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Review

New Therapeutic Targets for Alpha-1 Antitrypsin Deficiency

David A. Lomas, MD, PhD[1]

Abstract

Alpha-1antitrypsin deficiency (AATD) results from the intracellular polymerization and retention of mutant alpha-1antitrypsin (AAT) within the endoplasmic reticulum of hepatocytes. This causes cirrhosis whilst the deficiency of circulating AAT predisposes to early onset emphysema. This is an exciting time for researchers in the field with the development of novel therapies based on understanding the pathobiology of disease. I review here augmentation therapy to prevent the progression of lung disease and a range of approaches to treat the liver disease associated with the accumulation of mutant AAT: modifying proteostasis networks that are activated by Z AAT polymers, stimulating autophagy, small interfering RNA and small molecules to block intracellular polymerization, and stem cell technology to correct the genetic defect that underlies AATD.

Abbreviations: alpha-1 antitrypsin deficiency, **AATD**; alpha-1 antitrypsin, **AAT**; chronic obstructive pulmonary disease, **COPD**; forced expiratory volume in 1 second, **FEV_1**; recombinant adenovirus, **rAd**; recombinant adeno-associated virus, **rAAV**; Food and Drug Administration, **FDA**; small interfering RNA, **siRNA**; antisense, **ALN**; Arrowhead siRNA AAT, **ARC-AAT**; human-induced pluripotent stem cells, **hIPSCs**

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1 UCL Respiratory, Division of Medicine, University College London, United Kingdom

Address correspondence to:

David Lomas, MD, PhD
UCL Respiratory, Division of Medicine
Rayne Building
University College London
WC1E 6BN
London, UK
Email: d.lomas@ucl.ac.uk

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Introduction

The serpinopathies are characterized by the misfolding and intracellular polymerization of members of the serpin (serine protease inhibitor) superfamily.[1] The best characterized of the serpinopathies is alpha-1 antitrypsin deficiency (AATD).[2] Ninety five percent of severe deficiency of AAT results from the Glu342Lys mutation or Z allele. This mutation arose approximately 2000 years ago and is found in 1 out of 25 of the North European white population with 1 out of 2000 individuals carrying 2 Z alleles (homozygotes). The Z mutation causes approximately 70% of the synthesised protein to be degraded by the proteasome, 15%-20% misfolds to form ordered intracellular polymers and 10%-15% folds normally and is secreted into the circulation.[3] The intracellular polymers are sequestered within the endoplasmic

reticulum as Periodic Acid Schiff-positive, diastase-resistant inclusions[2,4] that are associated with neonatal hepatitis, cirrhosis and hepatocellular carcinoma.[5] AAT is a major circulating antiprotease that functions to regulate the proteolytic effects of neutrophil elastase within the lung. The lack of circulating AAT predisposes the Z AAT homozygote to early onset panlobular basal emphysema, particularly in individuals who smoke tobacco.[6] AATD is found in 1%-2% of individuals with chronic obstructive pulmonary disease (COPD)-a syndrome characterized by small airways disease and emphysema. COPD will be the third most common cause of death worldwide by 2020.

Current Therapies for Alpha-1 Antitrypsin Deficiency

AATD was first described by Laurell and Eriksson in 1963.[7] The most effective interventions are still behavioral.[8] In particular, individuals with AATD should refrain from smoking[6] and probably avoid passive exposures from dusty occupations.[9] This will help to preserve lung function. Individuals with AATD should also avoid excessive consumption of alcohol and excessive weight gain (which predisposes to a fatty liver). Both are additive to the liver damage caused by AATD.

The only specific treatment for AATD is augmentation with pooled plasma AAT.[10] This was launched in the United States in 1988 on the basis of biochemical data showing that infusions raised circulating levels of AAT and provided protection against proteases within the lung.[11] Two randomized clinical trials failed to demonstrate that augmentation therapy reduces the rate of decline in lung function as assessed by forced expiratory volume in 1 second (FEV_1).[12,13] However, augmentation therapy is associated with a reduction in sputum markers of inflammation[14] and in the frequency of respiratory tract infections.[15] The recent RAPID study funded by CSL Behring randomized 93 individuals (83 PiZZ, 2 PiSZ, 2 Z/Null, 6 other) to augmentation therapy and 87 to placebo (83 PiZZ, 0 PiSZ, 1 Z/Null, 3 other) (NCT00261833).[16] They were followed for 24 months. There was no difference in the annual rate of lung density loss at total lung capacity and functional residual capacity combined between the 2 groups. However, there was a significant reduction in annual rate of lung density loss at total lung capacity in individuals who received augmentation therapy (difference 0·74g/L per year [95% confidence interval 0·06–1·42], p=0·03) but there was no difference at functional residual capacity alone (difference 0·48g/L per year [–0·22 to 1·18], p=0·18). The significant benefit in one of the primary endpoints is encouraging but it is not clear how a difference in change of total lung capacity translates to clinical benefit or whether there is a particular subgroup that benefits from augmentation therapy. The study has resulted in approval for augmentation therapy in individuals with AATD by the European Medicines Agency. The United Kingdom National Institute for Clinical Excellence will now need to decide on the cost-benefit of this therapy and whether it represents value for money in the British health care system. This is important as augmentation therapy costs approximately $100,000/patient/year.

End stage lung disease in individuals with AATD may be suitable for transplantation. Indeed, lung transplantation for AATD-related emphysema accounts for 3.2% of all adult lung transplants and 10% of all transplants for emphysema.

There is no specific therapy for the liver disease associated with AATD other than conservative treatment that is used for all forms of chronic liver disease, and where suitable, liver transplantation. Transplantation for AATD accounts for 3.5% and 1.1% of pediatric and adult liver transplants respectively.

Novel Approaches to the Lung Disease Associated with Alpha-1 Antitrypsin Deficiency

Improved Alpha-1 Antitrypsin Replacement Therapy

Several approaches have been adopted to replace circulating AAT and therefore, protect against progressive emphysema that characterizes AATD. These include giving higher doses of intravenous AAT (120 rather than 60mg/kg per week), modifying recombinant AAT to increase its stability and delivering AAT as an inhaled preparation. The inhaled route requires significantly less material to inhibit neutrophil elastase but needs to access the alveolar space that is destroyed in emphysema. An alternative strategy is to use non-viral gene transfer, gamma-retrovirus, recombinant adenovirus (rAd), and recombinant adeno-associated virus (rAAV) vectors

to express AAT. These may be targeted to the lung epithelium as well as to hepatocytes, pleural and muscle cells. The challenge is to achieve long-term expression of large quantities of AAT. A phase II clinical trial that used rAAV vectors achieved only 3%-5% of the target level of AAT.[17]

Other Treatments for Lung Disease Associated with Alpha-1 Antitrypsin Deficiency

Individuals with AATD-related emphysema should receive the same therapies as individuals with COPD who have normal levels of AAT. These include: inhaled short- and long-acting beta2-agonists, inhaled corticosteroids and long-acting anti-cholinergics based on patient symptoms, lung function impairment and exacerbation frequency. Treatment with macrolide antibiotics and roflumilast should be considered for patients with frequent exacerbations. Individuals with AATD-related emphysema should also receive pulmonary rehabilitation and vaccination against pneumococcus and circulating strains of influenza. Attempts to regenerate alveoli damaged by emphysema with retinoic acid were effective in rat models of disease[18] but not in a clinical trial in humans.[19] The most effective therapy for individuals with severe airflow obstruction is lung transplantation. Younger patients with less comorbidity may have a higher benefit (increased survival) from double lung rather than single lung transplant. However, the supply of organs is limited. Thus, individuals with AATD may be considered for lung volume reduction surgery and endobronchial lung volume reduction as a bridge to surgery or for those who are not candidates for a major operation. Lung volume reduction surgery in individuals with AATD is inferior to that in patients with COPD and normal levels of AAT.[20,21] There appears to be a smaller increase in FEV_1 after surgery and a shorter duration of benefit.

Endobronchial lung volume reduction is performed by one-way valves placed by flexible bronchoscope. The data are limited to case series. The insertion of one-way endobronchial valves in 15 individuals with AATD resulted in an increase in FEV_1 of 54% after 12 months in 12 of the individuals, quality of life was much improved and 2 individuals were taken off oxygen therapy. There was no significant deterioration in lung function during the 4-year follow-up.[22] However, there were complications in 3 of the individuals: 1 developed a pneumothorax and had valve displacement and subsequent removal, 1 coughed up the valves after 2 months and 1 developed repeated and severe pneumonia and the valves had to be removed.

Novel Approaches to the Liver Disease Associated with Alpha-1 Antitrypsin Deficiency

Modifying Pathways and Proteostasis Networks That are Activated by Z Alpha-1 Antitrypsin Polymers

A surprising feature of the accumulation of Z AAT polymers as inclusions within hepatocytes is that, despite marked distortion of the endoplasmic reticulum architecture, they do not activate the unfolded protein response when expressed in cell models of disease.[23-26] However, they do activate NF-kB proteins and display a more marked unfolded protein response when stressed with a second hit.[24,26] This results from AAT polymers increasing the viscosity within the endoplasmic reticulum which reduces the mobility of chaperones and hence their ability to neutralize the effect of a second insult.[26]

The findings are different when assessed by gene profiling of hepatocytes from a transgenic mouse model expressing human Z AAT.[27] In this case, there was upregulation of genes associated with the unfolded protein response and cellular stress genes including c-JUN.[28] The expression of Z AAT upregulates JNK and c-JUN. Genetic ablation of JNK1 or JNK2 decreases AAT levels in vivo by reducing c-JUN mediated expression of AAT. Thus, JNK may be a therapeutic target for the liver disease associated with AATD. The difficulty is that JNK has pleotropic effects and is widely expressed in vivo. Thus, an inhibitor of JNK is likely to have off-target effects. Nevertheless, if validated in other studies, JNK may prove useful as a biomarker to develop agents that suppress the intracellular polymerization (and accumulation) of Z AAT.[29]

The intracellular polymerization of Z AAT occurs in the context of chaperone-mediated folding, proteasomal degradation and a cooperative proteostatic network. Chemical chaperones such as trimethylamine N-oxide, glycerol, erythritol, trehalose, and its breakdown product glucose, have been evaluated for their ability to stabilize the folding of Z AAT in vitro.[30,31] Four-phenylbutyric acid is effective in increasing the secretion of functionally active Z AAT in a cell and

animal model of disease[32] but was not effective when assessed in a clinical trial in individuals with Z AATD.[33] An alternative approach is to target the proteostasis network that responds to the expression of Z AAT. Defining this network may lead to the identification of key "nodes", signaling pathways and molecules that may be manipulated to increase folding of Z AAT to the monomeric protein and so facilitate secretion. This is illustrated by suberoylanilide hydroxamic acid which increases the secretion of Z AAT from epithelial cell lines by inhibition of histone deacetylase 7.[34] Such an approach has yet to be evaluated in animal models of disease or in humans. However, it provides support for small molecule or siRNA-based screens of cell lines or model organisms (for example in *C. elegans* and drosophila) to identify other pathways that are up or down regulated following the expression of Z or other mutants of AAT. These pathways may be targeted for pharmacological intervention to reduce intracellular inclusions and/or increase the secretion of the mutant protein.

Stimulating Autophagy to Clear Intracellular Inclusions

The landmark paper by Hidvegi and colleagues demonstrated that the Food and Drug Administration (FDA)-approved drug, carbamazepine, can stimulate proteasomal and autophagy pathways to clear intracellular polymers of AAT.[35] Carbamazepine is widely usually used to treat epilepsy. It was selected as it stimulates autophagy by a pathway that is independent of mTOR (mammalian target of rapamycin) and is known to enhance autophagic degradation of polyglutamine repeats. Moreover, the safety profile is well known in humans. Administration of large doses of carbamazepine (10-20 times the recommended dose for individuals with epilepsy) to a transgenic mouse model expressing human Z AAT reduced the intrahepatic PAS positive inclusions of Z AAT within 2 weeks of therapy and reversed hepatic fibrosis. These findings support the development of autophagy enhancers to treat AATD and the assessment of carbamazepine in a randomized, controlled clinical trial in individuals with severe liver disease (NCT01379469).[36] In this study, participants are started on 400mg/day carbamazepine and the dose increased weekly by 200mg/day until a stable therapeutic concentration is reached with a dose not exceeding 1200mg/day (or 1000mg/day in participants less than 15 years of age). The placebo group receives encapsulated tablets without carbamazepine. The primary endpoint is a significant reduction in the hepatic accumulation of Z AAT.

It is uncertain whether the effects will be seen at much lower doses of carbamazepine than those used in transgenic mice and whether the human liver has the same capacity to recover as that of the mouse. However, even if the trial is unsuccessful, there is impetus to assess other FDA-approved drugs (such as lithium and rapamycin) that also stimulate autophagy in cell and animal models of disease.[37] Rapamycin targets mTOR and so may have synergistic properties with agents such as carbamazepine and lithium that act by pathways that are independent of mTOR.[38]

Rapamycin (sirolimus) has been evaluated in the transgenic mouse that overexpresses Z AAT.[39] Daily dosing had no effect on autophagy. However, weekly dosing increased the number of autophagic vacuoles, reduced the accumulation of intrahepatic polymerized Z AAT and reduced markers of hepatocellular injury including hepatic fibrosis and cleavage of caspase 12.[39] An alternative approach is to drive autophagy with viral vectors that overexpress the autophagy regulator transcription factor EB. This also reduced the accumulation of Z AAT, hepatocyte apoptosis and fibrosis in the liver of the transgenic mouse that expresses Z AAT.[40] It also decreased activation of hepatic NFκB and IL-6 that drive the expression of Z AAT. Both rapamycin and overexpression of transcription factor EB reduce the burden of intracellular AAT and decrease hepatic fibrosis in a mouse model of disease. The challenge now is to demonstrate efficacy of these approaches in humans.

Small Interfering RNA to Silence the Expression of Z Alpha-1 Antitrypsin

The most exciting recent therapeutic intervention for the liver disease associated with AATD is the development of RNA interference- based approaches to silence Z AAT synthesis within hepatocytes.[41,42] This is being led by 2 biotechnology companies: Arrowhead Research Corporation and Alnylam Pharmaceuticals, Inc. Small interfering RNA (siRNA) constructs have been targeted against hepatocyte mRNA encoding human AAT. The administration of these agents reduces soluble and aggregated hepatic AAT and circulating levels of AAT in the transgenic mouse model of disease.[43] siRNA constructs arrest the progression

of liver disease in transgenic mice following short-term treatment and reverse liver disease after long-term treatment. Their administration to non-human primates reduced circulating levels of normal AAT by approximately 80%.

siRNA therapy for AATD-related liver disease has been evaluated in a phase I/II clinical trial (NCT02503683).[44] This was a randomized, single-blind, placebo-controlled, single ascending dose and multiple dose study that aimed to enroll 66 participants. It started in July 2015 and aimed to complete in April 2017. The study assessed tolerability, pharmacokinetics and pharmacodynamics of subcutaneously administered antisense (ALN) AAT in healthy adult participants and individuals with Z AATD liver disease. The primary outcome was the safety of siRNA targeting ALN-AAT as assessed by the proportion of participants experiencing adverse events, serious adverse events, and adverse events leading to discontinuation of the study drug. The secondary outcome was the effect of siRNA targeting AAT on serum levels of AAT. Preliminary reports suggest that the administration of ALN-AAT caused a dose-dependent and durable knockdown of the target protein.[45] A single dose of ALN-AAT (6mg/kg) knocked down up to 88.9% of circulating AAT with a mean maximal knockdown of 83.9±2.6%. Monthly treatment resulted in a mean knockdown of serum AAT of 75.0±1.2% at approximately 6 months. However, there was liver enzyme elevation at the highest dose in 3 patients and so the candidate ALN-AAT siRNA has been terminated. A new candidate is being developed.

The multi-center, randomized, placebo-controlled, double-blind, single-dose-escalation first-in-human, Phase 1 study in healthy volunteers and AATD patients to evaluate the Arrowhead siRNA AAT (ARC-AAT) enrolled 65 participants but was terminated in January 2017 (NCT02363946).[46] Data presented at the 2016 Annual Meeting of the American Association for the Study of Liver Disease reported that ARC-AAT was well tolerated and induced deep and durable reduction of the target AAT protein (up to 90%).[47] The follow-up study, an open-label, multi-dose, Phase 2 study to determine the safety, tolerability and effect on circulating and intrahepatic AAT levels of ARC-AAT as evidenced by changes in liver biopsy in patients with AATD has been withdrawn (NCT02900183).[48] Further details of this program are awaited.

Small Molecule Approach to Block Intracellular Polymerization

AATD results from the retention of polymers of mutant AAT within the endoplasmic reticulum of hepatocytes.[2,4] Polymers form as a result of the Z mutation perturbing protein folding, and the structure of the folded protein, to form an unstable intermediate that we termed M*[49] in which β-sheet A opens [2,49] and the upper part of helix F unwinds.[50-52] The loop of another molecule inserts into the patent β-sheet A to form a loop-sheet dimer, which extends to form longer chains of loop-sheet polymers.[2,49,53] Ninety-five percent of severe deficiency of AAT results from the Z allele (Glu342Lys) but hepatic inclusions of intracellular polymers and profound plasma deficiency are also seen in 3 other mutants of AAT: Siiyama (Ser53Phe),[54] Mmalton (ΔPhe52)[55] and King's (His334Asp).[4] We have shown that this process of polymerization also explains the deficiency of the mild S (Glu264Val), I (Arg39Cys), Queen's (Lys154Asn) and Baghdad (Ala336Pro) alleles of AAT.[52,56-58] However, the rate of polymer formation is much slower in keeping with mild plasma deficiency and the absence of liver disease.

Understanding the pathophysiology of AAT polymerization has allowed the development of novel strategies to block polymerization with the aim of curing AATD. Initial studies showed that peptides that are homologous to the reactive centre loop can bind to AAT and block polymerisation in vitro.[2,59,60] Smaller peptides were identified that had a similar effect but with greater specificity for Z, rather than the wildtype M, AAT.[61-64] However, it is not clear how these peptides can be delivered to hepatocytes in vitro, let alone in in vivo.

An alternative approach is to use our understanding of the structural biology of polymers to develop small molecules that block polymerization. The crystal structure of AAT identified a hydrophobic pocket that is bounded by strand 2A and helices D and E.[65] The cavity is available in the monomeric, native protein but is filled by movement of β-sheet A as it accepts an exogenous reactive loop peptide during polymerization. Polymer formation was reduced when this cavity was filled by the Thr114Phe mutation on strand 2 of β-sheet A. This mutation also increased the secretion of Z AAT from a *Xenopus* oocyte expression system.[66,67] Virtual ligand screening against this cavity identified 66 compounds as potential binders.[68]

Some of these blocked polymerization in vitro and in cell models that express Z AAT.[68] This proof of principle resulted in a "Discovery Partnership with Academia" partnership with GlaxoSmithKline to develop small molecules that are effective in blocking polymerization as an approach to treating AATD.[69]

Intrabodies as a Strategy to Block Intracellular Polymerization and Increase Secretion of Z Alpha-1 Antitrypsin

Monoclonal antibody technology has allowed the identification of antibodies that detect the polymeric[4] and latent[70] conformers of AAT and antibodies that can block[71] and accelerate[72] polymer formation. The 4B12 monoclonal antibody blocked AAT polymerization at a 1:1 molar ratio in vitro by binding to an epitope that encompasses residues Glu32, Glu39 and His43 on helix A and Leu306 on helix I.[73] This antibody identified a region that may be targeted for the rational design of ligands that can dynamically influence AAT polymerization. Moreover, the expression of a single chain-variable-fragment intrabody of mAb4B12 reduced the intracellular polymerization of Z AAT by 60% and increased the secretion of Z AAT that retained inhibitory activity against neutrophil elastase.[71] This demonstrates that monoclonal antibodies can block the transition of Z AAT to aberrant polymers without compromising inhibitory activity of the protein.

Cell Therapy for Alpha-1 Antitrypsin Deficiency

Hepatocytes that express wildtype AAT have a competitive advantage over cells that express the Z protein. Wild-type donor hepatocytes replaced 20%-98% of host hepatocytes in transgenic mice expressing human Z AAT.[74] Repopulation was accelerated by injection of an adenovirus vector expressing hepatocyte growth factor but spontaneous repopulation with engrafted hepatocytes occurred in the transgenic mice even in the absence of severe liver injury. Donor cells replaced both globule-containing and globule-devoid cells, indicating that both types of host hepatocytes have impaired proliferation relative to wildtype hepatocytes. These results suggest that wildtype hepatocyte transplantation may be therapeutic for individuals with Z AAT liver disease.

Dermal fibroblasts have been isolated from individuals with AATD and used to generate patient-specific human-induced pluripotent stem cell (hIPSCs) lines. These hIPSC lines were differentiated into hepatocyte-like cells using a novel and simple 3-step differentiation protocol in chemically-defined conditions. The patient-specific hIPSC derived hepatocytes recapitulate protein misfolding and the formation of pathological polymers that characterize AATD.[75,76] They were also used in a proof of concept study to show that the combination of engineered Zinc finger nucleases and a piggyBac donor vector gene editing technique can be used to restore normal structure, function and secretion of AAT in subsequently derived liver cells.[77] The derived hepatocytes secreted AAT when introduced into a mouse model of liver injury.[77] The challenge is to obtain cells that are more like the fully-differentiated hepatocyte and which are safe to use in humans.

More recently Baligar and colleagues[78] showed that the intra-splenic injection of LSK cells (Lineage negative, Sca-1 positive, C-Kit negative) into the transgenic mouse model of AATD could reduce the numbers of AAT globule-containing hepatocytes in the recipient liver. This cell therapy improved proliferation of host globule-devoid hepatocytes and donor derived cells and partially improved liver pathology as assessed by inflammatory response, fibrosis and apoptotic hepatocyte death. The findings suggest transplantation of allogeneic bone marrow derived stromal cells may be an effective therapy for individuals with AATD. If correct, then this offers the potential to recover normal levels of secreted AAT and thereby, simultaneously treat the associated lung disease.

Summary

Current respiratory management of individuals with AATD involves the routine care offered to all patients with COPD along with augmentation therapy. However, augmentation therapy is expensive and the cost-benefit ratio, and the decision of which individuals should be treated, is not clear. Some individuals may benefit from lung volume reduction surgery and the placement of endobronchial valves although the benefit from lung volume reduction surgery may be short-lived and there is relatively little data on long-term outcomes following valve placement. The management of the liver disease associated with AATD is currently limited to supportive measures. However, there is real promise with the development of siRNA technology and agents

that block the formation of polymers or stimulate pathways that accelerate their clearance.

Declaration of Interests

DAL is working with GlaxoSmithKline to develop small molecules that block the intracellular polymerization of AAT.

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