrypsin [67] (s4A single-strand model), wild-type antithrombin in low salt conditions [57] (s4A + s5A β-hairpin model, s5A in magenta), and α_1-antitrypsin constrained by disulphide bond formation [68] (s1C + s4B + s5B triple-strand model)

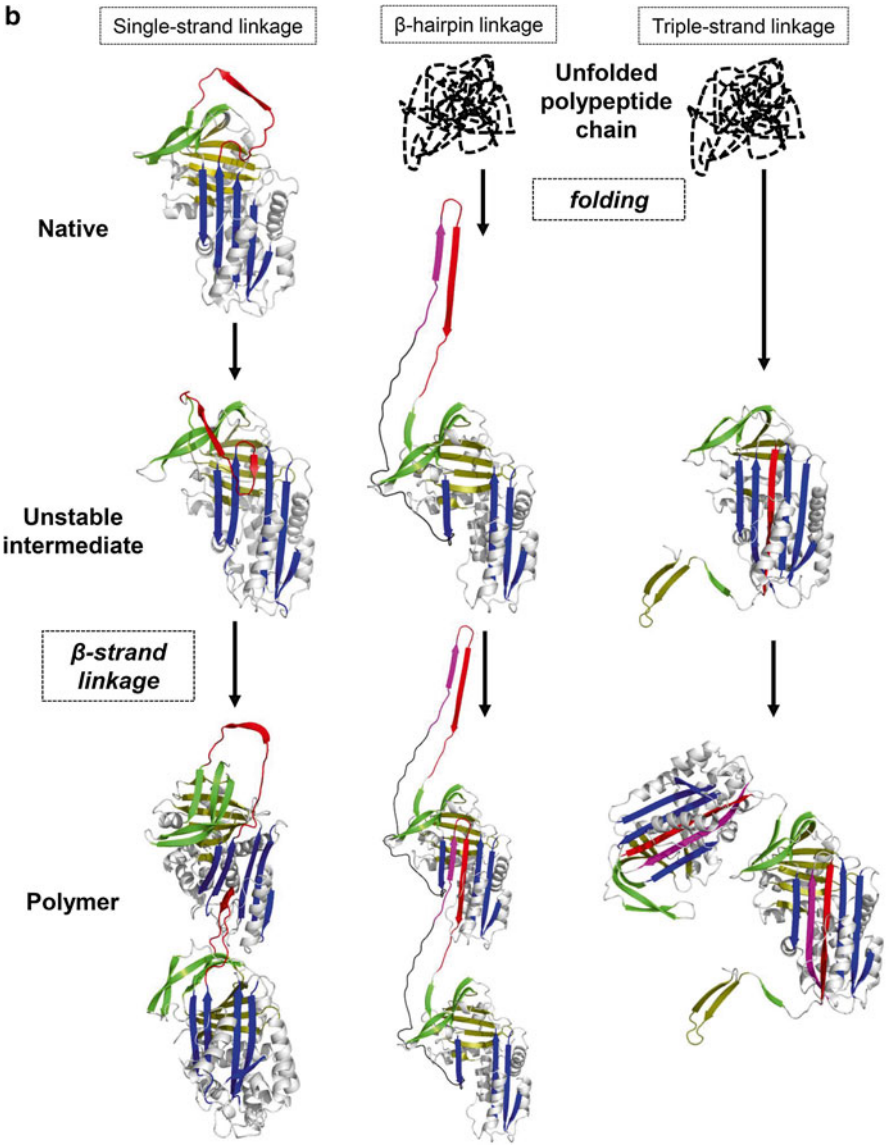

Fig. 3.3 (continued)

In recent years, most evidence has been adduced in support of three models of polymerisation (Fig. 3.3b and Table 3.1). The polymers form via intermediates that are folded to differing degrees relative to the native state. The intermediate in the triple-strand linkage model has a hydrophobic C-terminal sequence of 52 residues fully extruded into solution, in association with full intramolecular insertion of the reactive loop into β-sheet A [68]. This role of the C-terminal is of interest since

Table 3.1 Principal models of α_1-antitrypsin polymerisation currently proposed to represent the disease state

Model	How derived	Structural features	Supporting information
Single-strand linkage ("loop-sheet", "classical")	Extrapolated from X-ray crystal structure of the δ conformer of Leu55Pro α_1-antichymotrypsin [67]	Native-like intermediate characterised by partial intramolecular insertion of reactive loop into upper s4A position with opened lower s4A site. Polymer linkage via intermolecular insertion of reactive loop of one molecule into s4A of neighbouring subunit	Simplest model analogous to structures of stabilised monomeric states Peptide insertion into upper s4A site induces serpin polymerisation [70] Ion-mobility MS studies indicate dimer of 2C1 polymers formed from plasma M or Z α_1-antitrypsin has features most consistent with this model [71] NMR spectroscopy of mild deficiency variant indicates intermediate ensemble from which 2C1-positive polymers arise is highly native-like [45] Detailed biophysical studies of how Asp mutations in the reactive loop block intra- and intermolecular polymerisation steps [72]
β-hairpin linkage	Extrapolated from X-ray crystal structure of a self-terminating dimer of antithrombin formed at low salt conditions [57]	Highly unfolded intermediate characterised by extrusion of s5A to form a β-hairpin motif with s4A. To form extended linear polymers requires concomitant unfolding of hI as well as complementation of neighbouring subunit by β-hairpin insertion	Data from guanidine denaturation studies [57, 58] Requirement for high degree of unfoldedness consistent with polymers forming within the folding compartment (ER) However, polymers not well recognised by 2C1 monoclonal antibody [71]
Triple-strand linkage	Extrapolated from X-ray crystal structure of a self-terminating trimer of α_1-antitrypsin [68]	Intermediate state characterised by extrusion of a C-terminal sequence including s1C, s4B, and s5B that forms the complementary insertion motif between subunits. In the polymer, stability is achieved by intramolecular insertion of the reactive loop into the s4A position. This could occur before or after intermolecular linkage	Mutagenesis studies of formation of biochemically similar self-terminating trimers formed in yeast cell cytoplasm [68] Self-terminating trimer is recognised by 2C1 monoclonal antibody Similar C-terminal motif appears important in α_1-antitrypsin folding in studies of a two-chain construct [48]

insertion of this region has been proposed as a final step in folding to the native state in vitro [48, 54]. On the other hand, the intermediate and protomers of the single-strand linkage model have the most native-like structure [67, 71]. The modes of polymerisation that proceed via relatively unfolded states therefore seem less likely to occur physiologically (i.e. in the absence of chemically denaturing conditions) once the α_1-antitrypsin polypeptide has achieved the native conformation.

The α_1-antitrypsin intermediate conformer proposed for the single-strand linkage model is derived from the crystal structure of the δ conformer spontaneously adopted by a deficiency variant of α_1-antichymotrypsin [67]. It is consistent with data supporting the relevance of conformational change from the native state to polymerisation of Z α_1-antitrypsin and other deficiency variants in physiological conditions [8]. Polymers formed in denaturing conditions favouring β-hairpin linkage are not well recognised by the 2C1 mAb [71]. This recognises α_1-antitrypsin polymers found within hepatocyte ER and plasma in disease and those formed by heating, whose properties are most consistent with single-strand linkage [72]. However, a triple-strand linked, self-terminating (cyclo-trimeric) α_1-antitrypsin species is also well recognised by 2C1 [68]. These data suggest that single- and/or triple-strand linkage may underlie the polymerisation seen in disease.

The most straightforward approach to relate an X-ray crystal structure to a polymerisation mechanism is to extract the stabilising motif to model a preceding monomeric intermediate state. This is the basis for all the mechanisms discussed so far. An ingenious exception to this relates data from unfolding/refolding studies of α_1-antitrypsin in vitro to triple-strand linked polymer formation. This proposes that the intermolecular linkage (of s1C, s5B, and s6B strands) precedes final stabilisation through intramolecular insertion of the reactive loop in the s4A position.

The apparent contradictions between the different models of pathological polymerisation supported by X-ray crystallographic data highlight the challenges of relying strongly on this method to study a dynamic process occurring in solution. Moreover, in recent years it has become apparent that the effects of disease mutations upon dynamic behaviour of α_1-antitrypsin in solution are likely to be of particular importance [44, 45]. Since these behaviours may be critically affected by the experimental/environmental conditions, studying them in the appropriate pathophysiological milieu would be ideal. In general, it is increasingly feasible to study protein structure and dynamics in situ and ex vivo using methods such as in-cell NMR spectroscopy [60] or cryo-electron tomography [73]. However, applying them to define α_1-antitrypsin behaviour in disease at sufficiently high resolution to fully resolve these questions will likely remain challenging for some time. Pending such insights, the problem can be approached by using powerful but minimally perturbing methods to study the solution behaviour of the α_1-antitrypsin polypeptide in non-denaturing conditions as close to physiological as possible. NMR spectroscopy and native mass spectrometry may be particularly useful complementary techniques for this [45].

A more theoretical approach defines the issue in terms of whether in vivo polymerisation occurs during or subsequent to folding (misfolding vs. post-folding) or both. α_1-Antitrypsin polymers, like those of mutant neuroserpin in the serpinopathy

FENIB, accumulate within the folding compartment, i.e. the ER [8, 74]. Other conditions associated with the build-up of ordered protein aggregates seem to be associated with extensive non-native structure in protomers, e.g. amyloidosis [75–78]. These findings are believed to reflect abnormal folding processes, the conditions are therefore termed "misfolding diseases", and there are clear analogies between them and α_1-antitrypsin deficiency, e.g. tissue deposition of ordered protein aggregates with associated organ dysfunction. The principle that α_1-antitrypsin deficiency was strictly a misfolding disease was initially used to support the β-hairpin linkage model [57]. In particular, it justified relating polymerisation to data obtained from studies of α_1-antitrypsin partially denatured in guanidine [58]. Such species are poorly recognised by the 2C1 mAb that is highly specific for α_1-antitrypsin polymers found in vivo in hepatocyte inclusions [71, 79]. The assumption that polymers formed in denaturing conditions in vitro are of direct relevance to the disease mechanism of Z α_1-antitrypsin deficiency may therefore be of limited utility. However, these findings, together with those from other systems [75, 80–82], indicate that the same protein subunits can self-associate in ways that are energetically analogous but give structurally distinct polymeric or fibrillar architectures. Data related to denaturant-induced protein multimerisation therefore require validation (e.g. by studies in more pathophysiologically relevant conditions) before relevance to conformational disease is assumed.

On the other hand, α_1-antitrypsin has the potential to polymerise subsequent to its folding into a native state solution ensemble. In vitro α_1-antitrypsin deficiency variants polymerise from the native state at physiological temperature and concentrations in the order of hours, well within its half-life in the circulation [8, 22]. It therefore remains important to understand the proportion formed by association of native subunits extracellularly, compared with those formed within the ER and trafficked along the secretory pathway. Given the sensitivity of polymer morphology to experimental conditions in vivo, it is also important to ascertain whether polymers observed in the different locations (intracellularly, in the circulation, lung, skin, and renal parenchyma) are structurally identical.

Targeting of Misfolding and Polymer Formation for Drug Design

The immediate practical motivation for understanding the structural basis of α_1-antitrypsin polymerisation in disease is to optimally target therapeutic agents to disrupt this process and promote the native, functional conformation [61]. Precise definition of the most relevant linkage(s) is therefore ideal. However, the clinical and laboratory studies indicate key regions in terms of disease-relevant structural and dynamic behaviour of α_1-antitrypsin (Fig. 3.4). Moreover, common features of the different models already highlight a number of therapeutic approaches, and progress along these has been encouraging [59]. Hypothesis-based strategies target specific sites of importance to the intermolecular linkage itself or else sites that must remodel in the transition of the native to the polymerogenic intermediate state.

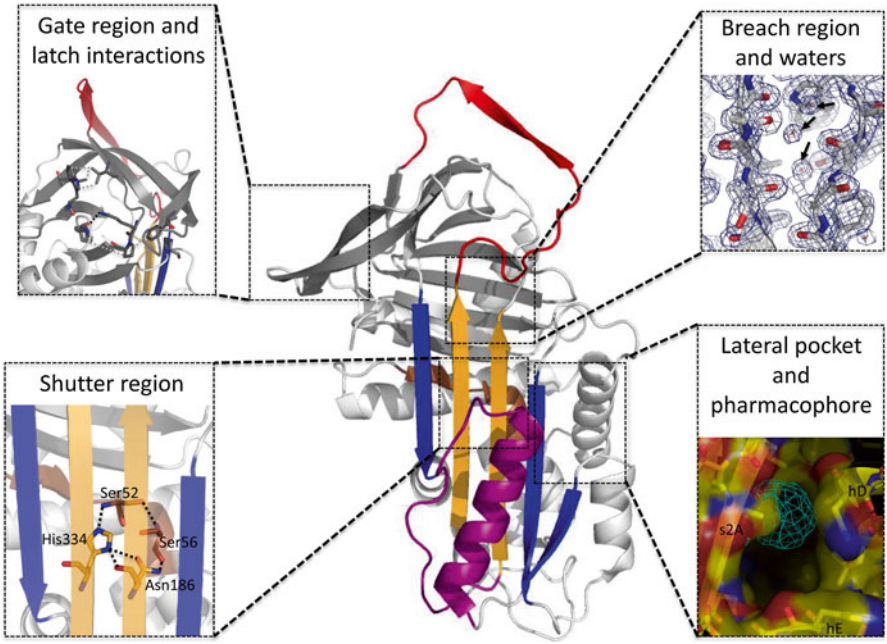

Fig. 3.4 Regions of α_1-antitrypsin structure whose importance is highlighted by deficiency mutations or therapeutic targeting. Additional colouring highlights the cryptic s4A site (neighbouring s3A and s5A strands in orange) that is filled in all three models of pathological polymerisation shown in Fig. 3.3b and the "clasp" region (hF + linker to s3A, *purple*). The clasp region remodels during polymerisation and is the site of polymerogenic mutations in α_1-antitrypsin and antithrombin [45, 62, 83]. Conversely, a stabilising mutation in this region helps limit the tendency of PAI-1 to spontaneously undergo the native to latent conformational transitional switch [66]. Boxed and zoomed regions show details of four other regions of interest in regard to disease and therapeutic interest. The breach region includes the top of the s4A site and underlying residues and interacts with the *N*-terminal base (proximal hinge) of the reactive loop to regulate intramolecular insertion. Its importance in disease is supported by the involvement of the site of the Z mutation (Glu342Lys) that occurs at the junction of s5A with the proximal hinge and may act by breach destabilisation. Zoom shows the $2fo-fc$ electron density map for the highest resolution structure of native α_1-antitrypsin [36]. *Arrows* highlight three water molecules coordinated within this otherwise hydrophobic region that prevent further H-bonding across the top of the breach and so likely allow readier initial insertion of the reactive loop via its P14 residue. The gate region provides a steric hindrance to the reactive loop and s1C movements required for the native to latent transition. This may also be the case for native to polymer transitions since s1C release is similarly required in both cases [84]. Moreover, the gate motif is stabilised in the native state to close a β-barrel formed by the *upper* strands of β-sheet B and β-sheet C (sheets shown in *grey*) that are linked at the opposite junction by continuous sequences. The hydrophobic (*grey dashed lines*) and polar (*black dashed line*) interactions responsible for this stabilisation are indicated. These are perturbed by mutations responsible for mild deficiency effects [20]. Other data also indicate roles for remodelling of helices A and G in polymerisation [20, 45, 85] and the importance of an arginine pocket that locally stabilises the inhibitory conformation of the reactive loop conformation in solution [86, 87]

Hypothesis-Based Targeting

s4A Site

Targeting of the s4A site [88–90] represents direct blockade of the intermolecular strand complementation step in the single-strand (and β-hairpin) linkage model. In the triple-strand linkage model, intermolecular linkage occurs via intermolecular C-terminal complementation. Here, blocking the intramolecular insertion of the reactive loop into the underlying β-sheet A by the same methods represents an allosteric action.

Annealing of peptide analogues of the reactive loop to the s4A site blocks polymerisation and may disassemble preformed polymers [8, 91]. Peptide insertion to fill s4A triggers the metastable to hyper-stable thermodynamic transition similarly to formation of enzyme-complexed, cleaved, latent, or pathological polymer states [92, 93]. The opening of the lower s4A site in particular represents a critical rate-limiting step in polymerisation [62, 94, 95]. Selective targeting of this region therefore permits the development of smaller peptides and mimetic molecules as polymer blocking agents, thereby improving their compliance with optimal ADME characteristics, e.g. as assessed by Lipinski criteria. In contrast, insertion into the upper s4A site that holds open the lower site favours polymerisation, presumably via single-strand linkage.

The strategy has therefore developed to identify peptides of reducing size that target the lower s4A site. Initial studies demonstrated that the FLEAIG analogue of the six reactive loop residues inserting in this region bound better to the Z variant than to wild-type, M α_1-antitrypsin, supporting the rationale of this approach in disease [90]. Subsequent studies reduced the peptide length further to four residues and explored the optimal binding sequence through a combinatorial approach [96]. To date, the tetrapeptide TTAI has shown the most promise in terms of binding and desirable effects in cell models [88].

Lateral Pocket

Expansion of β-sheet A is a common feature of all current models of pathological polymer linkage. In α_1-antitrypsin, the necessary movements of strands 1–3 of β-sheet A seal over a lateral hydrophobic pocket that is solvent exposed in the native conformer, and fill it with interacting amino-acid side chains [36, 97]. The pocket is highly druggable and extensive in silico, and in vitro screens have targeted it [62, 98, 99]. Site-directed mutagenesis and X-ray crystallographic studies have explored the potential of filling different regions of the pocket and adjacent areas. These demonstrated that it was possible to achieve a combination of thermodynamic stabilisation and polymerisation blockade relative to the wild-type protein whilst retaining enzyme inhibitory activity. This was achieved by filling a small region at the apex of the cavity by the introduction of a Thr114Phe mutation [62]. However, when added on the background of the Z mutation, the same mutation had no

significant effect on rescuing phenotype in cells. This is consistent with data supporting predominantly kinetic rather than thermodynamic destabilising effects resulting from the Z mutation [44]. Thermodynamic stabilisation effects on the wild-type protein may therefore not necessarily correlate with rescue of Z α_1-antitrypsin polymerisation. As a corollary, for drug design, screens that can identify interactions that increase the kinetic stability (i.e. reduce the conformational dynamics) of the native state ensemble may ultimately be more successful than those that focus solely on thermodynamic readouts.

A small but significant phenotypic rescue effect was seen when the nearby Gly117Phe mutation was used [62]. However, this was associated with still greater thermodynamic stabilisation of the native fold and its crystal structure demonstrated the Phe side chain at the mutation site interacted with packing of the F-helix, shifting it by half a turn, rather than filling the lateral pocket.

Unbiased Docking

Unbiased docking strategies work on the principle that the identification of compounds that can bind to native α_1-antitrypsin may include those that stabilise it against polymerisation in ways that would currently be challenging to predict [61]. They are therefore complementary to those based upon dynamic changes inferred from structural models of polymerisation, and begin with systematic studies of the native fold of α_1-antitrypsin to identify druggable targets [100]. The lateral pocket can be identified as the most druggable site by such methods. The s4A site is not highlighted in these studies since its existence requires the opening of a β-pleated sheet and exposure of hydrophobic core residues that is penalised by in silico modelling algorithms. However, other cryptic sites can be identified by simulating solution behaviour by computational approaches that can be relatively intensive (molecular dynamics) or simplified (e.g. TConCoord).

Polymerisation and Evolution: “Protective Polymer” and “Primordial Polymer” Hypotheses

The general risk of polymerisation of mutant α_1-antitrypsin is a consequence of the metastability of the native fold and this principle applies across the serpin family. Once a deleterious mutation has arisen, evolution tends to select against it. However, the Z variant appears to have arisen ~2000 years ago [101], and rather than being lost in the intervening period, it has become relatively common across European populations [102]. This raises the possibility that it may have been subject to positive selection pressure. The pro-inflammatory effects of extracellular α_1-antitrypsin polymers within the lung have been proposed to underlie a heterozygote advantage that may account for this [103]. The “protective polymer” hypothesis considers that,

in the pre-antibiotic, pre-smoking era, a mechanism to augment the neutrophilic inflammatory response to bacterial infections in the lung would confer a survival advantage. This would apply particularly in populations at higher risk of these diseases (pneumonia, acute bronchitis) due to the local climate. If associated with sufficient benefit, it would offset the homozygote disadvantage related to childhood liver disease that may have been relatively small relative to all-cause infant and childhood mortality.

More generally, a tendency to polymerisation may have been a prerequisite of all biomolecules from the origins of life, with misfolding and conformational diseases an inevitable consequence of the central role of proteins as effector molecules [104]. The "primordial polymer" hypothesis starts from the assumption that the building blocks of cellular life arose from diverse organic chemical reactions. Compounds would arise where energy gradients occurred in the presence of water, carbon, nitrogen, oxygen, phosphorous, and sulphur (e.g. hydrothermal vents). These small compounds would tend to disperse or be degraded by the same energy flows. However, compounds capable of stabilisation through self-association would tend to persist in aggregated states and remain in proximity with other polymerising systems. Self-association can arise through covalent and/or non-covalent interactions. Amino acids, sugars, nucleic acids, lipopolysaccharides, phospholipids, and their higher order, self-associated states would all be favoured by this process.

In such a scheme, a critical step to their integration within complex processes involves the development of a partitioning role between aqueous environments by a set of self-associating compounds. For cellular life, this role has been taken by lipid compounds; for viruses, protein assemblies perform an analogous function. Such partitioning gives rise to proto-cells that enclose a number of polymerising molecules. Enclosure of polymerising molecules within a proto-cell will generate dynamic equilibria between monomers and polymers and potentially between different types of molecule (e.g. amino acid and nucleic acid based). If transiently disrupted, the enclosing membrane will tend to restore its integrity either around a similar single enclosed unit or around "daughter units".

The biochemical composition within the derived proto-cells will be similar to the original and so will re-establish similar equilibria in this model. This allows the transmission of traits between proto-cells. Selection can then act to retain molecules that interact in a way that is beneficial for survival, e.g., through cytoskeletal (e.g. actins, tubulins) or catalytic (e.g. homohexameric enzymes) functions. The ability of the same protein subunits to self-associate in distinct ways depending on environmental conditions, with effects upon selectable traits, suggests that this may have allowed selection for factors influencing the intracellular environment as well as amino-acid sequence.

Ultimately, this hypothesis proposes that the templating nature of nucleic acid polymerisation proved optimal for specialising coding functions within RNA and DNA genetic systems. On the other hand, the range of amino acids utilised in polypeptide chains has led to the structural and functional diversity of protein folds and assemblies. Together with the capacity for specific modulation by co-factors, these properties have made proteins ideal effector molecules. Over time, more complex

life has co-evolved with the expansion of protein behaviour to develop chaperoning systems, degradation pathways, and folds that limit the tendency to self-associate and so allow monomeric protein functions to become important. However, the inherent propensity of proteins to adopt a range of polymerised states is unlikely to be lost if it is indeed a fundamental evolutionary mechanism, analogous to the vulnerability of DNA to mutation.

Conclusion

The misfolding and conformational mechanisms that underlie α_1-antitrypsin have been elucidated with the help of a battery of structural data. These are arguably unparalleled in terms of the quality and range of high-resolution crystallographic and complementary data. However, increasingly detailed data have led to areas of contention as well as resolution, with findings from studies in different conditions open to apparently contradictory interpretations relating to disease mechanism.

This likely reflects a wider, and previously under-appreciated, issue in the study of misfolding and conformational diseases. Proteins that are destabilised or partially unfolded may populate intermediate ensembles with the capacity to stabilise to a similar degree by self-association into more than one type of polymeric assembly. The nature of the predominant form is then determined by the environmental (e.g. biological compartment or experimental) conditions as well as mutations. This may underlie findings of heterogeneous polymer/aggregate/fibril morphology in other disease models, e.g. strain specificity in prion diseases. Understanding these processes in α_1-antitrypsin therefore establishes important contexts for data interpretation relevant to the wider range of diseases mediated by misfolding and/or conformational change.

On the other hand, the depth of biochemical and biophysical understanding that now exists for α_1-antitrypsin allows the development of both hypothesis-based and unbiased strategies for drug development. These sit within a general mission to develop strategies to preserve α_1-antitrypsin function, block formation of polymers, and aid their clearance. However, there are generic challenges involved in bringing drugs to market. Determining the nature(s) of the polymers that are of pathophysiological relevance, together with the underlying changes in protein structure and dynamics, remains key to maximising the range of therapeutic targets.

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Chapter 4
Managing the Adaptive Proteostatic Landscape: Restoring Resilience in Alpha-1 Antitrypsin Deficiency

Chao Wang and William E. Balch

Abbreviations

PN	Proteostasis network
ER	Endoplasmic reticulum
AAT	Alpha-1 antitrypsin
Q-state	Quinary state
APL	Adaptive proteostatic landscape
AATD	Alpha-1 antitrypsin deficiency
NE	Neutrophil elastase
COPD	Chronic obstructive pulmonary disease
RCL	Reactive center loop
WT	Wild-type
COPII	Coatomer protein complex II
G/ERAD	Glycan ER-associated degradation
ER-phagy	ER-associated autophagy response
BAL	Bronchoalveolar lavage
HSR	Heat shock response
UPR	Unfolded protein response
MSR	Maladaptive stress response

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ERAF	ER-assisted folding and glycosylation
QC	Quality control
NEF	Nucleotide exchange factors
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor

Protein nascent chains emerging from the ribosome generate a native conformation through multiple folding pathways and diverse energetic intermediates, defined by a funnel-like folding energy landscape that shapes protein function in the cytosol and endomembrane trafficking pathways [1–7]. It is now recognized that "folded" proteins also dynamically fluctuate among different energetic states [8, 9] and ~30 % eukaryotic proteins can be disordered transiently or permanently at different times in their functional lifespan [10–13]. De novo folding and local conformational interconversions in vivo are particularly challenging to the cellular folding program and prone to misfold and aggregate in the complex intra- [12, 14] and extracellular [15–17] environments unless protected.

To address multiple folding challenges defined in the inherited genome, biology has evolved a sophisticated program, referred to as *p*roteostasis [5, 6, 18–25], which faithfully manages protein structure and the multiplicity of dynamic native conformations required to fulfill function in response to diverse signals stemming from complex cell, tissue, and organismal demands. This proteostasis program is an evolutionarily ancient system [19] that integrates protein synthesis, folding, degradation, and membrane trafficking systems including both the exocytic [endoplasmic reticulum (ER), Golgi, cell surface] and endocytic (endosomes, lysosome) pathways harboring alpha-1 antitrypsin (AAT) [26–28]. The proteostasis network (PN) generates a buffer that can be used to form a "cloud" around each energetic folding species that functions as capacitor to define the dynamic "*q*uinary state (Q-state)" of protein fold [19, 26, 29–31]. The Q-state encompasses the highly dynamic noncovalent ionic, hydrogen bonding, hydrophobic, and van der Waals surface forces responsible for all biology. It responds to reversible covalent modifications such as acetylation–deacetylation and ubiquitin modifications of surface Lys residues and facilitates integration of the more structurally ordered secondary, tertiary, and quaternary states encoded by the primary sequence to define the structural and functional properties of the protein fold. In response to Q-state properties, PN components can regulate the multiple conformations defining the energetic states of each folding species and also determine the turnover and intra-/extracellular location of each folding species. The PN contributes to a complex and dynamic in vivo structural and functional landscape of folding proteome, referred as the *a*daptive *p*roteostatic *l*andscape (APL) (Fig. 4.1). Modulating the composition, concentration, and/or function of PN components remodels the APL, hence regulating the function of folding proteome in response to folding stress—both acute and chronic as occurs in inherited or evolving environmental-triggered disease [6, 30, 32–36]. APL provides a novel

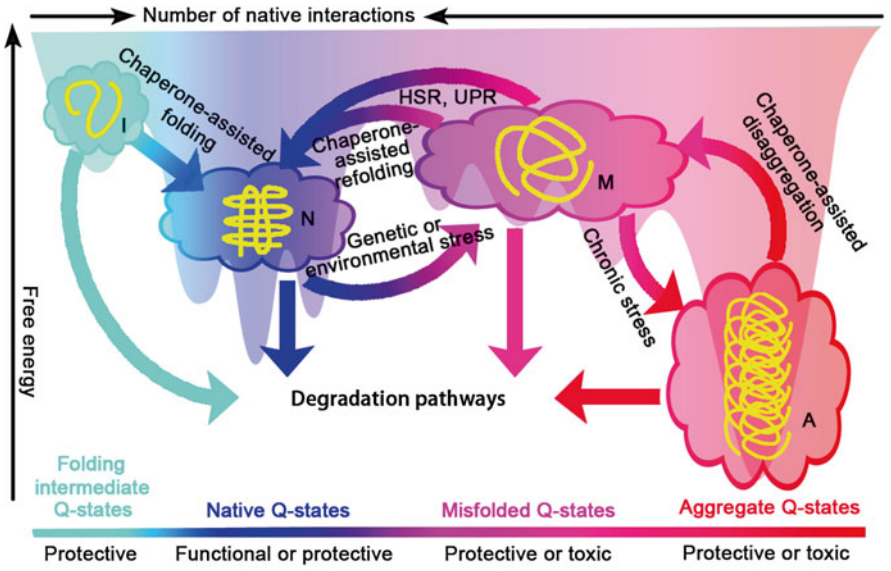

Fig. 4.1 A schematic diagram of APL. A protein adopts numerous energetic states illustrated here in a folding energy landscape with several states highlighted, such as folding intermediate (*I*), native state (*N*), misfolded state (*M*), and aggregate state (*A*). These states fluctuate among multiple local energy minimums. The PN recognizes these folding species harboring different thermodynamic and kinetic properties to define the Q-states and hence determines the functional environment (the Anfinsen physiological state) of each folding species. Here, a general "cloud" is used to represent the Q-state, but since the folding energy constantly changes, the Q-state likely also changes along the folding landscape, which is shown as a color scale from *cyan* to *red*. The conversion between Q-states and/or the turnover of folding species is highly dynamic and largely affected by both the PN and global stresses. Thus, modulating PN components could rebalance the APL from the stressed/diseased state to healthy state. HSR, heat shock response; UPR, unfolded protein response

therapeutic paradigm for all protein misfolding diseases [18, 20, 26, 30, 37] including *a*lpha-1 *a*ntitrypsin *d*eficiency (AATD) [23, 27, 38], which will be explored in this chapter.

AATD in the Context of Proteostasis Network

AATD is a consequence of point mutations produced in the inherited AAT gene [39–43]. AAT, which is mainly synthesized by hepatocytes, is the key antiprotease in serum that prevents the degradation of lung tissues by neutrophil elastase (NE) [40, 44–46]. The most common mutation triggering AATD is the Z-variant (Glu342Lys), which affects about 1 in 1600–3000 live births [47, 48]. We will primarily use this mutation to discuss the role of the PN in managing AATD, points which will equally apply to other AATD disease mutations [39, 40].

The Z-mutation alters the protein folding energetics of AAT. It produces misfolded and aggregated AAT that triggers serial responses from the PN, which differentially manages the APL in both the liver and lung in its attempt to maintain a healthy proteome for optimum biological activity [23, 27, 38], but sometimes fails [30]. When the capacity of PN is challenged in the liver, the increased polymerization/aggregation of misfolded AAT in the ER of hepatocytes leads to neonatal hepatitis [49], cirrhosis [50], and hepatocellular carcinoma [40, 51, 52]. On the other hand, the decreased pool of functional circulating plasma AAT [53] disrupts the protease–antiprotease balance in the lung and causes chronic obstructive pulmonary disease (COPD) and/or emphysema [27, 54–56].

In this review, we will first describe the proteostasis stress challenge in AATD and how the PN responds to the problem in the liver–plasma–lung axis. We will then discuss the rationale for therapeutically managing the APL by modulating the PN to mitigate folding stress and restore Z-AAT functionality and, finally, describe new potential therapeutic efforts by directly targeting the operational properties of the PN that are applicable to AATD.

The Folding Stress-Responsive APL in the Liver of AATD

Hepatocytes continuously synthesize and secrete a large amount of AAT to maintain a steady-state concentration range of 20–48 μM in serum [41]. To achieve a functional, folded form that can traverse the exocytic pathway, the newly synthesized AAT polypeptide chain needs to fold into a three-dimensional structure in the ER. Studies of the folding mechanisms of purified AAT in vitro revealed an intermediate folding state (*I*) between the unfolded state (*U*) and native state (*N*) (Fig. 4.2a), which is prone to misfold and polymerize [40–42, 57–62]. Furthermore, the native conformation of AAT is kinetically trapped in a metastable conformation with reactive center loop (RCL) transitioning through disordered and ordered conformational states during the AAT functional cycle [63, 64]. Therefore, the polymergenic intermediate state and the final metastable conformation render AAT with intrinsic misfolding and polymerization propensity—e.g., disorder. The Z-mutation (Glu342Lys) exacerbates this folding challenge seen for the wild-type (WT) AAT in the ER. Substitution of Glu342 by Lys slows down the folding rate of AAT [65] and deregulates the opening of β-sheet A, which leads to a misfolded intermediate (*M**) and an increasing tendency to form polymer (*P*) through a "loop-sheet" mechanism [40, 57, 58, 62, 66–68] (Fig. 4.2c). Additional models for AAT polymerization have also been proposed based on the crystal structures of a dimer of a serpin antithrombin [69] and a trimer of a disulfide mutant of AAT [70], suggesting that folding trajectories of AAT/Z-AAT could be diverse and likely dynamic, all subject to the local PN.

The PN has evolved specialized strategies for each energetic state of a folding species in order to maximize function. Abundant chaperones such as Grp78/BiP and Grp94/Hsp90 orthologs, calnexin, and folding enzymes [peptidylprolyl isomerase (FKBP) and protein disulfide isomerase (PDI) family members] in the ER, among

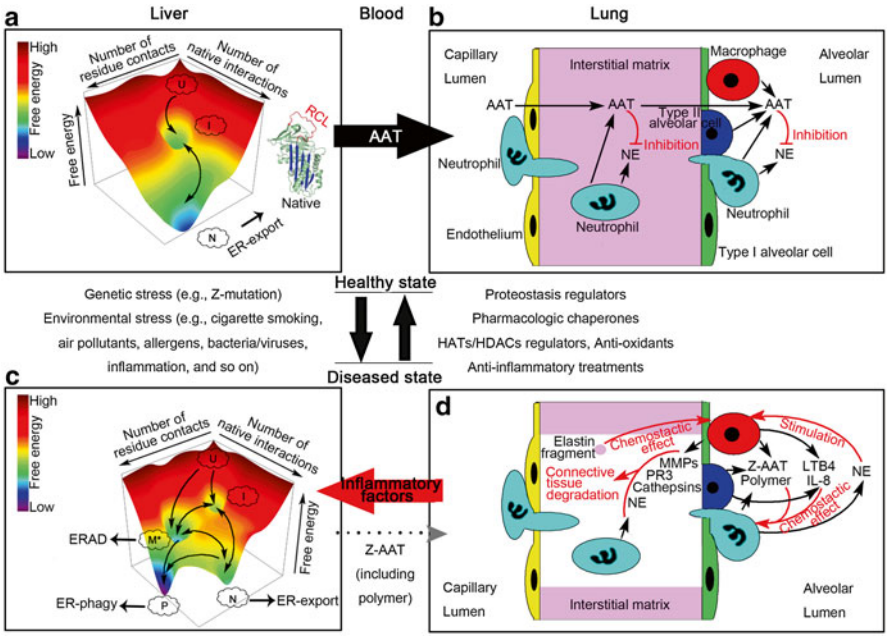

Fig. 4.2 The APL in the AATD context. (**a**) Newly synthesized AAT polypeptide (*U*) has high free energy and large entropy, which folds into native state (*N*) through intermediate state (*I*) in the ER and secrets into serum. Shown is the structure of the native conformation (1QLP) of AAT with reactive center loop (RCL) in red and β-sheet A in *blue*. (**b**) Abundant circulating AAT and locally secreted AAT inhibits NE to protect the integrity of lung tissue. (**c**) In ZZ-homozygous variants (or WT AAT in response to environmental stress), the folding of AAT may be trapped in a misfolded intermediate (*M**), which leads to accumulation of a polymer (*P*) in the ER. Here, the PN functions as a "cloud" surrounding each proteins folding state (the APL client) to help them traverse the free-energy barriers and/or sort different folding species to different destinies (i.e., lead the native folded monomeric state to the export pathway, a transiently misfolded intermediate to ERAD, and the polymer for degradation by the ER-phagy pathway). (**d**) Only 10–15 % Z-AAT is secreted into serum in AATD, which leads to continuous degradation of connective tissue by uninhibited NE, protease 3 (PR3), cathepsins, and metalloproteinases (MMPs). NE also stimulates the release of proteases (cathepsins and MMP-2) and chemotactic mediators (leukotriene B4 (LTB4) and IL-8) to accelerate lung damage. Digested elastin fragments and extracellular polymerization of Z-AAT contribute to lung disease through their chemotactic effect for monocytes and neutrophils, respectively. The trans-tissue inflammatory factors delivered from lung to liver exacerbate the folding challenge in the liver. We suggest that proteostasis regulators that modify the activity of the APL, chemical or pharmacologic chaperones that stabilize Z-AAT, HATs/HDACs regulators that adjust the Q-state and APL for a more productive folding event, as well as antioxidants and anti-inflammatory drugs, may provide therapeutic interventions to leverage the diseased APL back to a healthy APL and hence correct AATD

many others, prevent the aggregation of newly synthesized WT AAT polypeptides and help them reach a conformation that can be efficiently exported by the coatomer protein complex II (COPII) vesicle budding system [26, 71–79] (Fig. 4.2a). In contrast, because the folding of Z-AAT is extremely slow and thermodynamically compromised compared to WT AAT [65], the folding intermediates can be trapped

by the chaperones found in the ER [72, 80], a state which contributes to the increased concentration of intermediates susceptible to aggregation/polymerization—with only a small pool of Z-AAT apparently available secretion in its metastable state yielding a reduced serum load of 10–15 % that of WT.

We now appreciate that the ER PN has a set point that detects and partially targets the prolonged Z-variant intermediate for degradation through N-linked glycosylation pathways during its folding [75]. Here, polypeptides that cannot achieve sufficient thermodynamic stability in a biologically relevant time frame are processed by ER mannosidase (ERManI), removing several additional mannose units that typically occur in WT AAT processing for export. The energetics contributed by this processing event remains to be established. These intermediates are retrotranslocated to the cytosol for degradation by the proteasome through the glycan ER-associated degradation (G/ERAD) pathway [81–89]. In contrast, Z-AAT polymers/aggregates, which have been suggested in the past to be chaperome free (a point that requires further evaluation), cannot be degraded by G/ERAD pathway. One possibility is that the energy barrier for unfolding and/or retro-translocation across the ER membrane is too high. However, the PN has an alternative pathway to clear them by activating an ER-associated autophagy response (ER-phagy) [42, 43, 88, 90–96]. Therefore, these diverse folding species of Z-AAT with different biochemical/biophysical properties defining their Q-states are differentially recognized by the PN. This differential recognition defines their cellular fates and patient phenotype (Fig. 4.2c).

Remarkably, the ER APL is capable of resolving most proteostasis challenges in the liver brought on by the Z-mutation early in life, illustrating its robustness, since only about 8 % of ZZ-AAT homozygous newborns develop clinically significant liver disease [40, 42, 47, 97]. However, what changes during aging are the cumulative environmental and/or inherited/sporadic challenges leading to enhanced folding stress impacting APL function. This includes both genetic modifiers and environmental factors that either increase the Z-AAT folding challenge or decrease the proteostasis capacity [5] as well as the activity of the decaying stressed PN environment in response to disease [19, 30]. This contributes to late-stage liver and lung dysfunction characteristic of the aging AATD patient. Studies comparing the fibroblasts from “susceptible” and “protected” ZZ individuals reveal that a lag in degradation of translocated Z-AAT correlates with liver disease phenotype [40, 42, 82, 97], and a single nucleotide polymorphism that suppresses the translation of a key ERAD player, ERManI, was found to accelerate the onset of end-stage liver disease [98]. Thus, an increasingly impaired proteostasis program alters the APL and contributes to an early onset and/or a more severe form of the disease. It remains possible that the converse is also true—ZZ-AAT individuals with a more resilient PN may actually be protected and not be detected in the clinic until a very late stage of disease in response to either modifiers and/or management of the environment. It now becomes imperative to identify the role of the PN from the perspective of potential therapeutic targets reflecting the personalized proteostasis biology of the patient (see below).

AATD, Proteostasis, and Lung Disease

Unlike liver disease which can be detected in infants, the lung usually develops the first signs of AATD in early to late adulthood, suggesting that the increasing burden of misfolded/polymergenic Z-AAT derived from the liver and delivered via plasma to the lung generates an increasingly severe chronic PN challenge leading to pulmonary disease. Environmental pollutants (e.g., cigarette smoke) and pathogens (e.g., bacteria and viruses) that continually challenge the proteostasis program in the airway either directly through protein damage (e.g., oxidative modification) or indirectly through immune/inflammatory responses create additional stress that exacerbates onset of disease in the absence of protection by AAT [31]. For example, the risk of AATD lung disease is markedly increased by cigarette smoking, which can inactivate AAT (and other proteins [99]) through oxidation of the methionine residues in the RCL of AAT [99–103]. Moreover, cigarette smoking exacerbates the polymerization of both extracellular [104] and intracellular Z-AAT [105], decreases the transcytosis of circulating AAT from the capillary to alveoli [106], and upregulates the pro-inflammatory phenotype of lung cells that challenges the PN [105, 107]. Thus, cigarette smoking changes the structural/functional activity of AAT from multiple perspectives along the lung–plasma–lung axis to damage the proteostasis system of the lung independent of its impact on liver physiology, although increasing evidence suggests that cigarette smoke can contribute to liver disease.

The most prominent effect of the absence of AAT function in the lung is markedly increased NE activity leading to breakdown of the alveolar space. Specifically, inflammation in the alveolar wall induces the migration of neutrophils from alveolar capillary into the interstitium between the capillary endothelium and alveolar epithelium (Fig. 4.2b) [40, 42, 97, 108]. NE as well as protease 3 [109, 110], matrix metalloproteinases [111], and cysteinyl cathepsins [112] released from neutrophils degrade the lung interstitial matrix containing fibrillar collagens, elastic fibers, and proteoglycans and cause lung damage that leads to emphysema/COPD [23, 27, 113]. Although normally abundant circulating AAT enters the alveoli by transcytosis from circulating plasma [106, 114] to inhibit these proteases, local cells such as neutrophils [115], macrophages [116], and type II alveolar cells [117, 118] also produce AAT to protect the lung [40, 42, 97]. Thus, AAT functions as a component of a poorly understood PN network outside of the cell [17] to protect the integrity of the extracellular exposed lung proteome in response to inflammatory stress (Fig. 4.2b). However, in ZZ homozygotes, because the level of AAT in serum is only 10–15 % of normal healthy pool, this leaves the lung tissue subject to degradation by NE (Fig. 4.2d). Furthermore, because NE can stimulate the release of cathepsin B and matrix metalloprotease-2 from macrophages [119], this can exacerbate lung damage as the resulting degradation products are found to have a chemotactic effect for monocytes [120]. NE also upregulates the release of chemotactic mediators such as leukotriene B4 [121] and IL-8 [122], which leads to an increased concentration of neutrophils in the bronchoalveolar lavage (BAL) of AATD patients [40, 42, 97, 123]. Thus, the extracellular protease–antiprotease imbalance triggers a cascade of proteostasis mismanaged destruction of alveolar wall that causes defective alveoli (Fig. 4.2d).

Besides the loss-of-function mechanism, intracellular Z-AAT polymerization inside the monocytes [40, 67, 124] and/or lung epithelial cells [105] was shown to induce ER stress and release of inflammatory factors. Moreover, Z-AAT aggregates have been found in the BAL [125] and serum [53] of AATD patients. Given that polymer can be detected in patients that have undergone liver transplantation [126], these results potentially implicate a role of Z-AAT secreted from lung cells in polymer formation and accumulation in the lung. Some evidence suggests that extracellular polymerization of Z-AAT in the lung causes the influx of neutrophils into alveoli [126, 127] and further contributes to lung pathophysiology (Fig. 4.2d). Therefore, the misfolding/aggregation of Z-AAT is not restricted to its cell-autonomous impact on liver pathophysiology, but could play a prominent role in both cell-autonomous and cell-nonautonomous roles in the lung, confounding our understanding of the underlying proteostasis events responsible for the temporal (aging) and spatial (liver–plasma–lung axis) mitigation of disease.

Rationale for Managing the APL

The proteostasis problem is central for both liver and lung disease in AATD, so understanding the role of the PN is crucial for formulating new approaches for AATD disease intervention. The PN is a multilayered and fully integrated system composed of components that generate, maintain, regulate, and remove proteins [18, 20, 23, 26, 28, 30, 37]. As described in detail by Powers and colleagues [20], we define the innermost layer of the PN as the components that directly interact with and/or that affect the target protein including protein synthesis systems (transcription/translation machineries), folding/unfolding systems [chaperones and folding enzymes (the chaperome)], posttranslational modification systems (glycosylation, phosphorylation, acetylation and oxidation, among many others), trafficking systems (the exocytic, endocytic, and lysosomal pathways), and degradation systems including the ubiquitin–proteasome system (UPS) and the autophagy–lysosome axis managing the fate of cellular aggregates. The second layer of the PN is composed of multiple signaling pathways including the heat shock response (HSR), the unfolded protein response (UPR), and oxidative stress and inflammatory responses that modulate the composition, concentration, and function of the PN components comprising the innermost layer. These signaling pathways can trigger a cascade of events that promote cell/tissue health, reprogram the cell/tissue for repair or destruction, and/or support a malignant phenotype as found in neonatal liver disease [32, 128, 129]. The third, outermost layer includes environmental/physiological stressors, metabolites, and genetic/epigenetic factors that modify the first and second layer and are driven by the extracellular environment. Biology utilizes this sophisticated network to generally maintain the healthy state of the proteome that is susceptible to continuous genetic and environmental challenges, but is clearly challenged by the Z-variant and, for example, overwhelmed by a combined Z-mutation folding stress and an oxidative cigarette smoking-derived proteostasis

stress in AATD. To deal with the folding trajectory, it is now thought that the PN may recognize proteins based on their variable energetic standards contributing to a set point [19, 29, 30] that is sensitive to the unique proteostatic composition in each cell type and that is highly adaptable to the constantly changing stress that proteome misfolding places on proteostasis buffering (reserve) capacity. Moreover, recent studies show that proteostasis composition is under direct neuronal and neuroendocrine control [33, 130–132], leading to the recent suggestion that proteostasis cross talk between tissues, such as the liver–plasma–lung axis, could be integrated through the central and peripheral nervous system.

The above insights indicate that the conventional view that a single native protein conformation is "the" functional state is incorrect. It is likely that most proteins, particularly metastable AAT and its further destabilized Z-AAT variant, populate multiple intermediate functional states transiently associated with PN components throughout their lifetime, referred to as the "cloud" responsible for protein stability and evolvability [19, 35, 133]. Thus, it is the PN cloud that gives function to the dynamic Q-state of the protein fold in biology. In the case of WT AAT, while the cloud prepares the monomer to fulfill function as a NE inhibitor outside the cell, the polymer functions (in the context of the PN cloud) as a pro-inflammatory component that the tissue uses as a metric to call for support through immune responses. Besides the Z-mutation which dominates the current focus, there are over 100 different ATT mutations—more than 30 of which are found to affect either the level or function of AAT [134]. Interestingly, the thermodynamics of folding intermediates of most mutants (including Z-variant) are not dramatically different from that of WT AAT, but rather populate a series of modest free-energy wells in the folding energy landscape [135–137]. Where WT protein can easily traverse these energy wells with the help of the PN, AAT variants may become trapped and concentrated by local proteostasis cloud components because of either kinetic or thermodynamic problems contributed by the mutated Q-state structure (Fig. 4.2c). The fact that the diverse PNs in different cell types and compartments provide different levels of proteostatic capacity that are differentially sensitive to PN signaling pathways implies that the APL could be sculptable through reprogramming the PN [18, 20].

Z-AAT as a Sculptable Proteostatic Stress Modulator

Here, we use the folding of Z-AAT in the ER as an example to describe how the PN could modulate the APL and hence the disease phenotype. This description provides clues as to how we might leverage proteostasis to promote correction if we understand the mechanism(s) of action behind the interplay between the mutant protein fold and the local proteostasis cloud facilitating the folding trajectory.

While the tentative folding landscape of Z-AAT is shown in Fig. 4.2c, it is undoubtedly much more complicated. Each of the folding species dominating Z-AAT biology [e.g., *U* (unfolded nascent chain), *I* (on-pathway folding intermediate), *M** (off-pathway folding intermediate), *N* (native conformation), and *P* (polymer)]

is maintained by pathways that include PN-assisted ER translocation, ER folding and glycosylation [simplified as ER-associated folding (ERAF)] [19, 20, 138], degradation (ERAD, ER-phagy) [93], and ER export (COPII) [19, 138–141]. AAT is an acute-phase protein with its expression level increasing three- to fourfold in response to inflammation; thus, the ER import of *U* is highly variable and dependent on the signaling pathways dictating the functional needs of the cell, tissue. When the amount of *U* exceeds the capacity of ERAF that is critical to help stabilize metastable folding intermediates and prevent the aggregation of *U*, a process challenged by the thermodynamic/kinetic instability of all AAT mutants, the folding equilibrium of AAT, and particularly Z-AAT, will inevitably flow through the *M** and *P* pathways. Indeed, indomethacin, which activates IL-6-STAT3 cytokine signaling pathway and increases the expression level of Z-AAT, results in higher levels of Z-AAT polymers and increases liver damage in PiZZ mouse model [142]. Since Z-AAT has slow folding kinetics and low thermostability compared to WT AAT, the interconversion between *I*, *M**, and *N*, being concentration dependent, favors the conversion from *M** to *P* (or to a lesser extent from *N* to *P*). This process is unidirectional because once the polymer is formed, the stable conformation has an apparent large energy barrier for unfolding that the polymer cannot bypass [143, 144]. A solution that nature has evolved to remove such misfolded aggregates is to subcompartmentalize these thermodynamically stabilized structures in specialized, sealed degradation autophagic compartments [29, 43, 88, 90, 91]. This illustrates that by changing the APL through redefining the composition of the PN, the cell can flip the problem on its head to aggressively protect against aberrantly misfolded species.

The need for increased degradative capacitance to protect the cell from aggregated species is consistent with the observation that inhibiting ERAD without changing the capacity of ERAF and ER export increases the polymer level and contributes to AATD liver disease phenotype [82], reinforcing the interpretation that the dynamic folding equilibrium is under continuous control by the PN. Since our ultimate goal is to correct both liver and lung disease in AATD, we suggest that augmentation of ERAF and/or ER export machineries could increase the *I-N* folding pathway and decrease *M*-P* folding pathway, thus making the folding landscape of Z-AAT more like WT AAT as shown in Fig. 4.2a, possibly acutely or permanently correcting the fold by subtle changes in the APL [91, 145–147].

Proactive Proteostatic Signaling Pathways Activated by Z-AAT

The goal of proteostasis therapeutics is to use biology to fix biology by targeting the support pathways whose roots are 3.5 billion years old [19]. Remarkably, it is clear that biology, in response to the environment, has evolved many ways in phylogenetic diverse cell types to manage the fold and folding stress using common principles [19]. The versatility of the folding program should, in principle, be able to address challenges such as occurs in AATD-inherited disease through

reprogramming [18]. While HSR [3] is the universal response found to manage folding in all phyla, in eukaryotes the UPR [4] has evolved to handle misfolded proteins traversing endomembrane compartments. As such, each compartment relies on multiple but often unknown inputs to elicit highly variable and dynamic responses to handle folding challenges that either address the problem or found to be insurmountable and drive the cell down death pathways. Interestingly, Z-AAT does not activate the classic ER UPR signaling pathways that involve the IRE1, PERK, or ATF6 cytosolic transcription factors in hepatocytes [148–150]. The inability to generate an UPR response and promote complete degradation of misfolded Z-AAT through G/ERAD in hepatocytes could reflect the fact that the monomer and the polymer are actually considered as Q-state "normal" by the ER UPR signaling sensors, perhaps as a native but metastable "folded" structure that contributes to the dynamic "mouse-trap" mechanism [151]. However, the increased propensity to disorder in variants such as Z leads to a Q-state that largely defines a polymeric autophagic substrate and a modest substrate for G/ERAD.

In contrast to hepatocytes, Z-AAT induces UPR in monocytes where its expression is low [124]. Thus, in different proteostasis environments in which the aggregation phenotype may be reduced because of a smaller load of Z-AAT nascent chain whose polymer propensity is logarithmically proportional to concentration like amyloid, Z-AAT folding species may be sufficiently long-lived to be preferentially targeted to G/ERAD pathways [92]. These differences emphasize that diverse proteostatic dynamics found in different cell types can be targeted specifically by unique modifier therapeutics. Moreover, we have recently reported that the sustained expression of Z-AAT induces a maladaptive stress response (MSR) through chronic activation of the HSR pathway found in the cytosol [30]. A similar observation was reported in yeast where overexpression of a misfolded luminal protein CPY* was found to activate HSR [152]. Activation is unlikely due to UPR and/or altered G/ERAD because this phenomenon is observed in Ire1Δ strain in which both UPR and ERAD are impaired [152]. Importantly, we have shown that silencing HSF1, the HSR master regulator, either chemically or through the use of biologics (siRNA) restores the secretion of functional Z-AAT, indicating that the MSR confounds the disease phenotype in its attempt to solve it but cannot fix due to the chronic nature of the disease [30].

An additional proteostatic impact that has been noted in AATD is that the accumulation of Z-AAT activates oxidative stress [153] and inflammatory NF-κB pathways [27, 40, 148, 150] (or ER overload response [154]). How NF-κB pathway affects the PN is not well understood [27], but the inflammatory factors upregulated by this pathway, as well as trans-tissue inflammatory signals delivered from lung to liver (Fig. 4.2c, d), could increase the expression level Z-AAT, which may improve secretion but also could result in a higher production of polymer.

The above examples illustrate that proteostatic control in AATD could emanate from multiple and diverse signaling pathways reflecting the local cell and tissue PN response. A detailed understanding of the "personalized" folding of Z-AAT managed by proteostasis in these different cell, tissue, and host environments could

provide critical insights into unappreciated nodes in a correction network within the global PN impacting human disease.

Current Therapeutic Opportunities

AAT augmentation (replacement) therapy is the only specific noninvasive treatment for AATD [135, 155–159]. Replacement therapy is based on the concept that the patient should be able to restore the basal level of AAT by weekly intravenous purified human AAT, an approach that may slow or stop the progression of lung disease [135, 155–157, 160]. This treatment is indeed capable of contributing to the serum AAT pool above the protective threshold and to increase the anti-NE activity in BAL [155, 158, 161, 162]. However, the clinical impact of this treatment for lung function remains controversial [135, 156–159, 163–165]. The lack of a clear validated clinical impact by augmentation therapy could reflect the absence of well-controlled clinical trials to statistically assess apparent impact. Alternatively, the mechanism(s) responsible for lung disease could be much more complicated than just a shortage of circulating AAT below the protective threshold, perhaps reflecting the impact of the Z-AAT being produced by all cells (liver and lung), creating a proteostatic stress along the liver–plasma–lung axis by disrupting general proteostasis capacity and/or inhibiting the secretion of other unknown proteins/factors required for health. For example, issues such as acute-phase response and gain of toxicity of Z-AAT aggregates in the liver cannot be addressed by augmentation therapy. Moreover, the lung Z-AAT likely continues to create a burden that triggers inflammatory responses detrimental to lung health [134]. Furthermore, the continued production of Z-AAT could be a competitive inhibitor of perfused WT AAT function through as yet to be defined extracellular and transcellular proteostasis pathways [17, 19]. Environmental or genetic factors may also contribute to the efficacy of augmentation therapy from a personalized perspective that can only be resolved by understanding the steady-state dynamics of the APL in the liver, plasma, and lung and the impact of modifiers among the patient population.

In addition to augmentation therapy [135, 155–159], the liver and lung transplantation provide highly invasive but optional strategies for both early- and late-stage AAT-associated liver and lung disease [41, 166]. These treatments come with high risk and do not solve the global APL challenge from the perspective of continued Z-AAT production by other tissues. Progress on new therapeutic investigations reviewed elsewhere also comes from the perspective of gene therapy [167, 168], polymer inhibitors [40, 44, 53, 67, 68, 149, 169–172], and autophagy regulators [42, 43, 96, 146, 173, 174]. Below we will describe new opportunities for AATD drug discovery through proteostatic modulation of the APL.

Therapeutic Management Through the AATD Stress Chaperome

Chaperones (e.g., chaperonins, Hsp70, Hsp90 comprising the chaperome family) were initially described as proteins that assist non-covalent folding and assembly of polypeptides [2, 4, 14, 175, 176]. However, it is now recognized that chaperones also assist unfolding [177, 178], degradation [18, 21, 179–183], and trafficking [26] and direct the regulation of the biological function of thousands of client proteins including kinases driving cancer [128, 183, 184], DNA and RNA polymerase components [185, 186], transcription factors [187, 188], longevity factors [6, 189–191], most cell surface signaling receptors responsible for signaling proliferation (e.g., EGFR and steroid hormone receptors) responsible for metastatic disease [192], and cytoskeletal function [193, 194]. Chaperones also transmit the signal for misfolded proteins to activate UPR and HSR to manipulate the proteostasis capacity of the cell [4, 6, 18, 129]. Thus, chaperones provide the biological framework for regulation of the protein fold for function. They are potential key drug targets to manipulate both the dynamic Z-AAT folding Q-state and the signaling pathways controlling the PN that could alter folding dynamics to a more functional state to stabilize disease.

The major chaperones that bind Z-AAT in the ER include calnexin-UGGT system and GRP78/BiP (orthologue of the cytoplasmic Hsp70 family members) [4, 72, 80]. Through BiP, the secretory pathway is usually considered as a quality control system that only secretes the native conformer [22, 195, 196]. However, the concept of quality control is a misnomer. Rather than the native conformation per se, it is the global protein energetics and the variable PN that determine the success or failure of folding, export, and function and that is sensitive to genetic and epigenetic programs that define the physiology of each cell type [3, 30, 131, 139, 197, 198]. For Z-AAT, which is intrinsically metastable, competition for export and degradation components along the exocytic pathway will ultimately determine the final yield in the serum, emphasizing that it is not the AAT protein per se but the Q-state proteostatic cloud dominating trafficking pathways that dictates function driving liver and, ultimately, lung biology. While the ER export trafficking component ERGIC-53 has been suggested as a cargo receptor for WT AAT directing ER to Golgi exit, its interaction with Z-AAT is extremely low in the cell [44, 74, 199]. Multiple reasons could contribute to the low cargo receptor binding affinity for Z- compared to WT AAT including the following: (1) a large portion of Z-AAT is masked by the BiP/calnexin cycle; therefore, little Z-AAT is released for ERGIC-53 binding; (2) ERGIC-53 does not recognize the nonnative conformation of Z-AAT based on its thermodynamic or kinetic folding properties; and (3) the Z-AAT monomer–polymer balance in the ER is dominated by polymerization given its high concentration in the hepatocyte, an altered state that is not recognized efficiently by ERGIC-53. Thus, additional experiments are required to understand the underlying mechanism for ER export beyond the simple concept of quality control.

Other stress chaperones reported to interact with Z-AAT include GRP94 (orthologue of cytosolic Hsp90) [72, 200], GRP170 [72] and PDI [73], as well as numerous BiP and GRP94 co-chaperones—all poorly studied [40, 41, 201]. Collectively,

we refer to folding mediators as the ER "chaperome" that determines both the functional and degradative trajectory of the fold. Mutations in BiP lead to temporal impairment of ERAD of Z-AAT [179], and silencing of calnexin through siRNA increases the secretion of Z-AAT [202], which implies that these chaperones can alter the folding of Z-AAT in the context of their expression levels and the PN chaperome dynamics of a given cell type. This provides a simple demonstration that primary sequence, while important, provides a code that can be (re)interpreted by the chaperome/proteostasis system reflecting context.

The general mechanism used by chaperome biology is the dynamic binding and release of nonnative folding species to promote acquisition of the final folded state, presumably by preventing the formation of nonproductive, irreversible folding intermediate states (Figs. 4.1 and 4.2). Thus, it is not surprising that chaperones delay Z-AAT export from the ER since Z-AAT folds slowly and tends to misfold and therefore may reside more frequently in a "chaperone trap" by analogy to misfolded F508del CFTR [203]. However, the recognition that a substantial fraction of the Z-variant can escape the ER to yield a 10–15 % steady-state population in the plasma suggests that at a less populated state compared to WT AAT, Z-AAT occupies an energetically comparable folded state acceptable to the ER export machinery. Chaperones are also likely to be critical to manage the aggregation state of Z-AAT in the ER. However, no studies to date have addressed therapeutic management of Z-AAT through direct ER chaperome intervention.

What, then, could be the sequential events amenable to chaperome therapeutic intervention? We appreciate that the Z-AAT nascent chain enters the calnexin-UGGT cycle after co-translational removal of two glucose units by glucosidases I and II and then associates with BiP and ER export machinery components [75, 85]. ERAD is initiated when the binding of BiP reaches a peak. Thus, it seems that while the calnexin cycle is important for the initial folding and glycosylation of Z-AAT, BiP associates with Z-AAT in a late stage of folding and plays a central role in determining the balance of export, degradation, and aggregation. BiP alone is unlikely to be a direct drug target given its essential role for the folding of nearly all client proteins entering and/or exiting the ER and its critical role in controlling the UPR signaling pathway [4]. However, the function of BiP is regulated and diversified by its co-chaperone DNAJ proteins and nucleotide exchange factors (NEF) [72, 97, 204]. DNAJ proteins, which assist in client (AAT) delivery to BiP, often have distinct and specific client protein-binding properties due to their diverse structural organization in addition to the presence of the conserved J domain that interacts with BiP to promote function. After client protein binding, DNAJs recruit BiP, deliver the client protein, and stimulate BiP's ATPase activity to stabilize the client-BiP protein bound state. The release of the client protein from BiP is facilitated by NEFs (also called BAGs), which catalyze the exchange of ADP to ATP. There are seven DNAJ proteins and two NEFs that have been identified in the ER [205, 206]. However, there are few insights into their potential roles in WT and Z-AAT maturation. One NEF protein, GRP170, has been reported to interact with Z-AAT [72, 207, 208]. It would be interesting to see whether these co-chaperones play roles in determining the fate of Z-AAT delivery either to ERAD, aggregation, or export, since

targeting the binding interface between BiP and its co-chaperone and/or directly targeting co-chaperones is emerging as a new therapeutic opportunity. For example, targeting the cytosolic Hsp40-70 chaperome system for treatment of neurodegenerative disease and cancer is an active area of therapeutic development [2, 129, 181–183, 209, 210]. Consistent with this approach, we have suggested that elimination of chronic HSR (MSR) could restore the secretion of Z-AAT through MSR management [30]. These results point to the unanticipated direct or indirect importance of influence of the HSR on ER folding environment governed by the UPR and the cytosolic chaperome for modulating the APL of the Z-AAT. Though cytosolic chaperones are not directly involved in the folding of Z-AAT inside the ER, they have been shown to be critical for ERAD [211], autophagy [42, 91, 95, 96, 146, 212], and membrane trafficking [213]. Thus, it will be interesting to investigate how this proteostasis environment influences the general operation of the ER and downstream trafficking compartments in the management of Z-AAT proteotoxic stress in the liver and its function in the lung and whether they can be manipulated therapeutically through proteostasis regulators.

Therapeutic Management Through an Emergent Membrane Trafficking Proteostatic Code Driving Eukaryotic Biology

Numerous highly evolved endomembrane systems direct eukaryotic function [19, 26]. These provide optimal fold management systems reflecting the unique cellular, tissue, and organismal environmental challenges defining the APL. As such, trafficking has been proposed to be an extension of the proteostasis network [19, 26] with the trafficking machinery itself (the SNAREs, tethers, and vesicle-forming coats) defining the compartmentalized structure of folding pathways that facilitate delivery of protein to post-ER intracellular compartments [19, 26] and the extracellular space [17]. In the case of Z-AAT, folding species are exported out of the ER by COPII-coated vesicles [78, 214–218], which can mediate anterograde transport toward the Golgi and/or contribute to autophagosome formation [4, 42, 93, 94], divergent compartmentalization events impacting protein function [215, 219–221]. Exported material may be recycled back from Golgi compartments to the ER by the COPI coat system [78, 222, 223], refining the function of the ER folding environment and optimizing the composition of downstream Golgi and endocytic compartments [224]. The versatility of endomembrane trafficking design is consistent with the observation that Z-AAT can even exit the ER as a membrane-surrounded inclusion body, which is negative for autophagosomal and lysosomal markers, and can be inhibited by overexpression of calnexin [225]. Moreover, the ER has also been suggested to have COPII-independent mechanisms to dislocate large protein complexes involving the ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1) [226, 227], though the mechanisms remain unknown. Therefore, we consider membrane trafficking programs as dynamic modulators of the Anfinsen physiological state [26, 228], which utilizes developmentally specialized compartmentalization of

PN components to optimize use of the APL in each cell type, providing unique folding environments to respond to diverse proteostasis management signaling pathways that promote cell, tissue, and organismal survival.

Given the above, an untapped question is: how does the ER trafficking machinery recognize different energetic states of the incredibly diverse folding species exiting the ER to assess their specific destinations, such as autophagy, inclusion body, and/or ER-to-Golgi trafficking by COPII-dependent and/or COPII-independent mechanisms? Conventional ER export vesicles are coated by COPII complex, which have a typical size around 60–70 nm in diameter. Remarkably, we now appreciate that the size can be optimized depending on particular cargos, for example, collagen fibers (~300 nm) and lipoprotein particles (150–500 nm) require 300 nm vesicles [26, 215, 229–231] through the chaperome machinery. Cargo selection for export is initiated through the Sec23/24 adaptor complex that is subject to multiple posttranslational modifications for function [218, 232]. However, recent evidence suggests that the ability to generate diverse vesicle sizes that allow it to recruit large cargo involves additional trafficking components such as the adaptor escort proteins TANGO1 [233] and cTAGE5 [234] linked to the ER membrane. Moreover, it is sensitive to the ubiquitination state of one of the COPII components, Sec31 [26, 77, 229]. It will be interesting to see whether the size of COPII vesicles can be manipulated to differentially recruit WT and Z-AAT monomer or polymers for export as a therapeutic strategy. Even when Z-AAT successfully reaches in Golgi, resident proteins such as VIP36 and the KDEL receptor have been suggested to retrieve it back to the ER by interaction with its associated BiP [235]. VPS10 at the trans-Golgi has been shown to direct Z-AAT to lysosome for degradation [236], although it remains possible it has a normal function as an antiprotease inhibitor in the lysosome. Whereas considerable insight has been gained in understanding co-translational insertion and folding of the nascent chain in the ER, much less is known about the operation of the downstream trafficking compartments that could augment the protein fold through the COPI-mediated refinement and additional posttranslational modifications [78, 222, 223] that, when defective, are responsible for numerous pathological syndromes [26, 237]. Therefore, the unstable Z-AAT meets many challenges that can hinder its progress through exocytic pathway, highlighting the role of membrane compartments as proteostatic managers of the Q-state by generating sequential APLs. Additional studies may contribute to understanding the limitations restricting Z-AAT trafficking to serum for function in the lung.

Therapeutic Intervention Through the Acetylation–Deacetylation Network (HATs and HDACs)

We now appreciate that modifications of surface lysine (Lys) residues through competitive pathways of acetylation and ubiquitination can have a major impact on the Q-state responsible for protein stability and function by altering the response to the APL [19, 28–31]. The role of ubiquitination of surface Lys residues is complex and has been extensively reviewed [238]. Here, we focus on the impact of histone

acetyltransferase (HAT) and histone deacetylase (HDAC) in managing the APL in AATD.

HATs and HDACs mediate acetylation and deacetylation, respectively, modifications on the Lys residues of histones and nonhistone proteins. The acetylation status of a protein has a major impact on proteostasis pathways. While these modifications are well known to have an important role in the context of "epigenetics" that (re) program gene expression through histone modifications controlling nucleosome structure, we view a more general function of HATs and HDACs as "proteostatics" that modulate the charge status of surface Lys residues and, therefore, modify the Q-state for novel function in response to the local APL (Fig. 4.3). The importance of HAT/HDAC proteostatic activity is particularly evident not only in their impact in modulating posttranslationally the activity of many APL client proteins comprising the acetylome, but almost all PN components, including the core chaperones (Hsp90 [239], Hsp70 [240], Hsp40 [241], BiP [242, 243]), the HSR transcription factor HSF1 [244], degradation processes (by directly competing for Lys ubiquitination [245]), and membrane trafficking components including the assembly of microtubule and actin cytoskeletal polymers responsible for positioning and directing traffic through both the exocytic and endocytic pathways [246, 247]. Thus, HDACs globally impact proteostasis pathways by subtly modulating the thermodynamics and kinetics of the Q-state cloud biology in the context of the HDAC-

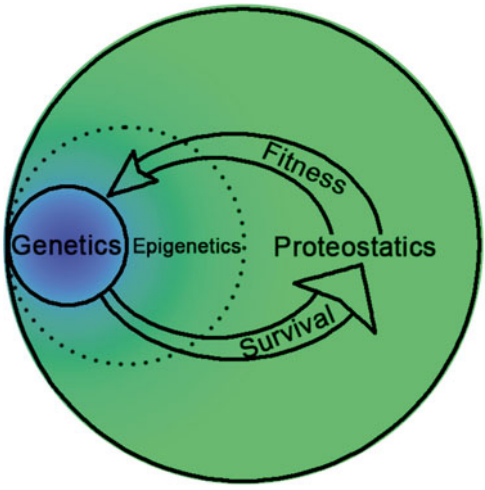

Fig. 4.3 The role of proteostatics in survival and fitness. Shown is the relationship between survival and fitness that is imprinted in genome through genetics, and epigenetic and proteostatic programs that are responsive to the environment. Genetics are defined by the inherited genome in the population that contributes to survival and fitness. Epigenetics, through histone modification of Lys residues by HATs/HDACs, alters the transcriptional environment of chromatin contributing to gene expression that promotes survival and "epi" (non-)genetic fitness in the population. We now appreciate that proteostatics encompasses not only the PN but includes epigenetics defined by HAT/HDAC-mediated acetylation–deacetylation pathways and contributes to the dynamic Q-state of the fold to promote daily survival in response to the environment and long-term fitness through modification of the genome

sensitive APL. In this view, proteostatic pathways that clearly encompass HDACs are potentially a therapeutic responsive real-time manager of protein folding imbalance in the cell [248] (Fig. 4.3).

Given the above, it is therefore not surprising that HDAC inhibitors (HDACi) have been shown to have beneficial effects for many protein misfolding diseases, such as cystic fibrosis [249], Gauchers disease [250], muscle atrophy [251], Niemann–Pick C [252, 253], and neurodegenerative diseases [254–258]. Specifically, we have shown that the pan Class I/II/IV HDACi suberoylanilide hydroxamic acid (SAHA) restores the secretion and activity of Z-AAT to ~50 % of the level that observed in WT AAT [27]. Currently, this correction appears to be mainly through inhibition of HDAC7, which is the same HDAC target that can be modified by SAHA to correct the folding of the most common mutation found in cystic fibrosis, F508del [249]. Incubation with SAHA or silencing of HDAC7 improves AATD through multiple pathways. For example, we have found that it upregulates the synthesis of Z-AAT, helps Z-AAT more efficiently exit calnexin chaperone cycle, modulates the ER redox environment by downregulating the expression of Ero1L, improves ER exit via COPII, and transits through the Golgi for improved secretion at the cell surface, as well as other pathways that we may not appreciate. This may simply be due to the fact that SAHA or silencing of HDAC7 produces a robust proteostatic encoded folding and trafficking environment (APL) for Z-AAT, which, interestingly, persists even after compound withdrawal following a chronic treatment regimen suggesting that imprinting occurs during epigenetic modification by HDACi [202]. Though SAHA-treated cells still produce polymeric Z-AAT, combining SAHA and polymer inhibitors [40, 169–171, 199, 259] may give an increasing yield of monomeric Z-AAT in plasma for lung protection.

In addition to its effect on Z-AAT folding and trafficking, SAHA exhibits anti-inflammatory properties (e.g., [260–264]), which could provide an unanticipated benefit in AATD by modulating inflammation in COPD [27]. However, the role of HDACs in lung biology is complicated. Class I and II HDAC activity is reduced in the lung of COPD patients even though the HAT activity remains unchanged [265, 266]. Moreover, reduction of HDAC2 causes corticosteroid resistance in COPD patients [267] leading to defective regeneration of airway epithelium [221], and reduction of HDAC3 increases the cytokine expression which would enhance inflammatory responses [268]. Besides the members of Class I and II, Zn^{2+}-dependent HDACs targeted by SAHA, SIRT1, one of the class III NAD (+)-dependent HDACs, is also reduced in lung of COPD patient [269]. Reduction of SIRT1 causes increased acetylation of histone H3 and NF-κB leading to upregulation metalloproteinase-9 expression affecting alveolar structure [270, 271]. In contrast, SIRT1 has been found to protect against emphysema by deacetylation of the FOXO3 transcription factor [272]. Consistent with these results, the SIRT1 activator resveratrol [262] and SRT1720 [273] were shown to alleviate lung inflammation.

In summary, by modulating the acetylation–deacetylation proteostatic balance driven by HAT-/HDAC-mediated Lys modification, it may be possible to both recover the shortage of functional antiprotease (AAT) production from the liver and decrease the threat from inflammatory pathways in the lung. However, it is clear that future

studies will be required to understand how the acetylation–deacetylation network managed by the HDAC population in the liver and lung tissue environments contribute to stabilization of function and to develop specific HDAC agonist/antagonists to optimize their impact on rescued Z-AAT in these environments. By using HDACi to alter the Q-state dictating protein folding stability and function, we may be able to synchronize the protein folding and function within the APL of multiple environments, an approach that could have an impact on disease etiology by using the same pathways biology nature has evolved to equilibrate the activity of diverse protein structures in a common proteostatics environment through Lys modification (Fig. 4.3).

Perspective: The Future of Proteostasis Management in AATD

We have described potential rationales and possible strategies to manage the APL and hence the function of the folding proteome responsible for human AATD. Though Anfinsen, who received the Nobel Prize in 1972 [228] for demonstrating that the primary sequence encoded by the genome plays a key role in generating the protein fold, he also posed a major challenge by suggesting that the biologically active conformation of a protein is actually determined by its environment—referred to as the physiological state. For the most part, the current practice of medicine ignores the underpinning dynamics of this physiological state as a focus for therapeutics. Given the evolutionary process [133], by definition we are all mutants. We achieve function as a consequence of proteostatic optimized evolvability [19, 34, 192, 274] that promotes daily survival and ultimately, through emergent proteostatics, fitness [19, 35, 275]. During the AAT functional cycle, the RCL needs to fully insert into the β-sheet A to flip the targeted elastase to another side of AAT [63, 64], which is likely thermodynamically and/or kinetically favored by the Z-variant mutation due to the increased metastability of the β-sheet A. However, this potential "functional" adjustment renders Z-variant polymerization propensity too great since the metastable β-sheet A also induces the intermolecular RCL insertion [40, 199, 276]. Such "off-pathway" mutations persist through evolutionary selection because of the highly evolved PN, which buffers these out-of-balance energetic states [19, 274]. Only when the normally robust PN is impaired in response to global stress or chronic aging does the "misfit" folding species begin to exhibit its proteotoxic function as occurs in AATD. We are now at the beginning of our efforts to remove the cloak masking the role of the PN in managing inherited genetic changes—proteostatic buffered changes that have worked in a positive sense and exceedingly well over the last 3.5 billion years to evolve and diversify extant life [19, 275]. We project that future studies defining proteostatic principles will provide insights and tools to adjust the dysfunctional APL found in AATD back to a healthy state, leading to reduced fragility and improved resilience of the liver and lung environments.

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Chapter 5
United States Targeted Detection Program for Alpha-1 Antitrypsin Deficiency

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Abbreviations

AAT	Alpha-1 antitrypsin
AATD	Alpha-1 antitrypsin deficiency
FEV_1	Forced expiratory volume in one second
COPD	Chronic obstructive pulmonary disease

Introduction

Alpha-1-antitrypsin deficiency (AATD) is an under-recognized hereditary condition characterized by low serum levels of alpha-1 antitrypsin (AAT) and an increased risk for the development of respiratory and hepatic disease [1–3]. Fewer than 10 % of the individuals with severe AATD are recognized clinically, typically after long diagnostic delays [4–6]. Its early detection is crucial as it may prompt specific interventions

Authors' Contributions Adriano Tonelli participated in the study design IRB application, statistical analysis, interpretation of data, and writing and revision of the manuscript.

Farshid Rouhani participated in the study design data collection, interpretation of data, and writing and revision of the manuscript.

Pam Schreck participated in the IRB application data collection and writing and revision of the manuscript.

Mark Brantly participated in the study design data collection, interpretation of data, and writing and revision of the manuscript.

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such as testing of family members, genetic counseling for patients and families, lifestyle changes (e.g., smoking cessation and occupation change), screening for other manifestations of the disease, and consideration of augmentation therapy [7].

An early diagnosis of AATD can be accomplished by screening the general population [8, 9] or by targeting high-risk groups. Population-based screening has the advantage of detecting clinically unaffected individuals at a younger age, but this modality is expensive and challenging to perform on a large scale [10]. Targeted detection is associated with higher recognition rates and lower costs. The diagnostic yield and costs of this approach depend largely on the characteristic of the individuals tested and the protocol used for processing the samples [11]. The ATS/ERS statement describes the conditions that should prompt testing for AATD, serving as the basis for targeted testing [1].

The gene that codes for AAT protein is located on the long arm of chromosome 14 (14q31–32.3). More than 120 variants of this gene exist. At least 32 of these variants are associated with altered, reduced, or absent serum AAT; however, the vast majority (~95 %) of AATD individuals with lung disease are homozygous for the Z allele [12, 13]. In addition, subjects heterozygous for the Z allele (PI MZ and PI SZ) have a modest increase in the risk of developing lung disease [1, 14–16].

Detection of AATD

Different algorithms for the detection of AATD that combine measurement of AAT protein in plasma, genotyping, and isoelectric focusing (IEF) have been proposed. The algorithm used in this study begins with genotyping for the two most common deficient alleles involved in the disease, namely PI*Z (Glu 342 *G*AG → Lys *A*AG) and PI*S (Glu 264G*A*A → Val G*T*A). Genotyping is less technically demanding than IEF and it allows us to identify the prevalence of the main genotypic variants in the population studied, a relevant analysis, since one of the main barriers for not testing for AATD individuals is the perceived low yield of the test [17].

Little is known about the demographic, clinical, and genotype characteristics of individuals who underwent target screening for AATD in the USA. We sought to determine the prevalence of the main AATD genotypic variants and describe the demographic and clinical characteristic of the patients who underwent testing in one of the largest targeted detection programs in the USA. In addition, we explored the relationship between age and genotype, gender, race, smoking history, year of testing, region of origin of the sample in the USA, and reason for testing.

National Detection Program

Data from subjects who underwent AATD testing as part of the National Detection Program for alpha-1-antitrypsin deficiency from December 2003 to July 2009 were retrospectively analyzed. Kits for alpha-1 antitrypsin detection, containing

a healthcare provider's guide to alpha-1 antitrypsin deficiency, the ATS/ERS statement on standards for the diagnosis and management of individuals with alpha-1 antitrypsin deficiency [1], and several blood collection cards, were distributed to respiratory physicians throughout the country. A total of 5195 US physicians ordered at least one AATD test. Each blood collection card was accompanied by a questionnaire asking demographic and clinical information, including age, gender, race, geographic data, smoking history, diagnosis of respiratory or hepatic diseases, and family history of respiratory diseases. Family history of respiratory diseases referred to the presence of AATD, COPD, or emphysema in parents or siblings. Although a more recent version of the questionnaire discriminated among these conditions, for consistency these respiratory diseases were grouped together.

We recorded the US State where the sample was originated and grouped them in Regions (Northeast, Midwest, South, and West) as defined by the U.S. Census Bureau [18]. We excluded from the analysis those samples in which the origin was not recorded or they originated from Canada or Puerto Rico ($n = 328$).

Although we suggested testing potential AATD individuals following the recommendations proposed by the ATS/ERS statement [1], in practice, individuals were tested according to the discretion of physicians who received the kits. To simplify the analysis, we divided the reasons for screening into five main categories: (1) presence of respiratory diseases such as COPD, asthma, and bronchiectasis, (2) presence of hepatic diseases (hepatitis or cirrhosis) or abnormal liver function tests, (3) combination of respiratory and hepatic diseases, (4) presence of family history of COPD or AATD, and (5) family history of respiratory diseases plus respiratory and/or hepatic diseases.

Fresh whole blood was collected by fingertip puncture on a 903 filter paper. Blood was allowed to air dry before mailing. Once the dried blood spot (DBS) sample was received, we processed it following the algorithm shown in Fig. 5.1. PI*Z and PI*S genotyping was performed with End-point allelic discrimination on an ABI Prism 7500 Fast System (Applied Biosystems) using 3.2 mm DBS punches. Assays were run in a 96-well format according to the manufacturer's instructions. Nephelometry determination was performed using methods described previously [19, 20]. For reflex testing, we performed single nucleotide polymorphisms (SNP) analysis and DNA sequencing using similar methodology to Ferrarotti et al. [21].

Dried blood spot collection methodology is minimally invasive, requires a small amount of sample, and is easier to preserve and ship [22]. In addition, we have demonstrated an excellent correlation between AAT protein concentration measured in plasma and in DBS ($n = 347$, $r = 0.95$) (Fig. 5.2).

Although this model confirmed the presence of S and Z alleles, and detected the majority of deficient individuals, it did not directly test for the common "wild-type" M variant. The presence of PI*M was assumed when genotyping for S and Z was negative and the DBS AAT protein level was concordant with this genotype. We used PI*M instead of PI non S-non Z (which is the correct term) for simplicity.

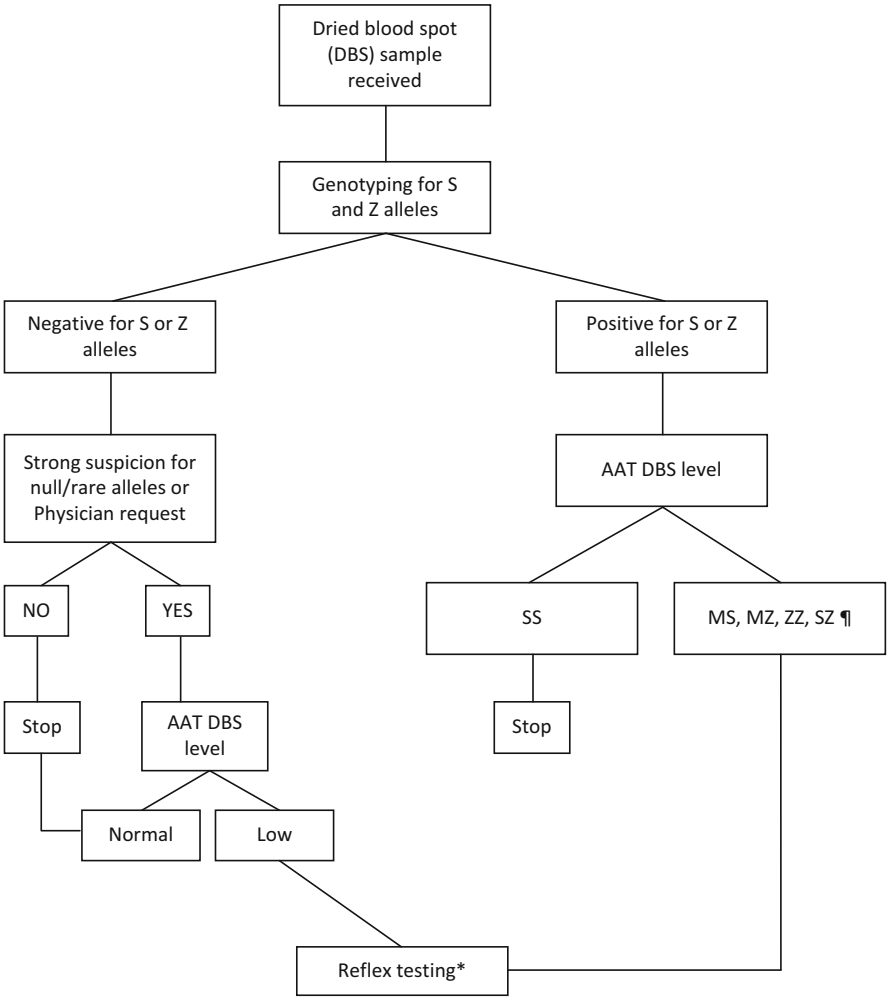

Fig. 5.1 University of Florida detection Lab Testing Algorithm. Heterozygous and homozygous individuals for PiZ undergo reflex testing for confirmatory purposes. In the case of PiMZ and PiMS individuals with low DBS AAT levels, reflex testing is performed to rule out the combination of a Z or S allele with a Null or Rare allele. *Blood sample is requested for PI typing by single nucleotide polymorphism (SNP) and DNA sequencing

Results of the National Detection Program

A total of 40,049 unique subjects underwent target screening for the presence of AATD from December 2003 to June 2009. For this analysis, we included adult patients (≥16 years) with an available genotype. Data from 37,708 individuals were used for further analysis (Fig. 5.3).

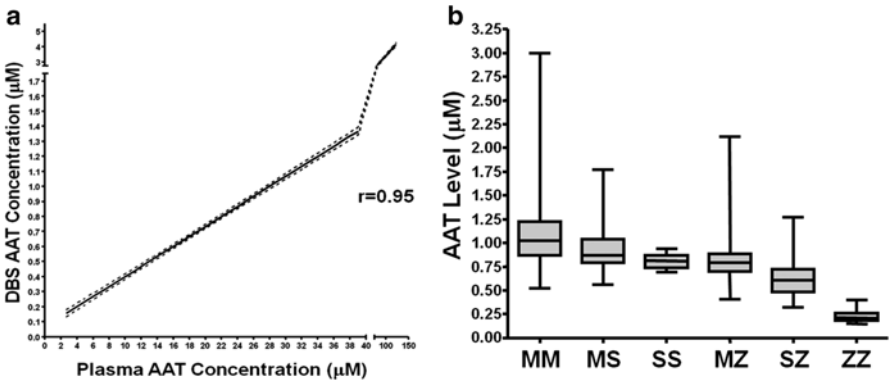

Fig. 5.2 Correlation between AAT protein concentration measured in plasma and DBS (panel **a**), and AAT protein concentration in DBS according to genotype (panel **b**) ($n=347$)

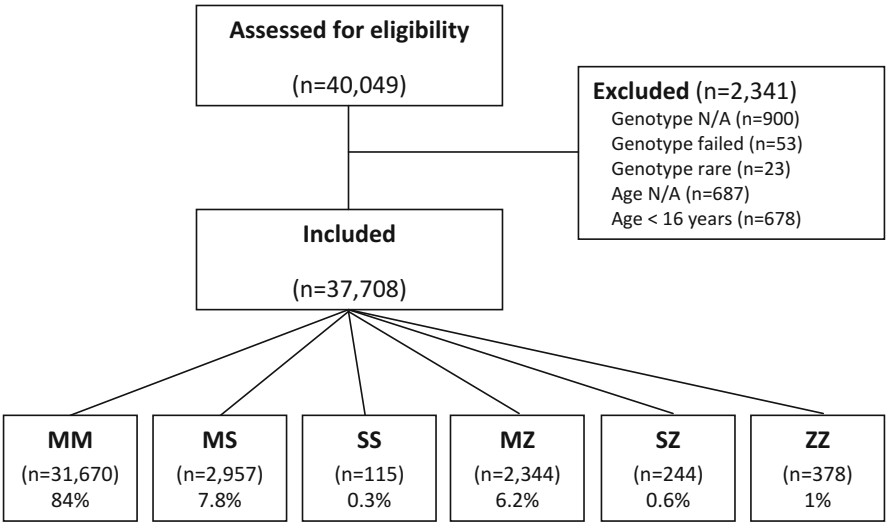

Fig. 5.3 Flow diagram of the study population and results of genotyping

The genotypes were MM in 84 % ($n=31,670$), MS in 7.8 % ($n=2957$), SS in 0.3 % ($n=115$), MZ in 6.2 % ($n=2344$), SZ in 0.6 % ($n=244$), and ZZ in 1 % ($n=378$) (Fig. 5.2). One in 6.25 individuals had a genotype other than MM. At least one Z allele was present in 7.9 % of the samples ($n=2966$). The S and Z allelic frequency was 4.55 % and 4.43 %, respectively.

Compared with data obtained from control cohorts of genetic epidemiological studies in the USA ($n=27,809$) [23], we found an increase in the detection rate of genotypes other than PI MM in our program. The detection rate markedly increased for the genotype ZZ by a factor of approximately 48 times (Table 5.1).

Table 5.1 Comparison between prevalence of PI ZZ in epidemiologic studies versus our targeted screening program

	Epidemiologic studies (%)[a]	Targeted screening (%)	Increase in detection rate
PI MM	91.14	84	0.92
PI*MS	5.88	7.8	1.33
PI*SS	0.095	0.3	3.16
PI*MZ	2.78	6.2	2.23
PI*SZ	0.089	0.6	6.74
PI ZZ	0.021	1	47.62
PI*Z allele	1.45	4.55	3.14
PI*S allele	3.07	4.43	1.44

[a]Data obtained from de Serres et al. who estimated the gene frequency according to the race and calculated the prevalence of different AATD phenotypes in the general US population [23]

Age

The age at the time of genetic testing for the entire cohort was 57.3 ± 15 years (Table 5.2). Individuals heterozygous for PI*Z were genetically diagnosed 3.8 (95 % CI: 0.3) years before subjects without any Z allele ($p < 0.001$). Individuals homozygous for PIZ were genetically diagnosed 1.96 (95 % CI: 0.7) years before subjects heterozygous for PI*Z ($p = 0.007$). PI*Z homozygous were genetically diagnosed 5.7 (95 % CI: 0.7) years earlier than subjects without any Z allele ($p < 0.001$).

The difference in age at the time of the testing between PI*MM and PI ZZ individuals was 5.73 years (95 % CI 4.2–7.2) (Table 5.3 and Fig. 5.4). PI ZZ individuals were genetically diagnosed at the age of 50 years or above and 70 years or above in 55 % and 9 % of the cases, respectively. The oldest individual diagnosed with the ZZ genotype was 87 years old.

Gender

Of all the subjects, 58 % were women. A lower percentage of women were observed in individuals heterozygous (55.2 %) and homozygous (49.2 %) for PiZ ($p < 0.001$) (Table 5.2). This female gender predominance was maintained for all genotypes, except for PI ZZ (Table 5.3).

In PI ZZ individuals, the age at the time of genetic testing did not differ between genders [men: 52.1 ± 11 years versus women: 51.7 ± 14 years ($p = 0.75$)].

Table 5.2 Characteristics of the individuals in the overall population and subjects with no Z allele, heterozygous and homozygous for PI*Z

	Overall population	No Z alleles	PI*Z heterozygous	PI*Z homozygous	p^*
n	37,708	34,742	2966	378	
Age (years) mean±SD	57.3 ± 15	57.6 ± 15	53.9 ± 16	51.9 ± 13	<0.001
Gender (women %)	58	58	55.2	49.2	<0.001
Race (%)					
White	80.2	79.4	90.3	89.1	<0.001
African-American	6.9	7.4	0.7	–	
Hispanic	6.1	6.4	2.3	3.3	
Asian-Pacific	0.8	0.9	0.1	–	
American-Indian	0.2	0.2	0.2	–	
Mixed	0.4	0.4	0.7	0.5	
Other	0.7	0.7	0.2	0.3	
Not reported	4.6	4.5	5.7	6.8	
Smoking (%)					
Current	20.6	20.9	18.2	11	<0.001
Ex-smokers	41.7	41.8	41.1	39.5	
Never-smokers	28.2	27.9	31.1	34.3	
Unknown	9.5	9.5	9.7	15.2	
Origin of the sample (US Region) (%)					
Northeast	13.2	13.1	14.1	12.9	<0.001
Midwest	18.9	18.7	21.1	16.8	
South	55	55.3	50.8	54.4	
West	12.9	12.8	14	15.9	
Reason for testing (1–5) (%)					
1	68.2	69.4	54.1	51.3	<0.001
2	2.3	2.3	2.6	3.4	
3	3.8	3.9	3.2	4.5	
4	1	0.4	7.8	7.7	
5	1.4	0.9	6.6	9.3	
Unknown	23.3	23.1	25.8	23.8	

$^*p$ for the comparison of No Z alleles versus heterozygous and homozygous PI*Z

Race

The race distribution of the entire cohort, individuals heterozygous and homozygous for PI*Z, divided by genotype, is shown in Tables 5.2 and 5.3. The PI*Z allele was present mainly in Caucasians and rarely in individuals of African-American, Asian-Pacific, or American-Indian descent. The PI*ZZ allele combination was not seen in the latter three groups (Table 5.2).

Table 5.3 Characteristics of the individuals divided by genotype

	MM	MS	SS	MZ	SZ	ZZ	*p*
Age (years)	57.6 ± 15	57.8 ± 15	55.2 ± 15	53.8 ± 16	54.8 ± 16	51.9 ± 13	<0.001
Gender (women) (%)	58	57	55	56	51	49	<0.001
Race (%)							
White	79.1	82.5	81.7	90.5	88.4	89.1	<0.001
AA	8	1.7	–	0.8	–	–	
Hispanic	6.1	2.2	13.9	2.1	3.7	3.3	
Smoking history (%)							
CS	20.8	22	24.8	18.5	14.5	11	<0.001
ES	41.7	41.7	46	40.1	50.6	39.5	
NS	28	26.9	20.4	31.7	24.9	34.3	
NR	9.5	9.4	8.8	9.6	10	15.2	
Origin of the sample (%)							
Northeast	13.3	11.1	10.5	13.9	16.5	12.9	<0.001
Midwest	18.8	18.2	21.9	20.8	23.6	16.8	
South	55.2	56.6	60.5	51	48.8	54.4	
West	12.7	14.2	7	14.3	11.2	15.9	
Reason for testing[a] (%)							
1	69.5	68.9	64.3	53.9	56.1	51.3	<0.001
2	2.3	2.5	3.5	2.6	2.5	3.4	
3	3.9	3.9	5.2	3.2	3.3	4.5	
4	0.3	0.8	0.9	7.6	9.8	7.7	
5	0.8	1.4	2.6	6.4	8.6	9.3	

[a]Reason was not available in 23.2, 22.4, 23.5, 26.4, 19.7, and 23.8 % of the samples for genotypes MM, MS, SS, MZ, SZ, and ZZ. Abbreviations: *F* Female, *M* men, *W* white, *O* other race, *NR* not reported, *CS* current smoker, *ES* ex-smoker, *NS* never-smoker

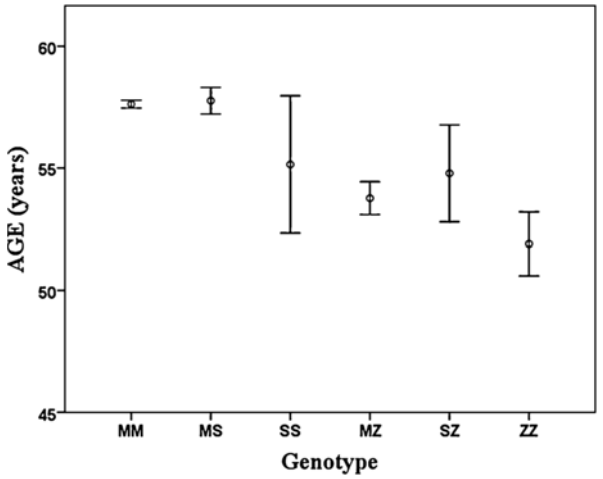

Fig. 5.4 Mean age (±2 Standard Error) according to genotype

Smoking History

The smoking history for the entire cohort, individuals heterozygous and homozygous for PI*Z and divided by genotype, is shown in Tables 5.2 and 5.3. The percentage of never-smokers increased and the proportion of current smokers decreased when comparing subjects with no Z allele to subjects with one and two PI*Z alleles ($p<0.001$) (Table 5.2). Interestingly, PI ZZ women included more never-smokers individuals than PI ZZ men (42.9 vs. 25.9 %, $p<0.001$). In contrast, PI ZZ men included more ex-smokers subjects than PI ZZ women (49.7 vs. 28.8 %, $p<0.001$).

Year of Testing

We tested 3142 samples in 2004, 3766 in 2005, 5257 in 2006, 7283 in 2007, 11,006 in 2008, and 7116 during the first 6 months of 2009. The corresponding mean ages at genetic testing were 54.4 ± 15 years, 58.2 ± 16 years, 57.3 ± 15 years, 57 ± 15 years, 57.6 ± 14 years, and 58.3 ± 15 years, respectively ($p<0.001$). In contrast, the age at genetic testing in PI*ZZ individuals was not significantly different for the years 2004–2009.

Origin of the Sample

Samples originated from US Postal Regions Northeast, Midwest, South, and West in 13.2 %, 18.9 %, 55 %, and 12.9 % of the cases, respectively. The age at genetic diagnosis of PI*ZZ was not significantly different among regions.

Reasons for AATD Testing

Patients were tested predominantly for respiratory diseases (68.2 %) (Tables 5.2 and 5.3). Heterozygous and homozygous for PI*Z had a higher proportion of individuals with family history of respiratory diseases than subjects without any Z allele ($p<0.001$).

Interpretation of the National Detection Program

In this study, which included a large number of patients who underwent AATD targeted detection in the USA, we observed a higher frequency of AATD alleles compared with population screening studies in the USA. We noted that patients

were genetically tested at a relatively advanced age. Homozygous and heterozygous variants of PI*Z were diagnosed at a younger age, a difference more noticeable in current smokers. Interestingly, no correlation was found between age at genetic diagnosis and gender, race, year of genetic testing, and origin of the sample in the USA in PI ZZ subjects.

Targeted detection is a tool to increase AATD recognition that has a higher yield and lower cost than population screening. Using the algorithm described in Fig. 5.1, we found that 16 % of the samples had a genotype other than PiMM, a figure almost twice the prevalence observed in the general population (8.8 %) [24]. In the present assessment, the detection rate for PI ZZ was 1 %, which increased to 1.1 % (401/37,731) and 1.71 % (645/37,731) if Z null/rare and PI SZ genotypes were sequentially included. When compared with general screening, the detection rate of PI ZZ individuals increased approximately 48 times with targeted detection. A review of the literature reveals a broad degree of detection rates for PI ZZ, ranging from 0.2 to 6.6 % [11, 17, 25–27]. This variation is likely to result from the different criteria used for testing of potential AATD subjects.

The use of the real-time polymerase chain reaction for genotyping, as the first test in our algorithm, allows the detection of the two most common deficiency alleles found in AATD individuals in a rapid and efficient way. If used alone, this test may result in the misclassification of rare deficiency alleles. For this reason, we measured AAT protein DBS levels in patients homozygous and heterozygous for PI*Z and PI*S, for confirmation and to avoid misclassification of a second deficient allele. In the event that AAT protein level was low in DBS, we perform PI typing as a reflex testing. In PI MM individuals, we only performed AAT plasma levels in those subjects considered to be at high risk (less than 50 years of age, presence of emphysema, and/or suggestive family history). The routine measurement of AAT plasma levels in all PI MM subjects would not be cost-effective, as a minuscule proportion of these subjects would have severe AATD. To prove this concept, we analyzed data from the Alpha-one coded testing (ACT) trial (unpublished) on 5670 individuals with genotype PI*MM who had measurement of DBS AAT protein level. In this study, only 27 patients (0.4 %) had an AAT DBS level below or equal to 0.6 μM (a conservative cutoff).

A similar approach to diagnosis was validated by other studies [28, 29] that used genotyping and AAT protein serum concentrations with reflex IEF if results are discordant. Snyder et al. [28] found that 4 % of the samples were discordant for genotype and AAT protein serum concentration, requiring reflex PI typing. Bornhorst et al. [29] observed that by adding genotyping, AAT protein serum concentration, and reflex PI typing, 1.6 % of the samples with two deficient alleles were misclassified..

Most of the patients who had PI ZZ genotype were 50 years of above, of white race, and had a history of cigarette smoking. AATD is largely under-recognized and there are significant diagnostic delays [4], due to the fact that many individuals do not have significant clinical impairment, especially if they do not smoke. This may in part explain why more than 50 % of AATD individuals were diagnosed after the age of 50 years. Other explanations are the relatively low percentage of patients wo

were tested for family history of respiratory diseases including AATD (17 %) and the relatively low rate of current smokers (10.6 %). The mean age at the time of inclusion of AATD individuals in different registries ranged from 46.1 to 51 years [2, 27, 30, 31], though this result may be biased as patients may have been diagnosed several years prior to the inclusion in these registries. PI ZZ individuals were diagnosed at a younger age than PiMM subjects. Similarly, Lieberman et al. [32] found that COPD individuals homozygous for PI ZZ were younger (55.9 ± 9.8 years) than PI MM subjects (64 ± 8.7 years, $p < 0.001$).

We observed a balanced gender distribution in PI ZZ patients, although more women were tested than men. Other studies found a slight male predominance (52.3–55 %), particularly in index cases [2, 30, 33].

Homozygous PI*Z individuals were only of Caucasian, Hispanic, or mixed race, even though a significant number of patients were of African-American, Asian-Pacific, and American-Indian race. In these latter races, few individuals were heterozygous for PI*Z. This marked white race predominance was observed in other studies, ranging from 96.2 to 99.2 % [2, 31]. de Serres et al. described that the highest risk for AATD is found in Caucasians, followed by Hispanics and African Americans. The lower prevalence is among Mexican Americans and Asians [34].

Homozygous or heterozygous individuals for PI*Z had less percentage of current smokers and more never-smoker individuals than the overall population studied. These findings are similar to the smoking status of other studies (current smoker ranged from 2.1 to 8.3 % [2, 12, 31]. In our study, PI ZZ smokers were diagnosed at a younger age than nonsmokers or ex-smokers, a finding supported by other author that demonstrated that nonsmoking AATD individuals have a delay of symptoms and most of them a normal life span [33, 35–38]. Interestingly, the duration of smoking also influenced the age at the time of testing, since individuals who smoked more than 10 years were diagnosed 7.4 (95 % CI: 2.6) years before than subjects who smoked less than for this duration of time ($p = 0.005$).

The number of subjects tested in our program has increased during the 6-year study period. The age at the time of testing has remained stable at around 57–58 years after an initial increase. Contrary to what one would have expected after the release of the ATS/ERS consensus statement in 2003 [1], we did not observe a reduction in the age of the subjects tested for the deficiency.

More than 50 % of the samples were originated in US Postal Region III, as we are the referral center for the State of Florida AATD detection program. We did not find a statistical difference in the prevalence of AATD in the samples tested when divided by Regions. Furthermore, the age of individuals tested from the four US Postal Regions was similar.

More than half of the individual were tested due to respiratory symptoms. In a small percentage (2.4 %) of the samples, a family history of respiratory disease and AATD was volunteered. This percentage is lower than the one reported by other studies [2, 30]. We did not find a correlation between reason for testing and age at the time of genetic diagnosis.

There are limitations to this study. The results only apply to individuals who underwent targeted detection and are not representative of all individuals with the

disease. Symptomatic individuals are more likely to undergo testing than asymptomatic subjects, leading to ascertainment bias. Single rare deficiency alleles were not detected by our algorithm and few homozygous null/rare individuals could have been missed. More than 50 % of the samples originated in US Region IV (other Regions were less represented), and a family history of AATD was not separated from a family history of respiratory diseases (COPD or emphysema). In spite of these limitations, this study provides a valuable insight into the prevalence and demographic and clinical characteristics of severe AATD in patients who undergo targeted screening in the USA.

The results of this study support the use of targeted detection programs to identify non-index and index individuals with severe AATD who can benefit from genetic counseling, risk-reduction behaviors, and/or augmentation treatment. Furthermore, the program provides opportunities to increase the awareness about this largely unrecognized disease.

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Chapter 6
Lung Disease of Alpha-1 Antitrypsin Deficiency

Robert A. Sandhaus

Introduction

A deficiency of the circulating glycoprotein, alpha-1 antitrypsin (AAT), can lead to clinically significant lung injury in a proportion of those who inherit this condition. The risk of lung disease is affected by the specific genotype of the SERPINA1 gene that codes for the AAT protein, risk factor exposures, and the individual's genetic background [1, 2]. Even those with the most commonly identified severely deficient genotype, PiZZ, may lead their entire lives without evidence of clinically significant, AAT deficiency-related disease. The types of lung injury that occur in individuals with AAT deficiency (AATD) are not unique; people with normal AAT genes can develop similar lung diseases with extended exposures to the same risk factors as those that accelerate damage in those with AATD. In some ways, AATD simply magnifies and amplifies the damage done to lung tissue by such risk factors, most prominently tobacco smoke exposure [3].

The two most common pulmonary conditions associated with AATD are pulmonary emphysema and bronchiectasis. The pulmonary emphysema tends to be panlobular in distribution and most prominent in the lower lobes but this is not invariable [4]. Upper zone disease and centrilobular distribution can be seen in AATD-related lung disease but this tends to be less common. Similarly, the bronchiectasis noted in those with AATD does not have unique characteristics except for its ubiquity. In one study [5], approximately 94 % of individuals with AATD had computerized tomography (CT) lung radiology consistent with bronchiectasis. In most cases, this bronchiectasis is asymptomatic, the so-called anatomic bronchiectasis.

Rarer lung conditions have been associated with AATD. Patients with ANCA-positive granulomatosis with polyangiitis (formerly Wegener's granulomatosis)

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appear to have a higher prevalence of abnormal AAT genotypes than the general population [6]. There is a biochemical logic to this since the ANCA antigen, proteinase 3, is inhibited by AAT.

It is important to identify individuals with AATD, especially those with lung disease, because progression of disease can be prevented, slowed, or halted by elimination of risk factors [3]. In addition, in many countries, individuals with emphysema due to AATD can receive intravenous infusions of wild-type AAT protein, derived from human plasma, a therapy shown to reduce the rate of emphysema progression. Potential future therapies for AATD-related lung disease include gene therapies, stem cell therapies, small-molecule protease inhibitors, and gene correction. While most of the pulmonary injury associated with bronchiectasis and emphysema is considered irreversible, future therapeutics may reverse or repair these injuries.

History

Lung disease and AATD have been intimately associated since the first description of this genetic condition. In 1963, the publications by Laurell and Eriksson identified a genetic deficiency of the AAT protein and described its association with "familial emphysema" [7]. A mechanism that might link this plasma protein deficiency with the alveolar destruction of pulmonary emphysema was unknown at the time.

At essentially the same time, Gross and associates found that intratracheal instillation of certain proteases produced pulmonary injury in rodents analogous to the histology of human pulmonary emphysema [8]. Further work by a number of investigators found that only proteases able to degrade elastin, a prominent lung connective tissue protein, were capable of inducing pulmonary emphysema in animal models [9, 10]. While a number of different proteases with elastolytic properties had been identified and tested with success, none of these appeared to have access to human lungs. This led to questions about the applicability of these animal models to human disease. Since 1964 was the year of the US Surgeon General's report linking cigarette smoking to lung diseases including pulmonary emphysema, investigators even evaluated tobacco and tobacco smoke for elastolytic activity, but none was found.

This picture changed in 1968 with Janoff and Sherrer's first description of a potent elastolytic protease isolated from the human polymorphonuclear neutrophilic leukocyte or neutrophil [11]. This "human neutrophil elastase" (HNE) proved to be quite potent at producing emphysema in a number of different animal species. Soon after the first descriptions of the effects of this elastase, reports appeared documenting that human AAT protein was an exceptionally potent inhibitor of HNE [12, 13]. While AAT is a broad-spectrum serine protease inhibitor (SERPIN), its inhibitory kinetics suggest that its preferred target is HNE. Finally, a potential mechanism for the emphysematous lung injury seen in patients with AATD was emerging.

The proposed mechanism for lung injury in AATD revolved around the protease-antiprotease balance in the lung parenchyma. In individuals with normal levels of AAT protein in the lung, neutrophils perform their function as the major acute inflammatory cells of the body with AAT protein acting as a barrier of sorts to protect normal lung connective tissue, especially elastin, from proteolytic degradation during lung inflammation. In individuals with a hereditary deficiency of AAT, there is insufficient anti-elastase protection and, over time, emphysema can result [14].

Soon after this scenario became accepted, a new wrinkle appeared in the story. Investigators noted that certain products of tobacco combustion were capable of inactivating the anti-elastase properties of AAT [15]. Simply bubbling cigarette smoke through a solution of AAT could completely inactivate elastase inhibition by the AAT protein. The mechanism of this inactivation proved to be oxidation of a methionine residue in the catalytic site of the AAT molecule [16]. The result is that the protease pathogenesis mechanism of pulmonary emphysema was extended to those with normal circulating AAT levels who smoke cigarettes.

Given this unifying theory of emphysema pathogenesis, how can one explain the fact that some with AATD never develop clinically significant lung disease? Individuals with the most commonly identified genotypes associated with AATD have plasma AAT levels that are approximately 10–15 % of the usual levels expected in individuals with the normal, PiMM, AAT genotype. It appears that under most circumstances in individuals with AATD, when there is no increased inflammation in the lungs and there is little oxidative inactivation of the AAT protein bathing the lungs, even this reduced level of AAT is sufficient to protect the lung from significant destruction. Events that can upset this delicate balance would include lung infection and exposure to agents that can increase inflammation or decrease the effectiveness of AAT as an elastase inhibitor, such as cigarette smoke, including secondhand smoke, and occupational exposures.

A group of AAT mutations, known collectively as “null mutations,” lead to the production of no AAT protein [17]. Virtually all individuals identified with two null SERPINA1 genes develop pulmonary emphysema as young adults and, without therapy, their disease progresses rapidly. This suggests that even having a reduced level of AAT protein in the lungs provides significantly more protection than a total absence of AAT protein.

In the early 1980s, armed with this understanding of the role of AAT in lung protection, a group of investigators in the Pulmonary Division of the NIH intramural facility in Bethesda, Maryland, sought to evaluate whether supplementation of the circulating levels of AAT protein might benefit patients with lung disease due to AATD [18]. They developed a methodology for the purification of AAT from the plasma of healthy individuals with normal SERPINA1 genes and infused individuals with AATD with this purified AAT concentrate. Further, they demonstrated that they could raise both the plasma and lung levels of AAT with these infusions. Unfortunately, AAT is cleared relatively rapidly from the circulation with a half-life of less than 1 week, so infusions with multiple grams of this “augmentation therapy” on a weekly schedule were required to maintain levels felt to be adequate.

Since this regimen failed to maintain a "normal" trough level through the end of a week, even once a steady-state level had been achieved, a "protective threshold" was defined, based on the evaluation of AAT genotypes that produced mildly decreased levels but seemed to have no or minimal increased risk of lung disease. The regimen of 60 mg/kg of body weight, given by weekly intravenous infusion, was found to keep patients studied above this protective threshold [19].

The US Food and Drug Administration (FDA) approved the marketing of the first AAT augmentation therapy based on this biochemical efficacy in December of 1987. Since it was appreciated that a significant percentage of individuals with AATD would never develop lung disease, it was directed that this therapy should be reserved for individuals with severe AAT deficiency who had documented lung disease. For 15 years, this was the only augmentation therapy product approved in the USA and was widely prescribed to treat AATD-related emphysema. Beginning in 2003, additional products gained marketing approval until, at the time of this writing, there are a total of four such products available in the USA and several products available in other parts of the world. In the USA, each of the three additional augmentation therapy products was approved based on small studies that demonstrated the newer products were not inferior to the original in safety and biochemical efficacy.

Liver injury in AATD is associated with polymerization and retention of AAT protein within hepatocytes (see appropriate chapters in this book). Recent work has demonstrated that these same polymers can be found in blood and within the connective tissue of the pulmonary parenchyma in deficient individuals [20, 21]. In addition, these polymers in the lung appear to have pro-inflammatory properties that may enhance the lung damage associated with AATD.

Clinical Presentation of AATD Lung Disease

Lung disease in AATD is mostly indistinguishable from chronic obstructive pulmonary disease (COPD) in general. Identification of AATD in a given individual depends on a laboratory diagnosis indicating a low level of circulating AAT protein and Pi-type or genotype revealing two abnormal AAT-coding genes [22]. Pi-typing evaluates circulating AAT protein using isoelectric focusing to reveal differences in protein migration patterns due to molecular isoforms. Genotyping evaluates the SERPINA1 gene for known mutations associated with deficiency. In rare cases, it is sometimes necessary to sequence the SERPINA1 genes to identify a rare or previously unknown genotype. To date, more than 400 mutations of the SERPINA1 gene have been reported. A minority of these is associated with a deficiency or dysfunction of circulating AAT protein.

While the definitive diagnosis of AATD is based on laboratory testing, there are clinical indicators that increase the likelihood of its presence. Precocious emphysema or emphysema out of proportion to smoking history often prompts testing for AATD. Similarly, a family history of emphysema, especially with lung disease out

of proportion to risk factors like smoking, can be suggestive and a family history of unexplained liver disease may also raise suspicion. It is increasingly common for radiologists to suggest a diagnosis of AATD based on chest CT evaluation showing lower zone, panlobular emphysema, often accompanied by bronchiectasis. Current guidelines and standards suggest testing all individuals diagnosed with COPD for AATD regardless of age and smoking history. Several studies have demonstrated an increased prevalence of undetected AATD (between 0.6 and 3 %) among the general COPD population [23, 24].

Finally, family testing of those identified with AATD will often reveal undetected, and often asymptomatic, AATD in family members. In the future, routine AATD testing of newborns may well be the norm. A focus on risk factor reduction in those identified at an early age may well prevent much of the disease we currently see in this condition.

A retrospective study of a single center's experience has suggested an increased incidence of lung cancer in individuals carrying at least one AAT deficiency mutation [25]. This work still requires confirmation.

Management of AATD Lung Disease

Once identified, an individual with AATD should be assessed for the conditions associated with this genetic condition including, most prominently, lung and liver disease. Individuals with normal lung function and no evidence of significant destructive lung disease should be educated about AATD and risk factors for disease. Smoking prevention and cessation are among the most important steps to prevent lung disease in AATD (as in many lung conditions). Currently, augmentation therapy with plasma-derived AAT protein is not indicated in those without evidence of destructive lung disease. Careful monitoring of lung function and radiology is suggested.

While there are no evidence-based recommendations for the follow-up of healthy individuals with AATD, in general, clinicians treating a large number of such patients do annual or biannual complete pulmonary function testing often accompanied by liver function studies. A baseline high-resolution CT of the chest without contrast can be considered (Fig. 6.1). Interval repetition of the chest CT is not recommended but is reserved for evaluating significant changes in an individual's respiratory status [26].

The clinical evaluation and follow-up of individuals with AATD-related lung disease is similar to the monitoring of healthy individuals with AATD. The frequency of evaluation is generally increased, however. Those with destructive lung disease usually have their lung function evaluated semiannually until stable and then are moved to annual visits unless an unexpected change occurs. Generally, those with lung disease have some evaluation of oxygenation included in their workup, such as an arterial blood gas, a 6-min walk with oximetry, or a formal pulmonary exercise study. Depending on risk factors and age, a cardiopulmonary

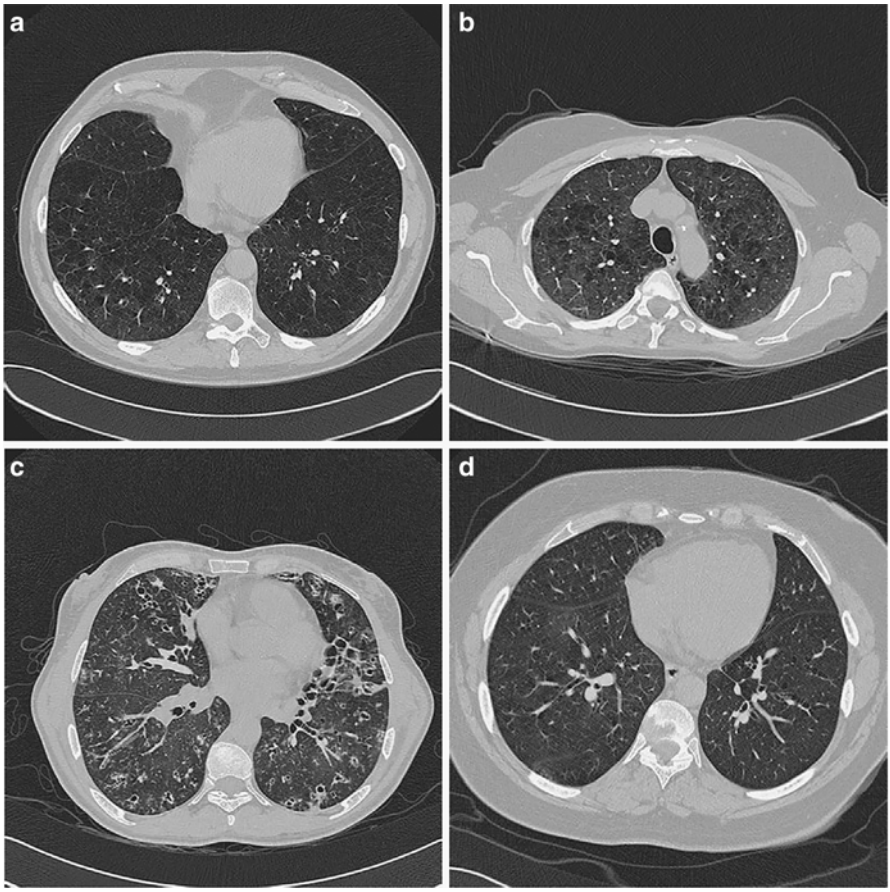

Fig. 6.1 Computed tomography of the chest in patients with AAT deficiency shows a broad range of manifestations. AAT deficiency has classically been associated with the development of basilar-predominant panacinar emphysema (Panel **a**). However, upper-lobe-predominant emphysema (Panel **b**) and bronchiectasis (Panel **c**) can also be observed, and sometimes the lungs are normal (Panel **d**) (reprinted with permission from Ref. [26])

exercise study may be considered. In those requiring supplemental oxygen, it is often reasonable to consider an echocardiogram to evaluate for right-sided heart disease and pulmonary hypertension.

The treatment of lung disease due to AATD broadly follows the treatment recommendations for individuals with non-AATD COPD and/or bronchiectasis. While clinical trials of medications for the treatment of COPD have generally excluded individuals with AATD and none of the usual COPD drugs have been specifically evaluated for effectiveness in AATD-related lung disease, it is generally assumed that AATD-associated lung disease responds to the same medications and other treatments that are used in treating typical, smoking-related COPD. The mainstays of therapy include long-acting inhaled beta-agonists, long-acting inhaled

anticholinergics, and long-acting inhaled corticosteroids. These medications are presumed to have the same beneficial effects on pulmonary exacerbation incidence and symptoms as in non-AATD COPD. As with these agents in any patient being treated for COPD, the goal is to use the lowest dose and fewest drugs that achieve the desired benefit.

Pulmonary exacerbations are common in AATD lung disease. Studies have suggested that on average AATD patients with lung disease have just over two exacerbations per year, with subpopulations within these studies that have no exacerbation and others with many more than two each year [27]. This latter group may benefit from the addition of a phosphodiesterase 4 inhibitor or chronic macrolide therapy, again based on studies in non-AATD COPD patients. For individuals with clinically significant bronchiectasis, airway clearance devices and techniques may be beneficial. Antibiotic therapy should be based on sputum culture and sensitivity results in this setting and resistant enteric organisms may colonize AATD patients with long-standing bronchiectasis, just as in this condition in those without AATD.

With respect to the treatment of an exacerbation, logic would suggest that patients with AATD would be at greatest risk of incremental lung destruction during a time of acute lung inflammation. Therefore, aggressive treatment of pulmonary exacerbations is recommended. Empiric therapy with antibiotics is recommended, if no recent sputum culture results are available to guide antibiotic choice. Addition of oral or parenteral corticosteroids may be required for severe exacerbations.

Other treatments initially developed for non-AATD COPD are also employed in the management of AATD lung disease. Pulmonary rehabilitation appears to benefit many with this condition. Immunization against organisms that commonly cause community-acquired pneumonia is recommended for those with AATD lung disease as is annual influenza immunization. Many clinicians recommend immunization against hepatitis A and B virus in order to reduce the risk of liver injury.

The use of AAT augmentation therapy should be considered in any AATD patient with documented emphysema. The goal of augmentation therapy is to prevent or slow the rapid decline in lung function found in individuals with lung disease due to AATD. The appropriate time to start augmentation therapy in an individual with AATD lung disease is an area of some disagreement. The first large study (large by rare disease standards) of patients with AATD was the NIH/NHLBI Registry of Patients with Alpha-1 Antitrypsin Deficiency (AATD) which enrolled 1129 individuals with AATD with a goal of evaluating the natural history of AATD [28]. Enrolled individuals were followed for approximately 5 years (3.5–7 years) with annual or semiannual testing of lung function, chest radiology, and blood work. The study began soon after the marketing approval of the first augmentation therapy product in the USA, and a majority of enrollees were treated with augmentation therapy for all or part of their time in the registry. A post-hoc analysis of the effect of augmentation therapy on mortality and lung function was performed [29]. A significant improvement in survival was noted in those who (1) received augmentation therapy during any of their time in the registry and (2) had an entry forced expiratory volume in 1 s (FEV_1) of less than 50 % predicted. In addition, there was a significantly decreased rate of decline of lung function, as assessed by FEV_1, in

individuals receiving augmentation therapy whose entry FEV_1 was between 35 and 49 % predicted compared to those who did not receive augmentation. Studies performed concurrently in Europe, with smaller subject numbers, confirmed many of the findings of the US registry study, although survival improvement could not be documented in those studies [30, 31].

Based on these studies, some clinicians elect to initiate augmentation therapy only once the FEV_1 of an affected individual declines to less than 50–60 % predicted. Others note the small number of healthier but declining patients, and thus the lack of power to detect improvement in healthier individuals, in the studies just cited. In addition, it can be pointed out that individuals who have emphysema due to AATD but have an FEV_1 greater than 50–60 % of predicted would be asked to wait until additional lung tissue was irreversibly destroyed if the former approach is used to guide the initiation of augmentation therapy. Once the decision to initiate augmentation therapy is made, the dose approved for the currently available therapies remains the same as initially described in the publications from the NIH intramural investigations of the 1980s: 60 mg/kg/week by intravenous infusion. There remains some controversy about whether this is the appropriate dosage and administration interval for all patients. At the time of this writing, there is at least one dose-ranging study enrolling subjects.

What remains controversial still, more than 25 years after the introduction of the first AAT augmentation product, is the measurable clinical benefit of this therapy. At the time of approval of the first product in this class, there were too few identified potential subjects to perform a well-powered, placebo-controlled, long-term prospective clinical efficacy study. Now that detection efforts have identified a sufficient number of potential subjects to perform such studies, the effectiveness of augmentation therapy is so widely accepted, at least within the USA, that a long-term placebo-controlled trial is virtually impossible to enroll because potential subjects fear randomization into the placebo group. Further, a growing number of ethics committees/institutional review boards (IRB) are finding ethical conflicts with studies that intend to enroll AATD patients and randomize some into placebo arms for prolonged periods. Several small, pilot, placebo-controlled prospective studies have been performed [32, 33], and, to date, one well-powered placebo-controlled trial of augmentation therapy has been completed [34]. The most recent studies have used longitudinal measurements of lung CT densitometry as the primary endpoint, since this appears to allow the most direct quantification of lung tissue destruction currently available.

The single well-powered study enrolled about 180 subjects with approximately half randomized to receive augmentation therapy at standard doses and the other half receiving placebo infusions for 2 years. This study demonstrated a significant preservation of lung tissue comparing the treated to the control group. In addition, an extension study allowed treated subjects to continue on therapy for an additional 2 years and rolled the placebo subjects onto treatment for this second 2-year period. The extension study demonstrated that, during the second 2 years of therapy, the rate of loss of lung tissue slowed even further in the group on 4 years of augmentation therapy. See Fig. 6.2. In addition, those initially receiving placebo showed a

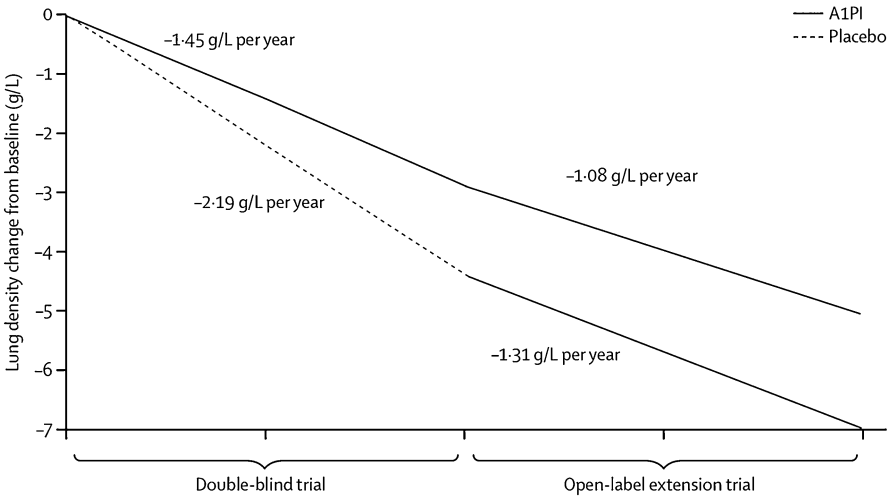

Fig. 6.2 Effect of augmentation therapy (A1PI) versus placebo on rate of lung density decrease during the double-blind and open-label portions of the trial in all patients. Values on graph are annual rates of decrease calculated from CT densitometry at total lung capacity. A1PI $n=92$; placebo $n=85$ during double-blind period. A1PI $n=50$; placebo $n=47$ during open-label period. $p=0.03$ during the double-blind portion (reprinted with permission from Ref. [32])

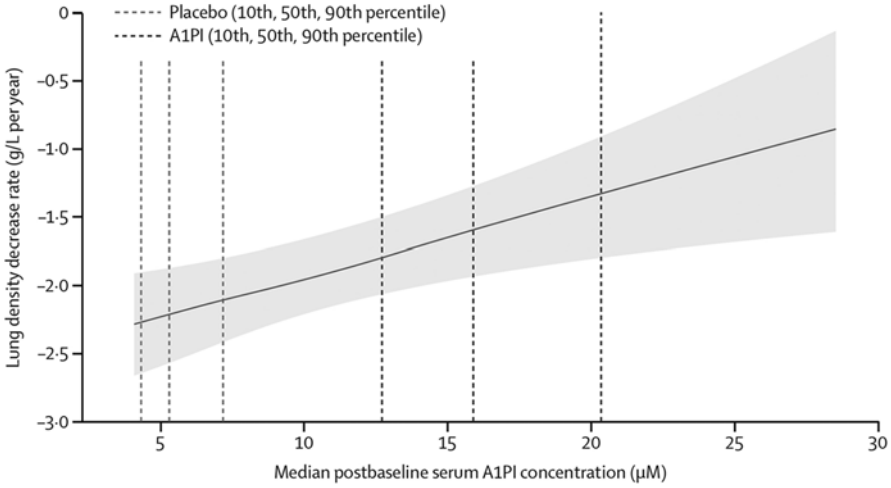

Fig. 6.3 Rates of lung density decrease at total lung capacity versus trough augmentation therapy serum concentrations achieved. Response-exposure curve. *Shaded area* represents 90 % confidence intervals. A1PI = α1 (reprinted with permission from Ref. [32])

decrease in the rate of loss of lung tissue once they were started on augmentation therapy for the 2 years of the extension study. An interesting finding in this study was that the trough blood levels of AAT were inversely correlated with the rate of lung tissue loss. See Fig. 6.3.

While it is encouraging to see the results of this trial, the use of CT densitometry to quantify lung tissue destruction is not accepted by all. In addition, the clinical significance of the magnitude of benefit demonstrated in these studies remains unclear. Still, in a condition that progresses over decades, even small annual improvements can have large long-term benefits. At this time, it can be said that augmentation is generally well accepted in areas of the world where it is available and a growing number of health systems are approving the administration of this class of product. Augmentation therapy remains the only specific therapy currently available to treat the lung disease of AATD. It should be noted that no benefit of augmentation therapy in granulomatosis with polyangiitis has been demonstrated.

Another powerful treatment for individuals with AATD is education. Education of affected individuals and their family members regarding the mechanisms of disease, risk factors, and treatment can be a powerful therapy to help prevent and treat lung disease in AATD. This has been demonstrated by the publications from the AlphaNet health management program, evaluating the benefits of patient self-management in a large group of lung-affected individuals with AATD [35]. Currently including nearly 5000 individuals, most on augmentation therapy, this program continues to involve those with AATD in their own care and prevention of disease.

An important unanswered question is the risk of lung disease in those who inherit a single abnormal AAT gene. For several common genotypes, there appears to be little evidence of an increase in risk. These include the S, I, P, and F genes when each is inherited with a normal M gene. Two heterozygote combinations deserve special attention: PiMZ and PiMNull. The blood levels of AAT with these latter heterozygotes can approach those seen to lead to increased risk of lung disease in those who are homozygote for a deficient gene and those who inherit a complex heterozygote, such as PiSZ. Studies have suggested that individuals with the PiMZ genotype have an increased risk of lung disease only if they smoke tobacco products with little or no increased risk seen in nonsmokers [36, 37]. It is possible that there is a subgroup of PiMZ individuals with significantly increased risk of lung disease but this has yet to be demonstrated conclusively.

Summary

Lung disease is the most common trigger for testing of individuals for AATD. Evidence suggests that the great majority of individuals with AATD remain undetected [38]. Whether they remain untested because of lack of significant illness or because the diagnosis remains unconsidered even in those with cardinal diseases is not clear. Based on family testing results, it appears likely that many of those with AATD who remain undetected are perfectly healthy. However, studies of those with the diagnosis of COPD suggest that there are a sizable number of individuals with undetected AATD within the COPD population.

Since there is specific therapy for AATD-related COPD that is distinct for that provided to patients with more usual COPD, identifying those with lung disease due to AATD can be clinically important. In addition, identifying an individual with lung disease due to AATD also identifies a family that is potentially at risk.

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Chapter 7
Liver Disease in α1-Antitrypsin Deficiency

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Introduction

Liver disease was first described in α1-antitrypsin deficiency (ATD) by Sharp and colleagues who discovered it in a child with cirrhosis in 1969 [1]. Since that time ATD has been widely recognized as the most common genetic diagnosis for children who undergo liver transplantation. Eriksson and colleagues showed in 1986 that adults with ATD also had a predilection for cirrhosis and hepatocellular carcinoma (HCC) [2], and onset of liver disease later in life is now known to be more common than previously recognized. The liver damage is predominantly characterized by fibrosis and ultimately cirrhosis with relatively limited inflammation. Nevertheless, there is wide variability in the incidence and severity of liver disease among individuals with the classical form of ATD, as shown by analysis of a cohort of Swedish individuals identified in a nationwide screening study by Sveger [3]. Only ~8% of the cohort had clinically significant liver disease over the first three decades of life. A greater proportion of this population will probably be impacted as they reach older ages, but we do know that a significant number of affected homozygotes completely escape clinical symptoms of liver disease throughout their lifetime [2].

Using a variety of model systems, we have come to learn that the liver is damaged in ATD by a gain-of-toxic function mechanism that is dependent on the accumulation of misfolded α1-antitrypsin Z (ATZ) within the early compartments of the secretory pathway [4]. Several theories for how this proteotoxicity leads to excessive collagen deposition and hyperproliferation in the liver have been proposed. Putative genetic and environmental modifiers are thought to play a role in managing the "proteotoxicity" and to determine susceptibility to and severity of liver disease among homozygotes [5]. Furthermore, our studies have implicated two types of

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mechanisms that are putative targets of genetic or environmental modifiers: cellular pathways for degradation of misfolded ATZ and signaling mechanisms that permit cellular adaptation to the presence of misfolded ATZ [5]. Indeed, several novel concepts for therapy that capitalize on the degradation pathways are currently being investigated.

In this chapter we will review what is known about the clinical characteristics of this liver disease, pathogenesis of fibrosis and carcinoma in the liver, and current and novel therapeutic strategies.

Clinical Characteristics of ATD-Associated Liver Disease

Liver disease may have onset in infancy, childhood, adolescence, or middle-aged adults, and it is likely that these represent four distinct forms of the disease. Jaundice is the first sign of the disease in infants. Usually this is when the physiologic jaundice of the newborn is prolonged to 4–6 weeks of age. These infants often have elevated serum conjugated bilirubin and transaminase levels, and in some cases hepatomegaly may be evident. ATD is an important entity to consider in the differential diagnosis of neonatal cholestasis, as the infantile presentation of ATD can closely mimic extrahepatic biliary atresia in terms of overall clinical appearance and laboratory evaluation. A study conducted by the Biliary Atresia Research Consortium (BARC) found that pathologists blinded to clinical information regarding infant liver biopsies under review for the assessment of cholestatic liver disease were not consistently able to distinguish between cases due to biliary atresia from those due to ATD [6]. Furthermore, neonates with classical ATD may not demonstrate biliary excretion on hepatoscintigraphy due to their significant cholestasis, making it difficult to differentiate the possibility of ATD from biliary atresia using this modality in such cases [7]. Occasionally, the infant with ATD is first recognized because of pruritus and hypercholesterolemia, making it important to distinguish ATD from inborn errors of bile secretion that cause infantile cholestasis. Severe manifestations reflective of portal hypertension such as ascites, splenomegaly with hypersplenism, gastrointestinal bleeding, and poor growth can rarely occur during infancy [8].

The typical presentation for this disease in childhood, in adolescence, or during adulthood is one or more of the complications of portal hypertension. In many cases it is enlargement of the liver and/or spleen together with leukopenia and thrombocytopenia indicative of hypersplenism. Some people will present with ascites, gastrointestinal bleeding from esophageal varices, or hepatic encephalopathy. Occasionally the diagnosis is made when symptoms of abdominal pain are attributed to gallstones, and imaging studies are suggestive of hepatic cirrhosis. A number of affected individuals will be diagnosed incidentally when a nodular appearance to the liver is noticed during abdominal surgery for another diagnosis.

There is an increased incidence of HCC in persons with ATD. This was first shown by Eriksson and colleagues by an autopsy study in Sweden [2]. Statistical

analysis indicated that the incidence of hepatic carcinoma in ATD was higher than expected from the general population and also significantly higher than what would be expected in association with cirrhosis alone. This study also showed that both cirrhosis and HCC could be present incidentally in individuals that had never been diagnosed with liver disease and had died of other causes. In most cases HCC is discovered at the time of diagnosis of ATD-associated liver disease or soon after that diagnosis has been made. It is almost always in the adult form of the disease although one case of hepatic carcinoma was diagnosed in a 12-year-old [9]. The incidence of liver cancer in ATD is still probably not fully appreciated clinically. In a review of liver transplants performed at the University of Pittsburgh Medical Center (UPMC) from 1991 to 2012 in which ATD was at least one of the pathology-confirmed diagnoses from review of the explant, we found that 9 out of 98 adult patients (9.2 %) had explants positive for HCC, and another eight patients (8.2 %) had explants that demonstrated evidence of dysplasia (unpublished data). However, as noted below, not all of these patients were homozygotes, and several had other causes of liver disease, including three with a history of alcohol abuse, one had a history of alcohol abuse and autoimmune hepatitis, and one had been diagnosed with hemochromatosis (unpublished data). In one series of 164 cases of primary liver carcinoma, 13 cases had tumors surrounded by intrahepatocytic globular inclusions immunostained with a monoclonal antibody to ATZ [10]. This was taken to mean that 7.9 % of primary liver cancers were associated with ATD, but these cases were not independently confirmed by AT phenotype determination.

Over the past two decades, there has been an increase in the number of adults who undergo liver transplantation with a diagnosis of ATD. Indeed, over the last 10 years, 85–90 % of all liver transplant procedures done in the USA for ATD are for adults, typically at around 50–65 years of age (United Network of Organ Sharing, personal communication). The apperent change from childwood to adult predominance is partially explained by a backlog of childhood cases that underwent liver transplantation prior to that time, but it may also be partially explained by improved recognition of ATD as a cause of chronic liver disease [11].

The most important study of incidence and severity of liver disease in ATD comes from a nationwide screening study carried out by Sveger in the early 1970s [3]. In this study, the investigators screened 200,000 newborns from which they identified 127 individuals who were homozygotes for ATZ. From this group of homozygotes, 14 cases exhibited prolonged obstructive jaundice, and 9 out of the 14 developed clinical manifestations of severe liver disease. Notably, slightly more than half of the remaining infants identified in this cohort were found to have elevated blood transaminase levels but otherwise exhibited no other signs of liver disease. In the four decades following the initiation of this study, this cohort has been followed relatively comprehensively, and the results of subsequent follow-up analyses have revealed that only about 8 % of the population has developed clinically significant liver disease [12]. Because this cohort has not reached the peak age for adult-onset liver disease, 50–65 years of age, and because liver biopsies have not been a part of the study, it is likely that the true incidence of liver disease in an unbiased background is greater than 8 %. However, the study shows that there is wide

variability in the hepatic phenotype of ATD. Furthermore, it provides powerful evidence that genetic and/or environmental modifiers are critical in determining whether a homozygote is susceptible to or protected from clinical liver disease.

The natural history of ATD-associated liver disease is also quite variable. The majority of infants diagnosed with ATD because of prolonged neonatal jaundice will experience slow resolution of jaundice and elevated serum transaminases over the first 6–12 months of life. Even when splenomegaly or other signs and symptoms of portal hypertension are present, the disease may progress very slowly. In one report of 17 patients with cirrhosis and portal hypertension, it took 4 years before liver transplantation was required in nine patients, and seven of the patients were living relatively healthy lives for up to 23 years after diagnosis [13]. In other patients the liver disease progresses more rapidly, but it is almost always within the category of chronic and not acute liver failure.

Liver disease has been reported in individuals with AT allotypes other than the homozygous ATZ allotype (Table 7.1). Compound heterozygotes for the S and Z

Table 7.1 Deficiency variants of α1-antitrypsin

			Clinical disease		
Variant	Defect	Site	Liver	Lung	Cellular defect
Z	Single base substitution M1 (Ala213)	Glu342-Lys	+	+	IC accumulation
S	Single base substitution	Glu264-Val	–	–	IC accumulation
$M_{Heerlen}$	Single base substitution	Pro369-Leu	–	+	IC accumulation
$M_{Procida}$	Single base substitution	Leu41-Pro	–	+	IC accumulation
M_{Malton}	Single base deletion	Phe52	+	+	IC accumulation
M_{Duarte}	Unknown	Unknown	+?	+	Unknown
$M_{Mineral\ Springs}$	Single base substitution	Gly57-Glu	–	+	No function; EC degradation?
S_{iiyama}	Single base substitution	Ser53-Phe	–	+	IC accumulation
P_{Duarte}	Two base substitution	Arg101-His Asp256-Val	+?	+	Unknown
P_{Lowell}	Single base substitution	Asp256-Val	–	+	IC degradation?
$W_{Bethesda}$	Single base substitution	Ala336-Thre	–	+	Accelerated catabolism?
$Z_{Wrexham}$	Single base substitution	Ser19-Leu	?	?	Unknown
F	Single base substitution	Arg223-Cys	–	–	Unknown
T	Single base substitution	Glu264-Val	–	–	Unknown
I	Single base substitution	Arg39-Cys	–	–	IC degradation
$M_{Palermo}$	Single base deletion	Phe51	–	–	Unknown
$MN_{ichinan}$	Single base deletion and single base substitution	Phe52 Gly148-Arg	–	–	Unknown
$Z_{Ausburg}$	Single base substitution	Glu342-Lys	–	–	Unknown
King's	Single base substitution	His334-Asp	+		IC accumulation
M_{pisa}	Single base substitution	Lys259-Ile	–	+?	IC accumulation
$E_{taurisano}$	Single base substitution	Lys368-Glu	–	+	IC accumulation
$Y_{orzinuovi}$	Single base substitution	Pro391-His	+?	–	IC accumulation

allele appear to have a risk of liver disease that is similar to ZZ homozygotes [12]. In a follow-up analysis of a subset of 26-year-old subjects from the Swedish screening/cohort study, 3 out of 32 individuals (9 %) with the SZ allotype had elevated liver transaminases compared with 4 out of 70 subjects with the ZZ allotype (6 %). A subsequent follow-up study of a subset of 30-year-old patients from the same cohort found that 4 of 39 (10 %) subjects with SZ had elevated alanine transaminase (ALT) compared with 4 of 89 (5 %) subjects with ZZ [14]. Patients with the SZ allotype were found to represent 4 % of pathology-confirmed adult cases of ATD in our study of the UPMC liver transplant database from 1991 to 2012 (unpublished data). Data from three liver transplant centers from 1987 to 2012 indicated that 50 ZZ and 23 SZ adults had undergone liver transplantation [15]. Interestingly, the patients with the SZ allotype were more likely than the ZZ patients to have another reason for liver disease. Liver disease in individuals with the SZ allotype is likely to be related to accumulation of misfolded protein in the intracellular secretory compartments because the S variant is known to be prone to intracellular accumulation, albeit to a milder extent than the Z variant [16]. A few cases of liver disease have been reported in individuals with the homozygous M_{malton} variant of AT [17, 18], probably attributable to the tendency for this variant to misfold and accumulate within cells in a way that is similar to the Z variant [19]. The variant S_{iiyama}, which also has a tendency to misfold and accumulate within cells, was reported in a patient with emphysema and hepatocyte inclusions, but the patient did not manifest clinical liver disease [20]. The W variant was reported in three consanguineous children homozygous for the allele, two of whom died during infancy of severe liver disease [21]. Fra et al. reported a novel variant, Yorzinuovi (Pro391His), which was associated with reduced serum AT levels and an increased tendency to form intracellular polymers with increased intracellular accumulation [22]. The patient was 46 years old and had a 10-year history of elevated serum transaminases, but there was no further characterization of the liver involvement and no indication as to whether the patient had been evaluated for other causes of liver disease. However, AT inclusions were not detected in the liver biopsy by immunohistochemistry [22]. Miranda et al. reported the novel King's variant that was discovered in a 6-week-old boy with prolonged jaundice [23]. Laboratory studies demonstrated a significant reduction in the patient's serum AT level and a liver biopsy that was characteristic of classic ATD with intrahepatocytic inclusions. The child was found to be compound heterozygous for the Z and King's variant. Biochemical studies showed that the King's variant forms polymers within the endoplasmic reticulum and accumulates within cells [23]. In reports of rare AT variants, it is important to note the difficulty in ascertaining whether the AT allele truly causes liver damage unless alcohol consumption, viral hepatitis, and autoimmune disease have been extensively characterized. Furthermore, based on what we know about the incidence of liver disease among ZZ homozygotes, we could only anticipate a subpopulation of individuals with each allotype to be susceptible to clinical effects on the liver. Nevertheless, one of the correlative concepts that have arisen from the association of AT alleles with liver damage is that all of these alleles have polymerogenic properties. This concept has led to theory that polymerization plays an important role in the pathobiology of hepatotoxicity.

A subject of some controversy has been whether MZ heterozygotes are at higher risk for developing liver disease. An obstacle to addressing this question has been the tendency in the literature to address this issue with retrospective studies that derive from single-center experiences and small case series and are therefore unfortunately biased in ascertainment. An early study of liver biopsies found a relatively higher prevalence of MZ heterozygosity in patients with cryptogenic cirrhosis and chronic active viral hepatitis [24]. A cross-sectional case–control study by Regev et al. did not find an overall association between MZ and the presence of chronic liver disease or cryptogenic cirrhosis, but they did find a significantly higher prevalence of MZ in patients with decompensated liver disease from all causes. Furthermore, they found that there appeared to be an association between the presence of MZ phenotype and the severity of liver disease in patients with hepatitis C and nonalcoholic fatty liver disease [25]. A retrospective study by Graziadei et al. at a liver transplant center found that 8.2 % of their patients who underwent liver transplantation were MZ heterozygotes, compared with 2–4 % of the general population based on their review of published studies [26]. A small study by Vecchio et al. that reviewed 80 consecutive cases of cryptogenic cirrhosis and chronic active hepatitis found no cases of MZ heterozygosity. Given the small number of cases, it is likely that this study was underpowered to detect cases of MZ [27]. Although it is still difficult to know from these studies whether MZ heterozygosity truly causes liver disease by itself, we have seen a number of cases of MZ individuals with severe liver disease and no other potential cause despite extensive evaluation. A number of adults with the MZ phenotype undergo liver transplantation each year. In our review of liver transplants at UPMC from 1991 to 2012, AT phenotypes were done on 89 patients with pathological features of ATD and 44 of these were MZ heterozygotes (unpublished data). Furthermore, according to our conceptual model for the pathogenesis of liver disease in ZZ homozygotes, it seems likely that some MZ heterozygotes who have a complement of particularly powerful negative genetic and/or environmental modifiers would be susceptible to liver damage from the proteinopathy.

There has also been extensive discussion in the literature about whether other causes of liver disease act in concert with ATD. One intriguing study investigated the possibility that ATD and cystic fibrosis (CF) occurs together and increases the risk of liver disease in each of the populations. This case–control study examined genotyping data for five candidate genes in patients with cystic fibrosis (CF) and severe liver disease with portal hypertension from more than 100 CF centers across North America and Japan and found as its primary conclusion that having even a single copy of the Z allele is a risk factor for incidence and severity of liver disease in CF. No ZZ homozygotes were identified in that study [28]. In adults with ATD who come to clinical attention for liver disease, there is often evidence for other causes of liver disease such as alcoholic liver disease, nonalcoholic steatohepatitis, viral hepatitis, autoimmune liver disease, or hemochromatosis that can confound their clinical picture. The degree to which any given hepatic comorbidity can interact with ATD to accelerate the development of cirrhosis and HCC is not fully understood. Furthermore the majority of studies on this issue are confusing because

patients with ATD include mostly heterozygotes grouped together with a small or even unknown number of homozygotes. In regard to ATD together with hemochromatosis, one study found that the MZ heterozygous phenotype was about ten times more common compared with the general population (20 vs. 2.2 %) [29]. However, other studies have not found an association between these two conditions [30, 31]. There are case reports of more rapid clinical progression when hepatitis C virus infection is encountered in the setting of at least one Z allele [32, 33]. However, studies by Simsek et al. and Elzouki et al. did not reveal a clear synergizing effect [34, 35]. There is still very limited data about liver disease in ATD in combination with nonalcoholic fatty liver disease. One survey of an uncontrolled registry suggested that obesity is common in ATD patients with active liver disease [36], and we have seen a number of middle-aged patients who are Z homozygotes with hepatic steatosis as a part of the liver histological picture.

In children there have also been studies exploring the possibility that heterozygosity for the Z allele is a risk factor for liver disease of other causes. In one retrospective study at a single pediatric liver disease center, AT heterozygosity was more common in children with other chronic liver diseases [37]. Specifically, when they compared their population to an American reference database, the Z variant represented 2.2 % of AT alleles in their pediatric liver disease population, compared with 0.56 % in the reference database. This difference remained true when their population was subdivided between biliary atresia and non-biliary atresia patients. They also found that in the subset of their patients with biliary atresia who were referred for liver transplantation, possession of non-M alleles was associated with a shorter mean age to transplantation compared to MM homozygotes (235 vs. 779 days, $p = 0.036$).

The diagnosis of ATD should be considered in any individual being evaluated for symptoms and signs of liver disease. In infants this includes jaundice or pruritus. In older children hepatomegaly or splenomegaly may be the only signs. Elevated serum transaminases in an otherwise asymptomatic individual should also lead to evaluation for ATD. Certainly any signs of portal hypertension should trigger diagnostic studies for ATD. The diagnosis of ATD is established by the determination of serum AT protein phenotype by isoelectric focusing (IEF), an electrophoretic technique that permits the identification of AT variants by separation along a pH gradient. Serum AT concentrations also play an important role in the diagnostic evaluation. They may be used for screening, with levels below normal (85–215 mg/dL) being suggestive of the presence of at least one abnormal AT allele. It is ideal to determine both the AT phenotype and serum concentration as these assays are complementary in their functions. A technical limitation of phenotypic analysis by IEF is that it cannot establish the presence of a null AT allele combined with a normal or mutant allele. Serum AT levels are crucial in these cases for raising suspicion of the presence of a null allele [38]. On the other hand, serum AT levels alone are inadequate. They may appear to be borderline normal in Z heterozygotes, and AT levels can in some cases be falsely reassuring since AT, as an acute phase reactant, may rise in the setting of an inflammatory response. AT levels may also be affected by blood transfusions [36]. PCR-based targeted mutation analysis is available for the most

common alleles (M, Z, and S), which may be helpful when a patient's phenotype analysis is ambiguous, but it is limited in that it will not detect less common alleles. In rare situations in which routine testing is inconclusive and a rare or novel variant is suspected, next generation sequence analysis may be of value [39].

The characteristic histologic finding on liver biopsy is periodic acid Schiff (PAS)-positive, diastase-resistant globules and/or granules within hepatocytes, often in the periportal regions, accompanied by fibrosis and mild inflammation. The inclusions are round to oval, eosinophilic, and 1–40 μm in diameter. There may also be evidence of hepatocellular necrosis and bile duct epithelial cell destruction [40]. The degree of inflammation may vary depending on the presence of comorbid conditions such as steatohepatitis or viral hepatitis. Although suggestive, the presence of PAS-D globules is not absolutely pathognomonic for the diagnosis of ATD, as these structures have been reported to occasionally occur in individuals without ATD [41]. Inclusions may also be scant or absent in some ATD cases in the first few weeks of life [42]. Immunohistochemical stains for AT are useful for confirming the nature of the inclusions. Liver histology may also reflect the presence of dysplasia or carcinoma.

Mechanism of Deficiency

The classical form of ATD is characterized by a point mutation that substitutes lysine for glutamate 342 in the mutant protein, ATZ [4]. Studies in several different model systems using pulse-chase radiolabeling have shown that there is reduced secretion and intracellular accumulation of ATZ when compared to the wild-type AT [43–47]. Immunostaining and biochemical analyses show that mutant ATZ accumulates in early compartments of the secretory pathway, including most notably the rough endoplasmic reticulum (ER), and perhaps other as yet undefined compartments that do not stain with classical markers of the rough ER [46]. It is important to note that in these model systems the secretion of ATZ is reduced, whereas it is completely absent for a variety of null variants when they are expressed in similar model systems [48–50]. This reflects what is seen in humans with serum levels of ATZ at 10–15 % of normal while those of the null variant are undetectable. Together, these observations make it possible to conclude that these model systems faithfully recapitulate the human disease and definitively prove that the primary abnormality is defective biogenesis. Furthermore, site-directed mutagenesis studies have shown that the single amino acid substitution of lysine for glutamate 342 is sufficient to cause the cellular defect in which the mutant ATZ is unable to efficiently translocate through the intracellular secretory pathway [43].

It is still not entirely clear how the amino acid substitution leads to diminished secretion and intracellular accumulation of ATZ. Lomas and colleagues have argued that the primary defect is polymerization [51]. These authors demonstrated polymers and insoluble aggregates in liver biopsy specimens and plasma from patients with ATD and provided evidence for a loop–sheet insertion mechanism of

polymerization. In this conceptualization of polymerization, the characteristic lysine substitution is at the hinge of the mobile reactive-site loop, and because it has more bulk than the glutamate that is ordinarily at that site, it prevents the reactive-site loop from relaxing into a known gap in the A sheet that is part of the flexible conformational changes of the AT molecule. This permits the reactive-site loop on another ATZ molecule to insert into the gap and begin the oligomerization and ultimately polymerization process. In an important study, Sidhar et al. showed that the secretory defect that characterizes ATZ was partially corrected by introducing a second mutation that suppressed polymerization [52]. More recent studies by Huntington and colleagues have suggested a different mechanism for polymerogenic and aggregation-prone properties of ATZ that involves at least two different domain-swapping phenomena [53–55].

Nevertheless, several lines of evidence suggest that misfolding is the primary defect responsible for impaired secretion/intracellular accumulation of ATZ, and in this conceptualization polymerization/aggregation is a result of rather than the primary defect itself. First, only 18 % of the intracellular pool of ATZ is in polymers at steady state in a mammalian cell line model that faithfully recapitulates the intracellular accumulation/fate of ATZ [49]. Second, a naturally occurring variant of ATZ which has the same E342K mutation as ATZ and a carboxyl-terminal truncation accumulates in the ER even though it does not form polymers suggesting that misfolding is sufficient to lead to intracellular accumulation of ATZ [48]. Third, the results of Sidhar et al. [52] do not exclude the possibility that diminished secretion of ATZ is partially corrected by the second engineered mutation because this second mutation also prevents the primary misfolding defect. Furthermore, in a very interesting study, a small molecule that prevents polymerization in vitro does not correct the secretory defect of ATZ in vivo but rather leads to enhanced degradation [56]. It is hard to know for certain about the applicability of this last study because there was no analysis of whether polymerization was prevented by the small molecule in vivo. Taken together, we believe that misfolding is the primary defect and that polymerization and aggregation are time-dependent effects of the accumulation of ATZ because of misfolding. This conceptualization is also consistent with the domain-swapping mechanism of polymerization described by Huntington in which polymerization is viewed as a "kinetic" result, or delayed folding, of monomeric ATZ and explains how some ATZ gets secreted.

Pathogenesis of Liver Injury/Fibrosis

In most cases, ATD liver disease is slowly progressive and the dominant pathology is characterized by fibrosis/cirrhosis and carcinogenesis [57]. The mechanism of liver injury is now known to involve gain-of-function processes wherein the accumulation of the mutant ATZ in the ER of hepatocytes triggers a series of events that eventually results in proteotoxic damage. The most compelling evidence for the gain-of-toxic function mechanism comes from mouse models of ATD in which

transgenic expression of the mutant human ATZ gene is sufficient to reproduce the features of liver disease known to occur in humans [58, 59]. The PiZ mouse, generated with a human genomic fragment encompassing all of the exons and introns of the human ATZ gene [59] and which is the most extensively characterized model, has intrahepatocytic globules reflecting accumulation of misfolded ATZ in the ER, slowly progressing fibrosis, low-grade inflammation, dysplasia, and increased prevalence of hepatic carcinoma [60, 61]. Because there are normal levels of AT and presumably other anti-elastases in these animals, as directed by endogenous murine genes, the liver injury in these transgenic mice cannot be attributed to a loss-of-function mechanism such as excess proteolytic activity.

There is still relatively limited information about how proteinopathy leads to liver injury. Although structural and functional alterations in mitochondria and caspase-3 activation have been observed in liver tissue from the PiZ mouse model and from ATD patients [62, 63], mitochondrial dysfunction probably has mostly a cytostatic effect because apoptosis, necrosis, and inflammation are not major pathological characteristics of the liver in ATD.

The mechanisms responsible for the hepatic fibrotic response to proteinopathy are also not well understood. Genomic analysis of a mouse model with hepatocyte-specific inducible expression of mutant ATZ (Z mice), ideal for elucidating signal transduction pathways that are activated by the proteinopathy, has identified a relatively rich network of downstream targets of the TGFβ pathway, including the upregulation of connective tissue growth factor [64], and this pathway is known to play a central role in fibrosis [65–67]. We have also demonstrated that activation of the NFκB signaling pathway is an important and specific effect of cellular ATZ accumulation [50], and the NFκB pathway has been implicated in tissue fibrosis [68]. However, our studies have indicated a select repertoire of downstream targets for NFκB signaling in the setting of ATZ accumulation [64], suggesting that it is a unique response designed to affect hepatocellular proliferation (see below).

Several recent studies have suggested that fibrosis results from proteinopathies in several different tissues. Lung fibrosis has been described in several rare diseases characterized by proteinopathy in respiratory epithelial cells, including surfactant protein C deficiency and Hermansky–Pudlak syndrome [69, 70]. Similarly myocardial fibrosis has been described for desminopathy that affects cardiomyocytes [71] and skeletal muscle fibrosis in inclusion-body myositis [72]. Interestingly, by enhancing the degradation of misfolded proteins, autophagy has been shown to mitigate cardiac fibrosis from desminopathy [71] and skeletal muscle fibrosis from inclusion-body myositis [73] just as it does for hepatic fibrosis in the PiZ model of ATD (see below).

Although polymerization may not be the primary cause of defective secretion and intracellular accumulation, there is no question that polymers and aggregates form in the ER as a result of ATZ accumulation, and there are several lines of evidence that the ability to form polymers correlates with susceptibility to liver disease. First, all of the AT alleles that have been associated with liver disease appear to have the capacity to form polymers. Second, polymerogenic variants of another serpin that is expressed in neurons, neuroserpin, cause a neuronal degenerative disease [74].

Third, liver damage occurs in another genetic disease, inherited hypofibrinogenemia, associated with the accumulation of fibrinogen polymers and aggregates in the endoplasmic reticulum of liver cells [75]. Interestingly, liver disease has not been described for polymerogenic variants of other serpins, like C1 inhibitor [76], but this may be explained by the fact that these serpins are expressed at much lower concentrations and/or in cell types that are able to tolerate the variant serpins at these lower concentrations.

It is very important to point out that there is absolutely no in vitro or in vivo data that ATZ polymers themselves are directly toxic to liver cells. It is still possible that other forms of ATZ that accumulate in liver cells, such as monomeric misfolded ATZ or soluble ATZ oligomers or even a combination of misfolded monomers and polymers, are responsible for the cytotoxic effects. This means that polymerization could be a process that is associated with the generation of a toxic intermediate, and, if that is the case, it would represent a very reliable marker for some toxic misfolding event. It is even possible that polymerization is itself a mechanism for protecting cells from toxic misfolded ATZ and always occurs when toxic misfolding is present but in some cases is insufficient to provide the necessary protection. In studies of another proteinopathy, Alzheimer's disease, there is evidence suggesting that soluble oligomers of amyloid-β peptide are toxic to neurons [77], whereas aggregates, and particularly denser aggregates, are protective [78]. Further studies of this issue will be very important for determining novel treatment strategies in the future.

In an effort to further understand the mechanism by which proteinopathy leads to liver injury in ATD, the Perlmutter laboratory has investigated the mechanisms by which misfolded ATZ undergoes intracellular degradation. We hypothesized [5, 45] that differences in efficiency of the intracellular ATZ degradation could explain the differences in onset and severity of liver disease among Z homozygotes that were identified by the screening/cohort studies of Sveger in Sweden [3, 12]; in other words, genetic and environmental modifiers of the hepatic phenotype of ATD target intracellular degradative mechanisms (Fig. 7.1). The hypothesis was initially validated by studies showing that ATZ was degraded more slowly in ATD individuals with liver disease than in ATD individuals without apparent liver disease, using skin fibroblast line models engineered for the expression of ATZ using retroviral-mediated gene transfer [45]. Recently, studies by Tafaleng et al. have provided further validation of this hypothesis by showing slower degradation of ATZ in iPS-derived hepatocytes (iHeps) from ATD individuals with liver disease as compared to iHeps from ATD individuals without liver disease [46]. Interestingly, the rates of ATZ degradation in iHeps were almost identical to those in skin fibroblasts published 20 years ago with a half time of disappearance of ~4 h in those affected by liver disease compared to ~2 h in those without apparent liver disease. Furthermore, Tafaleng et al. found that large intracellular globular inclusions were only seen in the iHeps of the liver disease patients. In addition to showing that degradation may be relatively impaired in ATD individuals with liver disease, these results suggest the iHeps may be used to make premorbid predictions of liver disease susceptibility.

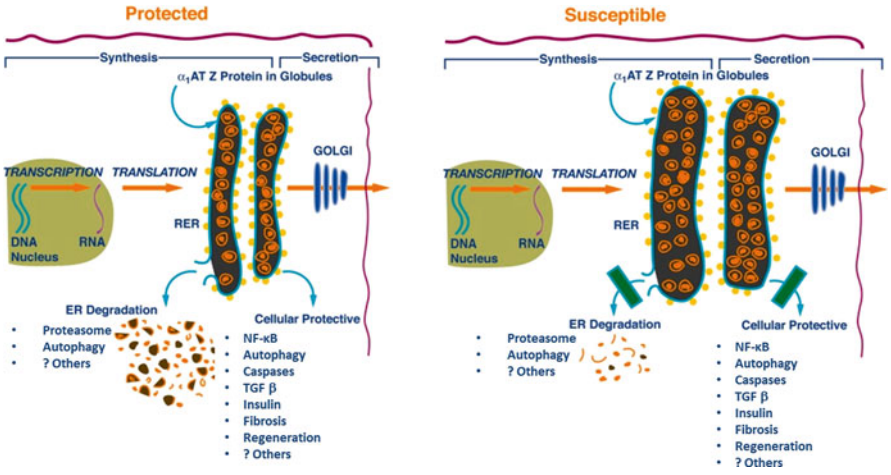


Fig. 7.1 Cellular factors that determine whether an AT-deficient individual is protected or susceptible to liver disease. In the susceptible host, there is greater accumulation of insoluble ATZ in the ER because of subtle alternations in putative proteostasis network regulatory mechanisms. Here these proteostasis regulatory mechanisms are envisioned as either ER degradation pathways or cellular protective responses. Reproduced from reference [57] with permission

Investigations of the mechanisms of ATZ degradation have shown that the proteasomal and autophagic pathways play critical roles. Early studies using yeast and mammalian cell lines showed that the proteasomal pathway participates in intracellular disposal of ATZ by a process that is now known as ER-associated degradation (ERAD) in which the substrate is extracted retrograde from the ER to the cytoplasm [79–81]. In fact, ATZ was one of the first identified substrates of the ERAD pathway [82].

Later, autophagy was discovered to be a second major pathway for disposal of misfolded ATZ [83]. Autophagy is an intracellular catabolic pathway by which cells digest subcellular structures and cytoplasm to generate amino acids as a survival mechanism. It is characterized by double-membrane vacuoles called autophagosomes which fuse with lysosomes for degradation of the internal constituents. Autophagy was firstly implicated in ATD when a marked increased autophagosomes were observed in human fibroblast cell lines engineered for the expression of mutant ATZ [83]. Increased autophagosomes were also observed in the liver of PiZ transgenic mice and in liver biopsy specimens from patients with ATD [83]. Definitive evidence for the role of autophagy in ATZ disposal was provided by genetic studies in which ATZ disposal was delayed in autophagy-deficient (Atg5-null) murine embryonic fibroblast cell lines [84] and Atg6-null yeast strains [85, 86]. Furthermore, by mating a transgenic mouse with liver-specific inducible expression of ATZ to the GFP–LC3 mouse that is characterized by GFP+ autophagosomes, we showed that accumulation of ATZ in liver cells is sufficient to activate the autophagic response [84]. The role of autophagy in intracellular ATZ degradation has been further validated recently by studies demonstrating that autophagy

enhancer drugs promote intracellular ATZ disposal and attenuate hepatic fibrosis in the PiZ mouse model of ATD in vivo [60, 87, 88]. One of the concepts originating from studies of ATZ disposal in autophagy-deficient yeast strains is that autophagy becomes particularly important at higher levels of ATZ expression [85]. These results taken together with the structural constraints of the proteasome have led to the supposition that the proteasomal pathway degrades soluble monomeric forms of ATZ, whereas autophagy is needed for soluble and insoluble polymers. However, it is also possible that autophagy also plays a role in the disposal of soluble monomeric ATZ that accumulates at levels of expression that exceed the capacity of the proteasome. Another important result from the studies by Kruse et al. in autophagy-deficient yeast also showed that a misfolded fibrinogen variant associated with liver disease in a rare inherited form of hypofibrinogenemia is degraded by autophagy in a manner almost identical to that of misfolded ATZ [86].

Several lines of evidence have suggested the existence of pathways for intracellular disposal of ATZ other than the proteasome and autophagy. Indeed, a sortilin-mediated pathway from Golgi to lysosome was recently shown to contribute to the degradation of ATZ in yeast and mammalian cell line models [85, 89]. Another non-proteasomal, non-autophagic pathway for ATZ disposal was recently identified in a powerful *C. elegans* model of ATD and found to be present in a mammalian cell line model as well [47]. Interestingly this pathway is suppressed by insulin signaling, and when upregulated by knocking down components of the insulin signaling pathway, it can completely mitigate ATZ proteotoxicity.

Studies designed to identify genetic and environmental modifiers of the liver disease phenotype in human populations have only recently been reported. In one interesting study, a single nucleotide polymorphism (SNP) in the *MAN1B1* gene was found to be overrepresented statistically in a series of infants with end-stage liver disease [90, 91]. This SNP was shown to decrease the levels of expression of the *MAN1B1* gene product, ER mannosidase I, and a recent study has provided evidence that such a polymorphism would theoretically impair ERAD/proteasomal degradation of misfolded proteins [92]. These results would appear to validate our hypothesis that intracellular degradation pathways would be targeted by modifiers of the liver disease phenotype. However, this SNP was not identified as a statistically significant modifier in another population [93] and will therefore likely require further population-based evaluation.

An SNP in the upstream flanking region of the AT gene has also been implicated in susceptibility to liver disease [94], but the nature of variant contradicted the rationale for how it might affect liver disease susceptibility. Furthermore, this study could lead to a different conclusion with legitimate alternate ways of classifying one of the populations used in the analysis.

Our hypothesis for variation in liver disease susceptibility also identifies signaling pathways that could increase or decrease ATZ proteotoxicity as potential targets for disease modifiers (Fig. 7.1). In order to begin to elucidate such pathways, we developed cell line and mouse models with inducible expression of ATZ as ideal systems. From these systems we now know that accumulation of ATZ specifically and selectively activates the autophagic response [84], NFκB signaling pathway and

cleavage of murine caspase-12 [50]. Gene expression profile analysis in the liver of the Z mouse with hepatocyte-specific inducible expression of ATZ implicated the TGFβ signaling pathway [64]. These signaling pathways could also act on ATZ proteotoxicity through a degradative mechanism. For instance, one of the genes most affected by the induction of ATZ accumulation in gene expression profile analysis was the regulator of G signaling RGS16 which likely acts by activating hepatic autophagy [64]. Insulin signaling could also affect ATZ proteotoxicity through an intracellular disposal mechanism [47]. Further studies of these types of proteostasis mechanisms could lead to the identification of additional modifiers in the future.

Pathogenesis of Hepatocellular Carcinoma in ATD

Although increased susceptibility to hepatic cancer was shown years ago [2], there have been only a few studies of potential mechanisms for carcinogenesis. Theorizing that hepatocellular hyperproliferation was likely to be involved, Rudnick et al. investigated the proliferation of liver cells by BrdU labeling in the PiZ mouse model of ATD [95]. In these studies hepatocellular proliferation was increased ~7-fold in the PiZ mouse compared to a wild-type control, and this degree of proliferation appeared to reflect the slowly progressing chronic nature of ATD liver disease. Most importantly, by using double immunohistochemical staining, it was shown that dividing hepatocytes were almost exclusively the ones that lacked intracellular ATZ inclusions, called the globule-devoid hepatocytes. Furthermore, it was shown that hyperproliferation of globule-devoid hepatocytes was driven by the number of adjacent

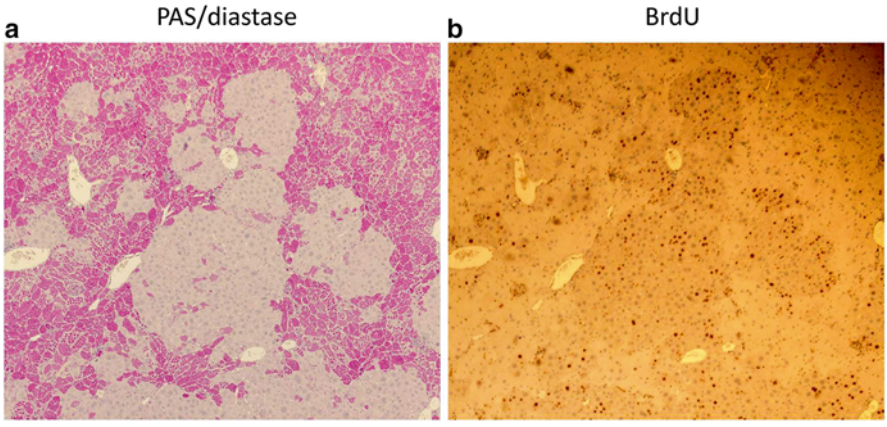


Fig. 7.2 Hepatocellular proliferation in the PiZ mouse model of AT deficiency. A liver section, stained with (**a**) PAS/diastase and (**b**) BrdU (brown staining of nuclei), shows proliferation only in globule-devoid hepatocytes or in inflammatory microabscesses. The tendency toward nodular areas of globule-devoid hepatocytes can be appreciated. Courtesy of Tunda Hidvegi, George Michalopoulos. Reproduced from Ref. [4] with permission

globule-containing hepatocytes. This last conclusion was based on the observation that the number of globule-containing hepatocytes was markedly increased in male PiZ mice or in testosterone-treated female PiZ mice, and this correlated directly with the degree of hyperproliferation of globule-devoid hepatocytes (Fig. 7.2).

Taken together these observations led to a theory that hepatocyte hyperproliferation was elicited by cross talk between globule-containing and globule-devoid hepatocytes ([96] and Fig. 7.3). The globule-devoid hepatocytes were viewed as younger cells capable of responding to trans-acting regenerative signals derived from the globule-containing hepatocytes. The globule-containing hepatocytes were viewed as having greater ATZ accumulation and proteotoxicity and unable to respond to the existing regenerative signals because of the proteotoxic effect on cell proliferation. Thus, the globule-containing hepatocytes are sick but not dead and stimulate the regeneration of the globule-devoid hepatocytes which have a selective proliferative advantage. Interestingly, the replicative defect in the globule-containing hepatocytes was shown to be relative because these cells could proliferate, as well as globule-devoid hepatocytes when regenerative stimuli were particularly powerful in PiZ mice that survived experimental partial hepatectomy [96]. The nature of the differences between the globule-containing and globule-devoid cells is not well elucidated. A study by Linblad et al. has suggested that the globule-devoid hepatocytes have lesser accumulation of ATZ [63], and this would be consistent with younger cells that have had less time to accumulate ATZ. It is also possible that globule-devoid hepatocytes are derived from globule-containing hepatocytes as they increase capacity for the degradation of ATZ. Several observations militate against this latter possibility. The number of globule-containing hepatocytes decrease with age [95], and Ding et al. [97] showed that transplanted hepatocytes have a selective proliferative advantage that also depends on the number of adjacent globule-containing hepatocytes in that it was much more evident in male PiZ mice that had significantly more globule-containing hepatocytes than female PiZ mice. This is associated with enhanced apoptosis of the host hepatocytes, hepatic repopulation with donor hepatocytes, and resolution of the liver fibrosis that occurs in untreated PiZ mice [97].

It is interesting to note that HCC develops with aging in male PiZ mice [61], and males with ATD were also disproportionately affected by hepatic cancer in the autopsy studies of Eriksson et al. [2]. Moreover, in cases of HCC associated with ATD, a staining pattern in which the carcinoma is negative for inclusions but surrounded by adjacent liver cells that are positive for inclusions (Fig. 7.4) is consistent with the theory proposed by Rudnick and Perlmutter [96].

We believe that the NFκB signaling pathway may be particularly important in the mechanism of hepatic carcinogenesis. This pathway is powerfully activated when ATZ accumulates in the liver [50]. However, genomic analysis of liver after controlled induction of ATZ accumulation identified a relatively limited repertoire of downstream targets of this pathway [64]. One of those targets, early growth response Egr1, was markedly downregulated [64], and it is known to be a transcriptional activator of hepatocyte regenerative activity [98]. This could mean that NFκB activation leads to decreased Egr1 expression and relatively decreased proliferation of globule-containing hepatocytes in ATD.

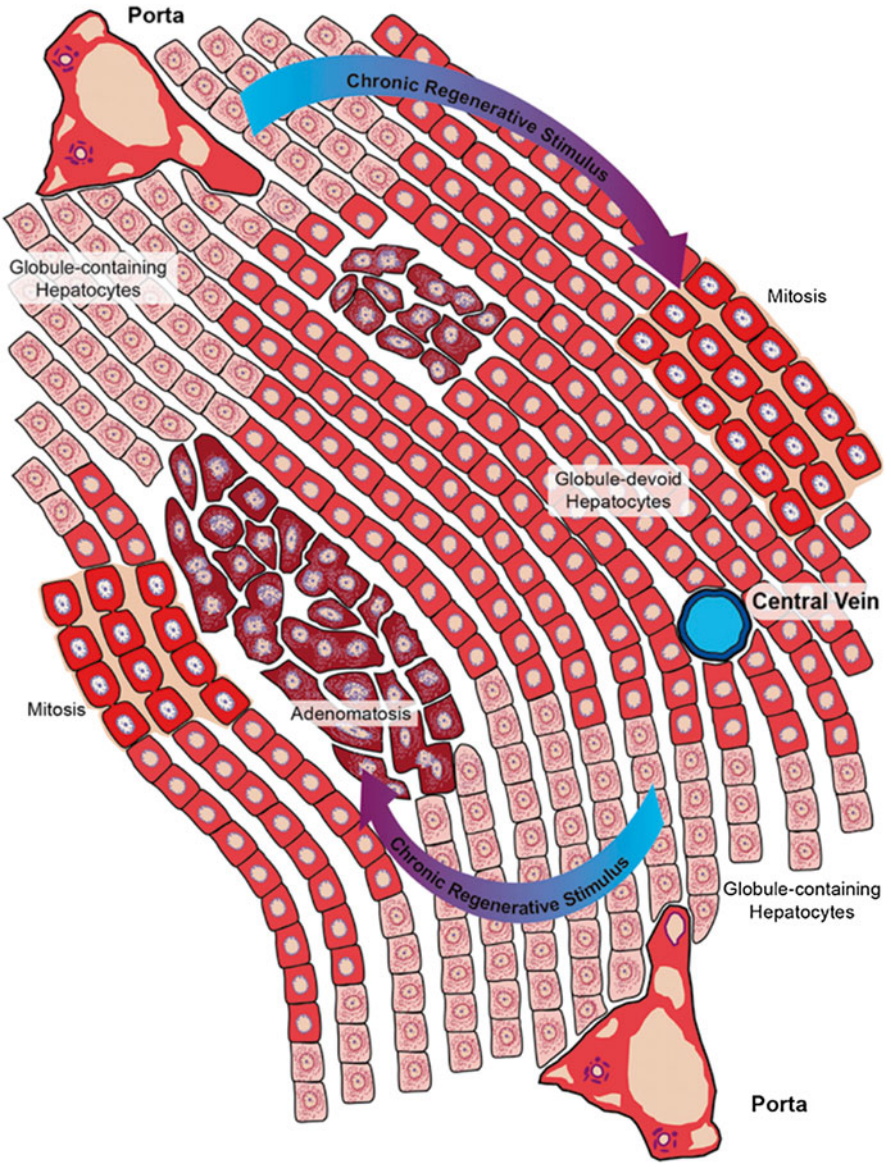


Fig. 7.3 Hypothetical model for hepatocarcinogenesis in ATD. Globule-containing hepatocytes (*pale pink*) tend to be periportal. They are "sick but not dead" and generate chronic regenerative signals which can only be received effectively in "trans" by globule-devoid hepatocytes (*deep pink*). The globule-devoid hepatocytes tend to be in the centrilobular regions. When regenerative signals are received by globule-devoid hepatocytes by this cross talk, it drives mitosis and ultimately carcinogenesis (*dark red*) in the globule-devoid regions. Reproduced from Ref. [93] with permission

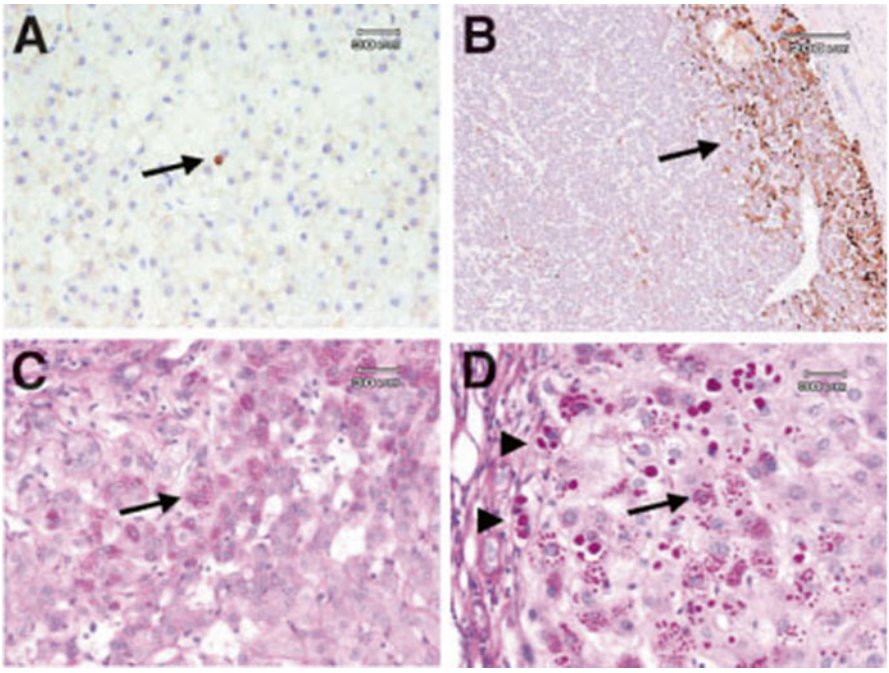


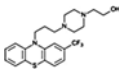
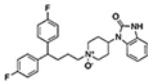
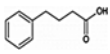
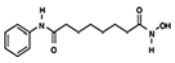
Fig. 7.4 Proliferation of hepatocytes in hepatocellular carcinoma associated with ATD. (**a**) The low proliferation rate of the tumor is demonstrated by the staining of only a single hepatocyte within the HCC (*arrow*) in this field (original magnification × 400); (**b**) immunohistochemistry for AT shows intracytoplasmic deposits in the normal hepatocytes at the periphery of the tumor; (**c**) and (**d**) PAS/diastase staining shows that there are deposits in the tumor cells, but they are fine granules (*arrow*) as compared to the globules of varying size in the surrounding normal hepatocytes (*arrowhead*) (**d**). Reproduced from Ref. [9] with permission

Therapeutic Strategies for Liver Disease of ATD

Orthotopic liver transplantation has been used for treatment of ATD-associated progressive liver dysfunction and liver failure with 5-year survival rates greater than 90 % for children and greater than 80 % for adults [99]. Interestingly, a number of patients with severe ATD liver disease experience low rates of disease progression and lead relatively normal lives for extended time period [13]. These individuals can therefore sometimes be observed and supported without transplantation, especially if living related donor transplantation is an option for them.

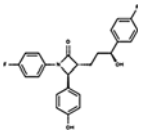
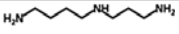
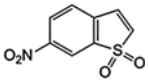
Several novel strategies for treatment of ATD liver disease that would obviate the need for organ transplantation and chronic immunosuppression are currently under investigation and at various stages of development. One of the relatively newer strategies involves autophagy enhancer drugs (Table 7.2). Autophagy was considered an excellent target because it is specifically activated when ATZ accumulates

Table 7.2 Therapeutic drug candidates

Name	Structure	Mechanism	Disease models tested	References
Carbamazepine (CBZ)		A well-known mood-stabilizing agent reported to induce autophagic disposal of insoluble ATZ and proteasomal degradation of soluble ATZ	Alpha-1 antitrypsin deficiency: mammalian cell lines; mouse model	Hidvegi et al. 2010 [57]
Fluphenazine (FLU)		Phenothiazine compound and identified as an autophagy inducer in high-throughput screening. Mechanism(s) of autophagic induction yet to be determined	Alpha-1 antitrypsin deficiency: high-throughput drug screen, *C. elegans* model; mammalian cell lines; mouse model	Gosai et al. 2010 [97] Li et al. 2014 [83]
Pimozide (PMZ)		Phenothiazine compound and identified as an autophagy inducer in high-throughput screening. Mechanism(s) of autophagic induction yet to be determined	Alpha-1 antitrypsin deficiency: high-throughput drug screen; *C. elegans* model	Gosai et al. 2010 [97]
4-Phenylbutyric acid (PBA)		Nonselective chemical chaperone that facilitates protein folding and reported to enhance secretion of ATZ	Alpha-1 antitrypsin deficiency: mammalian cell lines; mouse model	Burrows et al. 2000 [115] Teckman 2004 [116]
Suberoylanilide hydroxamic acid (SAHA)		Histone deacetylase 7 inhibitor, pharmacologically similar to PBA, and reported to enhance ATZ secretion and synthesis	Alpha-1 antitrypsin deficiency: mammalian cell lines	Bouchecareilh et al. 2012 [117]

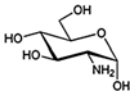
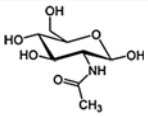
(continued)

Table 7.2 (continued)

Name	Structure	Mechanism	Disease models tested	References
Tat-beclin 1 peptide	YGRKKRRQRRR-GG-TNVFNATF EIWHDGEFGT	Novel autophagy-inducing peptide, which binds GAPR-1 releasing beclin-1 from Golgi, leading to increased beclin-1 in the cytosol which in turn induces autophagy	Infectious disease: mammalian cell lines; mouse model Huntington's disease: mammalian cell lines; mouse model	Shoji-Kawata et al. 2013 [108]
Ezetimibe		A cholesterol-reducing agent. Inactivates NPC1L1 by direct binding and reduces MTOR recruitment to lysosomes, thereby inhibiting mTORC1 activity, which leads to activation of autophagy	Alpha-1 antitrypsin deficiency: human primary hepatocytes	Yamamura et al. 2014 [110]
Spermidine		A naturally occurring polyamine; activates autophagy by altering cellular network of deacetylases and acetylases	Aging models: yeast model; mouse model; *C. elegans* model; mammalian cell lines	Eisenberg et al. 2009 [105] Morselli et al. 2011 [106]
Stattic		Chemical inhibitor of STAT3. It promotes PKR-dependent phosphorylation $eIF2^{\alpha}$ and consequent activation of autophagy	High-throughput drug screen; mammalian cell lines	Shen et al. 2012 [107]
Omega-6-polyunsaturated fatty acids		A class of PUFA that has been reported to activate autophagy. The molecular mechanism of autophagy induction not known	Aging model: *C. elegans* Mammalian cell lines	O' Rourke et al. 2013 [108]

(continued)

Table 7.2 (continued)

Name	Structure	Mechanism	Disease models tested	References
Glucosamine		An amino sugar which alleviates arthritic pain and improves skin quality. Induces autophagy in an mTOR-independent manner	Mammalian cell lines	Shintani et al. 2010 [109]
N-acetyl glucosamine		An amino sugar which reduces arthritic pain and improves skin conditions. Induces autophagy in an mTOR-independent manner	Mammalian cell lines	Shintani et al. 2010 [109]

in cells, and it also plays a critical role in intracellular disposal of ATZ. At the time when this approach was first investigated, several drugs which could enhance autophagic degradation of other misfolded proteins, such as mutant polyQ proteins that cause Huntington disease, were being described [100]. It had also become apparent that ATD liver disease could more frequently have its onset at 50–65 years of age coincident with the decline in autophagy function that is believed to trigger other age-dependent degenerative diseases associated with misfolded proteins. Hidvegi et al. first investigated the drug carbamazepine (CBZ), which is known for its widespread use in humans as an anticonvulsant and mood stabilizer, and found that it enhanced autophagic degradation of ATZ in mammalian cell line models of ATD [60]. Moreover, administration of this drug by oral gavage to the PiZ mouse model of ATD over a 3-week period significantly reduced hepatic ATZ load and hepatic fibrosis in vivo [60]. Because CBZ is already FDA-approved, it could be moved immediately into a Phase II/III clinical trial for the treatment of severe liver disease due to ATD.

The notion that autophagy enhancer drugs can mitigate proteotoxicity caused by intracellular accumulation of misfolded proteins has been bolstered by recent studies in *C. elegans* model system. High-throughput automated screening of a drug library (LOPAC) containing 1280 compounds in a *C. elegans* model system for ATD identified five hit compounds that decreased cellular ATZ load in a dose-dependent manner [101]. Four of these five compounds have autophagy enhancer activity and are already FDA approved for use in clinical practice. Interestingly, two of the hit compounds, fluphenazine and pimozide, belong to the phenothiazine drug family, which has structural similarity to tricyclic antidepressants, the structural family to which CBZ belongs. The phenothiazines were also previously shown to accelerate autophagic disposal of polyQ proteins [102, 103]. Fluphenazine has been

tested extensively in the *C. elegans* model of ATD, in mammalian cell line models, and also in the PiZ mouse. The results show that fluphenazine reduces hepatic ATZ load and hepatic fibrosis in vivo in all the model systems tested [87]. Altogether, the results from these studies show promising prospects for this type of high-throughput screening strategy and two novel strategies for chemical and computational-based drug discovery using the autophagy enhancer drug paradigm and the phenothiazine structure for discovering additional drugs for treating liver disease due to ATD [88, 104].

Several other drugs that increase autophagy have recently been reported and could therefore be candidates for treating liver disease due to ATD. Eisenberg et al. showed that spermidine induces autophagy in several model systems including yeast, worms, and mammalian cell systems and increases lifespan in flies, worms, and mice [105]. Furthermore, Morselli et al. has reported that spermidine and resveratrol can synergistically induce autophagy in mammalian cell culture, mouse, and *C. elegans* model systems by altering the cellular network of deacetylases and acetylases [106]. Using a high-throughput screening strategy for autophagy enhancers, Shen et al. discovered that chemical inhibitors of transcription factor STAT3 such as JSI-124, Stattic, and WP1066 were potent inducers of autophagy [107]. Similarly, omega-6-polyunsaturated fatty acid has been reported to enhance autophagy in mammalian cell lines and *C. elegans* model systems [108]. In a *C. elegans* model system, omega-6-polyunsaturated fatty acids promoted longevity through this mechanism [108]. Glucosamine and *N*-acetylglucosamine have also been shown to enhance autophagy in mammalian cell lines [109]. A cholesterol-reducing drug, ezetimibe, has been shown to activate autophagy. Studies by Yamamura et al. showed that this drug enhances autophagy specifically in hepatocytes and intestinal epithelial cells [110], consistent with the inhibitory effect of this drug on cholesterol-binding protein Niemann–Pick-type C1-like 1 (NPC1L1) [111]. The authors reported that inhibition of NPC1L1 by ezetimibe reduced recruitment of mTOR to the lysosomes, leading to inhibition of mTORC1 activity and hence activation of autophagy. Importantly, the authors show that ezetimibe reduced cellular ATZ load in primary cultures of human hepatocytes engineered for ATZ expression. Further studies are required to establish effectiveness of this drug in reversing the proteotoxic effect of ATZ accumulation in animal model systems. A novel autophagy-inducing peptide Tat-beclin 1 enhances degradation of mutant huntingtin and several invasive viral and bacterial pathogens in vivo [112]. This peptide, or drugs based on its structure, has the potential for treatment of a broad range of human diseases caused by proteotoxicity and infectious pathogens.

A number of relatively new gene therapy strategies are being investigated. One of these involves new methods for silencing gene expression using vectors that are also capable of encoding wild-type AT to address both gain- and loss-of-function sequelae of ATD, respectively [113, 114]. In one approach, Li et al. utilized adeno-associated virus harboring short-hairpin RNA to knockdown endogenous ATZ expression together with a codon-optimized wild-type AT transgene cassette [113]. In another approach, Mueller et al. utilized an adeno-associated virus harboring microRNA to silence endogenous ATZ gene expression together with a microRNA-resistant wild-type AT gene [114]. In each case hepatic ATZ load was

reduced and levels of human AT increased in the serum of a transgenic mouse model of ATD. However, the effect on liver fibrosis by this strategy was not as compelling, and hence further studies are required to test whether more potent and widespread silencing would be more effective. Another study using antisense oligonucleotides by systemic administration to silence ATZ gene expression had a more impressive effect in reducing hepatic fibrosis in the PiZ mouse model system [115].

Another potential gene therapy approach being investigated is the transfer of genes that activate autophagy and therein reduce ATZ accumulation and proteotoxicity. Pastore et al. have championed this approach using TFEB, a master transcriptional activator of the autophagolysosomal system [116]. Using helper-dependent adenovirus for systemic delivery of TFEB and targeting of its expression to liver, this approach significantly reduced hepatic ATZ load and liver fibrosis in the PiZ mouse model. In vitro studies also validated that TFEB reduces cellular ATZ levels in an autophagy-dependent manner [116]. Although it will not address the loss-of-function mechanisms associated with ATD lung disease, gene therapy with TFEB or drugs that target TFEB activation constitute exciting potential therapeutic strategies for liver disease associated with ATD.

Ultimately genomic editing will be considered to definitively correct the genetic defect that causes ATD. The most recent development in this area, CRISPR/Cas-9-mediated genome editing, has been used in a mouse model in vivo to correct the genetic defect in dystrophin (Dmd) that causes Duchenne muscular dystrophy (DMD) [117]. Using this approach, the Dmd mutation was corrected in the germline of the mdx mouse model of DMD and produced genetically mosaic animals containing 2–100 % correction of the Dmd gene. The authors reported that only a subset of corrected cells in vivo could perform a complete phenotypic rescue. Hence, the extent of phenotypic rescue in the mosaic mice exceeded the efficiency of gene correction. One possibility for this is that the corrected muscle cells have a selective proliferative advantage in the presence of injured muscle cells. If the technological advancement eventually provides a way for genome editing of postnatal somatic cells, in the future this strategy could be used for correcting other diseases caused by single gene mutation events including ATD.

Several research groups are exploring a "structure-based" screening strategy that aim at generating peptides to prevent polymerization of the mutant ATZ with the hypothesis that this would facilitate secretion. A small-molecule compound designed against a lateral hydrophobic cavity in ATZ prevented its polymerization; however, further experiments in a cell line model revealed that this compound enhanced intracellular degradation only, with minimal effect on secretion [56]. These results provide evidence that it is the misfolding of ATZ, independent of its tendency to polymerize, which is primarily responsible for impaired secretion. Another small molecule based on a peptide that targets the reactive center loop of AT has been designed and introduced in cell line model systems with evidence for improved secretion of ATZ [118]. However, the efficacy of this peptide in an animal model system for either increasing secretion or reducing liver damage in vivo remains to be tested. It also remains possible that this type of peptide binding changes the conformation of the mutant protein in such a way that both misfolding and polymerization are reduced independently.

Chemical chaperones that can nonselectively facilitate folding of diverse misfolded proteins have also been investigated as a potential therapeutic option for ATD liver disease. Glycerol and 4-phenylbutyric acid (PBA) were found to mediate a robust enhancement in the secretion of ATZ in a mammalian cell line model, and its oral administration in PiZ mice increased blood levels of human AT reaching 20–50 % of the levels present in PiM mice and normal humans [119]. However, a pilot clinical trial involving 10 patients with ATD-associated liver disease failed to reveal any significant increase in serum levels of AT after 14 days of treatment with PBA [120]. It is not clear why the drug lacked effect but the large doses required are known to be quite challenging to tolerate and so it may be worthwhile to test in the future if newer, more tolerable formulations are developed. Recently, suberoylanilide hydroxamic acid (SAHA), another drug which has many pharmacological similarities to PBA, has been found to enhance secretion of ATZ in cell line models of ATD [121]. However, SAHA has not yet been tested in animal models. Moreover, detailed studies are needed to delineate whether this effect is due to increased synthesis of ATZ or due to its ability to reduce ATZ accumulation in cells or both. If increased secretion of ATZ is because of, or even associated with, increased synthesis, the treatment could produce more, rather than less, cellular proteotoxicity.

Hepatocyte transplantation therapy has also been investigated as a potential treatment for ATD. It has been tested in the past as a treatment for several metabolic liver diseases [122, 123]. Compared to orthotopic liver transplantation, it has the advantage of being a minimally invasive procedure with little known morbidity and is considerably less expensive than protein (AT) replacement therapy or liver transplantation. Importantly, recent studies have revealed that wild-type donor hepatocytes can repopulate almost the entire liver of the PiZ mouse model of ATD [97]. In the PiZ mouse model, the donor cells replaced both globule-containing and globule-devoid cells, indicating that both types of affected hepatocytes have impaired proliferative capacity compared to wild-type hepatocytes. Because the transplanted hepatocytes have a selective proliferative advantage over ATZ-containing endogenous hepatocytes and can substitute for the latter in a diseased liver, this option of therapy may be considered for ATD lung and liver disease.

Another exciting therapeutic strategy in which genomic editing is combined with hepatocyte transplantation has been tested in a transgenic mouse model of ATD. Studies by Yusa et al. have shown that the mutation in the AT gene could be corrected in human induced pluripotent stem (iPS) cells derived from an ATD patient using a combination of zinc-finger nucleases and transposon techniques [124]. Importantly, the corrected iPS cell lines could then be engrafted into the liver of the transgenic mouse model system, and, based on the observations of Ding et al. [97], the corrected cells should expand significantly because they will have a selective proliferative advantage. This strategy, if it proves successful in further preclinical models, has the potential to address both the loss- and gain-of-function mechanisms of organ damage and the advantage of personalized treatment options without any need for immunosuppression.

Summary

A subgroup of individuals with homozygous PIZZ ATD develop liver disease characterized by fibrosis/cirrhosis and HCC. Genetic and environmental modifiers are probably responsible for rendering the subgroup susceptible to liver disease. This liver disease is more common in adults than previously recognized, and this may mean that it is part of a collection of degenerative diseases that are impacted by the age-dependent decline in proteostasis mechanisms such as autophagy. The disease that affects infants, older children, and adolescents probably results from particularly powerful, rare combinations of modifiers. An impressive number and repertoire of therapeutic strategies have recently moved into various stages of development, including one autophagy enhancer drug that is already in an active phase II/III clinical trial. We believe these efforts will soon begin to improve the outcome for individuals with severe liver disease due to ATD.

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Chapter 8
Alpha-1 Antitrypsin as a Therapeutic Agent for Conditions not Associated with Alpha-1 Antitrypsin Deficiency

Adam Wanner

Introduction

For many years, alpha-1 antitrypsin has been used as augmentation therapy in patients with COPD associated with alpha-1 antitrypsin deficiency. The primary goal of the treatment has been to raise circulating and tissue levels of alpha-1 antitrypsin thereby counteracting unopposed serine protease activity, notably neutrophil elastase, a putative factor in the pathogenesis of COPD. In the United States, regulatory approval by the Food and Drug Administration was obtained only for intravenous alpha-1 antitrypsin and only for lung disease associated with severe alpha-1 antitrypsin deficiency allele combinations; thus, the therapy has been restricted to a single condition.

Over the past 15 years, there has been a growing recognition of alpha-1 antitrypsin's broader anti-inflammatory actions beyond serine protease inhibition including immunomodulatory and anti-apoptotic effects. It has been shown that alpha-1 antitrypsin that has been modified to lose its antiprotease activity retains potent anti-inflammatory and immunomodulatory effects on human monocytes and in a mouse model of lung inflammation [1, 2]. Additional data have supported this concept by showing that alpha-1 antitrypsin is an endogenous inhibitor of cytokine production in whole human blood [3], that alpha-1 antitrypsin can activate phosphatases to abrogate inflammatory responses in the lung [4], and that alpha-1 antitrypsin inhibits IL-8 and neutrophil chemotaxis in the lung [5–7]. Of equal importance have been observations on alpha-1 antitrypsin's anti-apoptotic activity, likely owing to the inhibition of caspases, notably caspase-3 [8–11]. Antiangiogenic and antimicrobial effects have also been reported [12–14].

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Table 8.1 Alpha-1 antitrypsin-replete conditions under consideration for alpha-1 antitrypsin treatment

Type 1 diabetes mellitus[a]
Viral infections[a]
Graft-versus-host disease[a]
Cystic fibrosis[a]
Chronic obstructive pulmonary disease
Inflammatory bowel disease
Arthritis
Ischemic heart disease

[a]Including investigations that include early human trials

Given alpha-1 antitrypsin's diverse modes of action, it is not surprising that interest arose in using this protein to treat conditions such as inflammatory bowel disease [15], arthritis [16–18], ischemic heart disease [19], organ and cell transplant rejection, graft-versus-host disease, alpha-1 antitrypsin-replete chronic obstructive lung diseases, type 1 diabetes mellitus, and viral infection. The latter application is based on the role of serine proteases involved in viral entry into cells.

Most information has been obtained in type 1 diabetes mellitus, graft-versus-host disease, viral infection, and alpha-1 antitrypsin-replete lung disease, notably COPD and cystic fibrosis (Table 8.1). Furthermore, some of the related studies have not been limited to in vitro or animal observations but have also involved experiments in humans. In this chapter, we therefore will focus the discussion on the latter conditions.

If alpha-1 antitrypsin is administered to patients with normal serum alpha-1 antitrypsin levels, why should a therapeutic effect be expected? Why should supranormal circulating and presumably tissue alpha-1 antitrypsin concentrations be beneficial? In the absence of pharmacokinetic investigations in alpha-1 antitrypsin-replete humans receiving alpha-1 antitrypsin by intravenous infusion or inhalation, the question cannot be answered with confidence. However, in patients with alpha-1 antitrypsin deficiency, intravenously administered alpha-1 antitrypsin at a dose of 60 mg kg^{-1} has been shown to increase serum alpha-1 antitrypsin levels by ~120 mg dl^{-1} from ~60 mg dl^{-1} to a peak of ~180 mg dl^{-1}, with a gradual return to baseline within 7 days [20]. It is not known if similar increases would be seen in people with normal baseline serum alpha-1 antitrypsin levels, but assuming an increase in the same range would raise the levels from ~180 mg dl^{-1} to a peak of 300 mg dl^{-1}, with levels remaining above normal for a week. Alpha-1 antitrypsin is a positive acute phase reactant, and the above levels are well within the range of serum alpha-1 antitrypsin levels seen in conditions of stress such as acute coronary syndrome, abdominal surgery, and open-heart surgery [21–23] (Fig. 8.1).

Pro-inflammatory cytokines, especially interleukin-6, are thought to be involved in the acute phase response by inducing secretory acute phase proteins including alpha-1 antitrypsin in hepatocytes [24]. Inasmuch as the presumed role of alpha-1 antitrypsin in the acute phase response is to modulate the biological effects of acute inflammation, one could argue that maintaining serum alpha-1 antitrypsin levels elevated over longer periods of time by the administration of alpha-1 antitrypsin

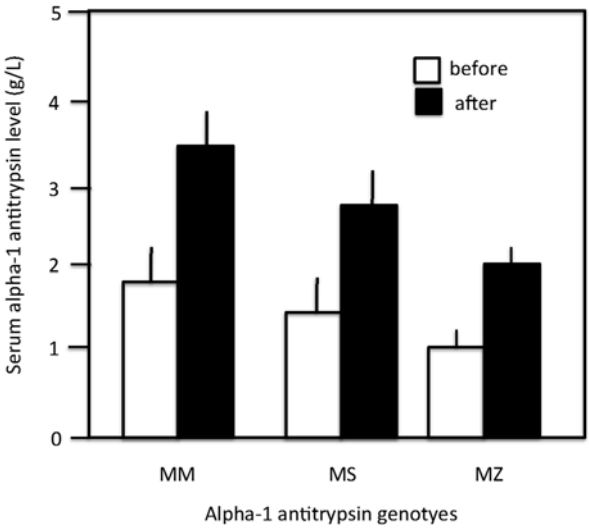

Fig. 8.1 Acute phase response in serum alpha-1 antitrypsin levels in patients before and 4–5 days after open-heart surgery. Means ± SD. Alpha-1 antitrypsin genotypes: MM ($n = 193$), MS ($n = 10$), MZ ($n = 5$). Note that the levels increased by about 100 % in all three groups. Graph constructed with data from Sandford et al., Am J Resp Crit Care Med 1999; 159:1624–1628

might be beneficial in chronic conditions involving inflammatory processes that may or may not include serine proteases.

Currently, only human plasma-derived alpha-1 antitrypsin is available for clinical use. The supply of this product is limited but apparently sufficient to meet the need in patients with an orphan condition such as severe alpha-1 antitrypsin deficiency, the only approved indication for alpha-1 antitrypsin therapy at the present time. Future research could identify new therapeutic target conditions for this protein; some of them may not be rare. Therefore, alternate sources and formulations of alpha-1 antitrypsin may have to be developed to meet the greater demand. Inhaled human plasma-derived alpha-1 antitrypsin has been experimentally administered as an aerosol to patients with alpha-1 antitrypsin-replete lung disease [25–27]. This mode of administration would reduce the amount of protein needed to treat the lung disease of alpha-1 antitrypsin-deficient and alpha-1 antitrypsin-replete patients. However, alternate sources of alpha-1 antitrypsin have been explored in an attempt to replace the human plasma-derived product. Several companies have used transgenic and recombinant approaches. A major challenge has been the requirement of mimicking the glycosylation pattern of native human alpha-1 antitrypsin to ensure adequate tissue penetration and acceptable plasma half-life values. Further studies are warranted to demonstrate the safety and efficacy of such products.

Finally, new formulations and applications of alpha-1 antitrypsin will require regulatory approval. For product licensing, low or lack of immunogenicity, efficacy, and safety will have to be demonstrated in the new target populations, and the trials will have to involve clinically meaningful, not just surrogate endpoints [28]. Meeting these requirements will have a major role in bringing the new alpha-1 antitrypsin products to the market and obtaining regulatory approval for them.

Type 1 Diabetes Mellitus

As an autoimmune condition, type 1 diabetes mellitus involves inflammation-related injury to beta cells in the pancreas, leading to fluctuating blood glucose levels. Alpha-1 antitrypsin is an endogenous anti-inflammatory molecule that could correct altered immunoregulatory pathways and modulate the inflammation associated with type 1 diabetes thereby improving blood glucose control [29]. This concept is strengthened by the fact that alpha-1 antitrypsin is locally manufactured in the pancreas under normal circumstances in various species including man. For example, the porcine pancreas contains alpha-1 antitrypsin as assessed by proteomic analysis, especially during development [30]. In humans, alpha-1 antitrypsin expression and secretion by islet cells has been investigated by immunofluorescence, Western blotting, and ELISA [31]. It was shown that alpha and delta cells are the primary source of synthesis and presumably secretion. Given the proximity of these cells to beta cells, this suggests the presence of a locally mediated biological function of alpha-1 antitrypsin by modulating immunologic and inflammatory processes that could be involved in type 1 diabetes. Furthermore, it has been reported that circulating alpha-1 antitrypsin is less active in diabetic individuals, possibly due to high glucose levels in hepatocytes where alpha-1 antitrypsin therefore is excessively glycated [32, 33]. Alpha-1 antitrypsin glycation also could occur in other alpha-1 antitrypsin-generating tissues, including the islet of the pancreas. So far, this has not been investigated.

Based on these considerations, there has been a considerable interest in studying the effects of exogenous alpha-1 antitrypsin or alpha-1 antitrypsin overexpression in the pancreas on glucose control in animal models of type 1 diabetes and in early human experiments. Underlying these studies is the expectation that above-normal levels of alpha-1 antitrypsin would rebalance immunoregulation in the pancreas. In support of this premise, it has been shown that a beta cell line stably transfected with human alpha-1 antitrypsin resists cytotoxic, T-cell-mediated apoptosis and inflammatory cytokine production [5].

Diabetic mice have been the most popular animal models of type 1 diabetes and have provided important information on the processes underlying the phenotype and their response to alpha-1 antitrypsin. The primary outcomes have been blood glucose levels and survival and function of beta cells transplanted into diabetic mice (antirejection action of alpha-1 antitrypsin). In a 4-week-old nonobese diabetic mouse strain, gene therapy with human alpha-1 antitrypsin using an adeno-associated viral vector prevented the development of diabetes [34, 35]. This was associated with an altered T-cell repertoire in spleen cells, suggesting a possible mechanism for the alpha-1 antitrypsin effect in the pancreas. A complementary study showed that in nonobese diabetic mice with early type 1 diabetes, alpha-1 antitrypsin therapy restored euglycemia and promoted beta cell expansion; again the effect was attributed to alpha-1 antitrypsin's broad anti-inflammatory actions [36]. In another murine model of streptozotocin-induced type 1 diabetes, an Fc-fused recombinant alpha-1 antitrypsin protein lacking anti-elastase activity prevented

hyperglycemia [32]. At least in this model, the anti-inflammatory action of alpha-1 antitrypsin could not be attributed to its anti-elastase activity, pointing to other immunomodulatory and anti-inflammatory actions. In particular, alpha-1 antitrypsin's known caspase-3 inhibitory and anti-apoptotic actions may have a role as shown in vitro and in vivo [37]. In that investigation, alpha-1 antitrypsin inhibited caspase-3 activity and prevented tumor necrosis factor alpha-induced apoptosis in a murine insulinoma cell line and reduced beta cell apoptosis as assessed by a TUNEL assay in streptozotocin-treated diabetic mice.

These results show that alpha-1 antitrypsin improves beta cell function and normalizes blood glucose levels in two different mouse models of type 1 diabetes and suggest that type 1 diabetes in humans could be a target for alpha-1 antitrypsin therapy. To date, a proof-of-concept study has raised the possibility that at least some patients could benefit from such treatment [5]. In 12 patients with type 1 diabetes and detectable blood C-peptide levels, the effect of 80 mg/kg alpha-1 antitrypsin, administered by intravenous infusion weekly for 8 weeks, on the C-peptide response to a mixed meal challenge was investigated. The C-peptide response (expressed as area under the curve) increased at 3 months in only five patients (Fig. 8.2), but there was an inverse relationship between the frequency of IL-1 beta-producing monocytes and the C-peptide response, indicating that specific inflammatory pathways sensitize patients to the effects of alpha-1 antitrypsin on beta cell

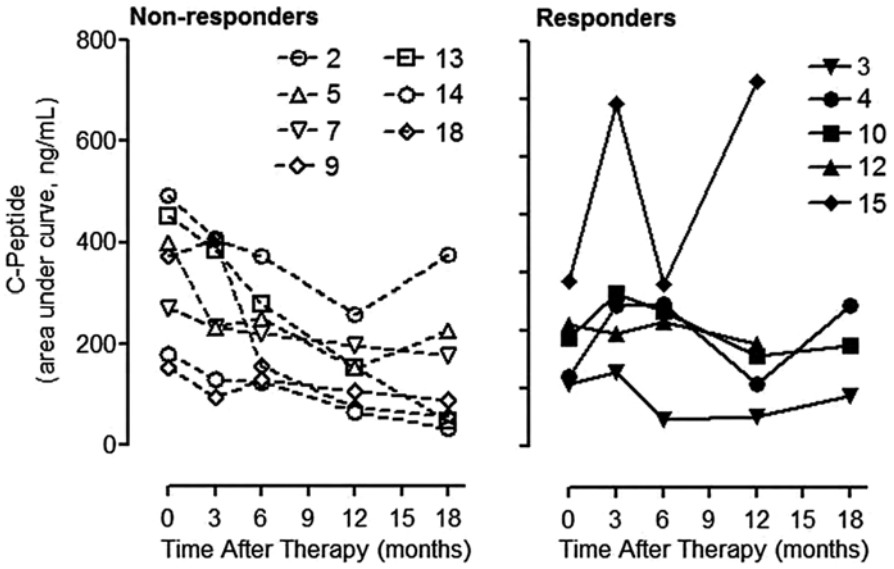

Fig. 8.2 C-peptide responses to a mixed meal tolerance test in 12 patients with type 1 diabetes and detectable blood C-peptide levels, before and at different times after 8 weekly infusions of 80 mg/kg alpha-1 antitrypsin. Note that in five patients, the response increased at 3 months irrespective of responder/nonresponder designation. Interestingly, there was an inverse relationship between the C-peptide response and the frequency of IL-1 beta-producing blood monocytes among all patients. With permission from Gottlieb et al., J Clin Endocrinol Metab 2014; 99: E 1418–1426

function. Three human trials using intravenous alpha-1 antitrypsin in patients with recently diagnosed type 1 diabetes have successfully been initiated; the primary outcomes are glycemic control or C-peptide response. Two trials are ongoing while one has been completed; as of November 2014, no results had yet been reported.

Alpha-1 antitrypsin also has been investigated in islet cell transplantation to treat type 1 diabetes. Essentially all reported studies in animal models have shown a beneficial effect of alpha-1 antitrypsin on beta cell survival and function. To our knowledge, alpha-1 antitrypsin thus far has not been administered to patients who have undergone islet transplantation for the treatment of type 1 diabetes.

Most studies have involved mice and were based on the hypothesis that alpha-1 antitrypsin would exert its effect by suppressing autoimmunity and protecting against graft rejection [38]. For example, islet allograft survival and euglycemia was extended by alpha-1 antitrypsin treatment in diabetic mice in one study; this was associated with a diminished release of tumor necrosis factor alpha from stimulated islet cells [39]. In a similar investigation, islet graft survival was improved by alpha-1 antitrypsin treatment in a syngeneic non-autoimmune mouse model of diabetes, again accompanied by a downregulation of tumor necrosis factor alpha [40]. In yet another study, single-dose T-cell depletion combined with alpha-1 antitrypsin treatment prolonged rat-to-mouse islet xenograft survival [41].

Other mechanisms implicated in limited graft survival also have been examined including deficient graft vascularization. For example, alpha-1 antitrypsin has been reported to stimulate vascular endothelial growth factor expression and release and to promote revascularization of islet cell allografts in an explantable compartment of the mouse pancreas [42]. Graft survival also could be limited due to graft injury by pancreatic proteases as pancreatic acinar cells have been shown to contaminate islet cell preparations and be co-transplanted with them. Acinar cell protease activity could then injure islet cells and shorten their survival. Supporting this possibility, it has been shown that the more impure the islet preparations, the greater their proteolytic activity, islet cell loss, and insulin depletion [43]. Incubation with alpha-1 antitrypsin protected the preparation from these changes, presumably by its protease inhibitory action.

The effect of alpha-1 antitrypsin on islet graft survival and function has also been investigated in a nonhuman primate, bringing the observation closer to the human condition. In subtotally pancreatectomized, streptozotocin-treated monkeys with autologous islet cell transplantation, treatment with alpha-1 antitrypsin during the peri-transplant period leads to functional islet mass expansion and improved graft function [44].

Viral Infection

The influenza A virus, coronaviruses, and the human immunodeficiency virus type 1 (HIV-1) use host serine proteases for cell entry and subsequent infection [45, 46]. The mechanism whereby serine proteases are involved in the initial steps of viral

entry has been clarified for the influenza virus A. Viral hemagglutinin activates hemagglutinin receptors expressed on the host cell membrane thereby activating host serine proteases that through their effect on the virus facilitate its entry into the cell [47]. Alpha-1 antitrypsin theoretically could suppress influenza A infection owing to its serine protease inhibitory action.

To date, the prophylactic or therapeutic effect of alpha-1 antitrypsin therapy in alpha-1 antitrypsin-deficient or alpha-1 antitrypsin-replete humans with influenza-A infection has not been examined. Yet the potential for alpha-1 antitrypsin's anti-influenza action deserves further exploration, especially with inhaled formulations. In this regard, it is noteworthy that influenza pneumonia in mice that overexpress human alpha-1 antitrypsin has been reported to be associated with a better survival than in wild-type control mice [28].

More information is available on the role of alpha-1 antitrypsin in HIV-1 infection. It has been shown that HIV-1 does not replicate in alpha-1 antitrypsin-replete whole blood but replicates well in alpha-1 antitrypsin-deficient blood [46]. Furthermore, alpha-1 antitrypsin has been reported to suppress HIV-1 production in chronically infected monocytes, to inhibit HIV-1 entry into a cell line designed to detect viral entry, and to reduce HIV-1 replication in human peripheral mononuclear cells [46, 48]. A 20-residue virus inhibitory peptide corresponding to the C-terminal region of alpha-1 antitrypsin has been shown to inhibit viral entry and may explain the anti-HIV action of alpha-1 antitrypsin [49].

These in vitro observations have been substantiated by an in vivo study involving human subjects. A 10-day infusion of the virus inhibitory peptide in treatment-naïve patients with HIV infection reduced the viral load by a factor of 12 [32] (Fig. 8.3). Conversely, an association has been reported to exist between HIV infection and reduced serum alpha-1 antitrypsin levels [50, 51]. Taken together, the currently available information provides a basis for future clinical investigations into the therapeutic potential of alpha-1 antitrypsin in HIV-1 infection. In contrast to influenza, HIV infection is of a chronic nature and as such provides a better target for interventional studies with alpha-1 antitrypsin.

Graft-Versus-Host Disease

Allogenic hematopoietic stem cell or bone marrow transplantation, while in clinical use for the treatment of leukemia, can lead to graft-versus-host disease [52]. Standard immunosuppressive therapy administered to attenuate the immunologically mediated attack of the graft against the host increases the risk of opportunistic infections and impairs the graft's antileukemia effect (graft-versus-leukemia response).

In search of agents that would suppress the immunological response of the graft against the host (graft-versus-host disease) without compromising its antileukemia effect (graft versus leukemia), several groups have investigated alpha-1 antitrypsin, a molecule with known anti-inflammatory and immunomodulatory profiles and

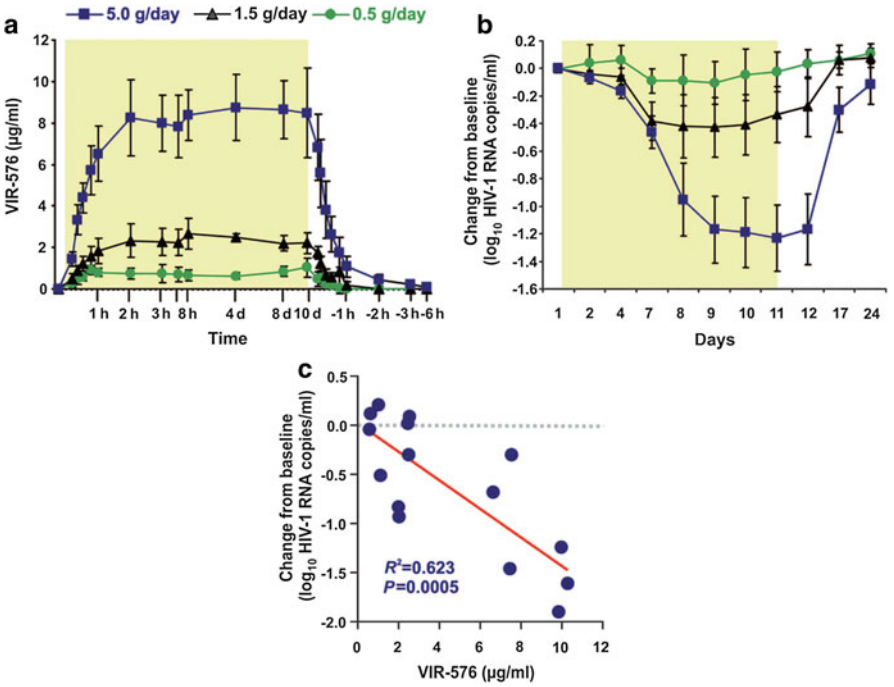

Fig. 8.3 Effect of short-term intravenous monotherapy with a natural 20-residue fragment of human a1-antitrypsin in HIV-infected patients ($N=15$). Values shown as mean (±SEM). (**a**) Quantities of the fragment (VIR-576) detected in the plasma of patients treated with three different doses. (**b**) Changes in $\log_{10}$ plasma viral load over time in the three dosing groups. (**c**) Correlation between the induced change in viral load and achieved VIR-576 plasma levels in all patients. With permission from Forssmann et al., Sci Transl Med 2010; 2: 63–70

excellent safety record. Again, the mouse served as the preferred model for this purpose. It has been reported that the administration of alpha-1 antitrypsin to mice undergoing allogenic bone marrow transplantation blunted graft-versus-host disease and prolonged graft survival [53]. The effect was attributed to inhibition of IL-32 activity. Other cytokines are likely to be involved as well as shown in three murine models of graft-versus-host disease in which the early administration of alpha-1 antitrypsin decreased mortality, an effect associated with a suppressed secretion of tumor necrosis factor alpha and IL-1 beta and upregulation of IL-10 [54]. Another study assessed the effect of alpha-1 antitrypsin on different T-cell populations and cytokines in a mouse model of graft-versus-host disease in which alpha-1 antitrypsin was introduced in the form of alpha-1 antitrypsin-modified donor cells [55]. The graft-versus-leukemia effect was not compromised in this model.

Human studies thus far have not been reported, but it has been shown that in patients who have undergone bone marrow transplantation, mRNA levels of IL-32 in blood leukocytes were higher in recipients with graft-versus-host disease than in those without [53]. As mentioned above, alpha-1 antitrypsin was found to inhibit

IL-32 activity in a murine model of graft-versus-host disease [53]. The time seems to be ripe for examining the effect of alpha-1 antitrypsin on graft-versus-host disease and graft-versus-leukemia responses in patients receiving bone marrow transplantation for the treatment of leukemia.

Alpha-1 Antitrypsin-Replete Obstructive Lung Diseases

Cystic fibrosis (CF) and COPD in patients without alpha-1 antitrypsin deficiency (common COPD) have been the primary targets for alpha-1 antitrypsin therapy where the goal is not to replace the protein but to raise its level beyond normal. There are at least two reasons for considering such therapy in these conditions. First, neutrophil elastase has been clearly shown to have a critical role in the pathogenesis of CF and common COPD [56, 57]. Alpha-1 antitrypsin is the most potent inhibitor of neutrophil elastase and, by suppressing neutrophil elastase activity, would be expected to ameliorate the disease process and prevent or slow progressive lung remodeling and functional impairment. Second, owing to alpha-1 antitrypsin's broader anti-inflammatory and anti-apoptotic actions, additional benefits might be seen in both conditions. Mechanistic investigations and phase 2 trials using aerosol alpha-1 antitrypsin carried out to date have provided a solid basis for further interventional studies using alpha-1 antitrypsin therapeutically in CF and common COPD.

Cystic Fibrosis

The lung disease of CF involves neutrophil elastase and its effects on mucus secretion, proteolytic destruction of airway and lung tissue, and facilitation of bacterial infection, notably by *Pseudomonas aeruginosa* [58, 59]. Neutrophils are recruited to lung tissue primarily by interleukin 8 (IL-8). Neutrophil elastase is released from neutrophils during migration, upon immune complex stimulation, and when they undergo necrosis or apoptosis [60]. In addition, IL-8 and tumor necrosis factor alpha promote neutrophil elastase release from neutrophils [63]. Alpha-1 antitrypsin is the most potent known inhibitor of neutrophil elastase, has other antiprotease activity relevant to CF, and is anti-apoptotic [8, 59]. However, its concentration in airway and lung tissue, even if above normal due to a presumably increased secretion by the liver, may not be sufficient to counteract the markedly augmented neutrophil elastase activity in the CF lung [56]. Given the importance of proteolytic activity in the inflammatory process associated with CF-related lung disease, it is not surprising that alpha-1 antitrypsin has been thought of as a therapeutic for this disease. Over 20 years ago, McElvaney et al. [58] administered aerosolized human plasma-derived alpha-1 antitrypsin to 12 patients with CF for 1 week. The authors showed that the treatment reduced neutrophil elastase activity in epithelial surface liquid (bronchoalveolar lavage fluid) with a critical alpha-1 antitrypsin

concentration of 8 μM; treatment also stimulated *Pseudomonas aeruginosa* killing by bronchoalveolar lavage fluid in vitro. Since then, major advances have been made in aerosol delivery technology with devices that can now deliver up to 70 % of the filling dose to the lungs [26, 27, 62]. In addition, high-purity liquid alpha-1 antitrypsin formulations are now available, and so far short-term aerosol alpha-1 antitrypsin treatment for up to 28 days has been found to be safe [26, 27, 50, 62].

Using the advanced aerosol delivery technology, two phase 2 studies have interrogated the effects of short-term aerosol therapy with alpha-1 antitrypsin in patients with stable CF. In a placebo-controlled investigation using between 125 and 500 mg alpha-1 antitrypsin daily for 4 weeks, there was a trend toward decreased neutrophil elastase activity in sputum as assessed by IL-8, myeloperoxidase, and neutrophil elastase-alpha-1 antitrypsin complexes [63]. In another interventional study, which was not placebo controlled, 52 CF patients were treated with 25 mg aerosolized alpha-1 antitrypsin daily for 4 weeks [64]. There were significant decreases in sputum neutrophil counts and IL-8 and unopposed neutrophil elastase activity from before to after the treatment. There were no concomitant changes in FEV_1.

These investigations have established the biochemical efficacy of inhaled alpha-1 antitrypsin in CF but thus far have not shown any clinical benefits. However, they provide a solid basis for phase 3 trials of longer treatment duration which will be needed to detect changes in clinical outcomes.

Common COPD

Although alpha-1 antitrypsin has broad immunomodulatory, anti-inflammatory, proteostatic, and anti-apoptotic actions [45], which theoretically could benefit patients with alpha-1 antitrypsin-replete COPD, no human studies with intravenous or inhaled alpha-1 antitrypsin have been reported in such patients to date. Yet, several in vitro observations and in vivo investigations in animals have shown that alpha-1 antitrypsin administration can attenuate experimental emphysema. Pemberton et al. [65] reported that a 6-month treatment with inhaled recombinant alpha-1 antitrypsin reduced airspace enlargement by 70 % in cigarette smoke-induced emphysema in mice. Additional experiments have shown that alpha-1 antitrypsin's anti-apoptotic activity could have a major role in this protective effect. Thus, in a murine emphysema model in which airspace enlargement was induced by pharmacologic inhibition of vascular growth factor receptors, mice overexpressing human MM alpha-1 antitrypsin were protected from the airspace enlargement; the protection was attributed to caspase-3 inhibition by alpha-1 antitrypsin [66]. A possible link between this observation and human cigarette smoke-induced COPD can be gleaned from an in vitro experiment in which alpha-1 antitrypsin purified from blood exhibited different anti-caspase activity in smokers and nonsmokers [7]. Alpha-1 antitrypsin derived from smokers inhibited executioner caspases (caspase-3, caspase-6, caspase-7) significantly less than alpha-1 antitrypsin from nonsmokers.

Neutrophil-dependent and neutrophil-independent inflammation and lung cell apoptosis are important players in the pathogenesis of CF and common COPD. In CF, phase 2 clinical trials have already been carried out and the prospect of phase 3 trials with inhaled alpha-1 antitrypsin in the future. In contrast, even early human studies with inhaled alpha-1 antitrypsin currently are lacking although the rationale for such treatment seems to be equally strong for both conditions. Awaiting the results of inhaled therapy with alpha-1 antitrypsin in patients with alpha-1 antitrypsin deficiency-associated COPD before investigating the effects of inhaled alpha-1 antitrypsin in alpha-1 antitrypsin-replete COPD may not be an optimal approach as raising alpha-1 antitrypsin levels toward or to normal may not be sufficient to modify the spectrum of inflammatory processes involved in common COPD. After all, alpha-1 antitrypsin is a positive acute phase reactant whose circulating level is increased above normal to modulate inflammation.

Conclusions

The data reviewed in this chapter lead to several conclusions about the prospect of administering alpha-1 antitrypsin to persons suffering from conditions not caused by alpha-1 antitrypsin deficiency. Promising are the results of investigations using animal models of human diseases such as type 1 diabetes, viral infection, graft-versus-host disease, cystic fibrosis, and alpha-1 antitrypsin-replete COPD. While other conditions including ischemic heart disease, inflammatory bowel disease, and arthritis have also been considered for alpha-1 antitrypsin therapy, the research addressing them may not have reached the same level of maturity.

There are at least two major reasons why these diseases have been considered for alpha-1 antitrypsin treatment. First, they involve immunologic and chronic inflammatory processes for which alpha-1 antitrypsin, owing to its broad immunomodulatory and anti-inflammatory activity, could clinically be beneficial. Second, alpha-1 antitrypsin is a positive acute phase reactant capable of modulating pro-inflammatory pathways; therefore, raising serum alpha-1 antitrypsin levels above normal in patients who are not alpha-1 antitrypsin deficient could mimic the natural anti-inflammatory actions of alpha-1 antitrypsin during inflammatory stress.

Early human studies have been conducted in type 1 diabetes, HIV infection, graft-versus-host disease, and cystic fibrosis. Although these investigations have established the feasibility, safety, and in some instances biological effectiveness of alpha-1 antitrypsin treatment, further trials are clearly needed to demonstrate its clinical utility.

With respect to new alpha-1 antitrypsin formulations, aerosol alpha-1 antitrypsin is the closest to becoming clinically available for patients with alpha-1 antitrypsin deficiency and potentially cystic fibrosis and alpha-1 antitrypsin-replete COPD. Significant challenges remain for the development of recombinant or transgenic alpha-1 antitrypsin as a substitute for human plasma-derived alpha-1 antitrypsin. However, both new applications and formulations of alpha-1 antitrypsin likely will continue to be explored and ultimately lead to a broader use of this protein in clinical medicine.

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Chapter 9
The Alpha-1 Constellation of Voluntary Health Organizations as a Paradigm for Confronting Rare Diseases

John W. Walsh

Introduction

The first individual to acquire a disease-causing mutation in their alpha-1 antitrypsin gene (*serpina1*) will never be known. The first documented individuals with disease due to this mutation were described in 1963 when alpha-1 antitrypsin deficiency (Alpha-1) was first detected in Sweden and tracked in subsequent studies there a decade later [1, 2]. Today, the full scope of the disease worldwide remains an imprecise figure, but many believe its frequency exceeds the number of cystic fibrosis patients [2].

In the United States alone, chronic obstructive pulmonary disease (COPD) exacerbations warranting activation of the 911 emergency medical system number at about 1.5 million per year, resulting in admissions of approximately 750,000 [3]. Between 1 and 3 % of COPD patients are believed to have Alpha-1 [1]. For many undiagnosed individuals, they either remain asymptomatic or are misdiagnosed at various stages of disease progression with signs and symptoms that may be indistinguishable from other conditions such as allergies, asthma, chronic bronchitis, emphysema, COPD, or cirrhosis of the liver, any of which could be due to Alpha-1. This ability to masquerade as more common conditions leads uninformed physicians to look no further for the genetic cause of their disease.

There is strength in numbers, but a patient population that is misdiagnosed or undiagnosed erodes that strength. Despite current findings and reasonable extrapolations to a larger, yet undiagnosed Alpha cohort, Alpha-1 is still considered a rare disease.

Today, the two primary VHOs in the Alpha-1 space, Alpha-1 Foundation and AlphaNet, are making progress in discovering [4], understanding, and supporting

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the Alpha-1 community. But before these organizations existed, to an Alpha, his illness might have appeared frustrating in its tenacity and uniqueness. A single Alpha's medical history might be useful to a healthcare provider in ameliorating that person's symptoms, but without further study, it could also be viewed in the wider context as a mere patient-specific anecdote. Yet when the histories of two Alphas are aggregated together, this can become the seed of a condition's natural history. Correlate the histories of three Alphas who are distantly genetically related, and patterns start emerging. In the Alpha-1 community, all it took was three Alphas and a rare disease voluntary health network was born.

Alpha-1 Community Genesis

It is possible, even understandable, to forget that in the study of disease, even a rare disease like Alpha-1, that illness is first and foremost a deeply personal and individual insult. An individual with Alpha-1 might take solace from the fact that others before him or her have heard the same difficult diagnosis and struggled to comprehend its meaning. It might help that sufferer, just as importantly, to know that his Alpha forebears have gone on to understand the full ramifications of the prognosis, disease process, and available treatments. The desire to connect with others who can truly empathize, and not just sympathize, is a powerful motivation for the recently diagnosed to reach beyond the doctor-patient relationship to connect with other Alphas in order to find solace; under the right circumstances, such a connection can quickly develop into concerted purpose, followed by action.

The step from diagnosis to a nascent rare disease network was almost instantaneous in my own case. When coping with a rare disease with a genetic basis, the ancestral nature of such a diagnosis has special meaning, I was to learn. In 1989, I was told that I might be an Alpha in a telephone call from my own twin brother, who had just received the results of an extensive battery of examinations culminating in the simple blood test that confirmed he had Alpha-1. Even more daunting to us, given the genetic component of Alpha-1, was the knowledge that some or all of our siblings would very likely share in the diagnosis. This turned out to be significant.

Indeed, testing revealed that I too was a person living with Alpha-1. Subsequently my older sister was diagnosed with Alpha-1 and my younger sister was diagnosed as an Alpha-1 carrier. In follow-up communications with my family, the common facets of our individual medical histories were brought into sharp relief. Respiratory illnesses, such as shortness of breath, and subsequent restricted activity, but also more serious hospitalizations for pneumonia, suddenly made a mordant kind of sense. And then there was the early death of our mother, who died at 46 years old, when my twin brother and I were only 13, of what was described as early onset emphysema. With a fresh look at her symptoms in this new light, it was clear that Alpha-1 had been a killer lurking in the midst of our family for at least three generations and very likely more.

My family members were all side by side in the trenches now. Driven by patients volunteering to be study subjects, intramural research performed at the National Institutes of Health (NIH) purified the protein (alpha-1 antitrypsin) and resulted in the development and approval of a therapy with demonstrated biochemical efficacy. Shared affliction and a desire to conquer a common enemy that had already cost so much encouraged us to participate in an important Alpha-1 study in the early 1990s sponsored by the National Heart, Lung, and Blood Institute at the NIH. It involved a patient registry following longitudinal Alpha-1 disease progression, a natural history study in other words, which was a phase IV requirement for the FDA approval of this first orphan drug approved for the treatment of Alpha-1 lung disease. Now we were not simply patients and certainly not victims. We were taking an active part in making a difference in the understanding of Alpha-1. We were helping with the discovery of treatments. This, in turn, helped us to transcend the difficulties that our own symptoms brought about because we felt we were part of the larger solution.

Then, despite the lethality of Alpha-1, and the suspected frequency of the disease, funding for further study in which we, and many other Alphas stood ready to take part, was terminated with the completion of the 5-year natural history study. In the grand scheme of research economics, Alpha-1 was deemed less important than other studies of diseases with a higher number of identified patients and the NIH advised us that they did not have the resources to pursue further studies. That decision, more than the disease itself, took our breath away that day. A dollar figure had been placed on our very lives and was found to be too costly.

My experience as a serial entrepreneur combined with my training as an Army Ranger triggered my reaction to find a way to continue when the study ended and funding was shifted away to research other diseases. The end of NIH-sponsored research inspired a small group of the study's alumni to reunite and seek new paths forward. I retained a fervent hope for advances in Alpha-1 treatment beyond ruinously expensive therapies and severely limited lung and liver transplantation, and I wanted a cure. We all did, the surviving Alphas and our loved ones. It was clear that I was not alone.

After all, the earliest and most ardent stakeholders in a rare disease voluntary health organization (VHO) might well be the patients themselves, if they are physically able to act on their own behalf.

Yet zeal is only the first qualification for founders of such a volunteer network. My stalwart partners at the inception of the Alpha-1 Foundation and AlphaNet were two remarkable, Alpha-1 affected women: Sandy Lindsey and Susan Stanley. Sandy Lindsey brought a core understanding of the workings and structure of nonprofit organizations from her efforts with the American Heart Association directly to the Alpha-1 cause. Susan Stanley worked for years in Seattle as a journalist. She had a fascination for, and a facility with, computers. She was an avid, early adopter on the bleeding edge not just of computer technology and software but also of the brave new cyberscape of the mid- to late 1980s, the Internet.

While participating in the NIH study, Alphas met at the 32 study sites around the United States and support groups were formed. A chain letter of the latest Alpha-1 news evolved into a computer-based listserv, as it was then called, which included

their caregivers and any clinicians and researchers with even the most remote interest in Alpha-1. A loose network of Alphas sharing their own experiences formed and evolved into the Alpha-1 Association (the first light in the constellation of Alpha-1 organizations). From there, advocacy and education efforts grew, and with realization that research to focus on solutions for Alpha-1 needed funding from sources other than the NIH, the next star in the constellation was formed, the Alpha-1 Foundation. Our community responded by taking responsibility to build the infrastructure to support research for therapeutic solutions to cure Alpha-1.

With such limited numbers diagnosed and aware of Alpha-1, and limited funding to support a research program, AlphaNet was formed to develop and provide a comprehensive health management program and, although a not-for-profit, generate recurring revenue to support the research mission of the Alpha-1 Foundation. AlphaNet became the third star to ignite. Orion the Hunter had his belt. The quarry was the cure for Alpha-1.

A rare disease VHO may begin with the smallest cadre of founders, but without the ability to combine the skills and knowledge of a few energetic partners and inform, educate, empower, and engage others to embrace the vision, take responsibility, and get involved, a network might collapse before it has truly begun to fulfill its mission.

The Research Engine and Its Fuel

Currently, websites like www.experiment.com and www.consano.org, crowdfunding platforms specifically designed for financing new scientific research, can be an important starting point for focusing urgently needed money toward the core mission of a health network: finding a cure. Such avenues were not open to the Alpha-1 Foundation at the time of its inception. Therefore, we helped wrap the early Alpha-1 network model in a financially sustainable framework. In simplest terms, in the mid-1990s, we proposed a risky, radical concept. Rather than constantly seek donations and grants to fund research, the Alpha-1 Foundation used funds provided by AlphaNet as a financial closed circuit. Far from running at a deficit, AlphaNet operates as a self-sustaining model where fees for services provided at no cost to Alphas are covered by biologic and pharmaceutical companies developing or manufacturing therapies to treat Alpha-1. Fees for such health management services are charged to these companies at industry-standard prices. It's the ultimate "recycling of our insurance dollars" to support research for the development of new therapies and a cure. AlphaNet's spectrum of services includes health management, clinical research management activities, and other specialized services while employing Alphas as health coordinators. Alphas *serving* Alphas. AlphaNet's revenues over expenses are redirected toward the work of the Alpha-1 Foundation, and AlphaNet has transferred more than $48 million to the Foundation at this writing. Alphas *funding* Alphas.

This model, so bold, and so successful, inspired the Health Foundation of South Florida, along with the Fraser, Fernandez, Cadwgan, Becker, and other family foundations, to throw their financial might behind the Alpha-1 Foundation to sustain the research momentum.

A detailed description of the complicated business relationships and legal requirements underpinning the not-for-profit Alpha-1 model falls outside the scope of this chapter. It is worth noting here that this model has been in operation for more than 20 years. With an eye toward customization, it could be adapted and scaled to support the needs of other rare disease VHOs. Anyone interested in applying the Alpha-1 Foundation/AlphaNet paradigm to other rare disease initiatives is invited to contact the Alpha-1 Foundation to learn more.

A Changing of the Guard

Early meetings with Sandy Lindsey and Susan Stanley were focused on establishing and maintaining contact with other Alphas and disseminating news of promising treatments as soon as they were available. Contact with near and far-flung Alphas soon became an open two-way line of communication. One early challenge facing all Alphas was that human plasma-derived alpha-1 antitrypsin, vital to slowing or arresting lung disease progression in Alphas, was often in short supply. We would learn when Alphas were unable to find or afford their augmentation treatment. Then we would move heaven and earth to bring needful Alphas the supplies they required to survive and thrive. Sometimes, most of us were only sub-therapeutically dosed and many could not stave off a regression to dangerously low alpha-1 antitrypsin levels, exposing us to accelerated lung destruction and, according to many, numerous exacerbation events.

Alpha-1 continued to erode Sandy Lindsey's and Susan Stanley's health, even as they were making great strides with building infrastructure to support research and engaging a growing number of other Alphas in the cause. Sandy Lindsey coming to the office with her supplemental oxygen in E tanks in tow meant a long, difficult period of catching her breath before she could settle into the many tasks at hand. When the trip to the office became too difficult, as with Susan Stanley, they would both carry on their pioneering work from their homes. As their conditions worsened, these dear and wise women planned for the grimmest eventuality, enrolling other members of the community to follow in their footsteps and to carry on their plans to expand the roles of the Alpha-1 Foundation and AlphaNet. After years of working together, I lost my treasured friends, my precious colleagues. Sandy Lindsey succumbed first in 2001, followed by Susan Stanley in 2002. Because of their foresight, though the transition was unavoidably gut-wrenching, it was not impossible.

In the early days of any rare disease VHO, key founders who are also part of the patient population are well advised to be multi-position players who intentionally recreate their skill sets in others through training. To this end, intra-office

communications must remain fluid and expanded engagement with others in the community must remain paramount. The rate of loss of these special cofounders and other staff members will be higher at the point of an organization's inception and remain so until the group turns the tide with the development of successful and available therapeutic interventions that begin to reduce morbidity and mortality among those critical workers and the community as a whole.

AlphaNet and the Alpha's Experience Today

AlphaNet has worked hard to stabilize as many Alphas as possible through the wide expansion of services the organization provides. Every Alpha who registers with AlphaNet is first assigned an AlphaNet Patient Services Coordinator (AlphaNet Coordinator or ANC), each of whom is also an Alpha, also known as a Peer Health Coach. An ANC or Peer Health Coach who shares the same Alpha-1 diagnosis maintains the more powerful bond of empathy with the individual, as opposed to just a sympathetic connection available with a non-Alpha. An ANC who has confronted his own Alpha-1 diagnosis will viscerally understand the individual's plight based on personal experience. An Alpha who knows his ANC is also an Alpha immediately has a reduced sense of isolation, which reduces anxiety and promotes wellness. For a recently diagnosed Alpha, the ANC might be the only other Alpha the individual meets until his personal Alpha-1 network grows. ANCs also stand ever ready to answer questions and provide support on a 24/7 basis.

An empathetic connection is only the beginning of an Alpha's relationship with their ANC. The coordinator may also assist the Alpha in receiving lifesaving medications and infusion supplies and in finding related home health services or infusion centers. The ANC also serves as a nexus of important information on research studies, clinical trials, legislative initiatives, and even lifestyle choices that will support an Alpha's long-term health. Just as important, the coordinator guides the Alpha toward opportunities for self-education on all the matters noted above [5].

AlphaNet's simplest core guiding principle is *Alphas serving Alphas*. Ultimately, a contingent of rare disease care coordinators who might not themselves be living with the relevant diagnosis can still offer significant and valuable help and guidance for a given patient population. That said, AlphaNet's experience with trained, dedicated Alphas providing frontline contact with, and support of, the broader Alpha-1 community has been supremely important and notably successful.

Similarly, members of other rare disease voluntary organizations might benefit by stacking the deck, so to speak, with personnel who are also living with the given rare disease diagnosis. The value of the support provided by an empathetic peer trained as a Peer Health Coach cannot be underestimated, both as a calming influence and as a provider of patient "hacks" that a non-Alpha case worker might not be aware of. Likewise, the unspoken example of the ANC living productively with the rare disease diagnosis might prove to be a source of encouragement and inspiration for the coach's Alpha-1 patient.

A rare disease VHO, regardless of whether care coordinators are themselves patients living with the disease, should consider offering what AlphaNet has found to be key services and products to patients of every stripe. These modules are described most simply as support, education, publications, and resources.

Specifically, the support component, provided by AlphaNet, is focused on meeting the immediate needs of the Alpha and his loved ones. AlphaNet serves as a referral source to the Alpha-1 Foundation that provides information on genetic counseling, the peer guides alluded to above, support groups for Alphas of all ages, information for parents of Alpha children, online or virtual support groups, and Alpha-focused events. Near to our hearts is the Alpha Angels In Memoriam list which serves two purposes: reminding us of the individual battles lost by friends and redoubling the sense of engagement and urgency of living Alphas.

The next crucial pillar in the Alpha-1 Foundation/AlphaNet network is education, in the form of numerous videos on the disease. They are available online for viewing on demand from both websites. The National Education Conference, Regional Education Conferences, and Support Group Meetings, organized by the Foundation are great sources that provide opportunities for fellowship, education, the latest information, and networking for Alphas.

In addition to video content available under the education heading, Alpha-1 Foundation's publications include relevant printed material that AlphaNet and the Alpha-1 Foundation have prepared or text resources available for downloading. The Alpha-1 Foundation publishes the quarterly *Alpha-1-to-One* magazine, the e-Alpha-1 News, and numerous collateral brochures and books created especially for younger Alphas. The importance of consistent communication about progress being made, shared experiences, information on community activities, and educational updates cannot be understated.

In the second century A.D. town of Oenoanda (in southwest Turkey), Diogenes commissioned the carving of the ancient world's largest inscription. Indeed, it was a kind of *prescription*, which covered a stone wall standing 12 ft high and stretching 200 ft through the town. The 25,000-word treatise referred to a cultural *disease* of ignorance and suggested the best *medicine* was the adoption of Epicureanism [6]. Fortunately, AlphaNet offers something more practical, useful, much more portable, and more widely available (though no less ambitious) to the Alpha-1 community, namely, the Big Fat Reference Guide (BFRG). The BFRG, is a vast (as the title implies) compendium of many valuable resources aggregated by AlphaNet to educate and support Alphas and their families as well as healthcare providers wanting to understand Alpha-1. Currently with important chapters published in nine languages, it covers topics such as testing, treatment, diet, exercise, coping, and insurance, to name a few. Education modules using the BFRG can even be personalized for the specific user, depending on the Alpha's situation and needs.

As a clearing house of resources, AlphaNet and the Alpha-1 Foundation offer testing information, rosters of healthcare providers, and opportunities to participate in research, among many other aspects directly pertinent to the complicated nature of Alpha-1, from care to cure. Any VHO or network hoping to successfully serve its rare disease patients might consider the Alpha- model and adapt its relevant modules to the particular needs of the patients and their families.

The Healthcare Provider Interface

In seeking to serve other Alphas and their loved ones to the best of the Alpha-1 Foundation/AlphaNet network's abilities, it became clear early on that physicians and other healthcare providers were also going to need support. Simply put, a rare disease might be overlooked by a general practitioner. For instance, hospital emergency department personnel might not order an Alpha-1 test if the patient presentation is consistent with asthma, generic COPD, or complications of cirrhosis of the liver.

The Alpha-1 Foundation and AlphaNet support healthcare providers with extensive data on testing and treatment. This includes helping to identify an Alpha-1 specialist when an individual tests positive. Informing physicians of augmentation therapy and its efficacy is paramount. Once again, the BFRG offers valuable information for healthcare providers to make available to their patients, and the Alpha-1 Foundation's Alpha-1 Healthcare Providers Guide provides information directed to the professional healthcare provider about evaluation, diagnosis, and management of Alpha-1.

Once a healthcare provider's awareness has been expanded to support greater vigilance for Alpha-1, the Alpha-1 Foundation provides follow-on training with 3 h of Continuing Medical Education (CME) and Continuing Respiratory Care Education (CRCE) which has been approved by the American Association for Respiratory Care (AARC) in accordance with the American Thoracic Society and the European Respiratory Society's standards for diagnosis and management of Alpha-1 for physicians and respiratory therapists, respectively, and AlphaNet provides a curriculum for Continuing Education Units (CEU) for infusion nurses [7].

Whether through funding of studies monitoring healthcare providers' adherence to important care guidelines [8] or the evolution of screening diagnostic protocols [9], the AlphaNet/Alpha-1 Foundation example of supporting healthcare providers might show great potential for adaptation to the needs of other rare disease voluntary organizations and networks.

Investigators

From inception, the Alpha-1 Foundation has been a true collaboration between the patient community and the scientific and clinical community. The principal investigators from the original NIH Study became the "Alpha-1 Docs," the definitive experts on Alpha-1 that continue to be a critical resource to healthcare providers. They partnered with Alphas across the country to form support groups and participated in shaping the vision and direction of the Alpha-1 Foundation and AlphaNet. Many remain an integral part of our scientific and clinical leadership involved with structuring our research agenda, participating in peer reviewing grant applications, and developing treatment guidelines and other clinical resources for both individuals with Alpha-1 and healthcare professionals.

The Alpha-1 Foundation research agenda continues to be carefully checked on an annual basis to determine the appropriate focus for research studies and clinical trials. The Foundation's initial research agenda was vetted in a thorough strategic planning initiative that included input from the NIH, the US Food and Drug Administration (FDA), and the Centers for Disease Control and Prevention. The Foundation's research funding priorities have included the molecular biology of alpha-1 antitrypsin expression, lung- and liver-focused clinical research, small-molecule therapies, gene therapy, and stem cell therapies. There are also new studies on the epidemiology and natural history of Alpha-1-related liver disease and new diagnostic technologies and techniques, as well as studies of the role of inflammation in the pathogenesis of Alpha-1 lung disease.

Without the push that this research funding provides, a rare disease network can barely reach beyond palliative care of its patients in the hope of discovering treatments and a cure.

It is a point of pride that the Alpha-1 Foundation attracts and retains a large community of researchers willing to do the extremely important basic science upon which to build the cure. In fact, several therapies for liver-related Alpha-1 are approaching readiness now because of basic science funded by the Alpha-1 Foundation in the 1990s. Such a close-knit relationship with the scientific and medical research community should be viewed as vital for any rare disease voluntary health network hoping to be effective in its mission.

Overcoming Stigma and Apathy

A VHO for a rare disease might have to contend with either stigma or apathy or even a destructive combination of the two. It is most likely that a rare disease's smaller diagnosed patient cohort neither excites significant attention from mainstream media nor from the broader marketplace to help rally legislative, research, and market-driven solutions. The Alpha-1 Association (now part of the Alpha-1 Foundation) struggled in the early days, but the difficulties eventually inspired the founding of AlphaNet and the Alpha-1 Foundation with their strong do-it-ourselves ethos. A significant facet of having a small identified patient group is that few outsiders are personally acquainted with an individual with Alpha-1. The brutally onerous nature of the disease and its debilitating toll are sequestered from the experience of most persons who do not themselves have Alpha-1. Out of sight, out of mind.

It is hard to imagine today, but at one time, HIV/AIDS was also considered a rare disease. In the public eye, the early 1980s witnessed the patient cohort quickly shunted from the shadows of apathy into the high noon sunlight of acute stigmatization. A stigma is a negative view of the patient body based on race, creed, color, sexual orientation, intelligence, physical ability, socioeconomic status, or other qualifications or sets of qualifications that delineate a minority population that exists amidst a larger or majority group. Unfortunately this larger group considers itself typical, average, or in some way entitled to enjoy the benefits of a position of

higher status than the stigmatized population, which often includes ignoring the plight of the beleaguered minority. Even though the virus that causes HIV/AIDS had been isolated for years, and though international media was warning of the true numbers of infected persons, as well as the disease's ongoing rapid and aggressive epidemic spread, it wasn't until 1986 that President Reagan even said the word AIDS in public [10]. VHOs like Act Up and the Gay Men's Health Crisis demonstrated the power of overcoming political, medical, institutional, and public inertia through vocal activism.

It may at first seem unlikely, but a common thread running between Alpha-1 Foundation and Act Up is that neither VHO allowed the media, the profit-driven corporate structures, or the political lobbying complexes at any level to dictate the narrative about patients living with the respective rare diseases. A rare disease VHO's underlying message in boardrooms and the halls of power was, is, and must remain that every life has value. Every life is worth saving. Every disease must have its cure. The moment a rare disease VHO capitulates to any other narrative, funding and then finding interim therapies and real cures are made more arduous.

Social Media

Building on the early "Listserv" outreach efforts that contributed to establishing the Alpha-1 Association, the Alpha-1 Foundation, and AlphaNet, today a rare disease VHO can leverage the power of social media. The staff of each rare disease VHO will have to assess social media's actual value in realizing its specific goals. Social media can be far-reaching and relatively inexpensive, and so it is well-suited to support awareness and education, the natural enemies of apathy and stigma.

That said, there is growing concern that social media is promulgating a phenomenon called *slacktivism* [11] described by the neologism formed by combining *slacker* and *activism*.

Slacktivism allows a social media user to *Like*, in the case of Facebook, or *Follow*, in the case of Twitter, as just two examples, to show support for a cause. This has potential use to build some awareness and even gain some sense extent of patient and public interest that could later be encouraged take an interest in participation, either with the organization or in clinical trials, for example. Unfortunately, a slacktivist might be satisfied with only making that mouse click show of support without making a greater investment such as responding to calls to action, making a donation, reaching out to legislators, or participating in other activities requiring expenditure of time, effort, and/or finances that can contribute to significant progress in achieving the goals of the VHO [12].

Where public-facing deployments of social media might inform, but not motivate, more private approaches, such as invitations to sign petitions, have been found to yield a greater likelihood of substantive future engagement and commitment of personal assets including time and money [12].

Website

Various social media pages and profiles for the rare disease VHO might best be thought of as spokes of a wheel leading to the hub, which is the organization's website. Where social media pages are managed under terms of service dictated, and often adversely modified, by the commercial needs of a separate hosting business entity, the content of websites, like those of the Alpha-1 Foundation and AlphaNet, may be more closely managed by the organization itself in accordance with its core mission, aims, and subsequent evolution.

The Alpha-1 Foundation's website is the official omnibus for the latest information on Alpha-1 including educational materials, referral networks, and research toward treatment and cure. It is intended to mirror the overall organization's structure and goal of being a one-stop entity for all services and support for Alphas, physicians, researchers, and any other person or institution that shares in the Foundation's concerns. It is a window for the press, as well. Ideally, the site serves to thoroughly answer initial inquiries and provide the direction for follow-on queries and more detailed information. The AlphaNet website provides access to the BFRG and coordinators and even goes so far as to include a glossary of important terms every Alpha needs to know and a simple pronunciation key.

There are software-based methods for tracking and data collection regarding the volume and demographics of websites' traffic. The use of tags and metatags can support search engine optimization, which supports a website's discovery by interested seekers. These tools can assist in everything from building public awareness to discovering a broader patient cohort, to supporting patients living with a given rare disease. It can also stand as a 24 h portal for securely accepting donations. A rare disease VHO is first and foremost made up of people, but social media used in conjunction with a discoverable, navigable website can be a powerful adjunct for disseminating information, as well as supporting engagement and feedback, which are crucial at all phases of a network's service trajectory.

The Alpha-1 Foundation and Advocacy

Individual action to improve awareness, and campaign for legislative change, especially the appropriation of funding for research and clinical trials, is important, but the Alpha-1 Foundation, like other VHOs for rare diseases, is a force multiplier. Uniting many voices into one, and raising the decibel level of alarm about the real number of lives at risk *now*, is one of the greatest strengths of a rare disease VHO. Like the Alpha-1 Foundation, other rare disease VHOs might find value in establishing themselves as a clearing house of basic information and emergent calls to action relevant to the illness in question.

Initially, the motivated Alpha volunteer must become familiar with the concerns of the moment that are pertinent to the efforts of the network. Once self-education

has begun, Alphas can avail themselves of ongoing advocacy alerts, bolstering their understanding with further training, focused printed materials and videos, conferences, and other advocacy activities local to the individual. For example, concerted outreach to Congressional leadership, which can be planned, organized, and timed by a central network coordinator, brings the common message to bear at critical times in the legislative calendar in support of passage of relevant bills and appropriations. Where the voice of an individual might be lost in the ambient din of lobbyists in Washington, D.C., the chorus of unity singing the anthem of progress cannot be ignored.

Ethical Legal and Social Issues

Today, the Alpha-1 Foundation has its own place around the anvil where national rare disease research policies are hammered out with such agencies as the FDA, the Centers for Disease Control and Prevention, and the NIH. This privileged position comes by dint of the Alpha-1 Foundation's assembly of an Ethical, Legal, and Social Issues (ELSI) Working Group, which identifies relevant issues and activities undertaken by the Foundation requiring bioethical or legal review. It then provides recommendations to the Medical and Scientific Advisory Committee on research study requests, clinical trial protocols, grant applications, informed consent, educational materials, and conflict of interest issues on an ongoing basis. With the ELSI Working Group, the Alpha-1 Foundation is able to guide and inform policy, rather than be subject to it. It might seem too esoteric for other rare disease voluntary networks to consider forming ELSI groups of their own, especially early in a network's trajectory. The ELSI Working Group was one of the first committees constituted during the early days of the Alpha-1 Foundation, and we are certain that today, the Alpha-1 Foundation would not enjoy the political clout that supports its mission had its ELSI group been established as an afterthought or forgotten altogether.

A Retrospective Informs the Future

The work of the Alpha-1 Foundation is not finished, but so much has been accomplished. The Alpha-1 Research Registry and Research Program together with the DNA and Tissue Bank are in and of themselves important milestones. Strategic partnerships with the American Thoracic Society, the American Association for the Study of Liver Diseases, the Genetic Alliance, the National Health Council, FasterCures, and the American Association for Respiratory Care, among other important professional organizations, vastly augment the intellectual capital available to fight Alpha-1 today.

Among the most important actions taken by the young Alpha-1 Foundation was the creation of the Alpha-1 Research Registry. The Registry stimulated additional

development of therapies and continues to be a significant resource for recruiting for clinical studies and expanding our knowledge of Alpha-1. The DNA and Tissue Bank and Repository provides much-needed samples to accelerate research. The Foundation's commitment to the Gordon L Snider Critical Issues Workshop Series and Biennial International Scientific Conference Series and the strategic liaison relationship with the FDA, NIH, and Centers for Medicare and Medicaid Services (CMS) support the ability to cross-fertilize investigator findings, address impediments to and gaps in research, and inform a constructive dialogue with federal agencies.

To date, the Alpha-1 Foundation has invested over $56 million toward research in 92 institutions in North America, Europe, Australia, and the Middle East. Therapeutic solutions and cures are within reach because of the balanced research portfolio and concentration on fostering research collaborations. Attaining financial stability should never be the final goal of a rare disease VHO. It was never our intent to create a lasting career of serving and financing the Alpha-1 Foundation in perpetuity. The Alpha-1 Foundation's fondest aspiration is to put itself out of business, ideally within the span of my own lifetime, because a cure is achieved. In the meantime, the Alpha-1 Foundation and AlphaNet are necessities, together forming a sturdy structure for progress, in the same way that scaffolding supports and surrounds the bold undertaking of constructing a tall building. In a perfect world, when the building is finished, the scaffolding comes down.

Conclusion

The Alpha-1 Foundation and AlphaNet, founded from simple beginnings, can continue to support their core services, helping to develop and deploy new diagnostics and therapeutics while standing poised to adapt to the future. One day will result in a cure for alpha-1 antitrypsin deficiency. All who work in the Alpha-1 Foundation and AlphaNet stand ready to help any VHO build on the model we have developed and nurtured over two decades. We're neither the oldest nor the newest, neither the smallest nor the largest. But our combined efforts have lifted a rare condition from obscurity and moved it toward awareness, detection, treatment, and a cure.

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