

Strategies to Provide Sufficient Anti-Protease Protection at Rest and During Acute Inflammation in ZZ Alpha-1 Antitrypsin Deficiency

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Strategies to provide sufficient anti-protease protection at rest and during acute inflammation in ZZ alpha-1 antitrypsin deficiency



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A thesis presented to the Royal College of Surgeons in Ireland, 123 St Stephen's
Green, Dublin 2, Ireland.

Submitted for the Degree of Doctor of Philosophy.

Supervisors: Prof. Noel G. McElvaney/Prof. Emer Reeves April 2022

Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of Degree of Doctor of Philosophy, is my own personal effort. I have not already obtained a degree in the RCSI on the basis of this work. Furthermore, I took reasonable care to ensure that this work is original and to the best of my knowledge does not breach any copyright law and has not been taken from sources except where such work has been cited and acknowledged within the text.

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Abstract

Alpha-1 antitrypsin (AAT) is a 52 kDa glycoprotein synthesized predominantly in the liver. AAT is a serine protease inhibitor but has also been shown to possess significant anti-inflammatory properties. AAT deficiency (AATD) is a hereditary disorder. The most common mutation causing AATD is the Z mutation, clinically characterized by the retention of polymerised Z protein in the liver, resulting in a toxic “gain of function.” This intracellular retention of AAT leads to decreased secretion of AAT into the circulation and lung, which leads to unopposed proteolytic action of proteases such as neutrophil elastase (NE) in the lung, resulting in tissue destruction and emphysema, a “loss of function.” The ideal therapeutic treatment for AATD would increase secretion of Z AAT out of the liver (removing the toxic “gain of function”) and into the circulation and lung, where it would protect the lungs (preventing any “loss of function”). In the setting of the worldwide COVID-19 pandemic, the intense acute inflammatory response observed in healthy individuals in response to SARS-CoV-2 infection highlighted that baseline M AAT levels would not be sufficient for ZZ individuals in an acute infection.

The aim of this study was to investigate the feasibility of a potential treatment option that would simultaneously treat both lung and liver disease, moving AAT out of the liver into the circulation and lung. The potential treatment was gentamicin and this study evaluated not only its ability to get Z AAT out of the cell, but also the amount of extracellular AAT required to protect the lung not only in quiescent times but also in time of severe inflammation such as SARS-CoV-2 infection. This study also evaluated the possibility that therapeutics aimed at increasing Z AAT extracellular secretion may also inadvertently cause increased intracellular Z AAT retention leading to inflammation and ER stress.

A variety of cell models of AATD were used, aiming to recapitulate the Z and M phenotypes. The results demonstrated that these cell lines did behave like M and Z cells but the secreted Z AAT response to gentamicin was insufficient when compared to the AAT levels observed in SARS-CoV-2 infection, even in its milder forms with the added problem that there was evidence of increased intracellular AAT retention and ER stress. In the next stages of this study various degrees of SARS-CoV-2 infection were used to determine what was driving the AAT response to inflammation and how much AAT was required to safeguard the lung and other

organ. The data showed that interleukin (IL)-6 was the main driver of AAT production and glycosylation of the AAT protein. The latter point is important as glycosylation materially affects the function of AAT as an anti-inflammatory specifically with regards to modulation of neutrophil migration.

Furthermore it was discovered that even with large amounts of AAT being produced by the liver in response to severe SARS-CoV-2 infection, there was still inadequate anti-protease protection in the airways due to the large NE burden, further evidence of the lack of utility of the commonly accepted putative protective AAT threshold of 11 μ M. These data also suggested a possible therapeutic option in SARS-CoV-2 ARDS, namely augmentation therapy with plasma purified AAT even in non-AATD MM individuals and led to a therapeutic trial of this medication in Beaumont hospital, the first randomised control trial of its type in the world. The data also suggested added caution is required in SARS-CoV-2-infected individuals who are treated with tocilizumab, an IL-6 receptor blocker as it blocks the AAT response to SARS-CoV-2 infection. Finally, SARS-CoV-2 infection trends in the ZZ AATD population were examined and using Astra Zeneca (AZ) vaccines as a surrogate for SARS-CoV-2 infection it was possible to demonstrate that while the anti-inflammatory response of ZZ AATD individuals is increased *in vivo* in response to SARS-CoV-2 infection and minimally to AZ vaccination it is inadequate and may be pro-inflammatory due to increased AAT production, retention and ER stress.

In conclusion, this research will contribute significantly to the body of knowledge regarding AATD, AAT's role in the inflammatory process and its potential therapeutic use outside of AATD. This study has explored the available in-vitro models, the target levels of secreted AAT to aim for, in particular during acute inflammatory periods and the potential downsides of increasing AATD production in ZZ AATD cells. It also suggests that while gentamicin may not be a feasible therapeutic option, the concept of getting Z AAT out of the liver and into the circulation, either by refolding or gene editing offers significant opportunities for effective treatment.

Dedication

I would like to dedicate this thesis to my family, in particular my parents who kept me on the straight and narrow, without them, without their support and encouragement I wouldn't be in the very fortunate position I'm in today. Finally I'd like to dedicate this to my dear friends who have passed but always encouraged me in my endeavours, Dr Shane Kenna and Joe Brady, who showed me how to get ahead in the RCSI.

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1. **McElvaney OF**, Asakura T, Meinig SL, Torres-Castillo JL, Hagan RS, Gabillard C, Murphy MP, Thorne LB, Borczuk A, Reeves EP, Zumwalt RE, Mikami Y, Carroll TP, Okuda K, Hogan G, McElvaney OJ, Clarke J, McEvoy NL, Mallon PW, McCarthy C, Curley G, Wolfgang MC, Boucher RC, McElvaney NG. Protease-anti-protease compartmentalization in SARS-CoV-2 ARDS: Therapeutic implications. *EBioMedicine*. 2022 Feb 22;77:103894. doi: 10.1016/j.ebiom.2022.103894. Epub ahead of print. PMID: 35217407; PMCID: PMC8861575. This relates to Chapter 1 and 4.
2. McElvaney OJ, McEvoy NL, Boland F, **McElvaney OF**, Hogan G, Donnelly K, Friel O, Browne E, Fraughen DD, Murphy MP, Clarke J, Choileáin ON, O'Connor E, McGuinness R, Boylan M, Kelly A, Hayden JC, Collins AM, Cullen A, Hyland D, Carroll TP, Geoghegan P, Laffey JG, Hennessey M, Martin-Loeches I, McElvaney NG, Curley GF. A randomized, double-blind, placebo-controlled trial of intravenous alpha-1 antitrypsin for acute respiratory distress syndrome secondary to COVID-19. *Med (N Y)*. 2022 Mar 11. doi: 10.1016/j.medj.2022.03.001. Epub ahead of print. PMID: 35291694; PMCID: PMC8913266. This relates to Chapter 4.
3. Palmas F, Clarke J, Colas RA, Gomez EA, Keogh A, Boylan M, McEvoy N, McElvaney OJ, **McElvaney OF**, Alalqam R, McElvaney NG, Curley GF, Dalli J. Dysregulated plasma lipid mediator profiles in critically ill COVID-19 patients. *PLoS One*. 2021 Aug 26;16(8):e0256226. doi: 10.1371/journal.pone.0256226. PMID: 34437568; PMCID: PMC8389414. This relates to Chapter 4.
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Clarke J, O'Connor E, Walsh S, Cho MH, Curley GF, McElvaney NG.
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 Erratum for: EBioMedicine. 2020 Nov;61:103026. PMID: 33181463; PMCID:
 PMC7654328. This relates to Chapter 4.

6. McElvaney OJ, O'Connor E, McEvoy NL, Fraughan DD, Clarke J, **McElvaney OF**, Gunaratnam C, O'Rourke J, Curley GF, McElvaney NG. Alpha-1 antitrypsin for cystic fibrosis complicated by severe cytokinemic COVID-19. J Cyst Fibros. 2021 Jan;20(1):31-35. doi: 10.1016/j.jcf.2020.11.012. Epub 2020 Nov 20. PMID: 33288475; PMCID: PMC7678455. This relates to Chapter 4.
7. **McElvaney OF**, Murphy MP, Reeves EP, McElvaney NG. Anti-cytokines as a Strategy in Alpha-1 Antitrypsin Deficiency. Chronic Obstr Pulm Dis. 2020 Jul;7(3):203-213. doi: 10.15326/jcopdf.7.3.2019.0171. PMID: 32503090; PMCID: PMC7857705. This relates to Chapter 4
8. Clarke J, Geoghegan P, McEvoy N, Boylan M, Ní Choileáin O, Mulligan M, Hogan G, Keogh A, McElvaney OJ, **McElvaney OF**, Bourke J, McNicholas B, Laffey JG, McElvaney NG, Curley GF. Prone positioning improves oxygenation and lung recruitment in patients with SARS-CoV-2 acute respiratory distress syndrome; a single centre cohort study of 20 consecutive patients. BMC Res Notes. 2021 Jan 9;14(1):20. doi: 10.1186/s13104-020-05426-2. PMID: 33422143; PMCID: PMC7796647. This relates to Chapter 4.
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List of Presentations/Awards

Presentations

RCSI Sheppard Prize Oral Presentation 2021: The role of AAT as an anti-inflammatory in SARS-CoV-2 infection.

American Thoracic Society 2022, San Francisco: Thematic Poster session: Secretion and retention of Z AAT in response to SARS-CoV-2 infection and similar stimuli.

American Thoracic Society 2022, San Francisco: Poster Discussion session: Protease-anti-protease compartmentalization in SARS CoV-2 ARDS: therapeutic implications.

Glossary of abbreviations

AAT: alpha-1-antitrypsin
AATD: alpha-1-antitrypsin deficiency
ANOVA: analysis of variance
ARDS: acute respiratory distress syndrome
Az: AstraZeneca vaccine
BCA: bicinchoninic acid assay
BSA: bovine serum albumin
CG: cathepsin G
CF: cystic fibrosis
COPD: chronic obstructive pulmonary disease
COVID-19: Coronavirus disease of 2019
CRP: C-reactive protein
CT: computed tomography
DPBS: Dulbecco's phosphate buffered saline
DMEM: Dulbecco's modified eagle medium
ELISA: enzyme linked immunosorbent assay
ER: endoplasmic reticulum
EMEM: eagles modified eagle media
FEV1: forced expiratory volume in 1 second
FRET: Florescence resonance energy transfer
FPLC: fast protein liquid chromatography
FVC forced vital capacity
g: grams
h: hours
HILIC: hydrophilic interaction liquid chromatography
HRP: horse radish peroxidase
IEF: isoelectric focusing
IL: interleukin
kDa: kilo-Dalton
LPS: lipopolysaccharide
LTB4: leukotriene-B4
mg: milligrams

ml: millilitre
NaCl: sodium chloride
NE: neutrophil elastase
NS: no significance
PBS: phosphate buffered saline
PR3: proteinase 3
PVDF: polyvinylidene fluoride
PWCF: People with Cystic Fibrosis
RPM: revolutions per minute
SARS-CoV-2: severe acute respiratory syndrome coronavirus 2
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Serpín: serine protease inhibitor
SLPI: secretory leukoprotease inhibitor
TA: Tracheal aspirates
TEMED: tetramethylethylenediamine
TNF- α : Tumour necrosis factor alpha
UNC: University of North Carolina
UV: Ultraviolet
WCL: Whole cell lysate

Chapter 1: Introduction

1.1 Alpha-1 antitrypsin and Alpha-1 antitrypsin deficiency

1.1.1 The role of alpha 1-antitrypsin and issues regarding the Z mutation

Alpha-1 anti-trypsin (AAT) is a glycosylated protein produced mainly in the liver (1). It first came to medical prominence in the 1960s when Laurel and Ericson, working in Malmo, Sweden discovered that the lack of this protein was associated with an increased risk for emphysema (2). This protein was initially named alpha-1 antitrypsin as it was shown to inhibit trypsin. Further work suggested that the major role of AAT in the lung was to inhibit neutrophil elastase (NE), an omnivorous protease produced by neutrophils, which is capable of digesting many structural components of the lung in addition to proteins involved in immunity and inflammation (3). This led to the development of the protease-anti-protease theory of emphysema in which the anti-protease protection in the lung, mainly provided by AAT, is markedly reduced, either functionally by cigarette smoke or in quantity, as in AAT deficiency (AATD), leading to the unopposed action of NE and subsequent lung destruction (4-6). AAT was first thought to fulfil a purely anti-serine protease role. In this regard, AAT was shown to inhibit NE, cathepsin G (CG) and proteinase-3 (PR3), the classic serine proteases produced by neutrophils (7). The role of AAT on non-serine proteases was ignored for some time. More recently work from this laboratory has shown that AAT can inhibit ADAM-17, a dismutase and metalloprotease, which has many anti-inflammatory abilities in addition to its anti-protease effects (8). AAT can also exert non-serine protease anti-protease effects by the downstream results of NE

inhibition. NE cleaves and activates other proteases, particularly the metalloprotease (MMP) group of proteases. Serine proteases (NE, cathepsin G, and proteinase-3) have all been shown to activate latent MMP-2 (9). MMP-2 activation by serine proteases is blocked by AAT, but not by a MMP inhibitor. In our laboratory we showed that not only does NE activate MMPs but it also up-regulates MMP-2 expression from monocytes in addition to cysteinyl cathepsin B (10). Inhibition of NE *in vivo* with aerosolized recombinant AAT in individuals with AATD, leads to a reduction in active cysteinyl cathepsins and metalloproteases in bronchoalveolar lavage (BAL), suggesting a hierarchy of proteases in the lung orchestrated, at least in part, by NE (11).

The anti-inflammatory effects of AAT have only recently come to the fore. Much of this information has devolved from studies in AATD. As early as 1991, Hubbard et al showed that BAL from people with AATD had increased number of neutrophils suggesting increased neutrophil chemotaxis into the lung (12). This was partly explained by spontaneous release of leukotriene B₄ (LTB₄) from macrophages in these individuals, and this group also showed that NE can induce LTB₄ release from macrophages. Further studies showed that treatment of AATD patients with intravenous AAT augmentation therapy reduced the levels of LTB₄ in the airways of individuals with AATD (13). Further studies evaluated the interaction between AAT and LTB₄, showing that AAT-LTB₄ binding occurs by protein–lipid hydrophobic interactions, preventing LTB₄ receptor engagement, in a process that does not affect the primary anti-NE function of AAT (14). Interestingly, docking experiments of various forms of AAT with LTB₄ showed three low energy sites indicative of likely binding interactions. One of these sites included the hydrophobic region located between strand 2 of the β -sheet A and helices D and E. As this is more exposed on the Z AAT molecule, further experiments showed that Z AAT could bind LTB₄ more avidly than M AAT, but Z polymers were unable to do so, suggesting that the AAT binding site for LTB₄ is located between strand 2 of the A β -sheet and helices D and E of AAT (14). This has relevance for AATD in two main ways as AAT can exist in polymer form and in latent form in plasma (15,16). The polymer form cannot inhibit NE and cannot bind LTB₄ while the latent form cannot inhibit NE. Importantly, it has recently been shown that some of the AAT in plasma purified AAT used for intravenous augmentation therapy also exists in a polymer or latent form and this may limit its efficacy.

LTB₄ is not the only neutrophil chemokine affected by AAT. AAT also interferes with neutrophil egress into the lungs by other mechanisms. One is through its inhibitory effects on the previously mentioned ADAM-17. Neutrophils isolated from stable AATD patients are characterized by low membrane expression of Fc γ RIIIb receptor and increased chemotaxis in response to interleukin-8 (IL-8) and soluble immune complexes (sIC) (8) (Figure 1.1). On exposure of the neutrophil to sIC, ADAM-17 is activated leading to the cleavage and shedding of the Fc γ RIIIb from the membrane. The sIC- Fc γ RIIIb complexes then crosslink with CR3, CD32a, or the fMLP receptor leading to migration of cells to the site of inflammation (8) (Figure 1.1). This system

is activated in AATD and is inhibited by exogenous AAT such as augmentation therapy, which results in decreased FcγRIIIb release from the neutrophil membrane and normalization of chemotaxis. ADAM-17 also orchestrates other signalling pathways including IL-6 signalling (see below, Figure 1.1). In cancer and inflammatory diseases, its proteolytic effects produce a supply of previously membrane-bound ligands for the epidermal growth factor receptor (EGFR), while it also promotes autoimmunity by cleaving the transmembrane protein TNF-α to make it systemically active (17,18).

AAT also affects IL-8-induced neutrophil chemotaxis (8). Neutrophil activation by IL-8 is accompanied by Akt phosphorylation and calcium (Ca²⁺) flux, leading to F-actin formation and cytoskeletal rearrangements (Figure 1.1). In a healthy individual, the resting circulating MM neutrophil is surrounded by a high concentration of AAT (approximately 27.5 μM). AAT binds IL-8 and prevents CXCR1 engagement. In AATD this inhibitory capacity is lost and there is increased neutrophil chemotaxis driven by IL-8. Interestingly the binding of AAT to IL-8 is through electrostatic bonds and is absent in non-glycosylated AAT.

Figure 1.1

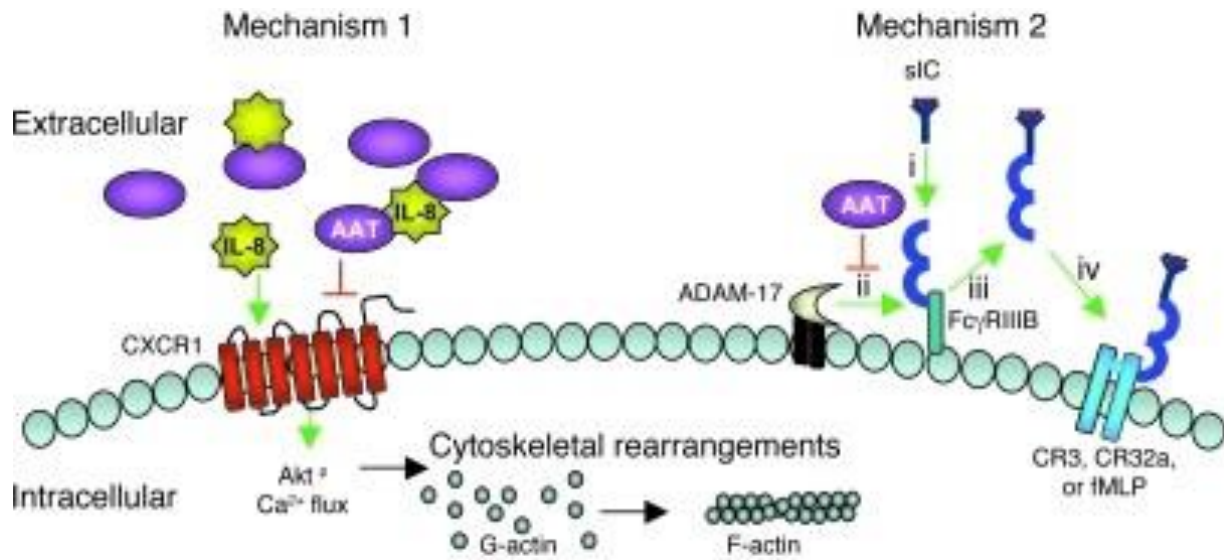


Figure 1.1

A. Role of AAT in interleukin (IL)-8 neutrophil signalling. **B.** Role of AAT in soluble immune complex (sIC) neutrophil signalling. Adapted from Bergin et al, J Clin Invest 2010 (8)

AAT glycosylation in IL-8 signalling was first evaluated in people with community acquired pneumonia (CAP) where it was shown that the glycosylation pattern of AAT changes during the course of infection and recovery (19,20). This was determined by means of isoelectric focusing and immune-blotting AAT during the course of infection. This technique, which is used to phenotype AATD also shows the various AAT bands. AAT has 9 glycoforms M0-M8 (Figure 1.2). In AAT's quiescent state there are 6 visible bands on isoelectric focusing immunoblots, the most obvious being M2, M4 and M6, but in times of inflammation M0 and M1 bands become more apparent. This is due to increased sialylation of the AAT protein. The M0, M1 bands denote a more negatively charged protein. This "hyper-glycosylated" AAT binds IL-8 and neutrophil activating protein (NAP)-2 more avidly than AAT without the M0M1 bands and prevents their interaction with their receptor thus down-regulating neutrophil chemotaxis (19). In CAP it has been demonstrated that M0,M1 bands appear on days 4-6 and no longer visible by day 8. When plasma was taken at the acute phase and recovery phase of CAP, the acute phase (ap) AAT, which contains little M0,M1 was less able to bind IL-8 than the recovery phase AAT (rAAT) which contained significantly more M0,M1 bands (19), (Figure 1.3. panel A). Thus, rAAT was shown to contain significantly more IL-8-AAT complexes than the ap AAT (19) (Figure 1.3. panel B, 1.3. panel C). This suggest an "off switch" in acute inflammatory processes such as CAP whereby the body dampens down the inflammatory response once it is no longer required thus preventing neutrophil mediated damage after the need for neutrophils to kill bacteria has passed.

Figure 1.2

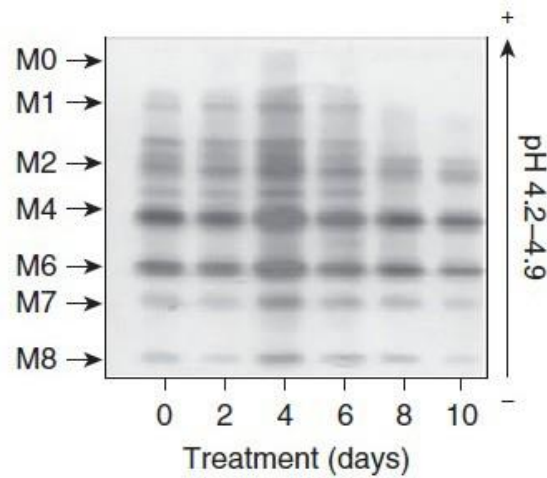


Figure 1.2

Isoforms of AAT in plasma during community acquired pneumonia (CAP) as determined by IEF appear at days 4-6 during recovery phase. (rp), disappearing at days 8-10. These changes are in line with changes in other inflammatory processes such as C - reactive protein (CRP). Adapted from McCarthy et al, Amer Jour Respir Crit care Med, 2018 (19)

Figure 1.3

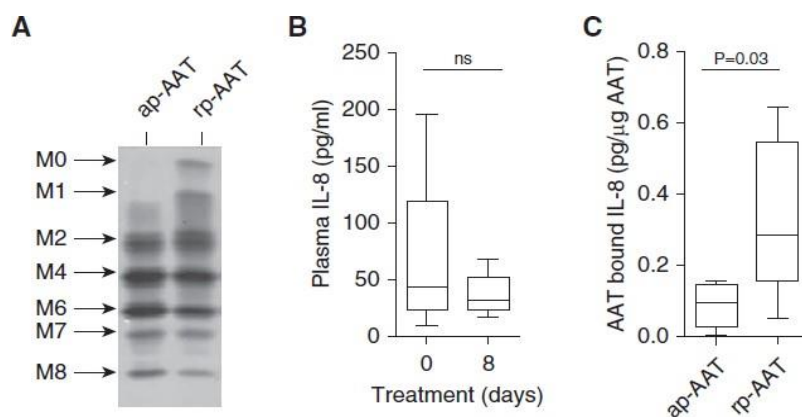


Figure 1.3

A. Isoelectric focusing gels from MM individuals with CAP during the acute phase (ap-AAT)) and recovery phase (rp-AAT) days 4-6. **B.** The levels of IL-8 in plasma of people with CAP, on day 0 and day 8 (no significant difference). **C.** The levels of ap-AAT or rp-AAT complexed to IL-8 with significantly higher levels bound to rp-AAT than ap-AAT. Adapted from McCarthy et al, Amer Jour Respir Crit care Med, 2018 (19)

However, in other chronic lung diseases such as cystic fibrosis (CF) there is dysregulation of this process (20). In CF we see a persistence of the M0M1 band even when an acute exacerbation has passed suggesting that the stimulus for increased glycosylation remains. This partly explains the persistent neutrophil dominated airway inflammation in CF, which is a prominent feature of that condition. This was further studied by looking at the means by which AAT is up-regulated *in vivo*. In general, while AAT can be increased by various inflammatory parameters, the physiological stimulus underpinning most of the acute phase AAT response is interleukin (IL)-6 (21). Not only does IL-6 up-regulate AAT mRNA and protein but it also up-regulates ST6 beta-galactoside Alpha-2, 6-Sialyltransferase 1 (ST6GAL1), an enzyme centrally involved in sialylation of proteins such as AAT (22). Thus, in both acute and chronic inflammatory lungs diseases such as CAP and CF the body responds by increasing the amount of AAT and also by changing its glycosylation profile. In CAP this works to shut off the inflammatory process, but in CF the inflammatory response is dysregulated and the persistence of sialylated AAT is not sufficient to suppress IL-8 induced neutrophil chemotaxis as the levels of IL-8 and other neutrophil chemokines in the lung subvert this protective process and overwhelm the attempts of M0,M1 AAT to bind and inhibit their inflammatory effects.

1.1.2 The pathogenesis of AATD

There is no putative role for NE in the pathogenesis of the liver disease associated with AATD (23). Work by Sharp and others revealed that the major cause of the low levels of AAT in the blood and lungs of people with the ZZ form of AATD was due to polymerization of the Z protein in the liver (24,25). This leads to endoplasmic reticulum (ER) stress, liver inflammation and reduced secretion of AAT into the circulation resulting in insufficient quantities to protect the lung (1,26,27). Thus, in the lung disease associated with AATD the problem is due to a lack of functional AAT in the lung, while in the liver disease there is a gain of function, i.e., too much mutant AAT in the liver, leading to liver inflammation. The obvious result from these insights is that the treatments of AATD will of necessity be different for the lung and liver unless a treatment is devised which would treat both simultaneously by promoting secretion of the mutant Z protein from the liver and into the lung.

1.1.3 The epidemiology of AATD

Much of the work in AATD has been on individuals homozygous for the Z mutation. Recent work suggests that individuals with heterozygote (MZ) (27) or compound heterozygotes (SZ) (28,29) have increased risk for COPD if they smoke, further stressing the important role of the Z allele in the propagation of COPD/emphysema. Individuals with the MZ mutation have also been shown to have an increased risk for liver disease, specifically liver steatosis and alcohol related liver disease (30), but as the risk from the MZ and SZ forms of AATD remains to be fully evaluated, the current study was designed to concentrate on the Z homozygote form of AATD, the form in which we have most information and which is specifically linked to disease even in the absence of a “second hit” such as cigarette smoking, excess alcohol or obesity. The prevalence of AATD has been greatly underestimated. In the United States it is estimated that it takes 7 doctors and 5 years before a patient is diagnosed appropriately with AATD (31). In addition, it is estimated that there are 3,000 ZZ individuals on the island of Ireland (8), 80,000 in Europe and a further 60,000 in North, Central, and South America (32,33), but less than 10% are diagnosed. In Ireland with a very intensive diagnostic, targeted detection program 400 ZZs have been identified to date, which is in excess of most other countries but still leaving many undiagnosed. This is partly due to these individuals being diagnosed as COPD, asthma or fatty or alcohol-related liver disease. This low number may also reflect the low or less severe clinical penetrance of ZZ AATD lung disease in the absence of smoking.

1.1.4 The Clinical Manifestations of AATD

In general, there are three major clinical manifestations of AATD: emphysema, liver disease and panniculitis (1,34,35). These can occur with all forms of AATD but are most severe and frequent in the Z homozygous setting. The major clinical manifestation of the lung disease associated with AATD is early progressive chronic obstructive lung disease, which can occur even in the absence of cigarette smoking, but which is greatly accelerated and exacerbated by smoking (1). Although felt initially to be mainly lower lobe in distribution it can manifest in the upper lobes and is generally indistinguishable from nonhereditary emphysema (36). This has led to considerable under-diagnoses or misdiagnosis of the condition (13).

This is a major problem as late diagnosis or misdiagnosis can delay lifestyle advice and lead to significant lung damage and morbidity. In the National Heart, Lung, and Blood Institute registry for severe AATD, although the average age at diagnosis was 46 years, the mean forced expiratory volume in 1 second (FEV1) and diffusing capacity of the lung for carbon monoxide (DLCO) were 47% and 50% predicted respectively, suggesting significant irreversible lung damage at an early age (37). The data for the Irish AATD Registry are similar with mean age of diagnosis at 44, mean FEV1 in ever smokers 53%, mean DLCO in ever smokers 48%. Interestingly the Irish Registry (38) contains a greater percentage of never smokers, mainly because of a very active family screening program. This leads to a large number of non-index cases, who are those detected, often in the absence of symptoms and in many cases early in the disease process. In this group at diagnosis, the mean FEV1 is 84% predicted and the mean DLCO is 71% predicted (39). This suggests a strong benefit of never smoking and also the benefit of a targeted detection program, which evaluates family members of people diagnosed with AATD. Despite that, these never smoking ZZ individual have evidence of airway obstruction on pulmonary function testing and a significant number have cough (39%) and sputum production (36%). AATD is also associated with liver disease. The major study of Sveger et al (40) evaluated 200,000 new-borns and identified 120 PiZZ, 48 PiSZ and one PiSS individuals. Of the PiZZ individuals, 14 had prolonged obstructive jaundice, 9 with severe clinical and laboratory evidence of liver disease. Approximately 15% of persons with cholestatic jaundice had progression to juvenile cirrhosis (41). The risk of death from liver disease among children with the PI ZZ genotype was 2 to 3%, but none of the survivors had clinical symptoms of liver disease at 12 years of age. In adults, clinically significant liver fibrosis has been shown on biopsy in 35% of adults with PiZZ AATD (42), and a large European analysis using non-invasive assessment showed clinically significant liver fibrosis in 20 to 36% of ZZ AATD individuals (43). AATD is also associated with panniculitis, an inflammation of the subcutaneous adipose tissue (34,35). This was initially thought to be rare affecting approximately 1% of people with AATD and only individuals with the severe phenotypes. Recent data would suggest otherwise. In one series, AATD was present in up to 15% of all cases of biopsy-proven panniculitis suggesting AATD-associated panniculitis may be more prevalent than previously thought and can have a severe presentation with a significant risk of death if not recognized.

1.1.5 Diagnosis of AATD

With these varying presentations it is obvious that early diagnosis is required. Diagnosis has been improved by moving away from the old paradigms of AATD diagnosis and towards targeted detection based on World Health Organization (WHO), European Respiratory Society (ERS) and American Thoracic Society (ATS) (44,45) guidelines which advocate AATD testing for all individuals with chronic obstructive pulmonary disease (COPD), poorly responsive asthma, cryptogenic liver disease, and first degree relatives of people with AATD. As a result, not only are more people with AATD being detected but they are being diagnosed earlier and at a time when lifestyle advice and specific treatments may be initiated in an effective fashion. The main diagnostic methods used are a combination of isoelectric focussing, genotyping and measurement of AAT levels with gene sequencing in rare cases (1).

1.1.6 General management and treatments for lung disease associated with AAT deficiency

The treatment options for AATD related lung disease may be divided into general treatments as for non-hereditary COPD (46) and specific treatments aimed at the underlying pathogenesis of AATD (1,47,48). The first option for every person with AATD is smoking cessation. This is especially true as we now have a much greater understanding of the lung disease risks associated with the various AATD phenotypes. In this regard, it is imperative to offer early and effective smoking cessation advice. The data to date shows that people with the MZ and SZ phenotypes do not have an increased risk for COPD if they do not smoke but their risk is significantly increased if they do smoke (27-29). Furthermore, in the SZ group the rate of decline in lung function post smoking cessation is the same as a never smoking SZ if airway obstruction has not manifested prior to stopping smoking, highlighting the need for smoking cessation programs in this cohort (29). There is a lack of knowledge on the natural history of the lung disease associated with the ZZ form of AATD and the effects of smoking and smoking cessation. As a result, it is unknown how much the risk for COPD is increased in smoking patients with the ZZ mutation versus non- smoking ZZ patients or whether non-smoking ZZ patients can get lung disease similar to smoking non-AATD MM individuals. This type of evaluation is best pursued, using family-based association testing (FBAT), a

technique pioneered in the pulmonary field by Silverman et al and which was used successfully to evaluate the risk of COPD in MZs (27) and later in SZs (28). This technique requires an initial identification of an index individual with AATD followed by evaluation of their family members. The technique concentrates on evaluating pulmonary function and the risk for COPD in these non-index ZZ and MM siblings. The diagnosis of AATD itself is a deterrent to smoking. In a recent study of people with AATD, current smoking was uncommon (2.5% v 17% v 16% for ZZ, SZ and MZ respectively) in those with ZZ being significantly less likely to be current smokers (49). In addition, in people with AATD, parental smoking is associated with ever-smoking status, higher cumulative tobacco consumption and more quit attempts to achieve smoking cessation among former smokers (49). Therefore, changing a smoking habit may result in positive results in subsequent generations. In addition to smoking cessation, influenza and pneumonia vaccination is recommended as is use of inhalers similar to those used in non-hereditary COPD.

1.1.7 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and its impact on AATD

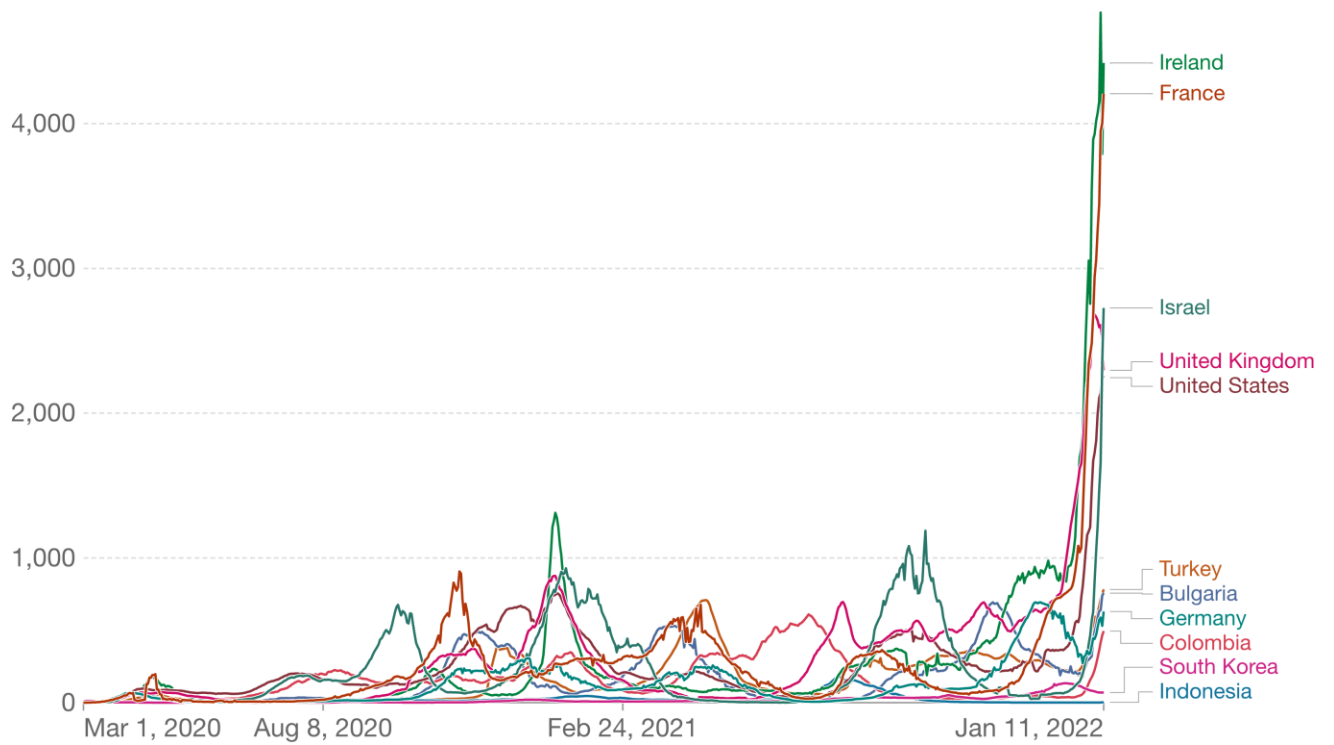
SARS-CoV-2 is a single-stranded RNA coronavirus, with a bat species or intermediate species thought to be its natural reservoir, similar to that of the SARS-CoV-1 virus that caused the 2002 SARS outbreak (50). SARS-CoV-2 causes the coronavirus-2019 (COVID-19) disease, a severe respiratory illness and major global health risk with few therapies available to modify its clinical course (50). SARS-CoV-2 is effective at human to human transmission predominantly via droplet or aerosol with more than 228 million laboratory-confirmed cases documented globally and over 4.5 million deaths since its initial discovery in Wuhan to September 2021(51). This research has been conducted over the entirety of the pandemic in particular during the three main waves which includes data prior to and post the advent of SARS-CoV-2 vaccination (Figure 1.4).

Figure 1.4

Daily new confirmed COVID-19 cases per million people

7-day rolling average. Due to limited testing, the number of confirmed cases is lower than the true number of infections.

Our World
in Data



Source: Johns Hopkins University CSSE COVID-19 Data

CC BY

Figure 1.4

Infection rates in Ireland throughout the duration of the pandemic as compared to other nations. Extracted from John Hopkins University CSSE COVID-19 Data

Alpha-1 antitrypsin (AAT), a major serine protease inhibitor and anti-inflammatory protein, may influence the trajectory of SARS-CoV-2 infection. SARS-CoV-2 is characterised by an IL-6 driven cytokinemia (52), the key cytokine in AAT up-regulation, and is associated with a rapidly developing acute respiratory distress syndrome (ARDS).

The role of IL-6 in acute phase reaction has been extensively studied during the COVID pandemic. For IL-6 mediated signal transduction to occur, IL-6 must first bind the IL-6 receptor (IL-6R), following which the IL-6/IL-6R complex must associate with a protein called gp130 (53, 54, 55) (Figure 1.5). Interestingly, IL-6R is a membrane-bound receptor expressed by relatively few cell types, most notably hepatocytes and some leukocytes such as macrophages, whereas gp130 is expressed by all cell types (54). In general, there are cells that possess both IL-6R and gp130 and are naturally IL-6-responsive, and those that express gp130 but not IL-6R and therefore cannot respond to IL-6 directly. IL-6 signalling via its membrane-bound cognate receptor is termed classical signalling and it is through this route that IL-6 up-regulates AAT and ST6GAL1. In pro-inflammatory states, IL-6R is cleaved from the cell surface and generates a soluble receptor, sIL-6R. The enzyme responsible for this cleavage event is the previously mentioned ADAM-17, (inhibited by AAT) which is upregulated in response to inflammation and infection. Soluble IL-6 receptor (sIL-6R) retains the ability to bind IL-6 (55). This IL-6/sIL-6R complex can then bind gp130. As gp130 is ubiquitously expressed, IL-6 when complexed to sIL-6R can therefore bind cells that are otherwise unresponsive to the cytokine and induce signalling. This process is called trans-signalling and explains why a large variety of cells which do not normally express the IL-6R, including epithelial cells, smooth muscle cells, and endothelial cells, respond to IL-6 in critical illness, while also identifying ADAM-17 as a potential therapeutic target. There is one further non-classical means by which IL-6 signalling can occur. This involves the antigen-specific interaction of a dendritic cell (which produces the IL-6 signal), and a T-cell (which receives it) resulting in the commitment of the T-cell to a pro-inflammatory phenotype. The anti-inflammatory activities of IL-6 are mediated via classical signalling, while the pro-inflammatory activities of IL-6 are mediated by trans-signalling, and occasionally by trans-presentation (Figure 1.5).

Figure 1.5

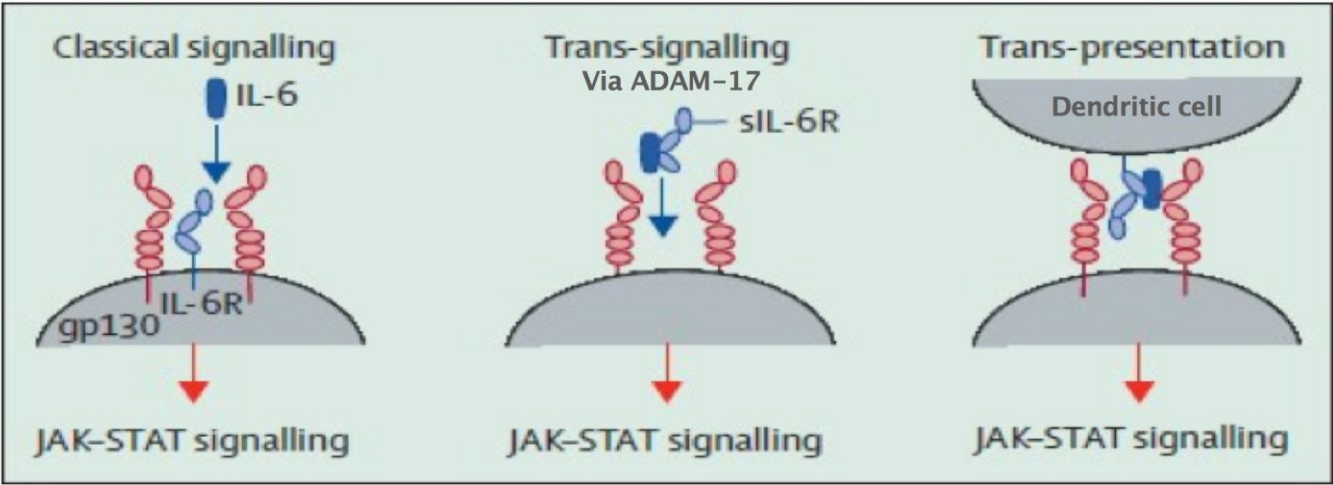


Figure 1.5

The three main forms of IL-6 signalling *in vivo*. Adapted from McElvaney et al, Lancet Respiratory Medicine, 2020. (55)

AAT has many anti-inflammatory and anti-protease effects, but despite the new data emerging in this area, NE and its effects remain central to the understanding of the lung disease associated with AATD. The unopposed actions of NE are manifold (1) (Figure 1.6). NE can cause direct epithelial damage (56), increase mucus production (57), cleave complement (58), immunoglobulins and complement receptors (59,60). In addition, NE can inactivate many of the body's innate defence mechanisms such as secretory leukoprotease inhibitor (SLPI) (61) and up-regulate and activate, other proteases such as cysteinyl cathepsins and metalloproteases which in turn inactivate defensins, SLPI, lactoferrin and other defence proteins (62-64). A NE knockout mouse has been shown to be relatively protected from cigarette smoke induced emphysema, and NE when instilled into the lungs causes a pathologic appearance similar to emphysema (65). Therefore, the role of NE and by extension AAT in the development of lung disease is well developed (66).

Figure 1.6

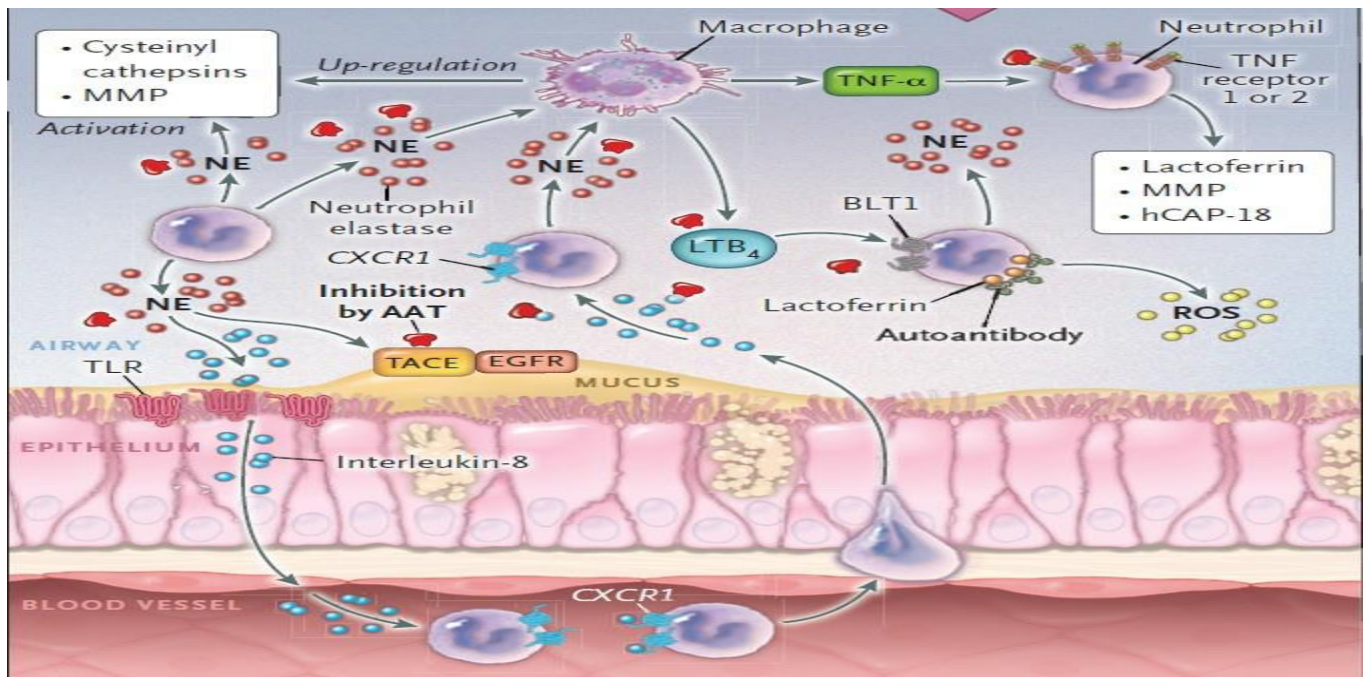


Figure 1.6

The many effects of unopposed NE activity. Adapted from Strnad et al. New England Journal of Medicine, 2020 (1)

- NE results in direct epithelial damage and can inactivate many of the body's innate defense mechanisms as seen in Fig.1.5
- NE activates other proteases such as cysteine cathepsins and metalloproteases which in turn inactivate defensins, SLPI, lactoferrin and other defense proteins
 - NE increases mucus production and cleaves complement immunoglobulins and complement receptors

The mechanism of an IL-6 driven acute phase response in SARS-CoV2 infection raises a specific concern for people with AATD, namely an inability to respond by increasing AAT secretion with subsequent increase in AAT intracellular retention and inflammation. Data, early in the COVID pandemic suggested that people with AATD may have an increased risk of contracting SARS-CoV-2 and a greater risk of lung damage in the event of SARS-CoV-2 infection. AAT directly inhibits TMPRSS-2 (67), the serine protease that cleaves the spike protein of SARS-CoV-2 which is essential for the virus to bind to its cell surface receptor (ACE2) to enter cells.). Other reports have demonstrated that AAT is the likely factor in BAL and sera which inhibits SARS-CoV-2 entry into cells (68,69), a finding confirmed by the ability of exogenous AAT to inhibit SARS-CoV-2 infection of airway epithelial cells. AAT has also been shown to have other significant anti-viral properties. In HIV infection, AAT interacts with gp41 to prevent HIV entry into CD4 lymphocytes and AAT also inhibits HIV replication by inhibition of NFkappaB activation and inducing prostaglandin synthase-2 release (70). A role for AAT in MERS-CoV infection through its effects on autophagy has also been proposed (71). In a recent paper by Nuno Faria et al a comparison was made between reported national estimates for the major AAT deficiency alleles PiZ and PiS (SERPINA1 rs28929474 and rs17580, respectively) with the Johns Hopkins University Coronavirus Resource Centre dataset. A significant positive correlation ($R = .54$, $P = 1.98e-6$) was found between the combined frequencies of the alpha-1 antitrypsin PiZ and PiS deficiency alleles in 67 countries and their reported SARS-CoV-2 mortality rates (72). In addition to COVID vaccination, it could be argued that timely initiation of antibiotic therapy is necessary in this group and raises the question of whether AAT supplementation or augmentation would be of benefit.

1.1.8 Augmentation therapy with plasma purified alpha-1 anti-trypsin

The only approved treatment specific for AATD is augmentation therapy with plasma purified AAT, which is specific for the lung disease associated with this disorder. This was first approved in 1987 following a number of seminal papers which showed that intravenous augmentation therapy with plasma purified AAT given at a dose of 60mg/kg once weekly could raise levels of AAT in plasma and lung epithelial lining fluid above a putative protective threshold (73,74). This was followed by further studies evaluating different dosing regimens and aerosol delivery of AAT (75). The

main thrust of these papers was to show a biochemical effect of augmentation therapy, i.e., a restoration of the anti-protease protection in blood and perhaps more importantly in the lung. It was always accepted that showing clinical efficacy would be more difficult given the relative rarity of the ZZ condition and the problems with using forced expired volume in one second (FEV1) as a clinical output (76). Despite this, a number of studies did suggest a clinical effect of augmentation therapy with intravenous AAT on FEV1 specifically in subgroups of the ZZ population (77,78).

The study by Seersholm et al evaluated annual change in FEV1 in AATD in a treated German group compared to an untreated Danish group (78). Overall, there was a difference in FEV1 change per year, but this was only statistically significant in those with an FEV1 between 31 and 65% predicted. The National Heart, Lung and Blood Institute (NHLBI) registry showed similar results with significant treatment effect in those with FEV1 between 35-49% predicted (77). This study also showed an effect of augmentation therapy on survival. Neither of these were randomized control trials and so clinical efficacy data continued to prove elusive. This process changed with the advent of utilizing lung density as measured by CT scan to estimate progression of emphysema in people with ZZ AATD. The first of these studies by Dirksen et al evaluated a relatively small Danish-Dutch cohort (n=56) with patients randomized to either AAT (250mg/Kg) or albumin (625mg/kg) at 4-week intervals for at least 3 years (79). This study showed an almost significant ($P=0.07$) difference between the AAT treated and albumin-treated groups and provided enough data to derive a power statistic to determine the number of AATD patients required for future trials to demonstrate a relevant clinical endpoint. It estimated that approximately 130 patients with ZZ AATD would be required in a randomised placebo-controlled trial to show significant protection against loss of lung tissue (1.07g/L) as measured by CT lung density. However, to demonstrate a corresponding (i.e., 50%) correction of the FEV1 slope, 550 AATD individuals would be required (79). This led to an increased interest in studies utilising lung density as an outcome measure in AATD. The EXAcerbations and Computed Tomography scan as Lung End points (EXACTLE) trial (80) followed which was a multi-centred, randomized, placebo-controlled trial evaluating 60mg/kg plasma purified AAT given weekly over two years compared to placebo. A number of statistical analyses were used in this study with p-values ranging from 0.049-0.084 but all trending towards efficacy in reducing progression of

emphysema. The most definitive trials to date in this area are the RAPID (81) and RAPID/OLE (open label extension) (82) studies.

Unlike the previous studies, the RAPID studies were powered sufficiently to show an effect of augmentation therapy on CT lung density. The RAPID study showed that intravenous administration of plasma purified AAT at a dose of 60mg/kg slowed down progression of emphysema, as measured by CT lung density at total lung capacity, by 33%. In this and the RAPID/OLE study it was further shown that when individuals who had received placebo in the RAPID study were switched over to active therapy their rate of lung tissue density loss declined. This was the first study to show definitively a clinical effect of augmentation therapy on lung in AATD. It did not however stop decline in lung tissue density and some of the results suggested that perhaps larger doses might be more effective, an area which is being actively pursued in upcoming studies. While these results were very encouraging the changes seen in CT lung density measurements were not associated with concomitant changes in measures of quality of life, pulmonary function or infective exacerbations. This has led the Food and Drugs Administration and other agencies to question whether a positive outcome in lung density measurements alone is sufficient proof of clinical efficacy if unaccompanied by other readouts. One of the potential reasons for the inability to see a change in spirometry could be that when the average patient is started on these studies the degree of lung function impairment is so great that only a minimal change might be expected which would be difficult to detect given the insensitivity of the measure. This was postulated in the original study by Dirksen et al (79) which showed that a significant protection effect by AAT augmentation therapy against the loss of lung tissue (as measured by CT lung density) would require evaluation of 130 patients over a period of 3 years, whereas demonstration of a corresponding (i.e., 50%) correction of the FEV1 slope would require 550 patients. The largest study to date, the RAPID study recruited 180 individuals (81,82), and took seven years to fully recruit.

1.1.9 Therapeutic aerosol delivery of AAT to the lung

Given that the standard intravenous dose of AAT is 60mg/kg and there is not an unlimited supply of plasma purified AAT, investigators have evaluated other forms of AAT and other routes of delivery. The initial work with recombinant AAT was disappointing as it quickly became obvious that non-glycosylated AAT as derived

from yeast-based systems had a very short half-life after intravenous administration (83). Later work would show that non-glycosylated AAT would also have a different anti-inflammatory profile (84,85). Transgenic AAT as produced initially in “Dolly” the sheep also suffered from glycosylation differences compared to human AAT with resultant effects on half-life on intravenous administration. With this as background, investigators turned to aerosol delivery of AAT as an alternative with lower doses required, ability to dose more frequently simulating what occurs in “real life” and less concerns about glycosylation impacting upon half-life (84,85). To date there has been one randomised controlled clinical trial on aerosolized AAT in AATD. The results showed an early increase in FEV1 compared to placebo but with a similar slope of decline thereafter (86). This suggested an anti-inflammatory effect of AAT but requires further study to determine a significant effect on emphysema. It should be noted that this study was with plasma purified AAT. The data from recombinant AAT has been variable. It was initially thought that administration of recombinant AAT to the lung would be safe and efficacious while avoiding the problems of shortened half- life due to differential glycosylation. Initial administration of recombinant AAT raised in yeast showed increased AAT on the lung epithelial surface with increased anti-NE capacity. This study also showed that the aerosolized AAT could cross the alveolar- capillary membrane and enter the blood, thus potentially providing some increase in systemic AAT and anti-NE capacity. There were some concerns about potential contamination of the AAT preparation with yeast. Following this, further studies took place with transgenic AAT, but these studies were cut short due to significant side effects characterized by an alveolitis, which was deemed due to co- precipitation of sheep alpha-1 anti-chymotrypsin along with humanised AAT from the sheep’s milk (87). As a result, most of the recent studies in aerosol delivery of AAT have utilised plasma purified AAT. A new opportunity has recently arisen with the advent of advanced recombinant technology which can “normalise” the glycosylation profile of AAT made in Chinese hamster ovarian (CHO) cells and which can be purified in sufficient quantities for human therapeutic trials. (88).

1.1.10 Use of AAT augmentation therapy outside AATD

Although AAT augmentation therapy by intravenous route or by aerosol was designed initially only with AATD in mind, the use of AAT as an anti-protease, anti-

inflammatory in other disease states has also been evaluated. The first of these was in cystic fibrosis (CF). CF is a hereditary disorder caused by mutations of the CF transmembrane conductance regulator (CFTR) gene. The major causes of morbidity and mortality in CF are related to lung disease. This lung disease starts in childhood and is characterized by production of thick mucus, airway inflammation and chronic airway colonization leading to airway damage, respiratory failure and death (89). CF is a classic neutrophil dominated lung disease. This neutrophil-mediated inflammation starts early in life and increases with age and with the acquisition of *Pseudomonas aeruginosa* in the airways. Much of the inflammation and bacterial colonization in CF can be attributed to the unopposed action of NE and this would seem to make it an ideal candidate for anti-NE therapies such as AAT (90,91). It should be noted that in CF the levels of AAT in plasma are normal or slightly elevated and completely active against NE. In the airways, however, the massive neutrophil and NE burden completely overwhelms AAT and other anti-proteases such as SLPI leaving the way open for unopposed NE activity (90). This was the first example of compartmentalisation of the inflammatory/protease processes in lung disease. AAT has been administered both intravenously and by aerosol to people with CF (PWCF) (92). With intravenous administration, doses ranging from 60-120mg/kg were administered once weekly but only the 120mg/kg dosage was able to inhibit NE in the airways and the effect was relatively short lived with NE reappearing on the respiratory epithelial surface (RES) within one week following administration.

Aerosol delivery of AAT in CF was also evaluated as CF lung disease is primarily an airway disease with little systemic consequences from the lung inflammation. In this context, AAT was administered at doses ranging from 1.5-3mg/kg. Once the levels of AAT on the respiratory epithelial surface (RES) reached 8 μ M or more, there was complete inhibition of NE, return of normal anti-NE capacity and restoration of neutrophil bacterial killing capacity. Later studies showed that AAT aerosol also prevents NE-mediated cleavage of the CXCR-1 receptor on neutrophils in CF and other inflammatory lung diseases, restoring *Pseudomonas aeruginosa* killing capacity (93). Further studies confirmed a dose-dependent inhibition of NE by AAT on the RES (94,95). These studies illustrated a number of challenges with AAT therapy in CF. Firstly the levels of NE in the lungs of PWCF vary. Secondly most

studies used sputum to evaluate NE and the methodology used was not standardized. Recent work has shown that sputum, either induced or spontaneously expectorated, when processed appropriately (96), shows similar NE levels to that found in bronchoalveolar lavage fluid (BA), the present gold standard investigative technique to evaluate the airways. This opens up the way for future studies in inflammatory lung conditions using relatively non-invasive sputum evaluations. One such condition is non-CF bronchiectasis (NCFB). This condition is also characterized by high levels of active NE in the airways and relatively normal or elevated AAT in the circulation. As with CF, the unopposed NE in the airways contributes significantly to the disease phenotype. A series of studies have attempted to inhibit NE in NCFB with mixed success but a recent study in the New England Journal of Medicine by Chalmers et al evaluated dipeptidyl peptidase 1 (DPP-1; also known as cathepsin C) in NCFB (97). Neutrophil serine proteases are activated during neutrophil maturation in the bone marrow by DPP-1, which removes the N-terminal dipeptide, thus allowing active enzymes to be packaged into granules before the release of neutrophils into the circulation. Brensocatib (INS1007) an oral, selective, competitive, and reversible inhibitor of DPP-1 was shown to inhibit neutrophil serine protease activity in the blood of healthy volunteers and when administered to people with NCFB over a 6-month period, showed significant ability to increase time to next exacerbation, in addition to reducing sputum NE levels.

Another condition in which exogenous AAT could have significant therapeutic benefit is in granulomatosis with polyangiitis (GPA, formally known as Wegener's granulomatosis (98). This condition is characterized by the presence of auto-antibodies against neutrophil cytoplasmic contents. These anti-neutrophil cytoplasmic antibodies (ANCA) are classically raised against NE, myeloperoxidase (MPO) or more commonly against proteinase-3 (PR3). AAT naturally inhibits both PR3 and NE. In epidemiological studies of GPA, there is significant over representation of the MZ AATD genotype and MZs, and in particular ZZs, have a worse prognosis with this condition. Previous work has shown that neutrophils from people with GPA express PR3 on their surface and, on exposure to ANCAs, produce an oxidant burst (99). This effect can be abrogated by the pre-treatment of these neutrophils with AAT. These studies illustrate that proteases may have a site-specific activity which they can exert even in the presence of normal or high circulation levels of AAT.

1.1.11 Gene therapy for AATD lung disease

As AATD is basically a monogenic disorder, gene therapy would seem to provide a potential therapeutic option. The most commonly employed gene-delivery vector to date is the adeno-associated virus (AAV), a single stranded DNA virus which can infect a variety of cells with low immunogenicity and stable long-term expression. In the first human study in AATD, an AAV vector containing the AAT cDNA was administered intramuscularly. The achieved level of AAT was well below the putative protective threshold (PTT). Patients developed neutralizing antibodies against the AAV capsid but the expression lasted for up to one year (100). A subsequent trial using a herpes-simplex virus-1 helper system showed a 10-fold higher level compared to the first trial but still below the PTT. This study required multiple intramuscular injections which patients found generally unacceptable. Subsequent evaluation showed that there was sustained AAT transgene expression of around 3% of the PTT with other downstream effects consistent with an increased anti-protease screen in blood (101). Other routes of AAV-gene transfer include pleural administration targeting mesothelial cells and liver targeting (102,103). An advantage in recent AAV- driven gene therapy is the use of the rhesus AAVrh.10 serotype that circumvents the problems associated with human immunity to AAV impacting upon repeat administration (103,104). A number of liver-targeted trials have been proposed. Liver targeting, while attractive in its ability to express high levels of AAT presents specific difficulties due to the intrinsic liver disease found in AATD which might be exacerbated by liver-directed AAV-vector administration. Other potential problems with AAV-usage is the possibility of insertional mutagenesis (105), which was noted in neonatal mice receiving AAV via injection (106). Despite the fact that the rate of integration of AAV genomes in the cells of non-human primates or humans is very low, integration remains a concern in on-going studies. Other viral vector systems include the adenovirus. This vector system has many desirable qualities; it is non- integrative, can work in both dividing and non-dividing cells, has a large DNA- insertion capacity and is relatively easy to produce in high quantities (107). In conditions such a cancers where the Ad vector can target cancer cells or in vaccine development (108) where its ability to express antigens can induce humoral responses, adenoviral vectors may have decided advantages (109). However, in AATD, transient gene expression and inflammation has dampened enthusiasm for

this approach. A potential way around this is to modify the Ad vectors to alter tropism.

One such effort involves using myeloid binding peptide (MBP) incorporating this into the Ad with resultant increased tropism for pulmonary endothelium and reduced tropism for hepatic cells, increasing lung levels of AAT while mitigating liver inflammation (110). Recent research has shown that the interaction between hexon (Ad major capsid protein) and factor X are the key mediators of Ad liver transduction after intravenous administration (111,112). This has led to another method to mitigate against liver inflammation using hexon swapping or hypervariable region swapping which will reduce liver sequestration of Ad (113,114). Even when the question of tropism is answered, the problem of limited duration of transgene expression is a major impediment to Ad-mediated gene therapy in the context of AATD. Various methodologies have been employed including use of non-human primate serotypes which have low sero-prevalence in humans and may be expected to have increased duration of expression with the opportunity for re-administration (115).

1.1.12 Treatment of AATD liver disease

For many years the only treatment for severe AATD-related liver disease was liver transplant. The early therapeutic attempts in AATD liver disease aimed at increasing AAT secretion from the liver or accelerating intracellular degradation. Chemical chaperones such as 4-phenyl butyrate were evaluated in the belief that they would improve folding of the Z protein and augment secretion. This approach was effective in animal models but in human studies, there was no significant effect on AAT (116). Other attempts using 4-mer and 6-mer peptides engineered to block polymerization have also been evaluated *in vitro* but with no *in vivo* success (117,118). Enhancing autophagy is another means of decreasing the Z AAT burden in the liver. A variety of such approaches have been used in animal models, but these would require excessive dosages to produce a clinical effect *in vivo*. Carbamazepine has been trialled in human subjects with ZZ AATD, but no clinical data is available at present (119).

Another option is to selectively knockout the production of ZAAT in the liver using RNA interference (RNAi). Two main ways of doing this are with small interfering RNAs (siRNAs) short double stranded RNA fragments and antisense

oligonucleotides (ASO) which are single stranded (120). The initial studies in this area borrowed from experience with hepatitis B infection (121). Various groups used liposome particles, or PEGylated nanoparticles to deliver siRNA but with little success. More recently, a polymer-based system, which included an amphipathic, endosomolytic polymer that was reversibly masked and only active in the acidic environment of the endosome has been used (122). A targeting ligand N-acetylgalactosamine (NAG) was attached to the masked polymer resulting in a hepatocyte-specific delivery via the asialoglycoprotein receptor present on hepatocytes. This allowed endosomal escape and cytoplasmic delivery of siRNA. The initial construct involved covalent attachment of the siRNA to the polymer via a bio-degradable disulphide link. This was changed on subsequent iterations so that now the targeted polymer is co-injected with a liver tropic-siRNA conjugated to cholesterol (chol-siRNA) (123). In preclinical studies this approach demonstrated decreased serum AAT levels in normal healthy volunteers. In the first-in-human study this approach showed knockdown of hepatic AAT production based on observed reduction in serum AAT concentration in healthy controls and people with AATD. The study was terminated early because of toxicity findings related to the delivery vehicle seen in a non-human primate study (124).

Since then, using a different delivery construct this group has published interim results from a phase-2 open label study which hopefully will be published in the near future. Several ASO-based drugs have been approved for different disorders; their use in AATD liver disease is still at the pre-clinical stage. Another option is the use of genome editors. The CRISPR/Cas9 system introduces a DNA double strand break at a specific locus targeted by a guide RNA (gRNA) sequence. These DNA strand breaks are repaired either by nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) (125). Using this system, a single dose of a CRISPR/Cas9 with a gRNA targeting human SERPIN1 delivered by an Ad vector normalized the pathologic liver phenotypes of PiZZ transgenic mice (126). The obvious drawback of this approach is that while it may effectively decrease the liver disease in AATD it also decreases the circulating AAT levels increasing the potential risk for lung disease.

1.1.13 Therapies aimed at both lung and liver disease in AATD

Because the pathogenesis of the lung and liver disease associated with AATD are so different, a single therapeutic approach aimed at both is rare. The attractive concept and indeed the “holy grail” of AATD therapy is to increase secretion of ZAAT from hepatocytes and in doing so, increase plasma and lung concentrations of AAT while also relieving liver stress. Intravenous administration of *Salmonella typhi* produced increases in serum AAT in people with the ZZ form of AATD but that approach was not considered as a long-term therapeutic option (127). Anabolic steroids can stimulate hepatocytes to increase secretion of AAT in MM individuals but not in ZZs (128). Its use was further hampered by the associated oestrogenic side effects. Later, a synthetic androgen, danazol was evaluated in this context. Danazol did increase AAT secretion in people with the ZZ form of AATD but only by approximately 37% over pre-treatment levels (129). None of these therapeutic approaches were evaluated for their effects on AATD liver disease and may even exacerbate it. The early attempts at preventing Z polymerization in the liver using targeted peptides have not been evaluated in humans. More recent developments, such as CRISPR/Cas9 may be of benefit. In its early usage in AATD, CRISPR/Cas9 aimed at targeting SERPINA1 and decreasing production of Z AAT. However, as noted above this will not alleviate and may increase the risk for lung disease. Recent approaches have used a dual approach inducing insertion and deletion mutations by NHEJ and gene correction by HDR at the SERPINA1 locus of the PiZZ mouse. This latter correction occurs at a much lower level with about 2-5% of the liver being corrected to the normal M-AAT while insertion-deletion rates were 5 times higher (130,131). With present technology it is likely that the concentration will be on HDR efficiency for production of M protein perhaps reducing the NHEJ frequency to prevent loss of function of the targeted gene despite the fact that this will also lessen the hepato-protective elements of the approach. A novel approach pioneered by Vertex pharmaceuticals built on their work in cystic fibrosis utilising correctors of abnormal protein folding (132,133). This led to the development of correctors aimed at ZAAT with the aim of correcting Z AAT folding and increasing its secretion into the blood stream. A series of such correctors developed by Vertex have been trialled in ZZ individuals and the results are awaited. Z Factor Limited another company using folding correctors has started a phase 1 double blinded randomized placebo-controlled single ascending and repeat dose trial evaluating healthy controls and

PiMZ individuals. In a recent study, a systematic library search identified a small molecule compound (GSK716) which was able to selectively correct Z AAT misfolding, decrease polymer formation and increase serum levels in PiZZ mice (134). This study failed to show any effect on intrahepatic AAT inclusions. Other approaches have targeted hydrophobic cavities in the ZAAT with the intent to prevent polymer formation, while others have evaluated intracellular antibody fragments (intrabodies). Classically these are single chain variable fragments, one heavy and one light chain variable domain linked by a synthetic peptide. *In vitro* evaluations have shown that this approach has the ability to reduce intracellular polymerization of ZAAT and increased secretion whilst maintaining anti- protease activity (135). The use of Ataluran and gentamicin in people with stop codon forms of AATD with some effect in M AAT cells has led to enthusiasm for evaluating this approach in ZZ cells. It is felt that this approach may increase AAT secretion, not by increased read through but mainly through RNA stabilisation. This has been tested in Null condition characterised by stop codons and in M cells but never in Z cells and never in humans (136,137). These approaches all raise the cardinal question in AATD therapeutics. How much AAT secretion is required from the liver to decrease liver inflammation while at the same time providing enough AAT to protect the lung?

1.1.14 The necessary amount of AAT for protease defence in AATD

One of the major problems facing treatment of the lung disease in AATD is the uncertainty as to what the target AAT level should be. Much of the risk stratification has centred on the concept of a “putative protective threshold (PTT)” of 11 μ M (0.57g/L) in serum. The origins of the PTT are unclear. It has been variously attributed to the AAT level associated with the SS mutation and in other publications to SZ levels. In 1991 it was stated that 11 μ M approximated the lower 10th centile of the SZ-AATD range (10 to 23 μ M (138), but recent data would suggest that 11 μ M is nearer the 40th centile for SZs [IQR 9.6-13 μ M] when levels are measured in the absence of a concomitant acute phase event (28,29). The only AATD mutation that straddles the 11 μ M level is the SZ mutation. The initial NHLBI registry of AATD used 11 μ M as the level for registry qualification (38). Most studies of augmentation therapy have used this level as one to aim for and in the RAPID and RAPID extension studies which showed significant effects of augmentation therapy on lung density as measured by CT scan, 60mg/Kg once weekly could keep people above

this PTT (81,82). However, recent studies show that people with the SZ form of AATD even with levels lower than 11 μ M have no increased risk for AATD if they never smoke (28). If they ever-smoke, they have a significantly increased risk for COPD compared to MMs or MSs who smoke a similar amount. The same data holds for MZs with levels significantly higher than 11 μ M who, if never smokers have no increased risk but in the event of ever-smoking have a 10-fold increased risk of COPD compared to MMs who smoke a similar amount (27). These data would suggest that the risk of COPD is not purely a manifestation of the AAT level but is also related to the inflammatory burden, some of which can be attributable to the “gain of function” associated with Z AAT retention in hepatocytes, monocytes, neutrophils and other cells. This might also explain why therapies aimed at restoring a PTT level of AAT may decrease rate of lung tissue loss but never actually stop it. It is also clear that levels of AAT increase significantly during infection which is the body’s natural acute phase anti- inflammatory response (139). Recent shows that during SARS- CoV-2 infection, the levels of AAT in MM individuals rise, but the extent of this rise in response to IL-6 is less in those with more severe SARS-CoV-2 infection than that seen in mild SARS-CoV-2 infection or in community acquired pneumonia and a high IL-6/AAT ratio represents a poor prognostic factor (Figure 1.7, 140).

Figure 1.7

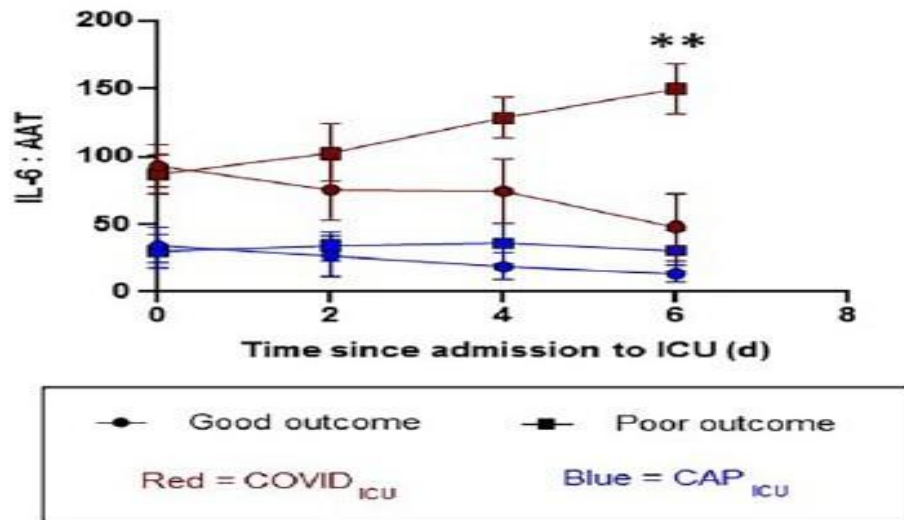


Figure 1.7

IL-6:AAT ratio as a prognostic factor in SARS-CoV-2 infection Adapted from McElvaney OJ et al. Am Jour Respir Crit Care Med. 2020 (ref 140)

1.1.15 The potential role of exogenous AAT in SARS-CoV-2 infection

These data and the previous data on a potential role for AAT in SARS-CoV-2 susceptibility have created an interest in the possible use of AAT as a treatment for SARS-CoV-2. To date there are three trials of AAT listed: NCT04547140 and NCT04495101 and another trial based in Beaumont hospital EUDRA CT number 2020-001391-15. In addition, there has been one non-randomized trial of AAT in SARS-CoV-2 infection (141). This study initially investigated the effects of AAT and other anti-proteases on SARS-CoV-2 access to human airway organoids and showed that AAT could decrease access of the virus into cells. They further showed definitively that AAT could inhibit TMPRSS2, a serine protease involved in modification of the spike protein on SARS-CoV-2 virus necessary for its interaction with the ACE2 receptor and entry into cells. Finally, they studied a small number of patients (n=9) who received AAT either by aerosol or by combined aerosol and intravenous route and showed a difference in clinical outcome compared to those receiving standard of care. It should be noted that this was a small study conducted in an open non-randomized fashion. The Beaumont hospital study (EUDRA CT number 2020-001391-15) was a randomised placebo-controlled trial of purified AAT given at a dose of 120mg/kg once weekly for four weeks or as a single dose. A potential hint towards efficacy of plasma purified intravenous AAT in this scenario is provided by a published single case report from this centre in which intravenous AAT was administered at a dose of 120mg/kg once weekly for four weeks to a person with CF (PWCF) who got SARS-CoV-2 infection (142). This PWCF was on a lung transplant list, had a tracheostomy and severely impaired lung function (forced expired volume in one second < 20%) prior to getting SARS-CoV-2 infection. The patient deteriorated rapidly and required artificial ventilation. The patient was not progressing on ventilation, but it was noted that the patient's BAL contained large amounts of NE and pro-inflammatory cytokines including IL1- β , IL-8, IL-6 and TNF α . This patient also showed systemic cytokinemia with increased levels of IL1- β , IL-8, IL-6 and TNF α . As a result and given the emerging data on a possible anti-inflammatory role for AAT in SARS-CoV-2 ARDS intravenous AAT was administered at the doses stated. Aerosol delivery was not considered due to the possibility of SARS-CoV-2 aerosol generation in an ICU setting. The patient recovered as evidenced not only by ventilator parameters but also by a very significant fall in inflammatory indices in plasma and BAL. In plasma these included significant drops

in IL-8, IL1- β , IL-6, TNF α and in addition in the lung, NE also fell significantly (142). One of the major questions raised by this series of evaluations was whether the PWCF was just getting a severe CF exacerbation unrelated to SARS-CoV-2 infection and that the main action of the intravenous AAT was on NE and not indicative of a particular anti-inflammatory anti-SARS-CoV-2 effect of AAT. This patient had been closely evaluated during a previous CF exacerbation and the levels of the inflammatory cytokines in blood and sputum at that time were significantly less than during the SARS-CoV-2 infection (142).

Figure 1.8

Inflammatory cytokines and neutrophil elastase activity levels relative to baseline.

Inflammatory mediators	Baseline	Most recent severe IECF*	COVID-19†
Blood cytokines (pg/ml)			
IL-1 β	12.1	20.7	141.4
IL-6	63.8	97.4	571.3
IL-8	88.3	119.9	238.7
sTNFR1	2870.9	3615.1	4092.5
Airway cytokines (pg/ml)‡			
IL-1 β	202.6	299.0	374.6
IL-6	104.7	198.4	633.9
IL-8	492.2	658.3	788.1
Airway NE activity (nM)‡	741.9	905.6	1298.8

* Also required intensive care unit admission.

† At time of admission to intensive care unit.

‡ Measured in bronchoalveolar lavage fluid IECF – infective exacerbation of cystic fibrosis IL – interleukins TNFR1 – soluble tumor necrosis factor receptor 1.

Figure 1.8

Cytokine and NE levels in plasma and airway secretions during SARS-CoV-2 ARDS and during prior CF exacerbation in PWCF (142)

These data suggested that a single once weekly AAT dose strategy would not replicate what happens in “real life” and would need to be increased particularly during times of infection/inflammation. Furthermore, the relevance of the protective threshold is challenged during times of infection and inflammation, specifically as high circulating levels of AAT as found in this PWCF did not reflect the severe inflammation and protease imbalance in the lungs. This is also shown by the inflammatory processes surrounding panniculitis. In this relatively rare complication of AATD, which can occur in most AATD mutations, albeit more commonly in ZZs, the response to intravenous AAT is striking but usually requires quite high levels of intravenous AAT (often in excess of 120mg/Kg), which may need replenishing. This was further highlighted during the COVID-19 pandemic, with healthy MM individuals producing 50-70 μ M of AAT in their acute responses (140). In these circumstances aiming for a level of 11 μ M would seem totally insufficient.

1.1.16 Aims of thesis

The ideal treatment for AATD should be one which simultaneously treats both lung and liver disease, moving AAT out of the liver into the circulation and lung with the ability to mount an acute phase response in times of inflammation. In this thesis, the feasibility of this option is outlined. In this introductory chapter 1, the present state of knowledge regarding the pathogenesis and natural history of AATD lung and liver disease is described along with the treatment options available as well as the potential use of AAT outside of AATD, including but not limited to SARS-CoV-2 infection. In chapter 2, the methodologies employed are described. In chapter 3, the *in vitro* AATD models are outlined specifically concentrating on transformed HEK-293 cells which have been modified to produce M and Z AAT, and HepG2 cell lines, one of which has been modified to produce Z AAT in contrast to the regular HepG2 cell lines which produce M AAT. Their advantages and disadvantages of these cell lines are outlined.

In chapters 4, the normal AAT acute phase reaction in blood and lung is evaluated utilising an MM population with SARS-CoV-2 acute respiratory distress syndrome (ARDS). This provided valuable insights into the potential therapeutic option of AAT administration to people with SARS-CoV-2 ARDS and directly led to a phase 2, multicentre, randomized, double-blind, placebo-controlled trial of intravenous

plasma-purified AAT (Prolastin, Grifols, S.A.) for ARDS secondary to SARS-CoV-2 infection, the results of which are presented in chapter 4. To further determine the AAT acute phase response, we also accessed a cohort of healthy individuals exposed to intravenous lipopolysaccharide, thereby simulating a bacterial response and giving further insight into how much gentamicin would be required to increase AAT secretion out of the cells to produce a standard acute phase response.

In Chapter 5, it was determined to evaluate MM and ZZ individuals' pre and post SARS-COV-2 vaccination. This was due to the small number of individuals with AATD presenting directly to our hospital with SARS-CoV-2 infection, with the attendant difficulty of assessing *in vivo* the acute phase AAT response in these individuals. This provided an opportunity to measure AAT levels in AATD patients pre and post an inflammatory insult and compare it to MM individuals receiving the same vaccination.

By answering these questions, we are confident that this thesis will contribute significantly to the body of knowledge regarding AAT deficiency, the therapeutic potential of AAT outside of AATD, the utility of *in vitro* models, the target levels of secreted AAT to aim for at baseline and during infection, and the potential downsides of increasing AATD production in Z AATD cells.

Chapter 2: Methodology

2.1 Materials:

2.1.1 Chemicals and Reagents

All chemicals and reagents used in this study were of the highest quality available and purchased from Sigma Aldrich (Dublin, Ireland) unless otherwise indicated.

2.1.2 Antibodies

The primary antibodies and secondary antibodies used for western blot analysis and immunohistochemical studies (IHC) are listed in tables 2.1-2.4

Table 2.1
Primary antibodies for Western blot analysis

Primary Antibody	Source	Manufacturer	Cat. No.	Working dilution
AAT	Sheep	Abcam	GR286990-3	1:2000
Beta-actin	Mouse	Santa Cruz Biotechnology	CO916	1:1000
Anti-hELA2	Mouse	R&D systems	MAB91671	1:500
SLPI	Goat	R&D systems	AF1274	1:2000

Table 2.2
Secondary antibodies for Western blot analysis

Secondary Antibody	Source	Manufacturer	Cat. No.	Working dilution
Anti-mouse IgG ₁ HRP-Linked Antibody	Mouse	Cell Signaling Technologies	7076S	1:1000
Mouse anti-goat IgG1 HRP- linked Antibody	Mouse	Santa Cruz Biotechnology	SC2020	1:1000

Table 2.3
Primary antibodies for immunohistochemical studies

Primary Antibody	Source	Manufacturer	Cat. No.	Working dilution
AAT	Rabbit	DAKO	A0012	1:800
Citrullinated histone H3	Rabbit	Abcam	ab5103	1:2000
Anti-hELA2	Mouse	R&D systems	MAB91671	1:500
SLPI	Mouse	Abcam	ab17157	1:4000

Table 2.4**Secondary antibodies for immunohistochemical studies**

Secondary Antibody	Source	Manufacturer	Cat. No.	Working dilution
Donkey Anti-MouseIgG H&L (Alexa Fluor® 647)	Donkey	Abcam	ab150107	1:200
Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Donkey	Abcam	ab150073	1:1000
Donkey Anti-Mouse IgG H&L (Alexa Fluor® 555) preabsorbed ab150110	Donkey	Abcam	ab150110	1:1000
Donkey Anti-MouseIgG H&L (Alexa Fluor® 647) preabsorbed	Donkey	Abcam	ab150111	1:1000

2.1.3 Florescence resonance energy transfer (FRET) materials

All FRET materials, including FRET substrate are included in the table 2.5

Table 2.5
Florescence resonance energy transfer (FRET) materials

Code & Company	Material
D4254, Sigma	DMF (N,N-dimethylformamide)
3230-v, Peptide Institute, Inc. 1mg Abz-Ala-Pro-Glu-Glu-II-Met-Arg-Arg-Gln-EDDnp MW = 1457 g/mol	FRET substrate for Neutrophil Elastase
TS563, Elastin Products 0.1 mg	Neutrophil elastase
Grifols	Human, plasma purified AAT

2.2 Methods

2.2.1 Patient selection and sampling

Patients from Beaumont hospital, with a laboratory confirmed SARS-CoV-2 diagnosis (via reverse transcriptase–polymerase chain reaction (RT-PCR) assay of nasopharyngeal swab specimens) requiring ICU admission for intubation and mechanical ventilation for hypoxemic respiratory failure were studied (SARS-CoV-2 ICU, n=20) (Chapter 4, Table 4.1, 4.2). Sample size was based on standard cut-offs of 0.05 for type I error and 0.2 for type II error, following observational studies of patients with non-SARS-CoV-2 ARDS (143). However, unfamiliarity with the potential breadth of presentations and outcomes in SARS-CoV-2 infection led to our decision to exceed requirements and recruit 20 patients.

Blood samples from the ICU cohort were collected by venepuncture into lithium heparin tubes on admission to ICU and every 2nd day following admission. Plasma was isolated from blood by centrifugation at 350 g for 10 mins. The same plasma collection techniques and inclusion criteria were used for the phase 2, multicentre, randomized, double-blind, placebo-controlled trial of intravenous plasma-purified AAT (Prolastin, Grifols, S.A.) for moderate-to-severe ARDS secondary to SARS-CoV-2 infection (Chapter 4, Table 4.3). nsARDS patients selected for tracheal aspirates (TA) samples (Chapter 4, n=6, Table 4.4) were chosen on the basis of a negative RT-PCR of a nasopharyngeal swab and moderate to severe ARDS as per BERLIN criteria, TA samples were collected aseptically, by opening the closed suction device, inserting sterile suction tubing and pulling back by no more than 37 cm to obtain an adequate sample. 8 plasma samples were collected from patients enrolled on the placebo arm of the KARE trial. TA samples from SARS-CoV-2 ARDS patients were obtained 1 day post intubation (n=15) (Chapter 4, Table 4.5), 10 from the University of North Carolina (UNC), 5 from Beaumont hospital. All of the TA control group samples from non-SARS ARDS (nsARDS) patients were also obtained 1 day post intubation (n=6). All sampled patients were selected at random from a list of medical record numbers, a computer-generated series of random numbers, corresponding to patients with a confirmed infected status. Patients were excluded if they were immunosuppressed, on long-term oral corticosteroids, anti-IL-1, anti-IL-6, or anti-TNF therapy, were pregnant, had active neoplasia, or a history of vasculitis or connective tissue disease. Experiments performed on the samples were undertaken by a blinded investigator who were excluded from the collection of samples and the

patient's clinical care. In ward patients with mild SARS-CoV-2 (n=24), plasma samples were taken pre- and 28 days post tocilizumab infusion (Chapter 4, Table 4.6). Plasma was also collected from healthy donors (n=11) (Chapter 4, table 4.7). Healthy, vaccinated ZZ AATD individuals were selected from the Irish AATD registry (n=19) and plasma samples from healthy individuals dosed with IV LPS were provided by Prof Simpson/Newcastle University and matched according to age and sex (n=12). All experiments involving patient samples were replicated a minimum of 3 times however the total number of samples taken varied depending on potential discharge or death of a patient.

2.2.2 Cell Culture

HEK-293 cell line

HEK 293 cells transfected with a dual reporter system using lentiviral vectors to express M and Z-AAT were kindly provided by colleagues at the Centre for Regenerative Medicine, Boston University. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) in combination with 10% (v/v) foetal calf serum (FCS), 1% (v/v) L-Glutamine and 0.2% (v/v) Primocin (InVivoGen). These cells were seeded in T-75 flasks at a density of 2.1×10^6 . When the cells reached 80% confluency the media was removed and discarded, the cells were then washed with phosphate buffered saline (DPBS) before detaching the adherent cells by adding 10x TryPLE (recombinant dissociation enzymes which cleave peptide bonds on the C-terminal sides of lysine and arginine) and incubating at 37°C for 5 min. The detached cells suspended in the TryPLE mixture were then transferred into a 15ml test tube with 10ml of fresh media and centrifuged at 300 x g for 5 min. A cell pellet formed at the bottom of the 15 ml tube and the residual media was discarded. The cell pellet was re-suspended in 1ml of fresh media. The cells were then assessed for viability. (136)

HEPG2 cell line

HepG2 cells (including Z-CRISPR-edited HepG2 cells) were cultured in Eagle's Modified Essential Medium (EMEM) supplemented with 10% (w/v) Foetal Calf Serum (FCS) and 0.2% (v/v) Primocin (InVivoGen). These cells were seeded in T-75 flasks at a density of 2.1×10^6 . When the cells have reached 80% confluency the media was removed and discarded, the cells were then washed with phosphate buffered saline (DPBS) before detaching the adherent cells by adding 10x TryPLE and

incubating at 37°C for 5 min. The detached cells suspended in the TryPLE mixture were then transferred into a 15ml test tube with 10ml of fresh media and centrifuged at 300 x g for 5 min. A cell pellet formed at the bottom of the 15 ml tube and the residual media was discarded. The cell pellet was re-suspended in 1ml of fresh media. The cells were evaluated for viability and seeded into 12 well plates for further experiments.

2.2.3 Trypan blue exclusion test for viability of cells

The cells were evaluated for viability, by the trypan blue exclusion method, and counted. 10µl of the cell suspension was added to an aliquot of 90µl of trypan blue, loaded onto a haemocytometer and viewed microscopically at 20 X magnification.

2.2.4 Cell line treatments

HEK-293 cell treatments

A 12 well plate, containing 2×10^5 cells/well in 1ml DMEM, was incubated overnight at 37°C, after which fresh media supplemented with HEK-293 cells were stimulated with FCS replete media supplemented with 0.5µg/ml of gentamicin.

HEPG2 cell treatments

HepG2 cells were stimulated with IL-6 (180pg/ml) (Abcam) for 6 to 36 hours to induce AAT expression. A 12 well plate, containing 2×10^5 cells/well in 1ml EMEM, was incubated overnight at 37°C, following which fresh media supplemented with IL-6 (180pg/ml) +/- Tocilizumab (50µg/ml, Roche) was added. Plasma sourced from patients with SARS-CoV-2 or from nsARDS patients on the first day of their ICU admission was used for stimulation of AAT production in HepG2 cells. A 24 well plate format was used, incubating 8×10^4 cells per well in 500µl of EMEM per well (overnight at 37°C), following which media was removed and replaced with 250µl of plasma from six SARS-CoV-2 ICU patients +/- Tocilizumab (50µg/ml) or from nsARDS patients +/- Tocilizumab (50µg/ml).

2.2.5 HEK-293 and HEPG2 cell line seeding and harvesting

Cells were seeded at 2×10^5 in triplicate in 12 well plates and allowed to adhere for 24 hrs. The media was then replaced with fresh media (+/- cell treatment for an experiment) to create our new time point zero and sampled at various time points that followed. The adherent cells were then washed with PBS before detaching them with trypLE, with this as our standard protocol we removed cell supernatant and stored at -80°C and the cells were washed x 3 in DPBS. TrypLe (500 μL) was added along with 500 μL of fresh media and then the cells were incubated for 5 min at 37°C . The cell suspension was added to 7 ml of fresh media in 15 ml tubes and centrifuged at $1000 \times g$ for 5 min, the media was removed, and the pellet was re-suspended in 1 ml PBS, the cells were counted using the method outlined above. The cell suspension was centrifuged again at $1500 \times g$ for 5 min at room temperature and the DPBS removed. Lysis buffer (300 μL) was added for every 1×10^7 cells (Lysis buffer contained; 10mM Tris HCL pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS containing additional protease inhibitors; 10 $\mu\text{g}/\text{ml}$ Na-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 1 $\mu\text{g}/\text{ml}$ phenylmethane sulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ pepstatin A and 10 $\mu\text{g}/\text{ml}$ leupeptin.) The lysed cell solution was then spun at $1000 \times g$ for 5 min to remove cellular debris (14). The lysed cells were used for further analysis via western blot analysis. Designated wells were reserved for mRNA extraction, after the supernatant was removed from the 12 well plates at the indicated timepoints RNA from transfected HEK cells was isolated with TRI reagent (1ml of Trizol to every 35mm well- in this case 250 μL to each well) (144).

2.2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

AAT in cell supernatant was measured using Human serpin A1 DuoSet ELISA (R&D systems). The recombinant Human Serpin A 1 standard was reconstituted with 0.5mL of Reagent Diluent (5% Tween in PBS, pH 7.2-7.4 filtered). A seven-point standard curve was created using a 2 fold serial dilution. The capture antibody (reconstituted in PBS) was diluted to its working concentration in PBS without carrier protein. A 96 well microplate was coated with 100 μL per well. The plate was sealed and agitated on a rocker overnight. Each well was then aspirated and washed with Wash buffer (0.05% tween in PBS) three times, and the ELISA plate was tapped on paper to remove any remaining droplets. The plate was then blocked with 300 μL of

reagent diluent per well and incubated at room temperature for 1 h, with a repeat aspiration step followed by standard and sample addition; 100µl of sample/standard per well was covered with an adhesive cover slip and incubated at room temperature for a further 2 h. The aspiration step was repeated, and the plate was moved to a dark room for the light sensitive stage of the experiment. Streptavidin-HRP was added to each well, which were then covered in an adhesive seal and wrapped in tin foil allowing it to incubate at room temperature for 20-25 min. The wells were then aspirated and 100µl of substrate solution was added to each well with careful attention to observe any change in the colour of the samples. Once this change had been noted throughout the standards, 50µl of stop solution (9.8 ml of deionised H₂O and 167µl of H₂SO₄) was added and the plate tapped to ensure mixing. The optical density of each well was determined using a microplate reader (SpectraMax M3) set to 540-570nm. IL-6 in cell supernatant or patient plasma was measured using Human IL-6 Quantikine ELISA (R&D systems). The human IL-6 standard was reconstituted with calibrator diluent RD6F for patient plasma and with RD5T calibrator diluent for cell supernatant samples. A 12-point standard curve was created using a 2-fold serial dilution. 100µl of assay diluent was added the pre-coated 96 well microplate which followed by standard and sample addition; 100µl of sample/standard per well was covered with an adhesive cover slip and incubated at room temperature for a further 2 h. Each well was then aspirated and washed with Wash buffer (0.05% tween in PBS) three times, and the ELISA plate was tapped on paper to remove any remaining droplets. 200µl of IL-6 conjugate was added to each well and allowed to incubate for a further 2 h. The aspiration step was repeated and the plate was moved to a dark room for the light sensitive stage of the experiment. 200µl of substrate was added to each well, which were then covered in an adhesive seal and wrapped in tin foil allowing it to incubate at room temperature for 20-25 min with careful attention to observe any change in the colour of the samples. Once this change had been noted throughout the standards, 50µl of stop solution was added and the plate tapped to ensure mixing. The optical density of each well was determined using a microplate reader (SpectraMax M3) set to 540-570nm.

2.2.7 Bicinchoninic acid (BCA) assay

Prior to performing western blot analysis on cell lysates, the amount of protein per sample was quantified by performing a BCA assay. 10 µl of standards and sample were added to the allocated wells on a 96 well plate. Reagents A and B (Thermo scientific, USA) were mixed and added to the samples and standards. The plate was then sealed and wrapped in tinfoil and incubated for 30 min after which the plate was read on the microplate reader (SpectraMax M3) at 550nm. The concentration of the samples was colorimetrically assessed based on a standard curve of known BSA protein concentrations (3000mg/ml - 0.00 mg/ml) (136).

2.2.8 SDS-PAGE gel

Samples, either cell lysates or supernatants were treated with 10x sample buffer (0.2% (w/v) bromophenol blue, 50% (w/v) sucrose, 1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) dithiothritol, 200mM EDTA 3M Tris-HCL pH 6.7) and then heated at 99°C for 2 min. The running gel was prepared using the following materials: 6ml of 1.5 M Tris pH 8.9, 7.6 ml of deionised H₂O (dH₂O), 10ml of protogel (National Diagnostics, USA), 240µl of 1% (w/v) SDS and finally added 6µl of Temed and 150µl Ammonium persulphate (APS) (stock solution: 0.1g in 1ml of dH₂O) as the last step. The gel was poured between the two glass plates and propanol was poured on top of the running gel and allowed to set, after which all excess propanol was poured off. This was followed by the addition of the stacking gel which was prepared with the following components: 630µl 0.5M Tris pH 6.8, 3.4ml dH₂O, 830µl protogel, 50 µl 1% (w/v) SDS and again at the final step before pouring 5µl of Temed and 50µl of APS was added. This was poured on top of the running gel, a comb was then inserted into the stacking gel and it was allowed to set. Once the gel was fully set, it was inserted into the protein electrophoresis rig and firmly secured. 1X running buffer (6g Tris (pH 8.9), 28.8g Glycin and 2g SDS and 200ml of deionised H₂O) was poured into the rig and the protein molecular weight ladder and samples/sample buffer mix were added to each well. The rig was then connected to a power box and set to 80 volts until the sample passed, the stacking gel at which point the voltage was increased up to 125 volts. After electrophoresis of the gel, the gels were stained in order to visualise the protein banding patterns using Coomassie blue stain (10% (v/v) acetic acid 45% (v/v) methanol, 45% H₂O, 0.2% (w/v) Coomassie brilliant blue) and subsequently de-staining to help visualise more

defined bands with Coomassie de-stain (10% (v/v) acetic acid, 25% (v/v) methanol, 65% H₂O)(136)

2.2.9 Western Blot Analysis

Western blot analysis was performed for specific proteins following the transfer of the gel onto PVDF membrane. The gel and the membrane were sandwiched between two sponges and two pieces of wattman paper soaked in Transfer buffer (14.4 g of glycine, 3.03g of Tris, 800 ml of dH₂O and 200ml of methanol) and placed into a transfer rig, which was filled with transfer buffer and an ice pack. A small magnetic stirrer was placed into the rig to keep it from overheating. The rig was then placed into the 4°C fridge, 33 volts were passed through the rig overnight. The membrane was then removed, reactivated with methanol, and washed. The membrane was blocked using Blocking buffer; 1g bovine serum albumin (BSA), 3g powdered milk and 100ml of PBS-Tween (500µl of Tween-20 per 1l of PBS). After the blocking stage the membrane was placed into a 50ml falcon tube with 6ml of Blocking buffer with the appropriate primary antibody (Table 2.1 AAT (1:2000 GR286990-3 Abcam), NE (1:1000 MAB91671 R&D systems) and SLPI (1:2000 AF1274 R&D systems) for 1h. Then if necessary probed with a secondary HRP-linked antibody post transfer (NE 1:1000 7076S Cell signalling technology and SLPI 1:1000 SC2020 Lot no. E 1313 santa Cruz biotechnology) The membrane was treated with immobilon western chemiluminescent HRP substrate. A single channel chemi-blot protocol was then entered into the ChemiDoc TM MP imaging system. After treating the membrane with chemiluminescent HRP substrate and developing the image of the membrane, it was then washed for 30 min in PBS-Tween and then re-probed for β -actin. The membrane was put back into Blocking buffer and exposed to the primary antibody (β - actin mouse monoclonal IgG C4 sc-47778). After this step, the membrane was again washed with PBS-Tween and transferred into anti mouse IgG HRP linked secondary antibody (136). The anti-actin membrane was then washed and developed as already described. Densitometry was performed on the western image using Image-lab software, the information was exported to the analysis table that can be opened as an excel file.

2.2.10 RNA isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After the supernatant was removed from the 12 well plates at the indicated timepoints RNA from transfected HEK-293 or HEPG2 cells was isolated with TRI reagent and chloroform (0.2µl per 1ml of Trizol) before being centrifuged at 12,000 x g for 15min at 4°C. Propranolol (125µl per .5ml of Tri reagent) was then added, following centrifugation the pellet was washed with 75% (v/v) ethanol, dried and finally re-suspended in RNA free water. Isolated RNA concentration was calculated using the Nanodrop 8000 spectrophotometer (Thermo scientific, Ireland). The RNA was stored in the -80°C for later use or it was converted to cDNA. Genomic DNA (gDNA) wipe from the QuantiTect reverse Transcription kit (Qiagen) was used to remove any contaminating gDNA and the RNA sample was heated at 42°C for 2 min then 1µl of RTase, 20µl of reverse transcriptase buffer and 1µl of Reverse Transcriptase primer mix were added to each 2µl sample to reverse transcribe RNA to cDNA. cDNA was then prepared on a PTC-200 thermocycler. cDNA was then subjected to 45 cycles of amplification for quantitative real-time polymerase chain reaction (qPCR) using the Master I LightCycler® 480 Kit (Roche) and specific primers targeting AAT (forward primer: 5'- ATGCTGCCCAGAAGACAGATA-3', reverse primer: 5'- CTGAAGGCGAACTCAGCCA-3') and ST6GAL1 Forward: 5'- AGG TGT GCT GTT GTG TCG -3' Reverse: 5'- TGT TGG AAG TTG GCT GTG G -3'). Analyses were performed on a LightCycler 480® instrument (Roche) and gene expression calculated with the $2^{-\Delta\Delta C_t}$ method using GAPDH as the reference gene (Forward: 5'- CAT GAG AAG TAT GAC AAC AGC CT -3' Reverse: 5'- AGT CCT TCC ACG ATA CCA AAG T -3') (144).

2.2.11 Fast Protein Liquid chromatography

M AAT or Z AAT was purified from plasma by fast protein liquid chromatography (FPLC) utilising Alpha-1 Antitrypsin Select Resin packed onto a Tricorn column and chromatographed on a ÄKTA prime plus. The plasma was then diluted with Buffer A (20mM of Tris, 150mM of NaCl pH 7.4) at a ratio of 1:3 and then loaded onto the column at a flow rate of 1.5ml/min. The bound AAT was eluted from the resin with a gradient from 0-100% Buffer B (2M MgCl₂) over 20ml. The AAT yield was determined by BCA assay and the presence of AAT was confirmed by Western blot

analysis (132). The AAT fractions collected were concentrated using Amicon Ultra centrifugal filters (Milipore) and then desalted into PBS using NAPTM-10 desalting columns (GE healthcare life sciences).

2.2.12 Batch Method

The batch method of plasma purification uses the same elution and binding buffer as in the FPLC method, however it does not have the same advantages of the FPLC such as two high precision pumps, a control unit, a column to contain the AAT select resin, a detection system and fractionator. This is a more manual procedure than FPLC. Another significant difference is the use of an AAT antibody resin, (stored in a 1:1 mix of resin and 20%(v/v) ethanol) which is not reusable. 400µl of M AAT plasma was made up to a 1:3 dilution with Binding buffer, the plasma was then filtered, and centrifuged at 10,000 x g for 5min. 200µl of the resin was washed by centrifuging at 500 x g for 1 min. The supernatant was then removed. It was then re-suspended in 1ml of Binding buffer, by vortexing. The plasma samples were added to their respective tubes gently inverted and incubated for 1 h at room temperature while being constantly agitated. This was then centrifuged again at 500 x g for 1 min and unbound supernatant was collected. The resin was then washed x 10 with Binding buffer, 500µl of Elution buffer was added and incubated for 2-5 min at room temperature, the supernatant was then collected and concentrated using Amicon Ultra centrifugal filters and then using NAPTM-10 desalted into PBS.

2.2.13 Isoelectric focusing (IEF)

IEF for AAT phenotyping utilises the Sebia Hydrays (Hydragel 18 A1AT). Plasma samples were diluted 1:10. The Hydrays system was set to a high voltage while performing isofocusing. Two sponges, one soaked in anodic and another in cathodic solution were placed onto the electrode carrier. Samples and standards were loaded onto the applicator while ethylene glycol was added to the temperature control plate and the agarose plate was then laid on top of the plate. The first migration step was 1h. After this step anti-serum (40µl A1AT-PER and 300µl of antiserum diluent) is spread over the agarose gel. Immunofixation was carried out over 10 min followed by repeated interspersed drying, washing and re-hydrating steps. wash. After washing a visualisation solution (4ml tetraiafulvalen (TFT) -1,2 solvent 100µl of TFT1 and TFT 2 and 4µl 30% (v/v) hydrogen peroxide) was added for 10 min and

then removed. The gel was then blotted and dried prior to the final washing and processing stage (136).

2.2.14 Sialylation of AAT

To analyse AAT sialylation in SARS-CoV-2 plasma, AAT was first isolated from plasma samples by use of an Alpha-1 Antitrypsin Select Resin packed into a Tricorn column as previously described. Samples were then chromatographed by fast protein liquid chromatography (FPLC) on an AKTA instrument equipped with UV detector and an automatic fraction collector. Fractions containing AAT were identified using UV profile and BCA protein assay kit (Thermo Fisher # 23225), pooled, and desalted into PBS using NAP-10 desalting columns (GE Healthcare # 17085402). The plasma samples were then denatured by incubation at 100°C with 1% (w/v) SDS and 1% (w/v) DTT before incubation overnight with PNGase F [LZ-rPNGaseF-kit] in NP-40 buffered solution to release N-glycans. The released glycans were then converted to aldoses with 0.1% formic acid, filtered through a protein binding membrane [LC- PBM-96] and dried. N-glycans released by PNGase F were labelled with procainamide. Procainamide labelled samples were analysed by HILIC-UPLC using an ACQUITY UPLC® BEH-Glycan 1.7 µm, 2.1 x 150 mm column at 40 °C on an ACQUITY UPLC H Class instrument with a fluorescence detector (λ_{ex} = 310 nm, λ_{em} = 370) controlled by Empower software version 3, build 3471, to produce % sialylation profiles on the SARS-CoV-2 samples as compared to healthy controls. (Ludger Ltd.).

2.2.15 AAT and NE activity analysis

SARS-CoV-2 patient plasma was used to evaluate SARS-CoV-2 patient AAT-mediated neutrophil elastase (NE) inhibition compared to non-SARS-CoV-2 ARDS. AAT was incubated at various concentrations with NE for 20 min at 37°C, added to a 96 well plate, and a solution of 2nM of FRET substrate Abz-Ala-Pro-Glu-Glu-Ile- Met-Arg-Arg-Gln-EDDnp (Peptide Inc. Osaka, Japan) added. Upon addition, the kinetic reactions were immediately measured at 37°C over 20 min with readings occurring every 20 seconds in a Spectra Max M3 microplate reader (Molecular Devices, Berkshire, UK). NE activity in SARS-CoV-2 TAs and TA sample NE inhibition activity by AAT was measured using a similar protocol. A seven-point standard beginning at 100nM of NE with further two-fold serial dilutions was used. Untreated TA samples were added to the plate with and without AAT (Prolastin 25mg/ml), a 2nM FRET

substrate Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln- EDDnp solution added, and kinetic reactions immediately measured at 37°C over 20 min with readings occurring every 20 seconds in a Spectra Max M3 microplate reader (Molecular Devices) (145).

2.2.16 Monocyte isolation

Using lithium heparin blood tubes whole blood was extracted from the patient. 10mL of whole blood was placed in a sterile 50 ml tube. A volume of whole blood was added to an equal volume of 1X saline (10ml:10ml). 10mL lymphoprep (STEMCELL technologies) was placed in a new sterile 50 ml tube then 20mL of blood-saline mix was carefully layered on top, using a 10ml pipette. The mixture was then centrifuged at 800 g for 10 min at room temperature, with the brake off. After centrifugation, a bright white cellular band at the plasma/lymphoprep interface was evident. Carefully this band was removed using a 1ml pipette and placed into a fresh 15mL tube. 5-10ml PBS was then added to the 15ml tube and centrifuged again at 500 x g for 5 min with the brake on to form a pellet. The supernatant was removed with a 10ml/Pasteur pipette, with care not to disturb the pellet, which was then re-suspended in 1ml EasySep Recommended Medium (PBS free of Calcium or magnesium, 2% FCS and 1mM EDTA STEMCELL technologies) and agitated gently. The 1ml suspension was transferred into a polystyrene falcon tube. After this 100uL of the CD 14 selection solution was added and the tube was agitated gently to mix. This mixture rested at room temperature for 10 minutes. At the 9-minute mark, the CD14 kit microsphere solution was vortexed and subsequently 100uL of the microsphere solution was added to the solution. After a further 3 minutes of resting the mixture was topped up to 2.5mL with the EasySep Recommended Medium (STEMCELL technologies). At this point the tube was placed inside the EasySep magnet for another 3 minutes. While the tube was within the magnet, the tube was inverted quickly so that the waste products are disposed of. Afterwards the tube was topped up to 2.5mL with the EasySep Recommended Medium and repeated 2 more times. At this point only monocytes were left inside the tube, which can be counted via haemocytometer (with trypan blue exclusion) and lysed accordingly for further study via Western blot analysis (146).

2.2.17 IHC in Non-disease Control and SARS-CoV-2 Autopsy Lungs

Non-disease control human lungs from individuals without a history of pulmonary disease and size mismatch unsuitable for transplantation were provided by the University of North Carolina (UNC) Tissue Procurement and Cell Culture Core (protocol #03-1396). SARS-CoV2 infected autopsy lungs (no hospital admission, no intubation) were provided by Dr Ross. E. Zumwalt (University of New Mexico, Albuquerque, NM), Edana Stroberg (Office of the Chief Medical Examiner, Oklahoma City, OK). Dr. Alain Borczuk (Weill Cornell Medicine, New York, NY) and Dr Leigh B. Thorne (University of North Carolina at Chapel Hill, Chapel Hill, NC). Immunohistochemistry was performed according to protocols as previously described (147). To probe for specific proteins, primary antibodies for SLPI (1:4000, ab17157, abcam), AAT (1:800, A0012, DAKO RRID), NE (1:100, MAB91671, R&D systems) and Citrullinated histone H3 (1:2000, ab5103, abcam) were used in 4% normal donkey serum in PBST (phosphate-buffered saline containing 0.1% Triton X-100). Secondary antibodies included biotinylated donkey anti-mouse IgG (1:200); Alexa Fluor 488 donkey anti-rabbit IgG (1:1000) RRID; Alexa Fluor 555 donkey anti-mouse IgG (1:1000) and Alexa Fluor 647 donkey anti-mouse/rabbit IgG (1:1000 dilution). For the immunofluorescence study, the Vector® TrueVIEW Autofluorescence Quenching Kit (Vector laboratories) was used to reduce background staining. Cover slipped slides were scanned using the following settings: Olympus VS200 whole slide scanner microscope with a 60X 1.4 NA objective, an Olympus confocal microscope with a 20X 0.75 NA / 60X 1.4 NA objective, or a Leica Stellaris5 confocal microscope with a 20X 0.75 NA / 63X 1.4 NA objective.

2.2.18 Statistical analysis

Statistical analysis was performed in Prism (v9.2, Graphpad). Results were presented as absolute numbers, means \pm standard deviation or as percentages as appropriate. Comparisons between two groups with normally distributed data utilized paired t tests. For comparisons of three or more groups, ANOVA was used. Multiple comparisons across multiple groups were by 2-way ANOVA, P values derived by Tukeys' post-hoc multiple comparison test. A value of $P < 0.05$ was considered statistically significant. Normality of data was tested using both the D'Agostino & Pearson test (sample size allowing) and Shapiro-Wilk test and was confirmed for each group. Multinomial logistic regression (R version 4.1.2) was used to assess the

association of the potential disease modifying or confounding factors, age, sex and AAT level at recruitment, with patient cohort and likelihood ratio testing was used to determine interaction between factors.

2.2.19 Ethics

Ethical approval for patient sampling was in accordance with the Declaration of Helsinki, Good Clinical Practices. Ethical approval for SARS-CoV-2 and nsARDS ICU patient plasma and TA sampling was received from the Beaumont Hospital Ethics Committee REC #18/52, #17/06 (amended specifically for SARS-CoV-2 ICU subjects). Further nsARDS patient samples were sourced from the placebo arm of the KARE trial (148).

Ethical approval for ward level patients treated with tocilizumab was approved by the St. Vincent's Healthcare Group Research Ethics Committee. TA samples from SARS-CoV-2 patients were obtained with informed consent and approval by the University of North Carolina Office of Human Research Ethics, (IRB 20-0822) Ethical approval for evaluation of ZZ patients and healthy control MM individuals was also received from the Beaumont Hospital Ethics Committee (BHEC, REC #20/38). The comparison group of healthy individuals dosed with LPS received ethical approval from the Newcastle & North Tyneside 2 Research Ethics Committee of the NHS Health Research Authority (REC reference number 12/NE/0196). Regarding consent, direct explicit consent was obtained in all those able to provide it, however in the case of intubated patients assent on their behalf by the next of kin and in the cases of the patient recovering delayed consent was obtained.

Chapter 3: The mechanism of Alpha-1 antitrypsin secretion *in vitro*

3.1 Introduction

The most common mutation causing Alpha-1 antitrypsin (AAT) deficiency (AATD) is the Z mutation. This accounts for >90% of severe deficiency of AAT. This mutation results from a single amino acid substitution resulting in a glutamic acid to lysine shift at residue 342. The consequence of this is disruption of salt bridges and polymerization of the Z protein in the liver, causing liver inflammation and decreased release of AAT into the circulation and lungs. This leads to liver disease due to endoplasmic reticulum (ER) stress and lung disease secondary to impaired protection against proteolytic damage to the delicate tissue of the airways (149). As discussed in the introduction chapter, the current mainstay of treatment for the lung manifestations of AATD is plasma purified AAT given intravenously once weekly at a dose of 60mg/kg. This approach has been shown to slow loss of lung tissue as evaluated by CT scan lung density measurements during the recent RAPID and RAPID OLE trials (81,82) but has no effect on the liver disease associated with AATD. Indeed, with regards treatment for AATD liver disease, transplantation is the only presently available option (150). With this as background, one potential novel treatment option for the Z form of AATD is to increase the secretion of AAT from the liver thus removing its toxic effect on the liver and at the same time increasing plasma levels of AAT to improve the anti-protease defence elsewhere in the body. A recent study from our laboratory has shown that in severe AATD due to a mutation caused by stop codons, secretion of AAT can be increased *in vitro* using read-through compounds such as ataluren and aminoglycosides such as gentamicin (136).

Aminoglycosides are antibiotics which have a 2-deoxystreptamine ring linked to multiple amino sugars (151). They bind to the bacterial ribosomal decoding centre which leads to mis-incorporation of near-cognate aminoacyl-tRNAs at both sense and stop codons, resulting in translational misreading and inhibition of protein synthesis at high concentrations. Differences in the eukaryotic ribosomal RNA sequence reduce the affinity of aminoglycosides for the eukaryotic decoding centre (151,152) and this permits these compounds to be used as antibiotics without inhibiting eukaryotic translation.

Aminoglycosides do not appear to induce significant misreading at sense codons in eukaryotes (153, 154) but a subset of them have been shown to act as read-through

compounds, which work by binding to eukaryotic ribosomes (155,156) resulting in misincorporation of near-cognate aminoacyl tRNAs at premature termination codons (PTC)s (157,158,159). Several studies have shown that some aminoglycosides can suppress translation termination at premature termination codons (PTCs) within various mRNAs and restore physiologically relevant levels of functional protein in mammalian cells (160).

Gentamicin is the aminoglycoside most commonly used for this type of nonsense suppression and has been shown to restore functional protein in short-term studies in a variety of mouse models.

Of particular interest from a pulmonary viewpoint are the data from a human trial of Ataluren (3-[5-(2-fluorophenyl)-[1,2,4] oxadiazol-3-yl]-benzoic acid) in people with cystic fibrosis (PWCF) . Ataluren is an orally bioavailable, investigational agent that promotes ribosomal read through of PTCs, and production of full-length, functional CFTR (161). This drug was tested in a clinical trial in PWCF who had a nonsense mutation in at least one allele of the CFTR gene. The results were non-significant. However, post hoc analysis of the subgroup of patients not using chronic inhaled tobramycin showed a 5.7% difference in relative change from baseline in % predicted FEV1 between Ataluren and placebo at Week 48 suggesting that tobramycin, an aminoglycoside similar to gentamicin was interfering with the read through efficacy of Ataluren. One of the problems with the gentamicin approach in lung disease is the toxicity associated with its long-term administration toxic side effects, which include hearing loss and kidney damage (162).

This toxicity is not attributable to gentamicin's ability to suppress PTCs but more likely due to interaction with off-target sites such as lysosomal membranes (163). In order to decrease the toxicity of gentamicin and other aminoglycosides a number of strategies have been used. Antioxidants, which can reduce free-radical formation resulting from aminoglycoside-induced toxicity have been utilised (164). Poly-L-aspartate which decreases nephrotoxicity (165) and ototoxicity (166) by reducing the interaction of aminoglycosides with lysosomal membranes has also been used with the added advantage that it increases cytoplasmic aminoglycoside concentrations, thereby enhancing PTC suppression (167,168).

The encapsulation of aminoglycosides into liposomes is another potential way to reduce aminoglycoside-induced toxicity. While liposomal forms of aminoglycosides may decrease side effects of aminoglycosides it is unclear whether it will also interfere with their ability to read through PTCs. In this regard, one study, utilizing a mouse model of mdx (muscular dystrophy) showed that intraperitoneal administration of gentamicin delivered in liposomes restored dystrophin protein expression more effectively in skeletal muscle than standard gentamicin (169). A rational design approach to generate aminoglycoside derivatives with enhanced nonsense suppression efficiency and reduced toxicity has also been used. This is based on developing structural forms incorporated onto an aminoglycoside chemical scaffold (170), which would be predicted to enhance aminoglycoside binding to cytoplasmic ribosomes while reducing aminoglycoside binding to mitochondrial ribosomes. This application has been used in mouse models of disease with some effect but not as effective as standard aminoglycosides. In this study, however, as proof of concept in a cell line experiment standard gentamicin was utilized. It is also noted that it has been shown that in some cases these compounds do not achieve increased secretion effect through increasing read through but most likely through mRNA stabilisation.

Previous work from our lab on a rare stop codon-based form of AATD called *NullBolton* evaluated read through compounds such as Ataluren and gentamicin on donor-derived *NullBolton* iPSC cells. As we have previously discussed, read-through should suppress translation termination at premature termination codons (PTCs) within various mRNAs and restore physiologically relevant levels of functional protein of appropriate size in mammalian cells. In the study cited, the data demonstrated that in response to these compounds, MM AAT and *NullBolton* AAT production/secretion increased. However, secreted *NullBolton* AAT was truncated, demonstrating that significant read-through had not occurred suggesting that the increased secretion was due to mRNA stabilization. These experiments suggested that if mRNA stabilisation could be achieved in cells expressing the Z protein, there would be increased secretion of Z AAT thereby potentially treating both the liver and lung manifestations of the condition.

Two cell lines were used to investigate the mRNA stabilization potential of gentamicin *in vitro*: Human Embryonic Kidney (HEK-293) cells transfected with a dual reporter system using lentiviral vectors to express both M or Z AAT (kindly provided by Andrew Wilson at the Centre for Regenerative Medicine, Boston University) and HepG2 cells a liver cell line (ATCC) and HepG2 cells which had been altered by CRISPR-Cas technology to produce Z AAT (gift from Mac Johnston, Vertex Pharmaceuticals Inc, Boston) were used. Although not a liver cell line HEK cells were used as these cell lines have previously been utilized in the lab and provided the opportunity to recapitulate the M and Z AAT response to gentamicin albeit not in a naturally AAT producing cell. HEPG2 cells, a liver cell line, was chosen to evaluate the ability of liver cells to respond to a naturally occurring inflammatory stimuli and assess how much AAT could be secreted from the cells as AAT is normally produced in the liver. An issue regarding HEPG2 cells is that they are representative of M AAT cells and not Z cells and therefore we accessed a (CRISPR altered) Z HepG2 cell line which had been altered to produce Z AAT to evaluate this response. Gentamicin was used as our read through compound.

These experiments coincided with the COVID-19 pandemic and it was observed, in our hospital and in other medical centres, that in MM AAT patients infected with SARS-CoV-2 there were elevated AAT, 2-3 times above the normal M range of 20-53µM. This raised the question of whether gentamicin or other inducers would be able to increase AAT secretion to these levels in addition to keeping a steady constitutive production of AAT. Recent literature has suggested that IL-6 is the major cytokine governing the inflammatory response in SARS-CoV-2 infection and we know that IL-6 is also involved in inducing AAT production and secretion (140). Therefore, transformed HEK cell lines were used to determine whether the M and Z secreted responses to gentamicin were similar. In addition, M and Z HepG2 cells were used to evaluate the levels of AAT and the speed of the AAT response that would be induced by a natural inflammatory stimulus, such as IL-6.

3.2 Aims for this chapter

- To determine utility of *in vitro* models of AAT production, secretion and retention in Z and M cell lines at rest and in response to stimuli
- To assess the ability of read-through compounds to increase secretion of AAT to levels commensurate with clinical effect
- To investigate if IL-6 is a sufficient inducer of AAT *in vitro* in M and Z cells

In order to achieve these aims the following objectives were set:

1. HEK-293 cells transfected with both M and Z constructs were assessed by ELISA and Western blot analysis to determine potential differing secretory responses to gentamicin
2. Lysates of HEK-293 cells transfected with both M and Z AAT constructs were evaluated for evidence of M or Z AAT retention
3. M and Z AAT HepG2 cells were assessed by ELISA and Western blot analysis to determine if they have differing secretory responses to gentamicin and IL-6
4. Lysates of M and Z AAT HepG2 cells were assessed to determine if increased AAT expression induced by gentamicin or IL-6 is associated with AAT retention and ER stress response

3.3 Results

3.3.1 Western blot analysis of cell supernatants and whole cell lysates of HEK-293 cells transfected with M or Z AAT constructs.

Western blot analysis revealed there was more AAT secreted into the supernatant of M AAT HEK-293 cells than Z AAT HEK-293 cells, particularly at 12h. (3.1, panel A). To determine what was happening intracellularly M and Z HEK-293 cells were lysed. In Figure 3.1, panel A, there is significantly more Z AAT retained intracellularly (in lysates) compared to M-AAT cells commensurate with the increased secretion in M cells seen in the supernatants. This is also reflected in densitometry (3.1 panel B) with the most statistically significant difference between M AAT and Z AAT intracellular retention being observed at 24h ($P=0.0139$). This M and Z HEK-293 cells used (passage 53 and 67 respectively) were seeded for 24hrs, as per the original protocol outlined in Chapter 2, with serum replete media (SRM) being replaced with fresh SRM in order to obtain samples at timepoints from 0h onwards. However, despite this result demonstrating AAT secretion and retention as seen in MM and ZZ individual *in vivo*, Western blot analysis of cell supernatants consistently demonstrated AAT protein at time point zero in M and Z AAT cell line supernatants. The original protocol, with its washing stages, its SRM replacement step lasting only seconds and the centrifuging of all samples collected made the possibility of cellular debris unlikely. This raised the possibility that foetal calf serum (FCS) in SRM which contains bovine AAT could cross-react with the AAT antibodies used thus accounting for protein at time point zero. To evaluate this, Western blot analysis was performed using the same AAT antibodies on cell culture media alone (containing 10% (v/v) FCS). A barely visible immunoband could be seen (Figure 3.1 panel C) suggesting that FCS cross-reactivity was unlikely and failed to explain why a defined band of protein was present at 0h. To further investigate this, a similar HEK-293 cell culturing protocol previously used in the lab was utilized. In this protocol, cells underwent a 48 h period of serum starvation at time point zero. An attempt was made to replicate this, however, cells quickly died when deprived of FCS, with the majority of cells lifting off the plate as demonstrated by the lack of protein per well in cell supernatants. (Figure 3.1 panel D).

To rectify this, the duration of serum starvation was reduced to 24 h with some slight improvement in cell survival but with the majority of cells dying within 4-6 h resulting in a similar lack of protein secreted into the supernatant (Figure 3.1 panel D).

Overall, despite the residual immunoband, these experiments demonstrated that using a protocol which utilized 10% (v/v) FCS throughout recapitulated a cell model of AATD in which HEK cells transformed with Z or M AAT constructs behaved in a fashion similar to that occurring *in vivo* with intracellular retention of Z protein and decreased secretion compared to less retention of M protein and more secretion.

This opened the way for experiments into increasing secretion of Z protein from the Z-HEK-293 cells with mRNA stabilisers such as gentamicin.

Figure 3.1

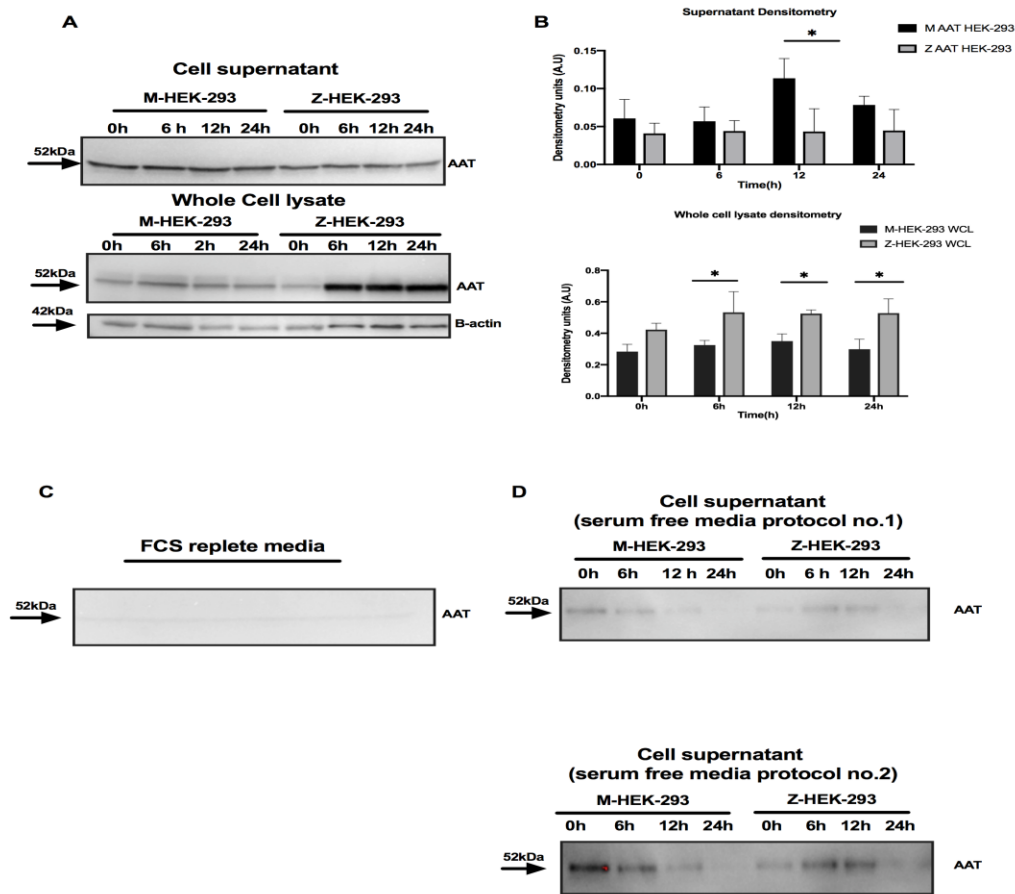


Figure 3.1

AAT Western blot analysis of HEK-293 cells transfected with both M and Z constructs.

- A.** Western blot analysis of cell supernatant and whole cell lysates (WCL) in both M and Z HEK-293 cell lines with sampling time points of 0, 6, 12 and 24h. This shows less secretion of AAT from the Z HEK-293 cell line compared to M HEK-293 cells and a corresponding increase in AAT retention in Z HEK-293 cells compared to M-HEK-293 cells. These are representative images; 6 Westerns were performed using the same samples and under the same conditions. **B.** Densitometry of supernatants and WCL in both M and Z HEK-293 cell lines. Supernatants of M HEK-293 demonstrate significantly increased levels of AAT protein secreted at 12h ($P=0.0175$). WCL of M and Z HEK-293 demonstrate significantly increased levels of AAT protein in Z cells as compared to M cells at 6h ($P=0.0290$) 12h ($P=0.0154$) and 24h ($P=0.0139$) (Two way ANOVA, Tukey's multiple comparisons test. * signifies $P<0.05$). **C.** Western blot analysis of FCS based cell culture media to determine if foetal calf AAT protein is picked up by the antibodies used at time point zero and other time points (this experiment was performed twice). **D.** Western blot analysis of cell supernatants using 48h serum starvation protocol at time point zero A Western blot analysis of cell supernatant using 24h serum starvation protocol at time point zero. These Westerns are representative images, 8 Westerns were performed based on the same serum free media protocols.

3.3.2 Enzyme-Linked Immunosorbent Assay (ELISA) and Western blot analysis of the pro-secretory effects of gentamicin on AAT.

HEK-293 cells were cultured using SRM supplemented with 0.5µg/ml of gentamicin. The AAT protein in cell supernatants was quantified using a human serpin A1 DuoSet ELISA (R&D systems). M HEK-293 cells produced 5841 pg/ml +/- 655.5 at the 24 h time point as compared to M-HEK-293 AAT production at 0h, 309.8 +/- 244.4pg/ml) (Figure 3.2 panel A, $P < 0.0001$). When exposed to gentamicin the AAT production further increased significantly to 8085 pg/ml +/- 871 as compared to untreated M HEK-293 cells at 24 (5841 pg/ml +/- 655.5) (Figure 3.2 panel A i, $P = 0.0082$). As expected, Z AAT secretion from Z HEK-293 cells at 24h was less than seen with M-HEK-293 cells but not significantly (4097pg/ml +/- 748, $NS = 0.0741$). When exposed to gentamicin Z-AAT secretion increased to just below the levels expressed by untreated M-HEK-293 (5285pg/ml +/- 336 Figure 3.2, panel A ii). Overall, the Z AAT HEK-293 secretory response to gentamicin at 24 h was significantly less than that seen in gentamicin treated M AAT HEK-293 cells ($P = 0.0006$). As shown in Figure 3.2, panel B intracellular AAT in M AAT HEK-293 cells decreased on Western blot analysis, corresponding with increased secretion, Z AAT HEK-293 cells over time demonstrated an increase in intracellular Z AAT as further confirmed by densitometry of immunobands (Figure 3.2 panel C). Z AAT HEK-293 cells at 24h had significantly more intracellular protein than M HEK 293 WCL ($P < 0.0001$). Z AAT protein secretion from Z AAT expressing cells was not significantly raised in response to gentamicin at 24h ($NS = 0.4833$) and was still below the gentamicin treated M-HEK-293 AAT levels showing that the transformed HEK cell lines behave as one might expect from Z and M transfected cells with intracellular retention of Z AAT and increased secretion of M AAT.

These results raised the possibility that HEK-293 cells, which do not naturally produce AAT, unless transformed, may not be a suitable *in vitro* model and that gentamicin may not be an effective inducer of AAT. This raised concerns that the AAT levels produced by Z HEK-293 in response to gentamicin would be insufficient in the case of a ZZ AATD patient who needed to mount an inflammatory response *in vivo*.

Figure 3.2

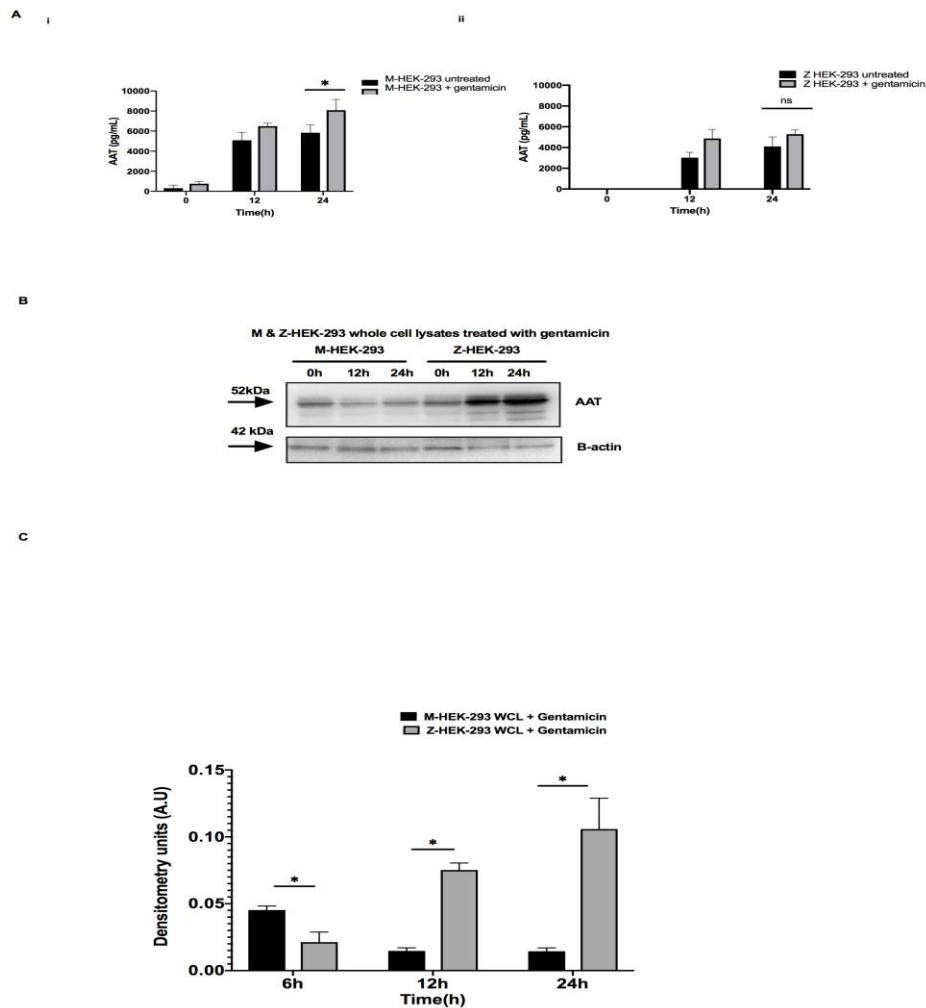


Figure 3.2

ELISA and Western blot analysis of M and Z-HEK-293 cell supernatant and WCL treated and/or untreated with gentamicin

A. At 24h M-HEK-293 cells exposed to gentamicin significantly increased AAT secretion compared to non-gentamicin treated cells. (8085 pg/ml \pm 871, 5841 pg/ml \pm 655.5, P value= 0.0082, Two way ANOVA, Tukey's multiple comparisons test * signifies P<0.05). At 24 h Z-HEK-293 cells exposed to gentamicin did not significantly increase AAT secretion compared to non-gentamicin treated cells (5285pg/ml \pm 336, AAT, increased from 4097pg/ml \pm 748, NS=0.4833, Two way ANOVA, Tukey's multiple comparisons test). At 24h there was significantly more AAT production in M-HEK-293 cells than in Z-HEK-293 cell (P=0.0006 Two way ANOVA, Tukey's multiple comparisons test. * signifies P<0.05). **B.** AAT in M-HEK-293 WCLs decrease over time on Western blot analysis, corresponding with increased secretion. Z-HEK-293 WCLs demonstrate a significant increase in retained intracellular Z-AAT over time. This is a representative image; three Western blots were performed using the same samples under the same conditions. **C.** Densitometry further demonstrates the increased intracellular protein in Z HEK-293 cells as compared to M HEK-293 cells at 12h (P<0.0001) and 24h (P<0.0001, Two way ANOVA, Tukey's multiple comparisons test. * signifies P<0.05).

3.3.3 Interleukin-6 (IL-6) as an inducer of AAT and ST6GAL-1 in HepG2 cells in the setting of an inflammatory insult (IL-6 / SARS-CoV-2)

HepG2 cell evaluation as a liver cell line, was deemed a more suitable model than HEK-293 cells as it better represented the organ most central to AAT production. AAT expression in hepatic cells, in response to IL-6 administration, at a concentration similar to that seen in SARS-CoV-2 ARDS plasma, is delayed, peaking at 24 hr after IL-6 administration (Figure 3.3, panel A). SARS-CoV-2 plasma also stimulated AAT mRNA production from hepatic cells in a similar fashion (Figure 3.3 panel B). These responses were largely blocked by tocilizumab, an antibody against the IL-6 receptor (Figure 3.3 panel B), intimating that IL-6 is the major component of SARS-CoV-2 plasma contributing to AAT upregulation. IL-6 in SARS-CoV-2 plasma also induced ST6GAL1, the enzyme mediating sialylation of AAT (Figure 3.3 panel C). This was also blocked by tocilizumab, confirming the contribution of IL-6 to sialylation of AAT, which is explored further in chapter 4.

Figure 3.3

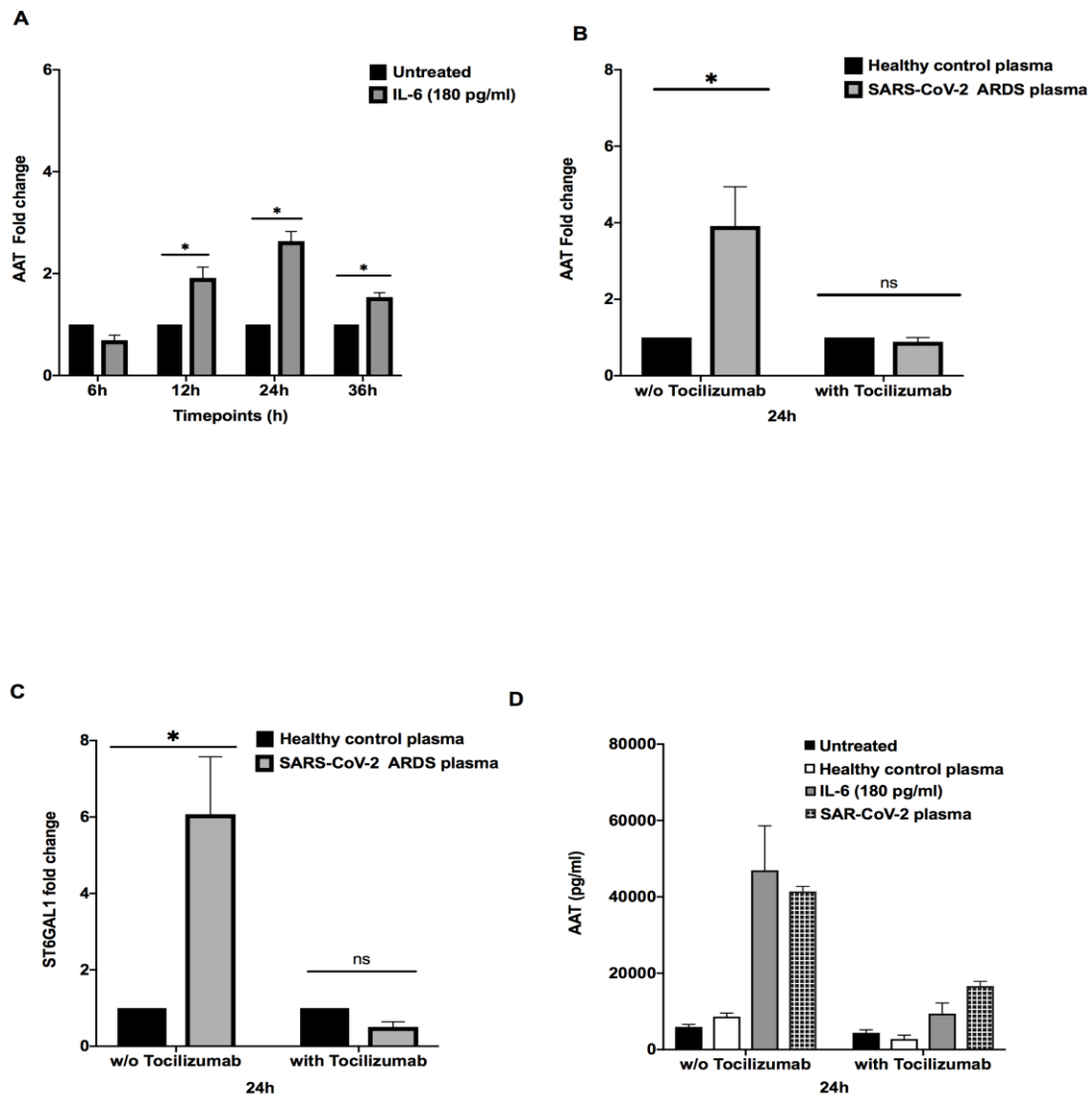


Figure 3.3

AAT production and retention in HepG2 cells in response to pro-secretory stimuli

A. AAT expression in HepG2 cells stimulated with IL-6 over time by RT-qPCR. AAT mRNA production is significantly and consistently raised from 12 h reaching its peak at 24h. Significance was tested for by two-way ANOVA with Tukey post-hoc test (* $P < 0.05$). **B.** AAT expression at 24 hr in HepG2 cells stimulated with SARS-CoV-2 or nsARDS plasma relative to expression in response to healthy control plasma in the absence (* $P < 0.05$) or presence of Tocilizumab. **C.** ST6GAL1 expression in HepG2 cells at 24 hr when stimulated with SARS-CoV-2 or nsARDS plasma, relative to expression in response to healthy control plasma in the absence (* $P < 0.05$) or presence of Tocilizumab. These experiments were repeated three times, with patient plasma being sourced from 20 SARS-CoV-2 patients admitted to ICU. Analyses were performed using a Two-way ANOVA, Tukey's multiple comparisons test. **D.** AAT protein secreted from untreated HEPG2 cells significantly increased when exposed to IL-6 and to SARS-CoV-2 plasma (P value < 0.0001). Analyses were performed using a Two-way ANOVA, Tukey's multiple comparisons test (* signifies $P < 0.05$).

3.3.4. AAT production and retention in HepG2 cells in response to pro-secretory stimuli.

The HepG2 cell model with or without CRISPR-Cas -9 editing, as a liver cell line, naturally produces AAT. In order to put previous experiments utilizing HEK-293 cells into context with regards retention and secretion of AAT we compared the M AAT and Z AAT secretion from both cell lines and as seen in Figure 3.4 panel A. There is a significant increase in the secretion of M AAT by HEPG2 cells as compared to the M HEK-293 model ($P < 0.0001$), and this is seen also in Z AAT production ($P < 0.0001$) by Z HepG2 cells. In the HEPG2 model the AAT secretion in the M cell line is also significantly higher than Z ($P < 0.001$). With this in mind, it was decided that the HEPG2 cell model would be the most realistic and accurate *in vitro* representation of what occurs *in vivo*.

Both M and Z AAT producing cells were exposed to several stimuli in order to elicit a pro-secretory response. Cells were treated with $0.5\mu\text{g/ml}$ of gentamicin as previously used in HEK-293-cell experiments. Another stimulus, IL-6, 180pg/ml , was also used to treat both M AAT and Z AAT HEPG2 cells. As seen in Figure 3.4 panel B neither M (>0.999) nor Z (>0.999) HEPG2 cells have a significant increase in AAT production in response to gentamicin but both had a significant increase, M AAT ($P < 0.0001$) and Z AAT ($P = 0.0024$) production in response to IL-6 (180pg/ml). This raised the question as to whether the Z AAT was retained intracellularly, thereby causing ER stress? To answer this Z and M HEPG2 cells exposed to gentamicin, IL-6 and SARS-CoV-2 plasma were lysed (WCL). Western blot analysis of WCL revealed increased intracellular Z AAT as compared to M AAT particularly in response to IL-6 and SARS-CoV-2 plasma. When probed for GRP-78, which denotes ER stress, Western blot analysis demonstrated increased ER stress in Z- HEPG2 cells, with the most notable stress observed in cells exposed to IL-6 or SARS-CoV-2 plasma (Figure 3.4, panel C).

Figure 3.4

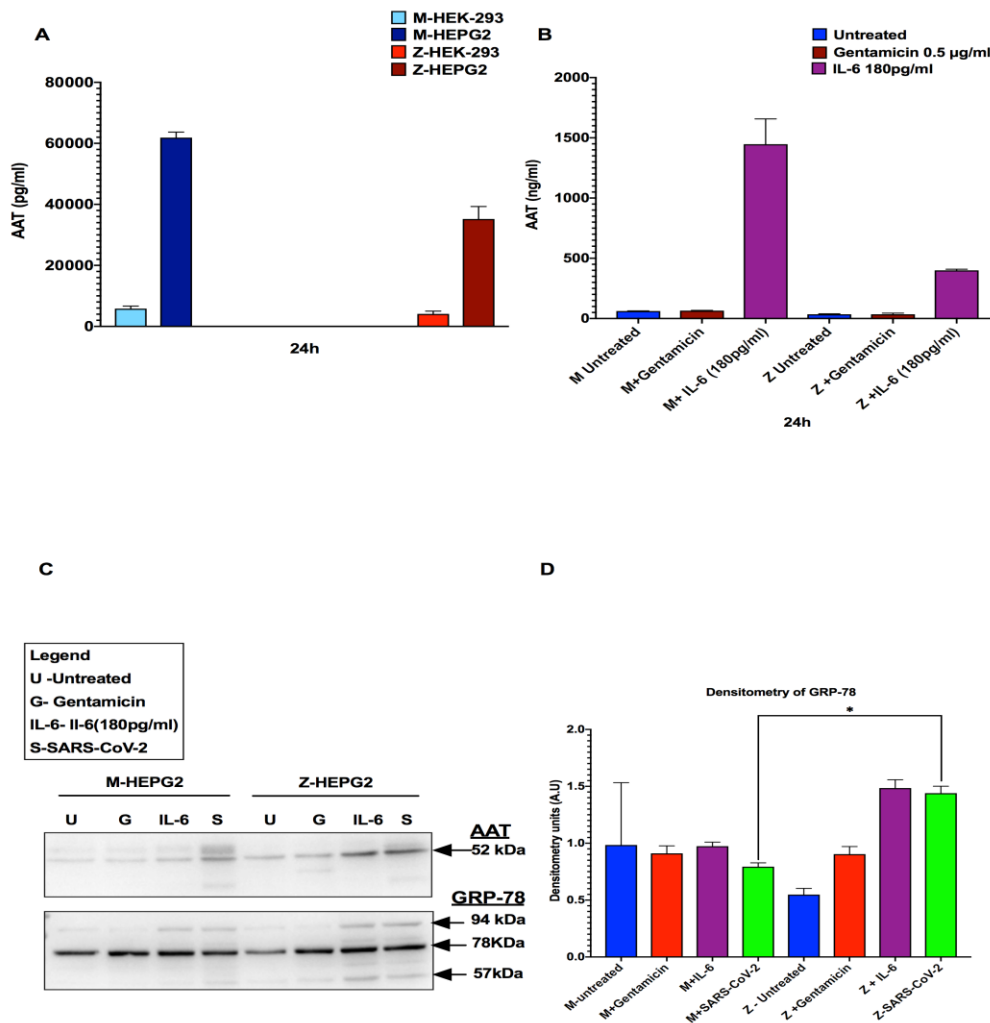


Figure 3.4

The comparison in AAT secretion and retention in HepG2 cells in response to pro-secretory stimuli

A. ELISA analysis shows significantly increased AAT levels being produced by HEPG2 cell lines at 24h than compared to HEK-293 cell lines in both the M ($P=0.999$) and Z ($NS >0.999$). A significant increase was seen when both M ($P<0.0001$) and the Z ($P<0.001$). Z AAT HEPG2 cell line produces less AAT than M AAT producing HEPG2 cells ($P<0.0001$). This was analysed by one way-ANOVA. **B.** The pro-secretory effect of gentamicin in HEPG2 cells was non-significant in both M ($NS >0.999$) and Z ($NS >0.999$). A significant increase was seen when both M ($P<0.0001$) and Z ($P=0.0024$) HEPG2 cell lines were exposed to IL-6 at 24h. **C.** Western blot analysis of HEPG2 WCL reveal increased intracellular AAT particularly in the Z-HEPG2 cell line, most notably in WCL treated with (IL-6 180pg/ml) or SARS-CoV-2 plasma. The WCL were also probed for GRP-78 and again in the Z-cell line particularly in the IL-6 and SARS-CoV-2 plasma this was observed. **D.** The increased GRP-78 in the Z-HEPG2 cell line, most notably in WCL treated with (IL-6 180pg/ml) or SARS-CoV-2 plasma is further outlined by densitometry factoring in our B-actin loading control ($P=0.0005$, One way ANOVA. * Signifies $P<0.05$).

3.4 Discussion

The two *in vitro* cell models used were quite unique and useful in different ways. The Z and M HEK-293 cell lines were developed by means of a novel dual-promoter lentiviral system to transfer normal human AAT cDNA into HEK-293 cells, which normally do not produce AAT. The cDNA was cloned from c-AT plasmid and PCR was used to add restriction sites. An amplicon was then ligated into the lentiviral constructs and M and Z constructs made (A. Wilson, Boston University). Although these cell lines follow a similar pattern of M and Z AAT retention and secretion, HEK-293 cells do not naturally produce AAT and the constructs' secretions are by definition, artificial and may not exhibit normal physiological responses. This is a possible explanation for the consistently high levels of baseline secreted Z AAT being produced. This issue, highlighted by Western blot analysis and ELISA, was most obvious with persistent AAT protein present at time point zero. It's possible that the lentivirus transfected cell lines, which were of an advanced passage number to begin with, were highly stressed when the media was being changed and secreted AAT as a response. In this context, even minimal serum starvation was found to be detrimental to cell viability as seen in Fig 3.1, panel D. When exposed to gentamicin, AAT secretion from M AAT and to a lesser extent, Z AAT does increase, however the majority of AAT in the Z HEK-293 cells appears to be trapped intracellularly, which is in keeping with classic ZZ AATD pathology. This also raises the possibility that treatments involved in increasing Z AAT production could potentially lead to increased endoplasmic reticulum (ER) stress due to the inability of the misfolded protein to pass through the ER and further inflammation. A potentially less injurious approach would be to use small peptides to inhibit polymerisation of AAT in the liver with subsequent increased secretion out of hepatocytes (117). or the recent corrector approach aimed at refolding AAT intracellularly and normalizing its secretion (120-123). Another question which remains is whether these approaches including gentamicin are capable of normalizing secretion of AAT sufficiently to deal with inflammatory demands.

In light of the data surrounding the AAT levels required in the face of SARS-CoV-2 infection or similar inflammatory conditions, the slight non-significant increase in Z AAT secretion by HEK-293 in response to gentamicin seems to be an inadequate response. In order to accurately access this in a model closer to "real life", liver cell

line HEPG2 cells, a cell line derived from hepatocytes, which normally produce the majority of AAT in the body were used. The M AAT producing HEPG2 cell line was able to mount a significant response to SARS-CoV-2 plasma demonstrating the amount of AAT that would be required in an inflammatory response, the subsequent and equally significant decrease in AAT production in response to tocilizumab suggests that IL-6 is the key cytokine driving AAT as well as ST6GAL1 production in SARS-CoV-2 plasma. Both of these are important as in addition to increased AAT levels the physiological response to inflammation involves altering the form and function of AAT by increasing its sialylation, which modulates neutrophil chemotaxis as described in chapter 4 and 5(19). To further evaluate this in the context of AATD, we utilised a Z AAT CRISPR edited HEPG2 cell line acquired from Dr Mac Johnston, Vertex. This was a unique cell model to determine how a ZZ individual would respond to pro-secretory and in the case of IL-6 pro-inflammatory stimuli. Our HEPG2 cell model demonstrated that in a liver cell line, naturally producing AAT, gentamicin did not significantly increase AAT production. However, in both M and Z HEPG2 cell lines exposed to IL-6 or SARS-CoV-2 plasma a significant AAT response was elicited, resulting in increased M and Z AAT secretion, although with less being produced by the latter, corresponding to increased intracellular retention of Z AAT in HEPG2 cells and subsequent ER stress.

In summary, in this chapter we evaluated 4 distinct cells lines, which were modified to represent the Z and M phenotypes. Both types of cells, modified HEK-293 cells and HepG2 cells behave in a manner expected of Z AAT and M AAT cells with decreased secretion from Z cells and increased Z AAT intracellular retention compared to M cells. The response of Z AAT cells to gentamicin was minimal and does not augur well for this as a future treatment option for Z AATD. This is particularly the case when the response of both M and Z cells lines to IL-6 and SARS-CoV-2 plasma is evaluated suggesting that the level of AAT changes according to circumstance and that the levels of AAT required even for non-inflammatory states will not be provided by gentamicin. This raised another series of questions. Is the large amount of AAT produced, even by M cells, sufficient to protect the lung in the setting of severe lung inflammation such as SARS-CoV-2 infection, or is the large AAT secretion in response to SARS-CoV-2 infection peculiar to an IL-6 driven viral induced inflammation and not required in the setting of bacterial infection.

This will be evaluated in Chapter 4. The question of whether the increased stimulus to AAT production in ZZ individuals in response to IL-6/SARS-CoV-2 infection will lead to increased AAT intracellular retention and ER stress will be addressed in Chapter 5.

**Chapter 4: The characterization
of anti-proteases during the
acute inflammatory response to
SARS-CoV-2 *in vivo***

4.1 Introduction

Alpha-1 antitrypsin (AAT), a major serine protease inhibitor and anti-inflammatory protein (1), may influence the trajectory of SARS-CoV-2 infection. Early work from our group showed that people with SARS-CoV-2 infection who failed to mount an adequate AAT response to IL-6 had a worse prognosis (140). The possible mechanisms subtending this are manifold. Early in disease, AAT may modulate the severity of SARS-CoV-2 infection via inhibition of TMPRSS-2 (67), the serine protease that cleaves the SARS-CoV-2 spike protein essential for cellular entry. Later in disease, AAT may inhibit inflammatory proteases, e.g., neutrophil elastases (NE), cysteinyl cathepsins, and metalloproteases downstream to SARS-CoV-2 infection, via direct interactions or via inflammatory cytokines that participate in airway pathologies (10). Interleukin-6 (IL-6) is a pivotal cytokine in SARS-CoV-2 pathogenesis and is associated with the morbidity and mortality of SARS-CoV-2-induced ARDS (140) (171). As noted previously, IL-6 is a major inducer of AAT production by the liver, (140) and this is mediated largely through the classic pathway whereby IL-6 interacts with the IL-6 receptor (IL-6R) on specific cells (172). The IL-6/IL-6R complex then binds to gp130, leading to activation of the JAK/STAT, ERK, and PI3K signal transduction pathways that regulate AAT transcription. As previously noted, IL-6 can also signal through other non-classical pathways especially via trans-signalling whereby the IL-6R is cleaved by ADAM-17 and is released into the circulation, binding IL-6 enabling it to interact with any cells containing gp130. It is recognised that much of the IL-6 anti-inflammatory effects are mediated through the classical signalling pathway while its pro-inflammatory effects are mediated mainly through trans-signalling. ADAM-17 is directly inhibited by AAT, yet another means by which AAT can influence inflammation in SARS-CoV-2 infection.

To evaluate the AAT response to SARS-CoV-2 infection, the AAT-NE balance was investigated in different compartments of the lung (n=8), airways (n=15) and plasma (n=20) of people with SARS-CoV-2 ARDS. The kinetics of IL-6 and AAT in the plasma were measured across the disease course, and the role of IL-6 in induction of hepatic AAT synthesis was explored along with its effects on beta-galactoside alpha-2-6 sialyltransferase1 (ST6GAL1) expression. ST6GAL1 mediates AAT sialylation during acute inflammation, promoting increased binding of AAT to

interleukin (IL)-8 and neutrophil activating peptide (NAP)-2, thereby regulating neutrophil chemotaxis (19). This was further explored by isolating and purifying AAT from SARS-CoV-2 patients with varying severity and varying degrees of sialylation and determining the ability of the differentially sialylated AAT to bind IL-8. The levels and activity of AAT in plasma, and AAT and NE in TAs from the airways of SARS-CoV-2 ARDS were characterized and compared to matched nsARDS controls. The localization of secretory leucoprotease inhibitor (SLPI), the other major airways anti-protease, was evaluated utilizing immunohistochemistry (IHC) of lungs from SARS-CoV-2 and control, non-lung disease patients and Western blot analysis of TA. IHC studies also investigated neutrophil localisation and AAT and NE levels and localisation in the alveolar compartment of SARS-CoV-2 autopsy lungs. Tocilizumab, an IL-6 receptor antagonist previously used in rheumatoid arthritis and systemic juvenile idiopathic arthritis but now increasingly being used as a therapy in SARS-CoV-2 infection was evaluated for its effect on AAT levels and sialylation *in vivo* (173). Finally, following on these results a phase 2, multicentre, randomized, double-blind, placebo-controlled trial of intravenous plasma-purified AAT (Prolastin, Grifols, S.A.) for moderate-to-severe ARDS secondary to SARS-CoV-2 infection was initiated (174). A total of 86 consecutive patients were screened, with 36 undergoing randomization having satisfied the criteria for entry to the study (Figure 4.1). Patients (n=36) were randomized to receive weekly placebo, weekly AAT (120mg/kg), or AAT once followed by weekly placebo. The primary endpoint was the change in plasma interleukin (IL)-6 concentration at day 7. In addition to assessing safety and tolerability, changes in plasma levels of IL-1 β , IL-8, IL-10 and soluble TNF receptor 1 (sTNFR1, a surrogate for circulating TNF- α), were assessed as secondary endpoints. Clinical outcomes were also recorded as pilot data to inform a larger phase 3 trial.

4.2 Aims for this chapter

- To determine the acute phase AAT response in plasma to SARS-CoV-2 infection
- To assess if there is a protease-anti-protease imbalance in the airways of people with severe SARS-CoV-2 infection, which would be potentially amenable to therapy with plasma purified AAT.
- To investigate if IL-6 receptor antagonists may be potentially harmful in the treatment of people infected with SARS-CoV-2
- To assess the effects of intravenous AAT in SARS-CoV-2 patients in a randomised control trial

In order to achieve these aims the following objectives were set.

1. To measure the levels, form and activity of AAT and anti-NE capacity in plasma in people with moderate and severe SARS-CoV-2 infection
2. To measure activity of AAT, SLPI and NE in tracheal aspirates (TA)s from the airways of people with SARS-CoV-2 ARDS.
3. To investigate the localization of AAT, NE and SLPI in the lungs of people with fatal SARS-CoV-2 compared to non-lung disease patients, utilizing immunohistochemistry (IHC) and confocal microscopy
4. To measure *in vivo* the levels of IL-6 and AAT in the plasma of mild – moderate SARS-CoV-2-infected subjects treated with a single dose of

- the IL-6 receptor antagonist tocilizumab
5. To conduct a phase 2, randomized, double-blind, placebo-controlled trial of intravenous plasma-purified AAT as an anti-inflammatory therapeutic for patients with ARDS secondary to SARS-CoV-2.

4.3 Results

4.3.1. Patient population characteristics

The demographics of the patient populations are shown in Tables 4. 1- 4.6; control population in table 4.7. Healthy controls (n=11) were similar in age to patients admitted to ICU with SARS-CoV-2 (n=20), (49 ± 16 vs 51 ± 13 years) and, though the other cohorts studied were older on average, age was not seen to associate significantly with any cohort over controls (61.3 ± 8 years, $P = 0.457$, and 60.8 years, $P=0.285$, for nsARDS (n=11) and SARS-CoV-2 infection requiring non-ICU hospitalisation, respectively). Patient gender was not significantly associated with any of the studied conditions.

Our study population was of similar age in comparison to other international patient cohorts with severe SARS-CoV-2 infection requiring intensive care recruited before vaccination availability, (51 ± 13 vs 58 ± 9 years (175). Patients had similar profile with respect to risk factors (BMI > 30: 55% vs 61%; hypertension: 55% vs 48%) and comparable symptoms on presentation of cough, dyspnoea and fever (176) albeit fewer patients in our study were diabetic (10% vs 24% (175). The cohort with moderate SARS-CoV-2 infection requiring hospitalization (n=30) was well-matched to similar populations elsewhere (61 ± 11 vs 63 ± 16 years, N=80 (176) 63 ± 18 years (177); 56 ± 14 years (178). Of the moderate cohort, 66% were male in the present study, which is comparable to studies elsewhere (59%, N=377 (178).

In our randomised control trial, baseline characteristics of the study groups at the time of randomization are shown in Table 4.3 The study groups were matched for age, BMI and clinical severity as assessed by $\text{PaO}_2:\text{FIO}_2$ (122.5 ± 40.5 mmHg in the placebo group vs 129.7 ± 38.2 mmHg in the treatment group) and SOFA score (7.8 ± 3.3 vs 7.2 ± 3.4). Patients were receiving lung-protective ventilation with average tidal volumes of 6.3 ± 0.6 ml/kg/IBW in the placebo group and 6.5 ± 0.8 ml/kg/IBW in the treatment group. A significant number of patients were either overweight or obese, with an average BMI of 33.4 ± 8.1 kg/m² in the placebo group and 35.2 ± 11.0 kg/m² in the treatment group. Two-thirds of the total population studied had a BMI ≥ 30 kg/m². Just over one-third required vasopressors at the time of randomization. Almost three-quarters of patients were receiving dexamethasone, most likely due to dissemination of preliminary results from the steroid arms of the

RECOVERY and REMAP-CAP trials on preprint servers while the study was ongoing. All patients studied had systemic inflammation, with comparable circulating levels of IL-6 (259.9 +/- 206.5 pg/mL in the placebo group vs 266.9 +/- 206.9 pg/mL in the treatment group), leukocyte count, C-reactive protein (CRP), lactate, D-dimer and fibrinogen (Table 4.3, Table 4.8). AAT levels at randomization were identical between the groups (Table 4.8).

Table 4.1

Demographics of patients with SARS-CoV-2 ARDS for plasma studies.

Demographics of patient cohort (n=20)	
Age in years	51.1 +/-13.4
Male/female	17/3
Days since onset of symptoms	8.7 +/- 3.9
CRP on admission to ICU	293 mg/L+/-104.9
Symptoms at admission (%)	
Fever	11 (55)
Dyspnea	13 (65)
Cough	13 (65)
Sputum production	2 (10)
Myalgia	3 (15)
Fatigue	2 (10)
Anorexia	2 (10)
Nausea	3 (15)
Vomiting	1 (5)
Diarrhea	2 (10)
Chest pain	1 (5)
Circumstances surrounding infection	
Close contact with infected person	3 (15)
Community-acquired	17 (85)
Comorbidities	
Hypertension	11 (55)
Coronary artery disease	1 (5)
Diabetes mellitus	2 (10)
Obesity	11 (55)
Smoking history	
Current	2 (10)
Former	1 (5)
Never	14 (70)

Table 4.2

Clinical status of patients with SARS-CoV-2 ARDS for plasma studies.

Clinical features of patient cohort at time of ICU admission	(n=20)
Temperature >38°C	9 (45)
Heart rate >100 beats per minute	12 (60)
Respiratory rate >20 breaths per minute	10 (50)
SaO ₂ <80% or requiring FiO ₂ ≥60%	16 (80)
Acute confusion	1 (5)
PaO ₂	9.5 +/- 1.6
Mean arterial pressure	91.6 +/- 16.1
SOFA score	8.05 +/- 2.3
PaO:FiO ₂ on arrival to ICU	160.5 +/- 60.8

Table 4.3
Characteristics of the Phase 2 trial cohort at randomization

Phase 2 clinical trial demographics		
	Placebo	AAT
Total number	11	25
Age in years	57 +/- 13	59 +/- 11
Male/female	9 (82) / 2 (18)	13 (52) / 12(48)
BMI (kg/m²)	33.4 +/- 8.1	35.2 +/- 11.0
SOFA score	7.8 +/- 3.3	7.2 +/- 3.4
Mean arterial pressure (mmHg)	87.7 +/- 12.1	87.6 +/- 15.3
Tidal volume (mL)	411.4 +/- 37.2	427.9 +/- 64.6
Tidal volume (mL/kg IBW)	6.3 +/- 0.6	6.5 +/- 0.8
PEEP (cm H₂O)	13.8 +/- 4.1	11.7 +/- 2.9
Plateau pressure (cm H₂O)	27.7 +/- 4.4	26.0 +/- 5.7
PaO₂ (kPa)	10.9 +/- 2.7	10.4 +/- 1.8
FIO₂ (%)	70.9 +/- 16.7	64.2 +/- 16.8
PaO₂:FIO₂ (mmHg)	122.5 +/- 40.5	129.7 +/- 38.2
Oxygenation index	12.3 +/- 3.6	10.6 +/- 4.4
Medications of interest		
Dexamethasone	8 (73)	18 (72)
Remdesivir	0 (0)	0 (0)
Tocilizumab	0 (0)	0 (0)
Hydroxychloroquine	0 (0)	0 (0)
Receiving vasopressors at randomization	4 (36)	9 (36)
Circulating inflammatory markers		
Interleukin-6 (pg/mL)	259.9 +/- 206.5	266.9 +/- 206.9
Alpha-1 antitrypsin (g/L)	2.3 +/- 0.6	2.3 +/- 0.5
C-reactive protein (mg/L)	155.4 +/- 118.4	154.4 +/- 135.5
Known comorbidities		
Hypertension	4 (36)	12 (48)
Ischemic heart disease	2 (18)	4 (16)
Diabetes mellitus	4 (36)	6 (24)
Obesity	7 (64)	17 (68)
Chronic lung disease	3 (27)	8 (32)
Chronic kidney disease	3 (27)	7 (28)
Smoking history		
Current	0	1 (4)
Former	3 (27)	14 (56)
Never	8 (73)	10 (40)
Vaping history		
Current	0 (0)	2 (8)
Former	0 (0)	1 (4)
Never	0 (0)	22 (88)

Table 4.4

Demographics of non-trial patients with non-SARS-CoV-2 ARDS (nsARDS) sampled for TA studies

Demographics of ICU TA samples	nsARDS TA n=6
Age	61.3+/-7.9
Days since symptom onset to ICU admission	9+/-6
Positive/Negative Respiratory microbiology since ICU admission	0/6
Mortality Y/N	0/6
PaO2:FiO2	156+/-28.7

Table 4.5

Demographics of non-trial patients with SARS-CoV-2 ARDS sampled for TA studies.

Demographics of ICU TA samples	SARS-CoV-2 TA n=15
Age	60.2+/-13.2
Days since symptom onset to ICU admission	10+/- 7.2
Positive/Negative Respiratory microbiology since ICU admission	7/8
Mortality Y/N	3/12

Table 4.6
Demographics of ward level patients receiving SOC+/- tocilizumab

SARS-CoV-2 patients receiving standard of care (SOC) + /- Tocilizumab	SOC	SOC + Tocilizumab
Number of patients	6	24
M/F	4/2	16/8
Age	60.8+/- 11.1	60.8+/-13.6
Mortality Y/N	0 (0)	2 (12)

Table 4.7
Demographics of healthy control MM individuals.

Demographics of healthy MM patients	(n=11)
Age in years	35+/-9.9
Male/female	4/7
Symptoms of COVID-19 at time of sampling (%)	
Fever	0
Dyspnea	0
Cough	0
Sputum production	0
Myalgia	0
Fatigue	0
Anorexia	0
Nausea	0
Vomiting	0
Diarrhea	0
Chest pain	0
Smoking history	
Current	1
Former	3
Never	7

Table 4.8
Biochemical characteristics at randomization in Phase 2 trial

Biochemical characteristics at randomization			
	Placebo+/- SD.	AAT+/- SD.	Normal value or range
Circulating interleukin-6 (pg/mL)	259.9 +/- 206.5	266.9 +/- 206.9	<7.0
Circulating AAT (g/L)	2.3 +/- 0.6	2.3 +/- 0.5	0.9-2.0
Hemoglobin (g/dL)	12.5 +/- 2.2	12.4 +/- 1.9	11.5-16.5
White cell count (10⁹/L)	12.7 +/- 3.3	12.3 +/- 5.0	4.0-11.0
Neutrophils	11.4 +/- 3.2	11.0 +/- 4.8	2.0-7.5
Lymphocytes	0.8 +/- 0.3	0.7 +/- 0.4	1.0-4.0
Monocytes	0.4 +/- 0.2	0.5 +/- 0.3	0.2-1.0
Eosinophils	0.02 +/- 0.04	0.03 +/- 0.05	0.04-0.4
Platelets (10⁹/L)	290.6 +/- 61.5	298.6 +/- 106.1	150-450
C-reactive protein (mg/L)	155.4 +/- 118.4	154.4 +/- 135.5	0-5
Lactate (mmol/L)	1.4 +/- 0.6	1.6 +/- 0.3	0.5-1.0
Albumin (IU/L)	32.2 +/- 3.5	31.4 +/- 4.5	35-52
Ferritin (ng/mL)	1330 +/- 906	1419 +/- 951	13-150
D-Dimer	14.8 +/- 22.7	15.9 +/- 35.5	0-0.5
Fibrinogen (g/L)	4.83 +/- 2.14	4.65 +/- 1.96	2.0-4.0
Urea (mmol/L)	9.5 +/- 3.4	10.7 +/- 5.6	2.8-8.1
Creatinine (μmol/L)	86.4 +/- 22.9	86.5 +/- 41.2	45-84
Arterial pH	7.4 +/- 0.1	7.4 +/- 0.1	7.35-7.45

Figure 4.1

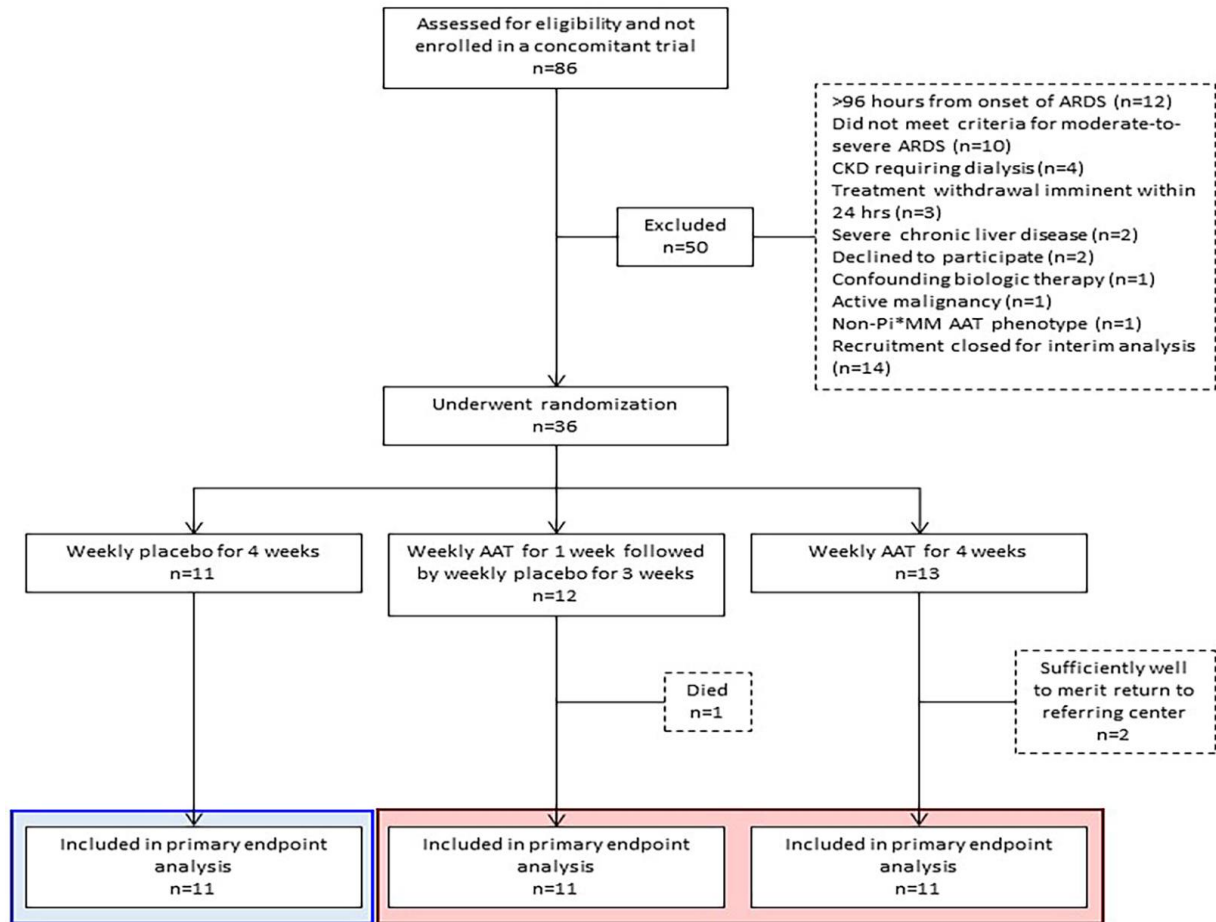


Figure 4.1.

Consort diagram.

Of 86 consecutive patients screened, 36 underwent randomization having satisfied the criteria for entry to the study. Of the 33 patients included in the primary endpoint analysis (change in circulating IL-6 concentration at one week), 22 had received a single infusion of AAT (highlighted in red) and 11 had received a single placebo infusion (highlighted in blue). Patients in the AAT treatment group subsequently received either weekly AAT or weekly placebo as part of a pilot safety and feasibility assessment.

4.3.2 Plasma AAT levels in non-trial SARS-CoV-2 ARDS

Plasma AAT levels in SARS-CoV-2 patients on admission to the ICU (3.29 ± 0.80 g/L) were significantly higher than in healthy controls (Mean: 1.74 ± 0.11 g/L, $p < 0.001$, Figure 4.2 panel A) but similar to nsARDS subjects (Mean: 2.9 ± 0.80 g/L, $p = 0.5273$, Figure 4.2 panel A). The higher levels of AAT in plasma in both ARDS groups persisted throughout their ICU stay (Figure 4.2 panel B,C). Elevated AAT was prominently associated with each of the conditions, as expected due to its nature as an acute phase protein (SARS-CoV-2 vs healthy controls: log odds 10.93, SE 4.3, $P = 0.011$).

Figure 4.2

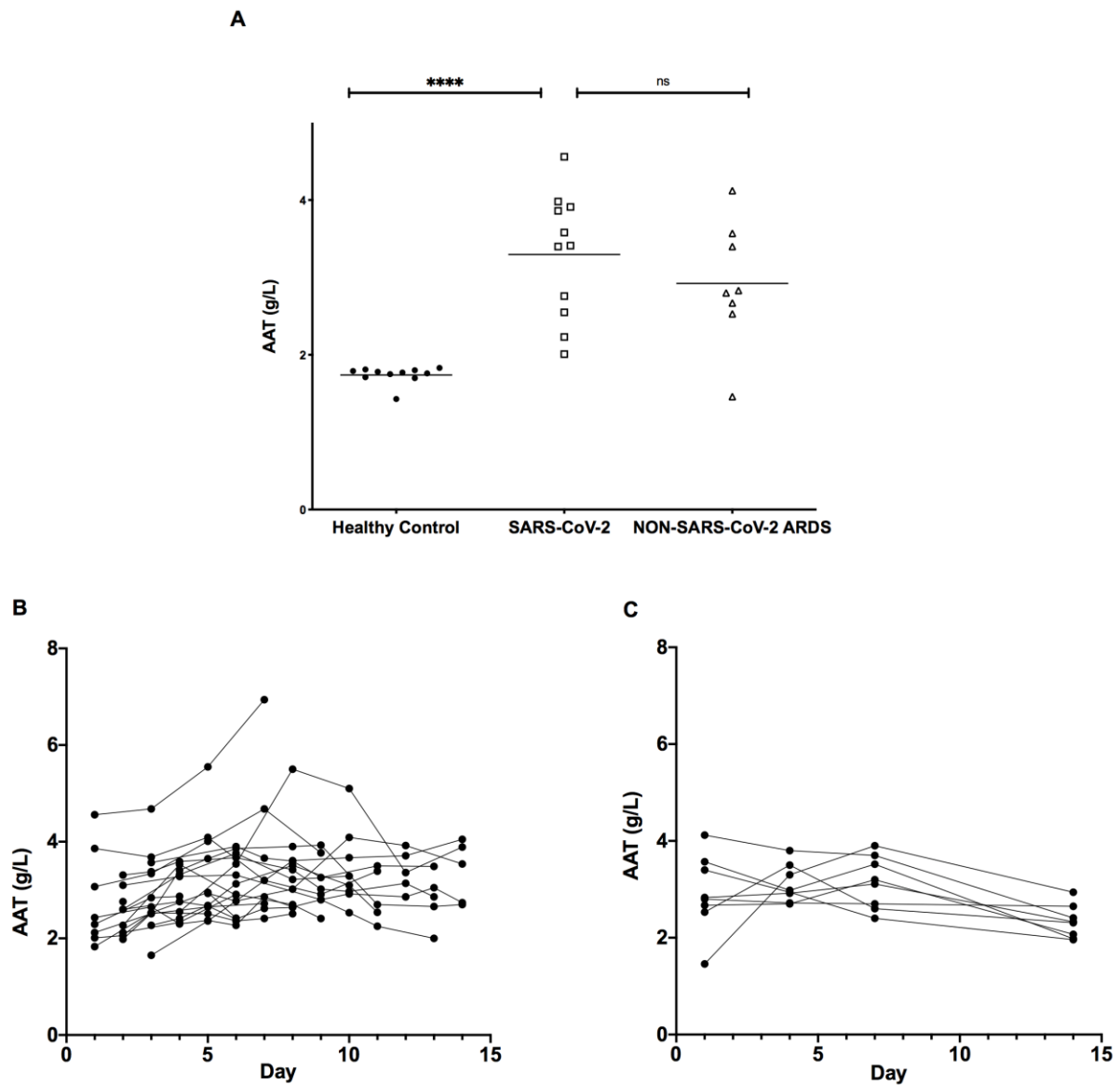


Figure 4.2

AAT levels in plasma of healthy donors and patients in ICU with SARS-CoV-2 or nsARDS.

A. Plasma AAT levels on first day of admission into ICU with SARS-CoV-2 (n=11, 3.294 g/L \pm 0.768) or nsARDS (2.923g/L \pm 0.7984) as compared to healthy controls (n=11) (1.73g/L \pm 0.104), $P<0.0001$, $P=0.0013$ respectively with no significant difference between SARS-CoV-2 and nsARDS at day 1. Significance was tested for by one way ANOVA with Tukey multiple comparison test (* $p<0.05$). **B.** Plasma AAT levels in SARS-CoV-2 patients over the first 15 days of ICU admission (Mean: 3.2 g/L \pm 0.7 n=20). **C.** Plasma alpha-1antitrypsin (AAT) levels in non-SARS-CoV-2 ARDS patients over the first 15 days of ICU admission (2.87g/L \pm 0.607) (n=8).

4.3.3 Form and activity of AAT, NE and SLPI in non-trial SARS-CoV-2 plasma and TA

Plasma AAT was not complexed or cleaved (Figure 4.3, panel A) and was fully active against NE (Figure 4.3, panel B). TA from intubated subjects with SARS-CoV-2 (n=15) and nsARDS (n=6) contained cleaved AAT and AAT-NE complexes on Western analysis (Figure 4.3.4. panel A i). In the SARS-CoV-2 TA, the AAT was cleaved to a greater extent than the nsARDS TA, and significant free NE was detected by Western blot analyses as well as cleaved SLPI in contrast to nsARDS (Figure 4.4 panel A ii and iii respectively). Of all 6 nsARDS TAs only 1 had free NE (Figure 4.5). As 10 of the SARS-CoV-2 TA samples were in 6M urea, NE activity could not be directly measured as 6M urea interferes with NE activity assays. Five of the SARS-CoV-2 TA samples had no 6M urea added and sufficient volume for FRET analysis. This showed active NE, in all five which was totally inhibited by the addition of plasma purified AAT (Figure 4.4, panel B).

Commensurate with the Western analysis, active NE was detected by FRET analysis in only one of the TA from nsARDS patients. The NE burden in SARS-CoV-2 infected airways compared to nsARDS was further demonstrated by iso-electric focusing with immunoblotting for AAT in TAs (Figure 4.6). Glycosylated AAT is present and well defined in the tracheal aspirates of individuals with nsARDS who have no active NE, but AAT is significantly degraded in SARS-CoV-2-ARDS TA samples where free NE was detectable.

Figure 4.3

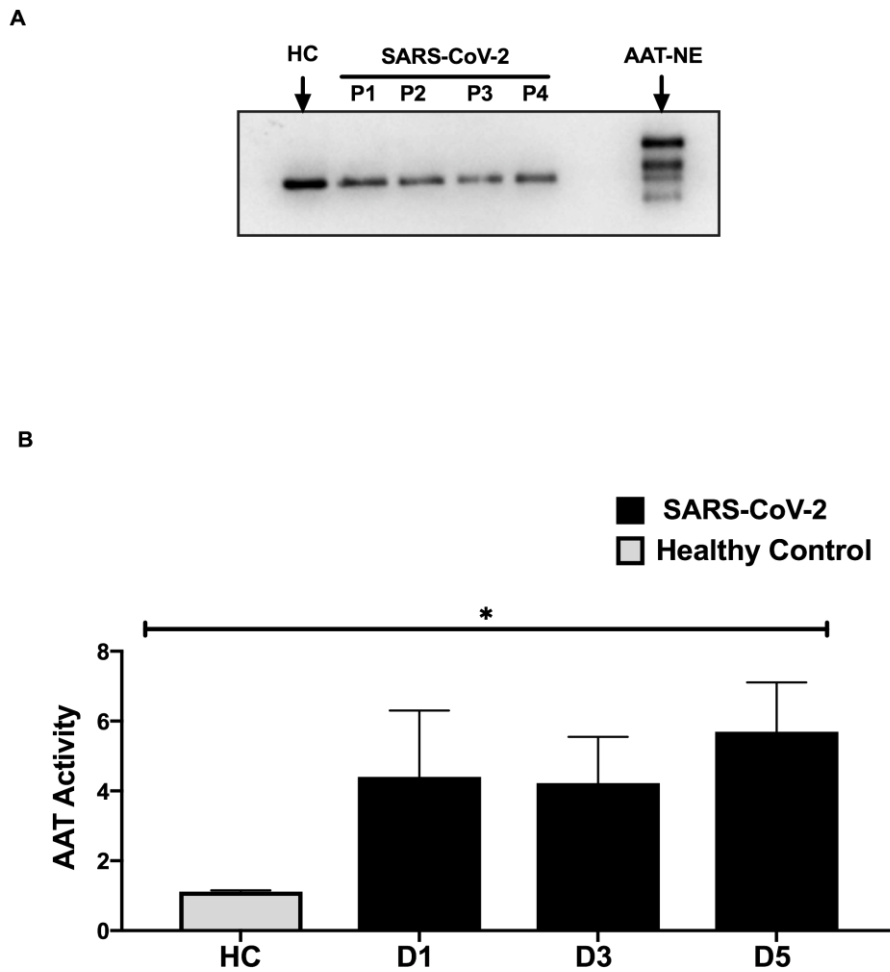


Figure 4.3

Form and function of AAT in the plasma of SARS-COV-2 ARDS patients

A. Western blot analysis for AAT in SARS-CoV-2 plasma from individual SARS-CoV-2 ARDS patients labelled P1, P2,P3 or P4 shows AAT at the appropriate molecular weight of 52 kDa without AAT-NE complexes or cleavage. **B.** FRET analysis demonstrate that AAT in the plasma of SARS-COV-2 patients is active and increased compared to healthy controls. $P < 0.0001$. Data represents mean AAT activity as the reciprocal of NE activity \pm SD. Significance was tested for by ANOVA; * $p < 0.05$.

Figure 4.4

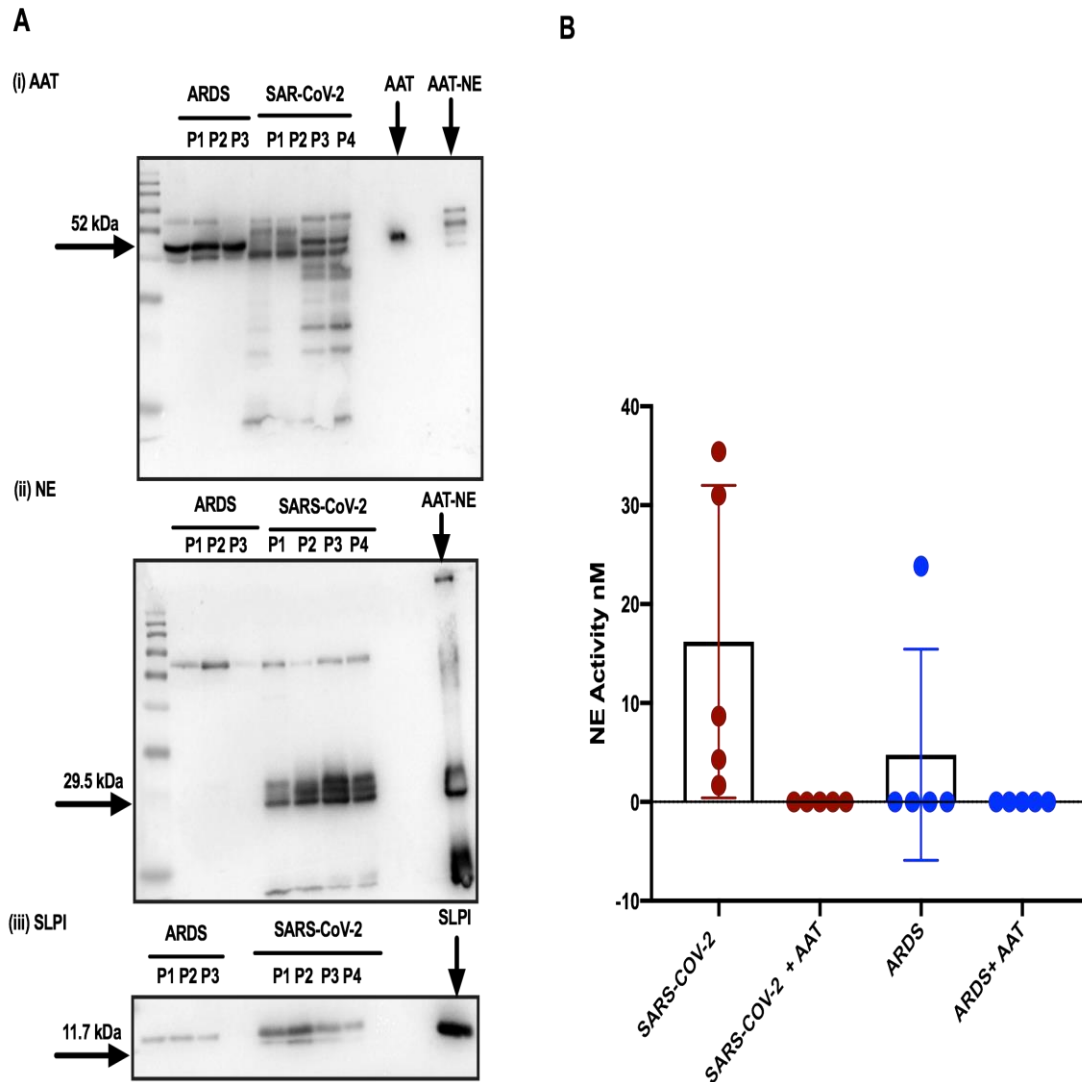


Figure 4.4

Form and function of AAT, NE and SLPI in plasma and tracheal aspirates (TA) of SARS-COV-2 ARDS patients

A (i). Western blot analysis of AAT in SARS-CoV-2 TAs from individual SARS-CoV-2 ARDS patients labelled P1, P2, P3, P4 and the control group of nsARDS patients labelled P1, P2, P3 demonstrates cleavage of AAT and AAT-NE complexes with significantly more cleavage of AAT seen in the SARS-CoV-2 TA samples. **(ii) (iii)** Western blot analysis of NE in the TA of the same individual patients show free NE and cleaved SLPI in SARS-CoV-2 TAs in contrast to the nsARDS control group. **B.** FRET analysis reveals active NE in all 5 TA from SARS-CoV-2 patients on their 1st day of ICU admission and inhibition with exogenous AAT. Only 1 of the 5 nsARDS TA samples demonstrated active NE which was inhibited by exogenous AAT. Significance was tested for by paired T test; * $p < 0.05$.

Figure 4.5

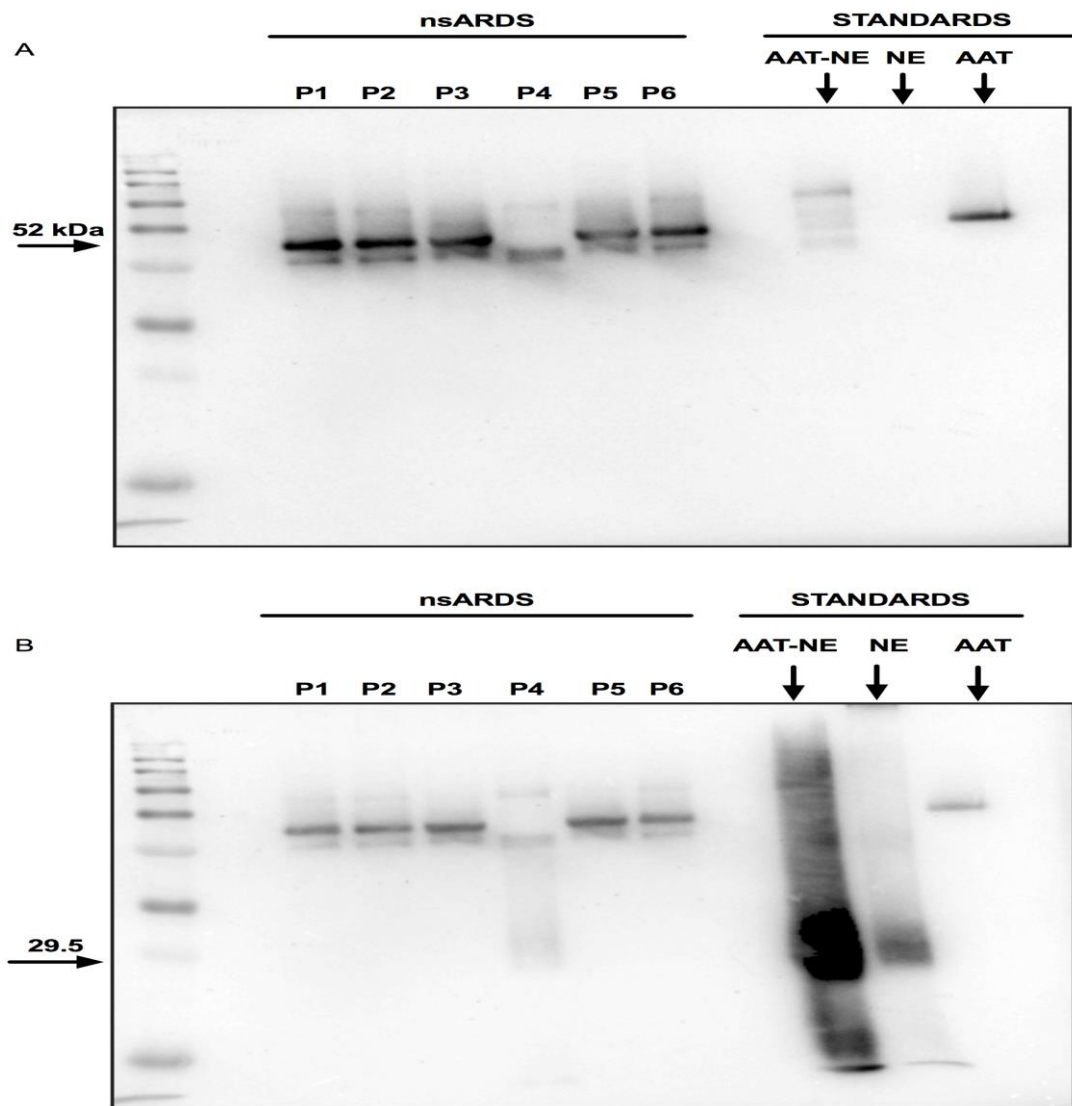


Figure 4.5
Western blot analysis of the NE and AAT present in nsARDS patient TA samples.

A. Western analysis of AAT in nsARDS TAs from individuals P1-P6. All samples demonstrate NE-AAT complexes and AAT cleavage, with P4 showing increased degradation and complexation of AAT. Below this western analysis is the image of the same blot re-probed for NE in all of the nsARDS TAs from individuals P1-P6. Only P4 demonstrates free NE.

Figure 4.6

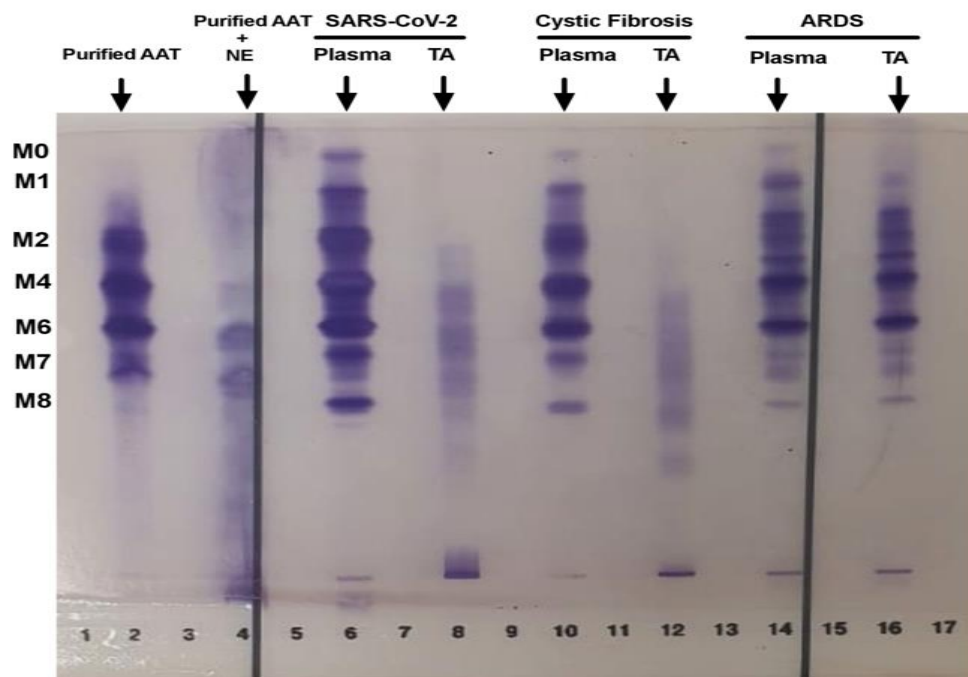


Figure 4.6

IEF gel of SARS-CoV-2 plasma and TA compared to different patient control groups, including cystic fibrosis and nsARDS

Representative IEF gel with the following samples in order from left to right: 1) Purified AAT, 2) Purified AAT plus NE, 3) SARS-CoV-2 plasma, 4) SARS-CoV-2 TA, 5) Cystic fibrosis plasma, 6) Cystic fibrosis TA, 7) nsARDS plasma, 8) nsARDS TA. With no change in the structure of the hyperglycosylated plasma samples from patients throughout but significant smearing of AAT in the context of neutrophilic disease states such as SARS-CoV-2 and cystic fibrosis. The normal glycosylation pattern seen in the ARDS TA corresponds with the lack of free or active NE as seen in western analysis and FRET analysis.

4.3.4 IHC analysis of autopsy SARS-CoV-2 lungs

All immunohistochemistry (IHC) studies were performed in the University of North Carolina using antibodies supplied by us and under our direction. IHC of control lungs identified SLPI in both superficial epithelia and submucosal glands (SMGs) (Figure 4.7 panel A). In contrast, in SARS-CoV-2 autopsy lungs, SLPI expression was decreased in both superficial epithelia and SMGs (Figure 4.7 panel A). Based on previous data demonstrating SARS-CoV-2 infection of superficial airway epithelia, these data are consistent with virus-induced suppression of SLPI expression (179). AAT and NE were localized in lungs from 8 subjects who died from SARS-CoV-2 infection. An increase in immuno-detectable AAT was observed in the SARS-CoV-2 autopsy lungs, mainly as free AAT filling alveolar lumens (Figure 4.7 panel B). There was no increase in neutrophil numbers in the SARS-CoV-2 lungs compared to controls as quantified by ELANE IHC (Figure 4.7 panel B). AAT appeared by IHC to be in excess of NE in SARS-CoV-2 lungs as indexed by increased AAT/NE (SERPINA1/ELANE) ratios (Figure 4.7, panel B, iv) and the increased number of AAT/NE double-positive cells (neutrophils associated with AAT) (Figure 4.7 panel B iv). Notably, a fraction of neutrophils in SARS-CoV-2 autopsy lungs, particularly in the interstitium, were characterized by NET formation as indicated by positive staining for citrullinated histone H3 (Figure 4.7, panel C, iii).

Figure 4.7

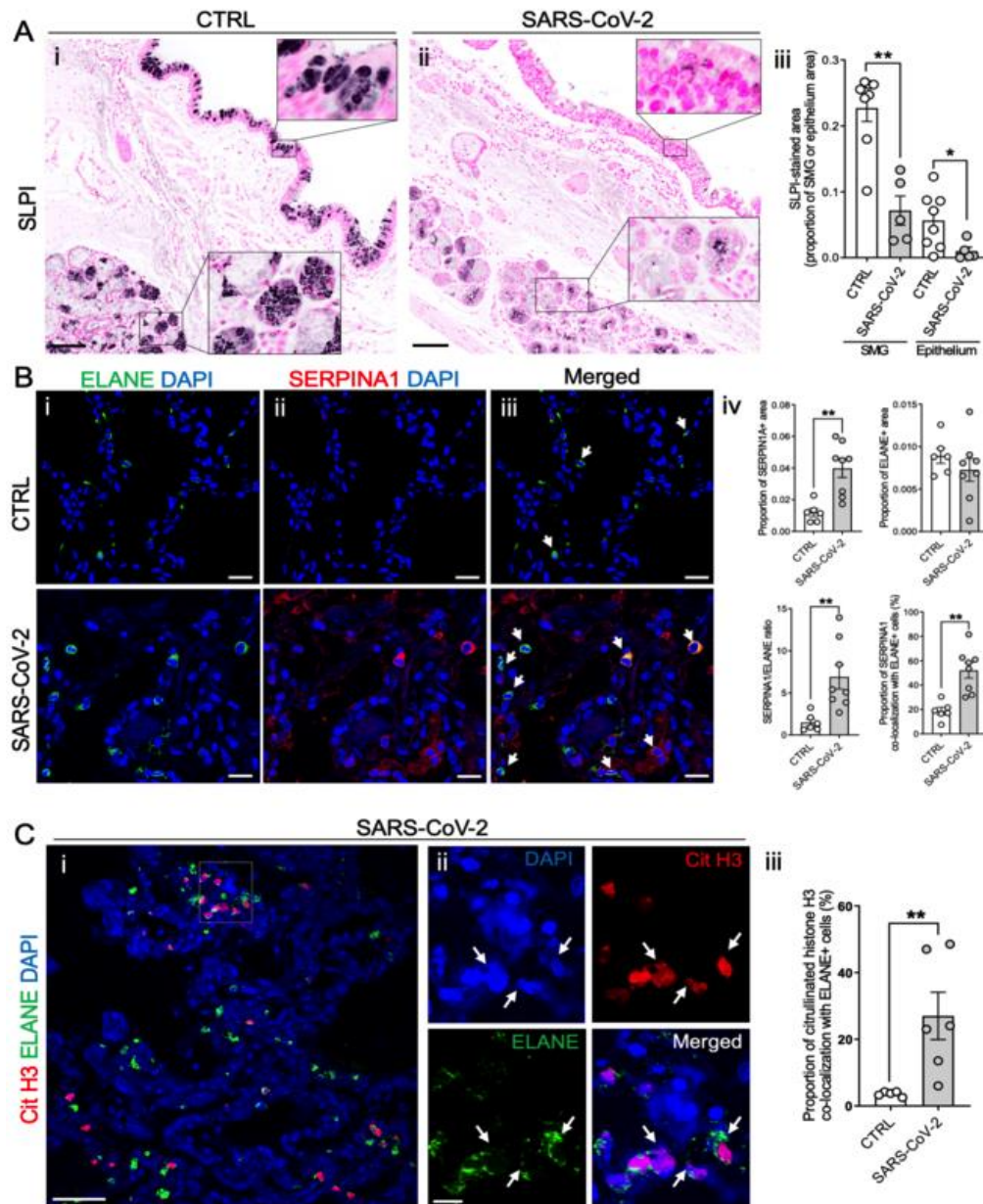


Figure 4.7

Protein expression in control and SARS-CoV-2 autopsy lungs by immunohistochemistry.

A. SLPI protein localization in large airways of control (n=4) (i) and SARS-CoV-2 autopsy lungs (n=8) (ii). Quantification of SLPI protein-stained area in each submucosal grand and epithelium (iii). **B.** Fluorescent immunohistochemistry of NE (ELANE) and AAT (SERPINA1) in alveoli of control and SARS-CoV-2 autopsy lungs (i-iii). Quantification of each protein-stained area, AAT/NE ratio, and colocalization between AAT and ELANE (iv). **C.** Fluorescent immunohistochemistry of NE (ELANE) and citrullinated histone H3 (Cit H3) in alveoli of control and SARS-CoV-2 autopsy lungs (i-ii). Arrows showed colocalization of ELANE and Cit H3 (ii). Quantification of co-localization between ELANE and Cit H3 (iii). Histogram bars and error bars represent mean \pm SEM. *P<0.05, **P<0.01. Scale bars = 100 μ m (a); 20 μ m (b); 50 μ m (ci); 10 μ m (Cii). Significance was tested for by Mann-Whitney test (U-test).

4.3.5 Alterations in glycosylation and sialylation of AAT in SARS- CoV-2 ARDS patient plasma.

The % sialylation of AAT in plasma from subjects with severe SARS-CoV-2 infection collected 8 days into ICU admissions was increased compared to healthy control plasma ($P= 0.0211$) (Figure 4.8, panel A). Concurrent with this, there was also an increased abundance of M0 and M1 bands detected in SARS-CoV-2 patients (Figure 4.8, panel B), and this increase in AAT sialylation glycoforms persisted throughout the ICU stay.

Figure 4.8

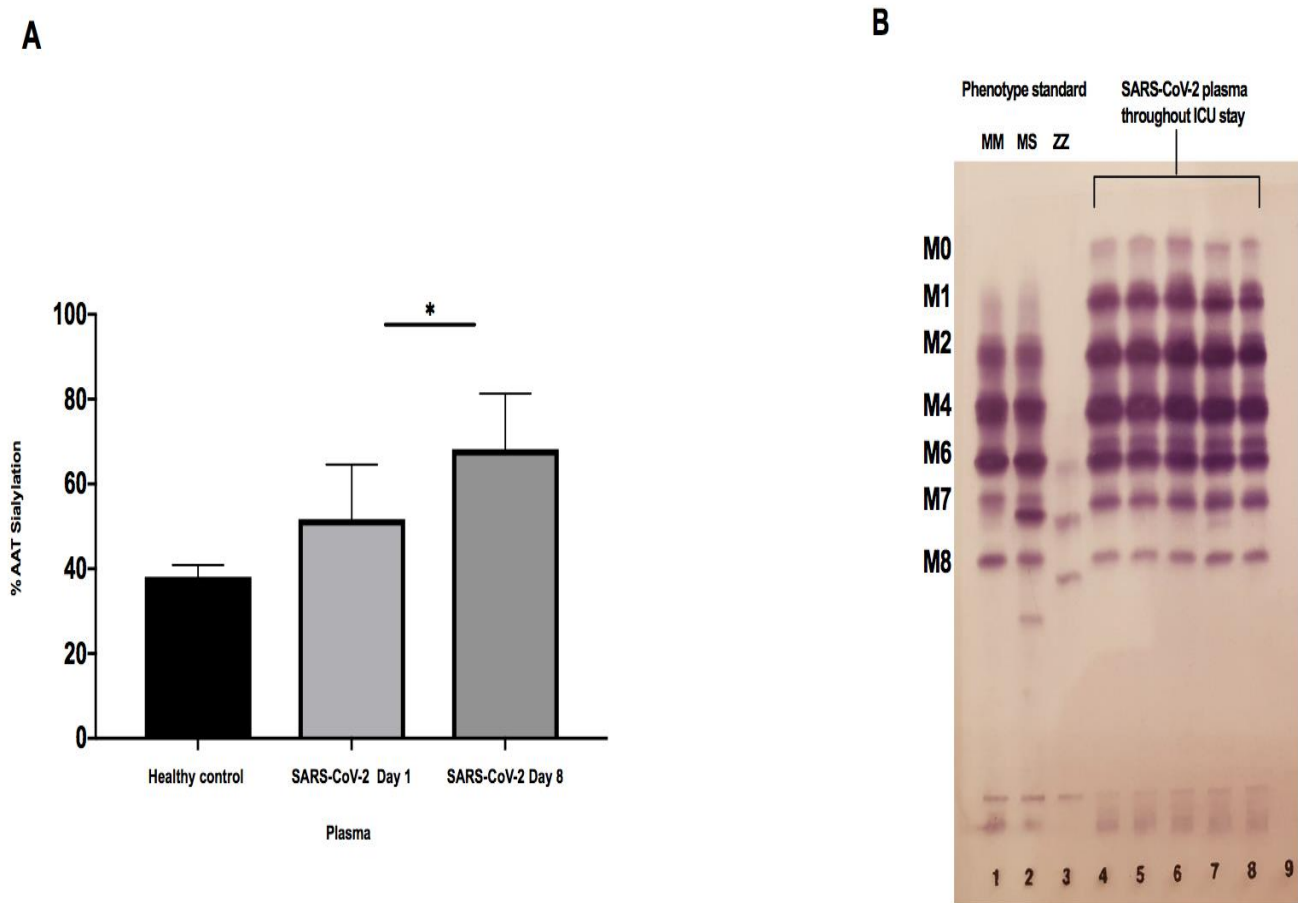


Figure 4.8

Plasma AAT sialylation and glycosylation in response to SARS-CoV-2 ARDS

A. Percentage of plasma AAT showing sialylation, from healthy controls compared to SARS-CoV-2 ARDS patients on day 1 (ns, =0.3) and day 8 of admission to ICU (P = 0.02) Analyses were performed using a two-way ANOVA, Tukey's multiple comparisons test. **B.** Representative isoelectric focusing gel shows presence of highly sialylated M0 and M1 AAT glycoforms in SARS-COV-2 plasma on day 1 post-ICU admission.

4.3.6 AAT and IL-6 levels pre and post pan-IL-6 receptor antagonist

Given the role of IL-6 signalling in regulating AAT secretion, the effect of tocilizumab on AAT *in vivo*, during acute SARS-CoV-2 infection was examined. Plasma samples were collected from ward level care patients with confirmed SARS-CoV-2 infection pre- and post-tocilizumab infusion. The initial plasma AAT level ($2.469 \text{ g/L} \pm 0.2$) was significantly higher than healthy controls ($P < 0.0001$). Patients were dosed with 8 mg/kg tocilizumab ($n=24$, table 4.6) (180). A significant decrease in AAT plasma levels was noted 28 days post tocilizumab ($1.3 \text{ g/L} \pm 0.225$, $P < 0.0001$) (Figure 4.9, panel A). In contrast, IL-6 levels remained elevated throughout the 28-day course in these patients (Figure 4.9 panel B, $NS=0.0998$). These changes were reflected in the increased IL-6/AAT ratio at day 28 post tocilizumab ($P=0.046$) (Figure 4.9, panel C), which suggested that IL-6 was no longer regulating AAT production. In ward level COVID-19 patients who received standard of care treatment (SOC) without tocilizumab ($n=6$), AAT levels remained elevated ($2.54 \text{ g/L} \pm 0.42$ to $2.27 \text{ g/L} \pm 0.39$ at day 28, $NS=0.1856$) (Figure 4.10, panel B). Similarly, the IL-6 levels in SOC patients showed no significant decline from their first recorded levels ($19.016 \text{ pg/ml} \pm 10.35 \text{ pg/ml}$ compared to $15.823 \text{ pg/ml} \pm 13.08$, $NS=0.885$) (Figure 4.10, panel A). The SOC patients exhibited AAT hyperglycosylation at the beginning and end of their treatment denoted by M0 and M1 bands, whereas the tocilizumab treated patients at day 28 shows no M0 or M1 bands despite initially manifesting a hyperglycosylated AAT profile prior to treatment (Figure 4.9 panel D). The degree of AAT/IL-8 binding in SARS-CoV-2 samples post tocilizumab infusion was significantly reduced compared to pre-infusion binding ($P=0.0011$) and in comparison to healthy controls ($P=0.0002$), reflecting the diminished glycosylation of AAT upon tocilizumab treatment (Figure 4.11).

Figure 4.9

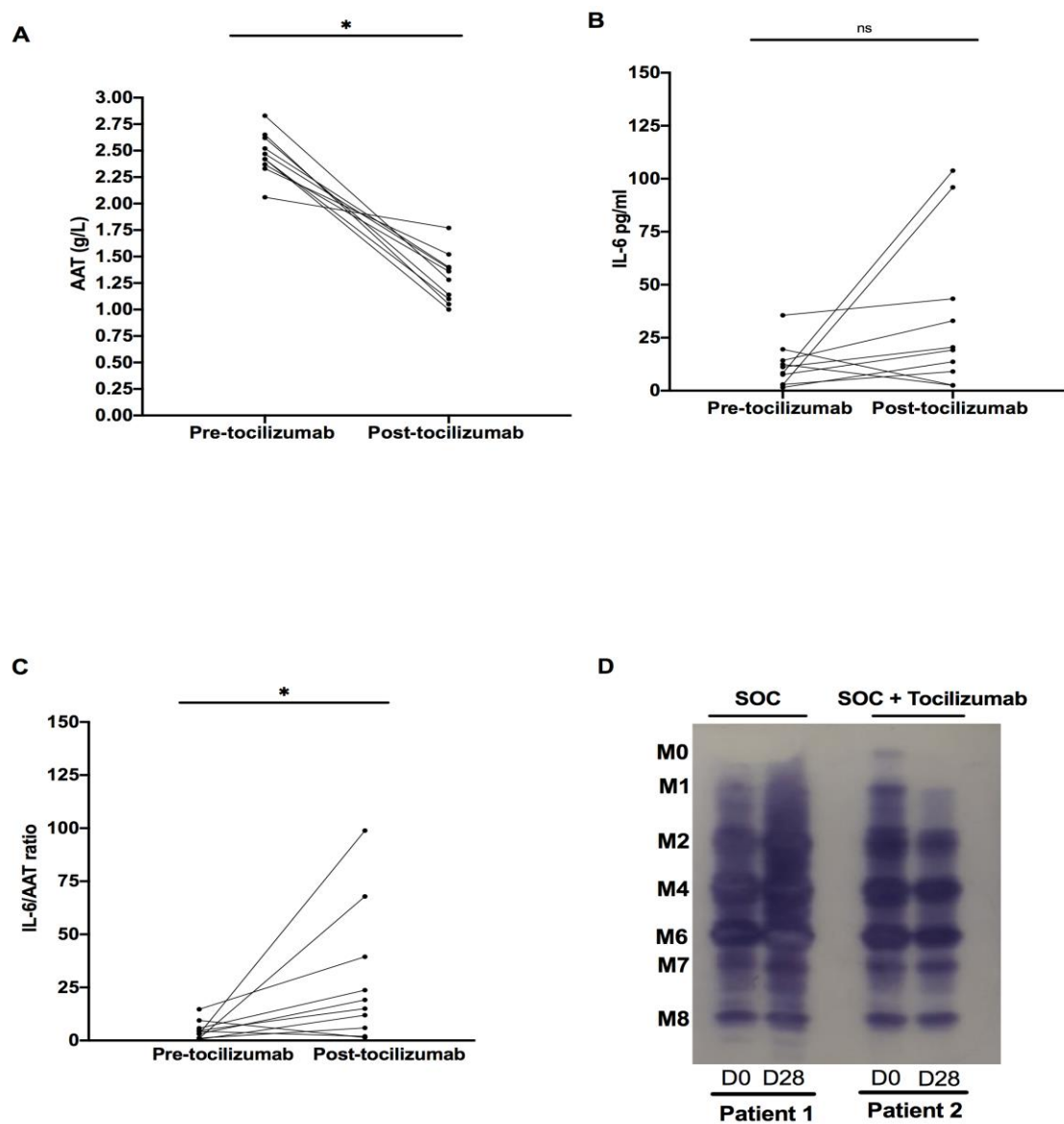


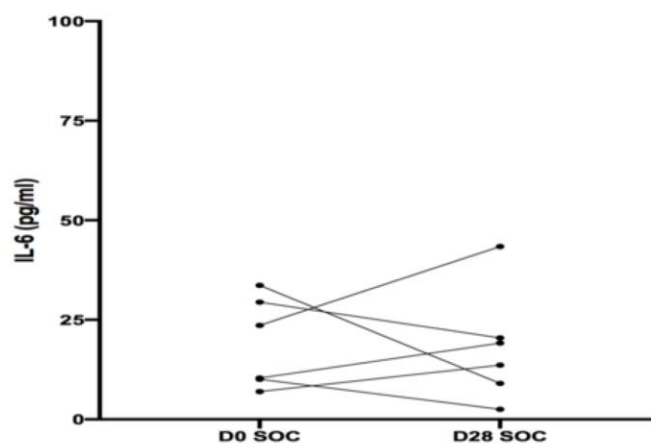
Figure 4.9

AAT and IL-6 levels pre and post pan-IL-6 receptor antagonist

A. Decrease in AAT plasma levels in ward level patients post tocilizumab (n=10) ($P < 0.0001$) (paired T test). **B.** Maintenance of elevated IL-6 plasma levels in the same cohort of ward level patients throughout their 28 day course (ns=0.099, paired T test). **C.** Increased IL-6/AAT ratio at day 28 post tocilizumab ($P=0.046$, paired T test). **D.** Glycosylation pattern pre- and post- tocilizumab infusion as compared to ward level patients receiving standard of care (SOC), matched for a similar AAT level and inflammatory profile sampled at the same timepoints.

Figure 4.10

A



B

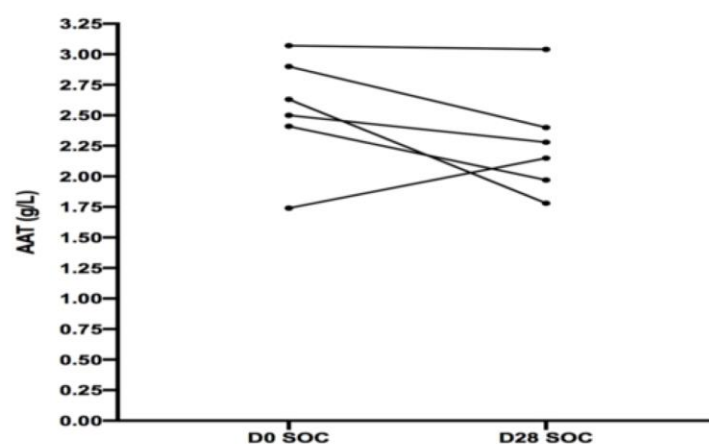


Figure 4.10

IL-6 and AAT levels of ward level SARS-CoV-2 patients receiving standard of care (SOC)

- A. AAT levels did not drop from their initial levels ($2.54\text{g/L} \pm 0.42$ to $2.27\text{g/L} \pm 0.39$ at day 28, $NS=0.1856$ paired T test). B. IL-6 levels in SOC patients did not decline from their first recorded levels ($19.016\text{ pg/ml} \pm 10.35$ to $15.823\text{ pg/ml} \pm 13.08$, $NS=0.885$ paired T test).

Figure 4.11

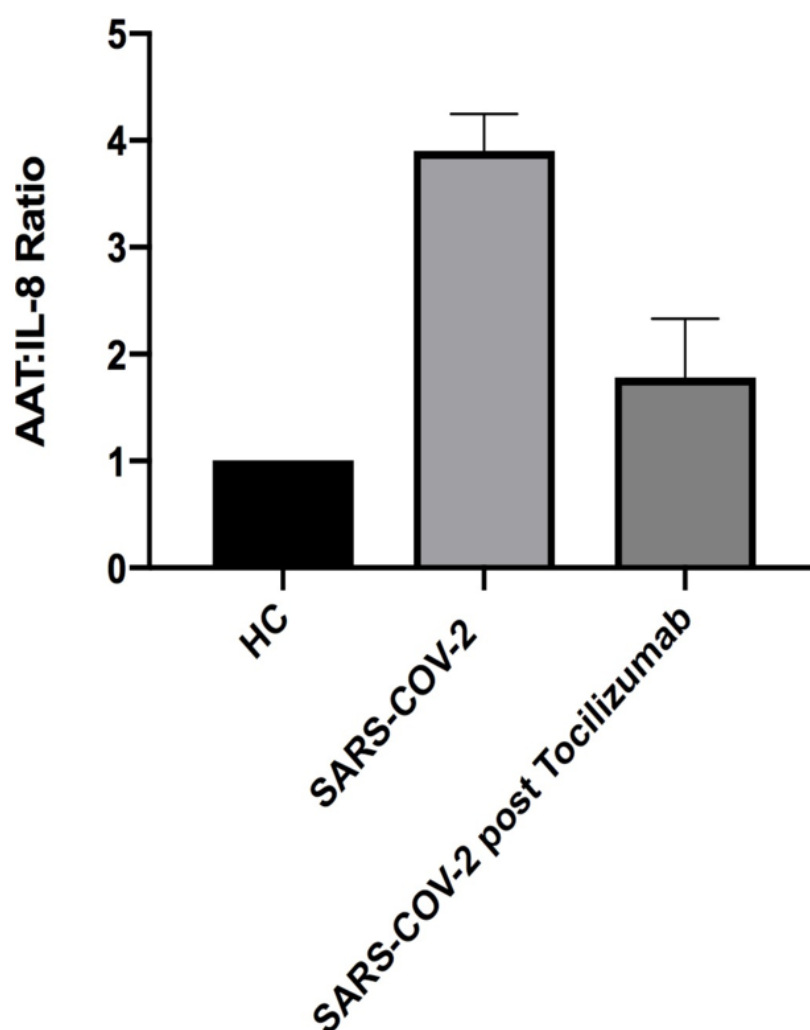


Figure 4.11

The AAT/IL-8 ratio of hyperglycosylated AAT in SARS-CoV-2 patients as compared to the same patient group post tocilizumab infusion and healthy controls.

Binding of IL-8 by AAT was increased in patients with SARS-CoV-2 infection compared to healthy controls ($P=0.0002$), and in comparison to AAT-IL-8 binding post tocilizumab infusion ($P=0.0011$). Significance was determined by one way ANOVA, Tukey multiple comparison test $*p<0.05$)

4.3.7 Plasma AAT concentrations in response to treatment with IV AAT in phase 2 trial

In SARS-CoV-2 ARDS patients receiving Prolastin, plasma AAT concentrations were significantly increased 2 days post-infusion (figure 4.12). Of note, AAT levels in the treatment group were still increased at day 7 compared to baseline, suggesting that weekly administration may result in a stacking effect. In contrast, no change in AAT levels was observed in those receiving placebo. At day 2 and day 7, plasma AAT concentrations were significantly higher in patients receiving Prolastin compared to those in the placebo group (both $P < 0.0001$).

Figure 4.12

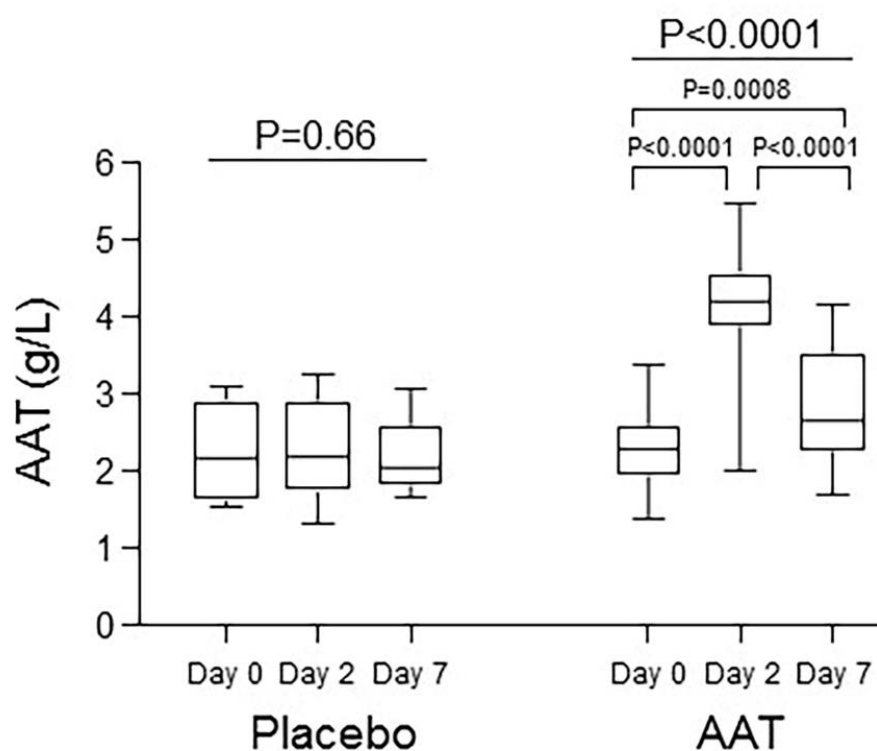


Figure 4.12

Alpha-1 antitrypsin levels over time.

Circulating alpha-1 antitrypsin (AAT) levels on day 0 were similar in the treatment group (AAT; 2.3 +/- 0.5g/L, n=22) compared to the placebo group (2.3 +/- 0.7g/L, n=11). AAT levels at day 2 were significantly higher in the treatment group (placebo: 2.3 +/- 0.7 g/L, AAT: 4.2 +/- 0.7g/L; P <0.0001). At day 7, the AAT level in the treatment group (2.8 +/- 0.8g/L) was increased compared to day 0 (P=0.0008).

4.3.8 Primary endpoint of phase 2 trial

Patients receiving Prolastin demonstrated a decrease in circulating IL-6 at day 7 compared to day 0 (day 0: 296.0 +/- 219.7 pg/mL, day 7: 217.7 +/- 168.7 pg/mL; Figure 4.13, panel A P=0.003), whereas an increase in IL-6 levels was observed in the placebo group (day 0: 259.9 +/- 206.5pg/ml, day 7: 348.2 +/- 264.0pg/ml; Figure 4.13 panel B; P=0.04). Similarly, the change in plasma levels of IL-6 at 1 week compared to baseline was -78.3 +/- 112.1 pg/mL in the treatment group compared to +88.3 +/- 125.8 pg/mL in the placebo group (Figure 4.13 panel B; P=0.002), thereby satisfying the primary endpoint for the study. The percentage change in plasma IL-6 from baseline followed a similar pattern, with a mean reduction of 17.4 +/- 42.3% in those receiving Prolastin versus a 37.8 +/- 56.6% increase in patients assigned to placebo (Figure 4.13 panel C; P=0.01).

Figure 4.13

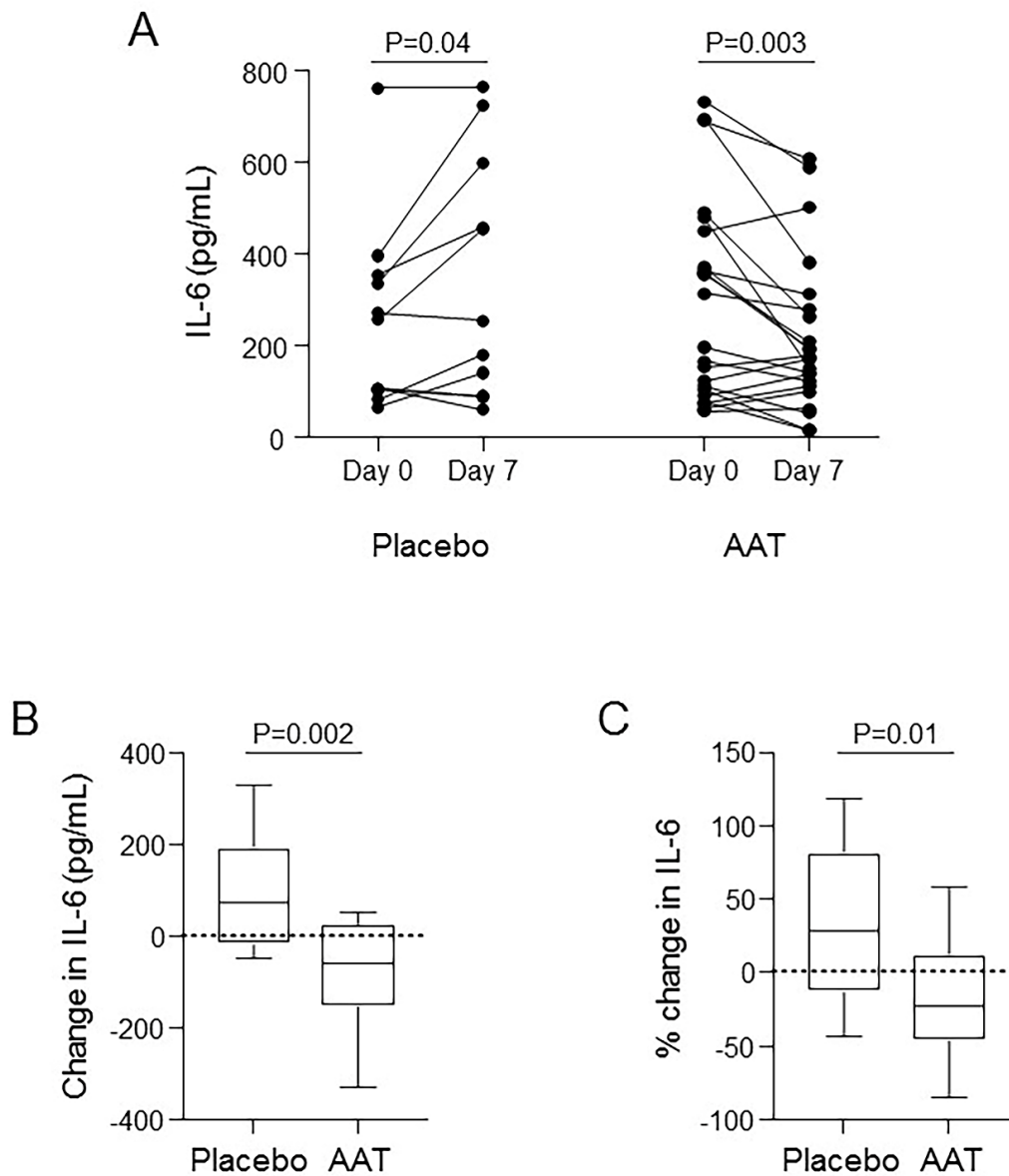


Figure 4.13

Decreased circulating IL-6 following treatment with IV AAT.

A. Plasma was obtained at day 0 and day 7 from patients receiving placebo (n=11) and patients receiving AAT (n=22). IL-6 levels were increased at day 7 compared to day 0 in the placebo group (day 0: 259.9 \pm 206.5 pg/ml, day 7: 348.2 \pm 264.0 pg/ml; $P=0.04$) and decreased at day 7 in the AAT group (day 0: 296.0 \pm 219.7 pg/ml, day 7: 217.7 \pm 168.7 pg/ml; $P=0.003$). **B.** The 7-day change in plasma levels of IL-6 from baseline was +88.3 \pm 125.8 pg/ml in the placebo group compared to -78.3 \pm 112.1 pg/ml in the treatment group ($P=0.002$). **C.** Patients assigned to placebo demonstrated a 37.8 \pm 56.6% increase in plasma IL-6 at day 7 compared to a mean reduction of 17.4 \pm 42.3% in those receiving IV AAT ($P=0.01$). IL = interleukin; AAT = alpha-1 antitrypsin.

4.3.9 Secondary biochemical endpoints of phase 2 trial

Changes in other circulating cytokine concentrations in response to IV AAT were investigated. The measurement of TNF- α in blood is complicated by its short half-life and rapid turnover. In plasma, concentrations of sTNFR1 act as a reliable surrogate marker for TNF- α levels (181, 182). As for IL-6, a significant reduction in plasma sTNFR1 concentrations was observed in the treatment group at day 7 (day 0: 4947 \pm 2605pg/ml, day 7: 4131 \pm 2207pg/ml; Figure 4.14; $P=0.0009$). Circulating levels of sTNFR1 in the placebo group were unchanged. The absolute 7-day change in plasma sTNFR1 was 507.6 \pm 1552pg/ml (an increase) in the placebo group, compared to -815.8 \pm 989.0pg/ml (a decrease) in study participants receiving IV AAT (Figure 4.14 panel B; $P=0.02$). The percentage change in plasma sTNFR1 was also significantly different between the groups (placebo: +23.1 \pm 37.7%; AAT: -15.3 \pm 19.6%; Fig. 4.14 panel C, $P=0.008$). These results are consistent with previous studies identifying AAT as a key regulator of TNF- α signalling and gene expression, and an inhibitor of TNF- α -induced nuclear factor κ B (NF- κ B) activation (179). It also illustrates an effect of intravenous AAT augmentation on ADAM-17 activity *in vivo*. Definitive effects of AAT therapy on levels of IL-1 β (Fig. 4.14 panel D-F), IL-8 (Fig. 4.14 panel G-I) and IL-10 (Fig. 4.14 panel J-L) at 1 week were not observed. However, post-hoc data analyses did demonstrate significant within-week differences in levels of IL-1 β and IL-8 in response to IV AAT (Table 4.9). Within-week effects on IL-6 and sTNFR1 were also present, with the reduction in plasma levels of these cytokines greatest at day 2 post-infusion, coinciding with peak circulating AAT levels.

Figure 4.14

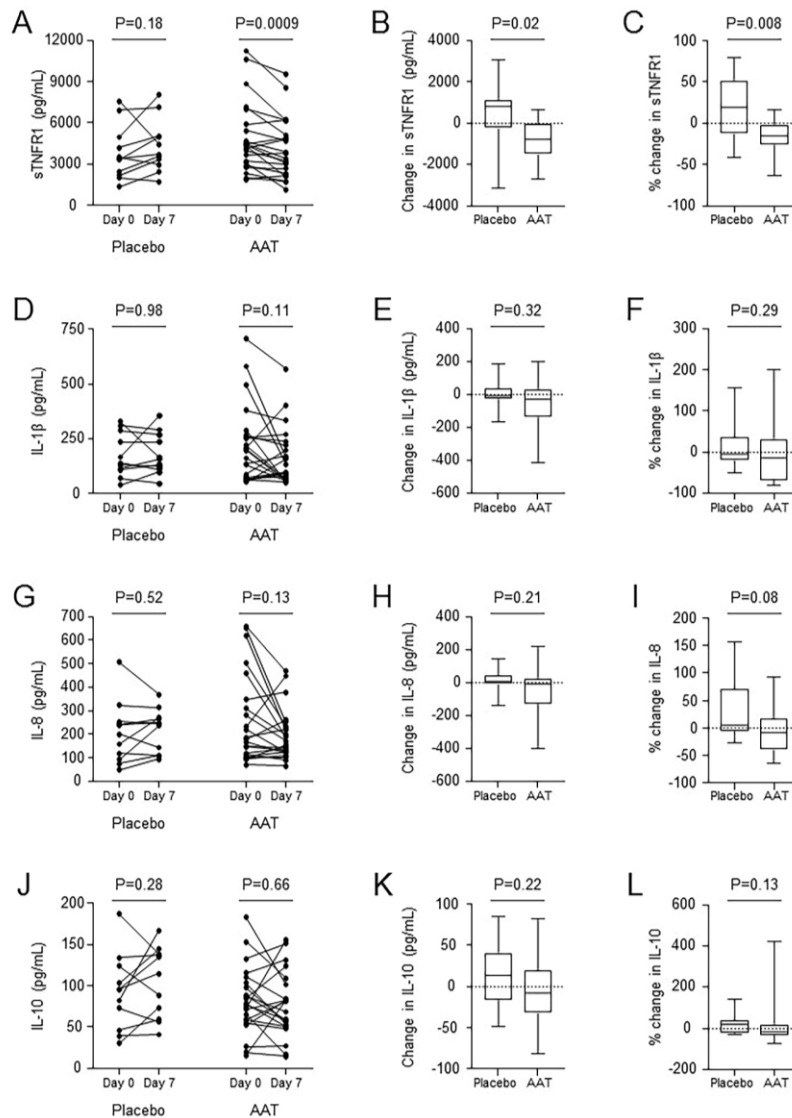


Figure 4.14

Secondary biochemical endpoints.

- A.** At day 7, levels of sTNFR1 were not significantly different in the placebo group compared to day 0 (day 0: 3808 +/- 1989pg/ml, day 7: 4315 +/- 1919pg/ml; P=0.18), in contrast to the AAT group, where sTNFR1 levels were decreased at day 7 (day 0: 4947 +/- 2605pg/ml, day 7: 4131 +/- 2207pg/ml; P=0.0009). **B, C** Compared to placebo, patients receiving AAT demonstrated a greater absolute change (placebo: +507.6 +/- 1552pg/ml; AAT: -815.8 +/- 989.0pg/ml; P=0.02) and the percentage change (placebo: +23.1 +/- 37.7%; AAT: -15.3 +/- 19.6%; P=0.008) in sTNFR1 at day 7. **D** No difference in IL-1β levels was detected at day 7 in either the placebo group (day 0: 174.7 +/- 99.8pg/ml, day 7: 179.9 +/- 94.9pg/ml; P=0.98) or the AAT group (day 0: 224.7 +/- 177.6pg/ml, day 7: 164.9 +/- 132.7pg/ml; P=0.11). **E-L** Treatment with AAT did not result in a greater absolute change (P=0.32) or percentage change (P=0.29) at day 7. **G-L** Similarly, no differences were observed for IL-8 on day 0 versus day 7 in either group (placebo: 205.2 +/- 131.8pg/ml vs 218.8 +/- 91.3pg/ml, P=0.52; AAT: 264.3 +/- 193.0pg/ml vs 198.1 +/- 110.1pg/ml, P=0.13) or for IL-10 (placebo: 92.1 +/- 46.0pg/ml vs 105.0 +/- 12.9pg/ml, P=0.28; AAT: 82.3 +/- 40.8 vs 76.8 +/- 39.4pg/ml, P=0.66). For both IL-8 and IL-10, the 7-day change in concentration failed to reach statistical significance, either in absolute (IL-8: P=0.21; IL-10: P=0.22) or percentage terms (IL-8: P=0.08; IL-10: P=0.13). IL = interleukin; sTNFR1 = soluble tumour necrosis factor receptor 1.

Table 4.9

Within-week changes in cytokine levels following IV AAT therapy

Within-week changes in cytokine levels following AAT therapy					
Marker (pg/mL)	Day 0 (n=25)	Day 2 (n=25)	Day 7 (n=22)	Day 0 vs Day 2	Trend over time
IL-6	267.0 +/- 220.9	197.7 +/- 164.7	217.7 +/- 168.7	P=0.004	P=0.002
sTNFR1	4604 +/- 2626	3804 +/- 2163	4131 +/- 2207	P=0.003	P=0.001
IL-1β	204.5 +/- 175.7	125.7 +/- 112.4	164.9 +/- 132.7	P=0.002	P=0.01
IL-8	245.0 +/- 188.8	189.3 +/- 115.5	198.1 +/- 110.1	P=0.007	P=0.04
IL-10	76.8 +/- 41.9	71.6 +/- 35.02	76.8 +/- 39.4	P=0.29	P=0.62

Data presented as mean +/- SD. For comparisons between cytokine levels at day 0 and day 2, P-values represent paired analyses, with a paired t-test used in the event of normally distributed data and Wilcoxon matched-pairs signed rank test used for comparisons between groups that failed normality testing. For the assessment of the trend in a given cytokine over time (day 0 to day 2 to day 7) a mixed effects analysis with Geisser-Greenhouse correction was used.

4.3.10 Secondary clinical endpoints of phase 2 trial

The study was not powered to detect meaningful effects on clinical outcomes such as mortality, but data on these outcomes were collected as part of a safety and feasibility assessment. No difference in mortality was observed between patients who received AAT and those in the treatment group (Fig. 4.15). IV AAT did not significantly reduce the time-to-extubation as assessed at the end of the 28-day study period compared to placebo (Fig. 4.16) though the point estimates favoured the treatment group, and merit further investigation in a larger study. Similarly, the number of ventilator-free days, sequential organ failure assessment (SOFA) score, PaO₂:FIO₂ ratio ICU length of stay and hospital length of stay were numerically improved in patients receiving AAT, without reaching statistical significance (Table 4.10, Table 4.11). No increase in secondary bacterial infection was observed in the treatment group, and no rebound effect on safety or clinical outcomes was seen in patients transitioning from AAT to placebo at one week. A summary of clinical outcomes of interest is available in Table 4.10.

Figure 4.15

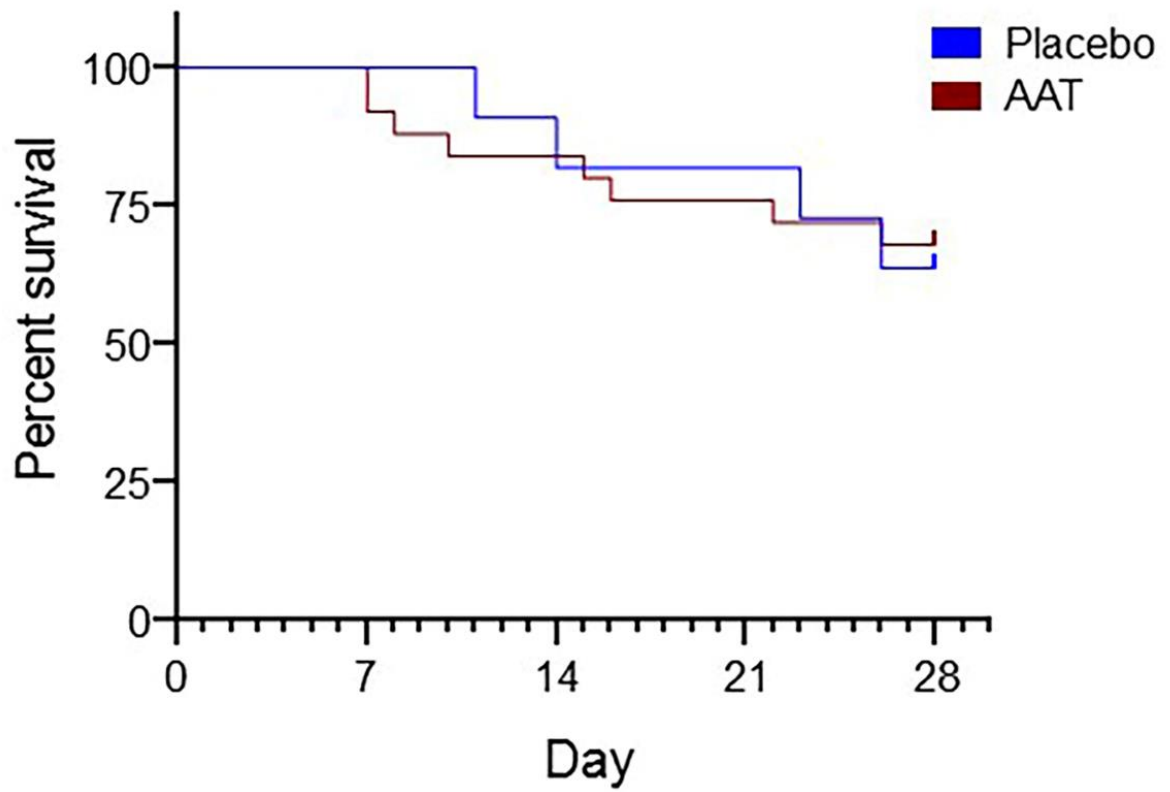


Figure 4.15

28-day mortality.

Kaplan Meier curve depicting mortality in the placebo (n=11) and treatment (n=25) groups. No significant difference was observed between the groups when assessed at day 28 (Log-rank $P=0.91$).

Figure 4.16

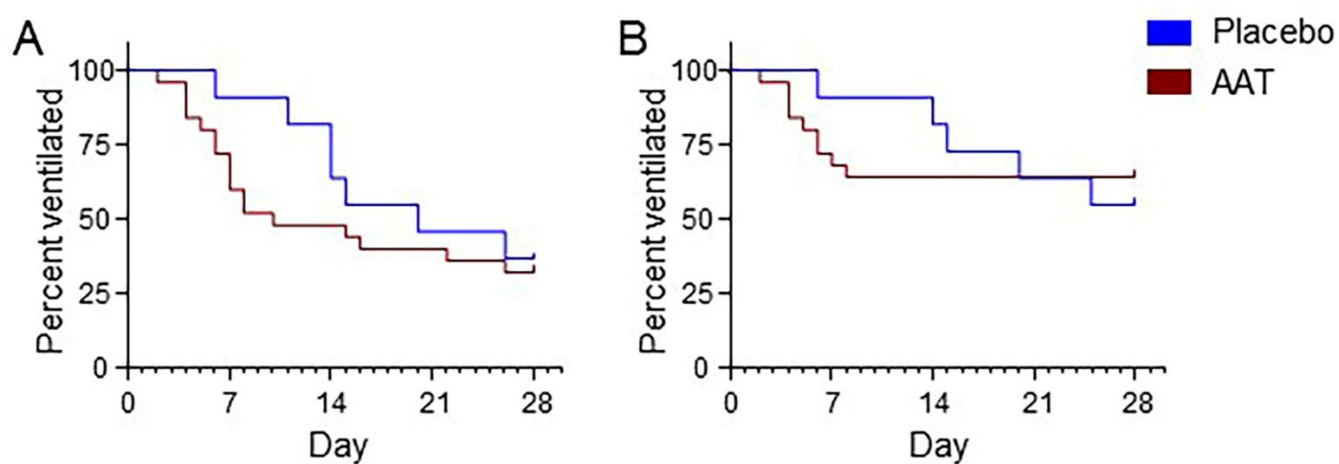


Figure 4.16

Time-to-extubation.

A. Kaplan Meier curve depicting time-to-extubation in the placebo (n=11) and treatment (n=25) groups. No significant difference was observed between the groups when assessed at day 28 (Log-rank $P=0.44$). **B.** Time-to-extubation in survivors only (Log-rank $P=0.89$).

Table 4.10
Clinical outcomes from IV AAT trial

Clinical outcomes of interest				
	Placebo	AAT once	AAT weekly	Any AAT
SOFA score at day 7	8.1 +/- 3.5	7.3 +/- 3.9	4.5 +/- 4.0	5.8 +/- 4.1
PaO₂:FIO₂ at day 7 (mmHg)	127.7 +/- 53.7	132.4 +/- 69.1	221.4 +/- 126.6	186.7 +/- 135.1
Mortality	4 (36)	6 (50)	2 (15)	8 (32)
Ventilator-free days	3.6 +/- 5.7	5.6 +/- 10.1	10.7 +/- 11.3	8.2 +/- 11.3
Acute kidney injury *	8 (72)	9 (75)	8 (62)	17 (68)
Secondary bacterial pneumonia *	7 (63)	6 (50)	5 (38)	11 (44)
ICU length of stay	21.4 +/- 5.9	16.4 +/- 9.3	15.6 +/- 10.8	16.0 +/- 9.9
Hospital length of stay	24.6 +/- 6.2	18.4 +/- 8.2	20.1 +/- 9.3	19.3 +/- 8.7

Data presented as number (%) or mean +/- SD. All variables assessed at day 28 unless stated otherwise.

SOFA – sequential organ failure assessment

PaO₂:FIO₂ – ratio of partial pressure of oxygen in arterial blood to the fraction of inspired oxygen

ICU – intensive care unit

* number of patients with an event over duration of study

Table 4.11
Inter-group statistical analyses for clinical outcomes

Inter-group statistical analyses for clinical outcomes of interest				
	Placebo vs AAT once	Placebo vs AAT weekly	AAT once vs AAT weekly	Placebo vs Any AAT
SOFA score at day 7	P=0.59	P=0.03	P=0.10	P=0.13
PaO₂:FIO₂ at day 7 (mmHg)	P=0.86	P=0.03	P=0.04	P=0.29
Ventilator-free days	P=0.95	P=0.23	P=0.22	P=0.50
ICU length of stay	P=0.22	P=0.30	P=0.73	P=0.19
Hospital length of stay	P=0.04	P=0.38	P=0.64	P=0.10

Data presented as number (%) or mean +/- SD. All variables assessed at day 28 unless stated otherwise. P-values represent paired analyses, with a paired t-test used in the event of normally distributed data and Wilcoxon matched-pairs signed rank test used for comparisons between groups that failed normality testing.

SOFA – sequential organ failure assessment

PaO₂:FIO₂ – ratio of partial pressure of oxygen in arterial blood to the fraction of inspired oxygen

ICU – intensive care unit

4.3.11 Safety and tolerability of IV AAT in SARS-CoV-2 ARDS

No adverse events (AE)s or severe AEs (SAEs) were considered to be related or probably related to the study drug. One AE (atrial fibrillation in a patient with known paroxysmal AF) was judged to be possibly related to IV Prolastin. One SAE was deemed to be possibly related to IV Prolastin (hypertension persistent for >30 min post-infusion) and resolved without sequelae. No AE or SAE resulted in discontinuation of treatment.

4.3.12 Preservation of classic IL-6 signalling in SARS-CoV-2 ARDS patients treated with IV AAT

CRP is induced via physiologic classic IL-6 signalling – but not by pathologic trans-signalling or trans-presentation – as part of the acute phase response (183, 184, 55, 185), and serves as an inflammatory biomarker in critical illness. At day 7 post-infusion, patients treated with IV AAT displayed decreased levels of circulating CRP, proportional to the decreases observed for plasma IL-6 (Fig. 4.17 panel A). However, CRP levels post-AAT were still elevated above the normal range (0-5 mg/L), indicating that the classic signalling pathway remained intact in patients receiving IV AAT at 120 mg/kg.

In addition to upregulating the production and release of endogenous AAT during the acute phase response, IL-6 also induces a change in the glycosylation and sialylation of AAT (19). This shift, which has previously been described in community-acquired pneumonia, results in the emergence of pro-resolution M0 and M1 AAT glycoforms on serum protein electrophoresis, a phenomenon that is specific for an IL-6-mediated acute phase response via classic signalling (19). Monoclonal antibodies against the IL-6 receptor such as tocilizumab do not discriminate between classical or trans-signalling, and therefore abolish both the pathological and physiological effects of the cytokine. Immunofixation of plasma glycoforms from patients in the AAT treatment group by isoelectric focusing gel electrophoresis confirmed the presence of M0/M1 AAT glycoforms (Fig. 4.17 panel B), consistent with preservation of classic signalling. In contrast, when plasma from matched SARS-CoV-2 ARDS patients receiving tocilizumab was analysed, M0, M1 glycoforms were absent, in keeping with inhibition of classic signalling.

Figure 4.17

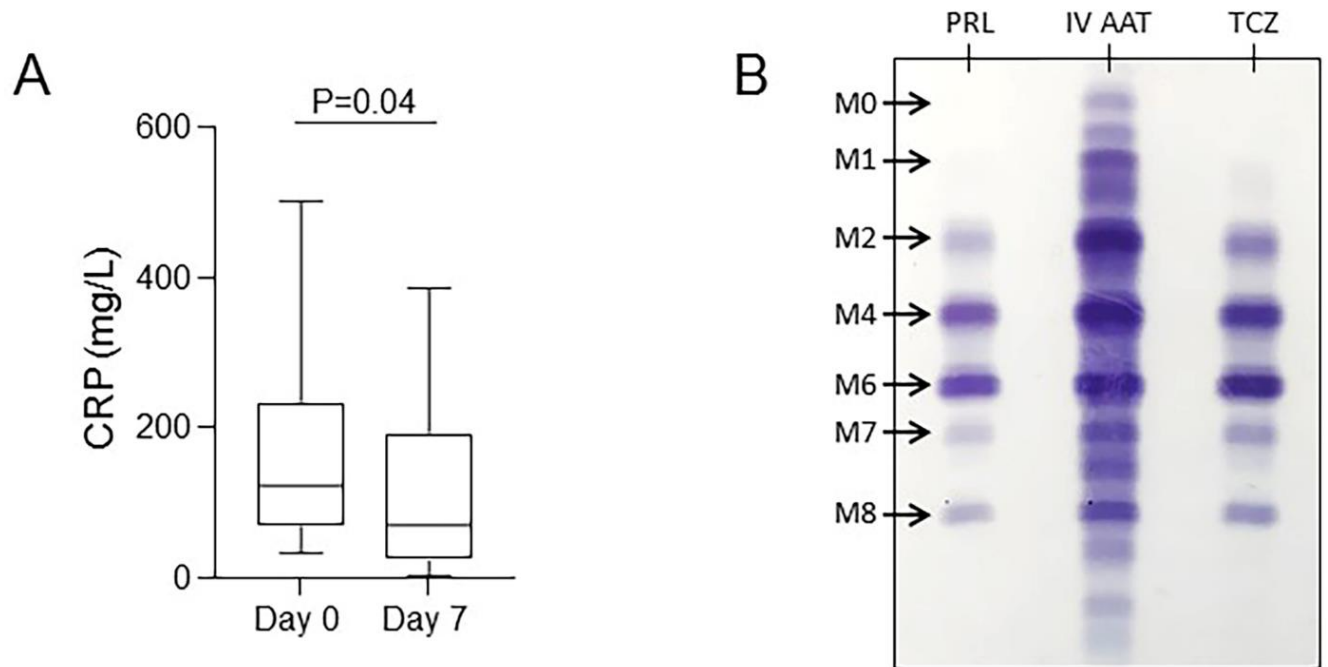


Figure 4.17

Physiological properties of IL-6 are preserved in patients treated with IV AAT.

A. Circulating CRP levels were measured in patients receiving IV AAT at study commencement (day 0) and again at one-week post-infusion (day 7, n=22). CRP was decreased – but not abolished entirely – at day 7 compared to day 0 (day 0: 168.6 +/- 138.0 mg/L, day 7: 110.4 +/- 117.2 mg/L; P=0.04). **B.** AAT protein phenotypes in plasma from patients in the treatment group (day 7 post-infusion; “IV AAT”) and patients receiving tocilizumab for COVID-19 ARDS (“TCZ”) were determined by immunofixation of glycoforms via isoelectric focusing gel electrophoresis. Exogenous Prolastin (“PRL”) was also assayed, to demonstrate an absence of M0/M1 bands in the infused study drug. Endogenous M0/M1 AAT glycoforms were present in patients receiving exogenous IV AAT, but were absent in patients receiving tocilizumab (representative image).

4.4 Discussion

SARS-CoV-2-infected subjects exhibited evidence of body compartment-specific, protease–antiprotease balances and imbalances. For example, patients with severe SARS-CoV-2 exhibited an acute phase plasma AAT response similar to patients with severe nsARDS (Figure 4.1 panel A). AAT in SARS-CoV-2 patient plasma was active against NE (Figure 4.2 panel B) and exhibited the increased sialylation (Figure 4.8 a and b), important for regulating neutrophil chemotaxis (19). The increased AAT levels in the plasma of subjects with severe SARS-CoV-2-ARDS or nsARDS was regulated largely by circulating IL-6, as shown by *in vitro* and *in vivo* inhibition by tocilizumab (Figures 3.3 A,B,C and 4.9 A). *In vitro* data suggested that approximately 24 hours is required for IL-6 in plasma to increase AAT levels (Figure 3.3 A), potentially rendering the lung with insufficient anti-protease protection during this time.

While patients with severe SARS-CoV-2 infection exhibited high levels of active AAT in excess of NE in their plasma, protease–anti-protease balance in the lung varied with compartment (airways versus alveolus; Figure 4.7 B). In all the TA from SARS-CoV-2-infected subjects, a protease imbalance was observed with AAT mostly cleaved or complexed with NE, and with detectable active free NE (Figure 4.4). On average, these TA were collected 11.1 +/- 6.9 days from onset of symptoms and 1 day post ICU admission. In contrast, only one of the patients with nsARDS demonstrated active NE in their TA (airways) which differentiates this syndrome from SARS –CoV-2 ARDS (Figure 4.4 B). This finding suggests that the AAT acute phase response in patients with SARS-CoV-2 ARDS is not sufficient to protect the proximal airways from NE-induced damage. A similar airway protease imbalance has been described in community acquired pneumonia (186) and cystic fibrosis (187).

AAT was not the only antiprotease affected by SARS-CoV-2. SLPI, the major locally produced anti-protease in the airways, was cleaved in TAs from SARS-CoV-2 unlike the uncleaved intact form of SLPI seen in nsARDS (Figure 4.4 a). There was also reduced SLPI protein expression in airway epithelia of acutely infected SARS-CoV-2 lungs (Figure 4.7 A). SLPI downregulation has been previously reported with suppression of Nrf2 signalling in SARS-COV-2 infections (188)(189) and with other viral infections (190), and its absence diminishes the airway antiprotease protective shield (191). Thus, in subjects with severe SARS-CoV-2 infection, the proximal airway anti-protease and anti-inflammatory protection afforded by locally (airway)

produced SLPI, in addition to systemically available AAT, is inadequate to protect against tissue damage. The potential deleterious consequences of this unopposed NE activity include direct tissue damage, impaired ciliary motility, increased mucin secretion, inactivated anti-inflammatory proteins, cleaved complement, complement receptors and immunoglobulins, and initiation of a cycle of inflammation in the lung (1). These sequelae are also seen in CF which has led to a series of studies into anti-protease therapy in this condition. Already a number of small non-randomised controlled trials have suggested some benefit of AAT administration in SARS-CoV-2 infection using both aerosol and intravenous routes of administration (141). In the phase 2 trial that this lab has undertaken it was determined that treatment with IV AAT was safe, feasible and biochemically efficacious. Following administration of a single IV infusion of Prolastin, plasma concentrations of the pro-inflammatory cytokine IL-6 were significantly decreased, an effect mirrored by increases in circulating AAT levels. Levels of sTNFR1 were also substantially decreased at one week in patients receiving Prolastin. Levels of the anti-inflammatory cytokine IL-10 and differential M0/M1 glycosylation of endogenous AAT were preserved, indicating that the decrease in inflammation observed did not come at the cost of pro-resolution mediators. This raises a number of questions such as which route of administration would be preferable. In the context of its anti-protease effects and the site of protease-anti-protease imbalance in SARS-CoV-2 ARDS, aerosol delivery of AAT would seem most beneficial but it should also be noted that AAT possesses significant anti-inflammatory effects (1) and in the context of the severe systemic inflammation seen in SARS-CoV-2 infection an intravenous delivery option would also merit consideration. Another concern is when in the course of the condition such administration should take place and whether it should be given to all the different SARS-CoV-2 clinical phenotypes. That would require further study specifically to determine whether AAT therapy in SARS-CoV-2 infection mediates its effect mainly through its anti-protease, anti-inflammatory capabilities or through its ability to decrease SARS-CoV-2 entry into cells via its actions on TMPRSS2.

If AAT synthesis and secretion are beneficial responses to SARS-CoV-2 infection, this raises the question as to whether decreases in AAT synthesis secondary to IL-6 inhibitors be harmful? Monoclonal antibodies directed against the IL-6R, e.g., tocilizumab, have been tested in SARS-CoV-2. Reports of tocilizumab efficacy in SARS-CoV-2 have been mixed. A retrospective analysis revealed an association

between tocilizumab administration and increased mortality (192), ICU admissions, mechanical ventilation, and length of stay in subjects with SARS-CoV-2 infection. However, while the recently published results of the RECOVERY and REMAP-CAP trials showed no benefit from tocilizumab alone, they did suggest a benefit from the addition of tocilizumab to high-dose dexamethasone (193). Tocilizumab does not discriminate between classical or trans-IL-6 signalling and, therefore, blocks many anti-inflammatory effects of IL-6, including AAT induction. As observed in the present study, the acute phase AAT response may be required to dampen airway inflammation and prevent airway damage during SARS-CoV-2 infections. The noticeable decrease in plasma AAT levels in patients treated with tocilizumab and the coinciding increase in their IL-6 /AAT ratio at day 28 of treatment demonstrates disruption to the normal IL-6-AAT signalling pathway (Figure 4.9). Not only does this downregulate AAT production *in vivo* but also ST6GAL1, as seen by the resolution of hyperglycosylated M0, M1 AAT bands in patient plasma (Figure 4.9 D). This finding also denotes a reduction in the ability of AAT to bind IL-8, essential for regulating chemotaxis during inflammatory processes, as shown in this study. These data suggest that in future IL-6 blockade trials, special care has to be taken to ensure that inhibition of AAT induction does not worsen an already precarious protease-anti-protease balance in the lungs. In this regard, a more targeted approach, perhaps aimed at IL-6 trans-signalling, using compounds such as soluble gp130, could be considered that would leave the classical IL-6-hepatic AAT synthesis/secretion pathway unaffected or alternatively therapies aimed at inhibiting IL-6 should be accompanied by concomitant administration of exogenous AAT.

A limitation of this study is that our patient samples were collected from several different centres. With regards airway samples, the majority of TA samples were from UNC (n=10), who treated them with 6M urea for safe handling. As a result, FRET analysis of protease activity analysis was not possible in these samples. Five additional TA were collected from Beaumont hospital which were suitable for NE activity analysis by FRET. No difference was observed on Western analysis of AAT or NE in the UNC 6M urea treated TA samples compared to the TAs from Beaumont hospital, both showing similar patterns of AAT degradation and non-complexed NE. A suspension of bronchoscopy services during the first wave of the SARS-CoV-2 pandemic prevented us from obtaining small airways lavages. Therefore, the alveolar compartment of SARS-CoV-2 patients was evaluated in UNC by

immunohistochemistry of post-mortem specimens. As the vast majority of patients in our ICU at the time had SARS-CoV-2 infection, we had minimal access to nsARDS samples necessitating our using samples from a prior study conducted in a different centre. However these samples were amenable to quantitative and protease activity analysis and thus provided a suitable disease comparison group. Our centre was not using tocilizumab as a therapeutic at the time of the study, so we accessed patient samples from a sister hospital in Dublin (SVH).

In summary, this research demonstrates that AAT responses to SARS-CoV-2 infection are compartmentalized with an appropriate increase in plasma and alveoli but deficient responses in airways. These data suggest administration of intravenous or aerosolized AAT to SARS-CoV-2- infected subject airways may be an appropriate therapeutic option (92) (194). Finally, according to these data caution should be exercised in SARS-CoV-2-infected individuals with respect to administration of IL-6 blocking therapies.

Chapter 5: The anti-inflammatory response of individuals with ZZ-Alpha-1 antitrypsin deficiency to various stimuli; SARS-CoV-2 infection, SARS-CoV-2 vaccination and LPS.

5.1 Introduction

In this chapter the *in vivo* response of MM and ZZ individuals to various inflammatory insults such as SARS-CoV-2 infection, bacterial infection (LPS) and SARS-CoV-2 vaccination was investigated. This will determine if therapies aimed at increasing secretion of Z AAT are feasible in their ability to increase AAT secretion while at the same time decreasing intracellular accumulation and inflammation. An ideal therapy would resolve the lack of protease defense of the lung while simultaneously removing the toxic “gain of function” which results in hepatic cell damage (as well as having an effect on other non-hepatic cells such as monocytes and neutrophils), resulting in liver cirrhosis and hepatobiliary carcinoma in ZZ individuals. It was necessary to elucidate how much AAT is needed at baseline and during acute inflammatory responses to protect the lungs, as this ability to increase AAT secretion in reaction to inflammatory insult should be incorporated into any therapeutic option. This potential to vary the AAT response represents “real world” rather than a fixed putative protective threshold.

In chapter 4 it was determined that AAT responses to SARS-CoV-2 infection are compartmentalized with an appropriate AAT increase in plasma and alveoli but insufficient responses in the airways. This also showed that a robust anti-inflammatory response characterized by increased AAT levels is an important protective reaction in viral infections (195,196), raising the question of how a ZZ AATD individual would respond to SARS-CoV-2 infection or similar inflammatory stimuli. In previous literature published from our lab, MM individuals with severe SARS-CoV-2 infection resulting in ICU admission demonstrated a blunted AAT response to SARS-CoV-2 during a major IL-6 driven cytokinemia. This highlighted that SARS-CoV-2 has a distinct inflammatory phenotype, one that may be associated with altered immune-metabolism (140). With this in mind it was hypothesized that the ZZ population would also have a blunted, ineffectual anti-inflammatory response to SARS-CoV-2 or indeed other infections leaving the lung even more open to proteolytic damage. The main treatment target and risk stratification for those with ZZ AATD

has been centered around the long-accepted theory of a "putative protective threshold" of 11 μ M in AAT in plasma (197). The rationale behind the 11 μ M level remains unclear and recent evidence suggests that it is no longer fit for purpose (198). The current SARS-CoV-2 pandemic challenges this theory as MM-AAT replete individuals have to mount a significant acute AAT response of 50-70 μ M in plasma in an attempt to counter SARS-CoV-2 infection and even then, there is evidence of active NE in the airways suggesting that the protective threshold is a moving target based on the degree of inflammation in the system.

5.2 Aims of this chapter

It was hypothesized that those with ZZ AATD would not be able to mount an appropriate inflammatory AAT response to SARS-CoV-2 infection and in their efforts to do so may cause increased intracellular retention and inflammation. The aim of this part of the study was to monitor the Irish ZZ AATD population for prevalence of SARS-CoV-2 infection, determine infection severity and the extracellular and intracellular inflammatory response in these individuals. Currently there are 227 ZZ AATD individuals enrolled on the Irish national AATD registry. Of these, 203 individuals were deemed suitable to take part in our research. Participants were invited to complete a customized Google form survey, focusing on SARS-CoV-2 infection rates, exacerbation frequency and cocooning (self-isolating/protective) habits (see appendix 1).

From our *in vitro* work it is clear that AAT production in ZZ AATD is mediated by the same IL-6-AAT pathway as in healthy MM individuals but in those with the ZZ phenotype, the misfolded Z-AAT protein is retained in the ER and is not secreted from the liver in sufficient quantity to provide adequate anti-protease and anti-inflammatory protection (199). Furthermore, the retained Z-AAT has the capacity to cause intracellular inflammation through ER stress mediated inflammatory pathway. We have shown *in vitro* that IL-6 which is upregulated in acute SARS-CoV-2 infection, increases the production and retention of misfolded Z-AAT in liver cells *in vitro* causing ER-stress. In order to evaluate this *in vivo* we

examined the IL-6 –AAT relationship in ZZ AATD patients with SARS-CoV-2 infection and in ZZ individuals receiving non-replicating viral vector SARS-CoV-2 vaccination, which to an extent mimics mild SARS-CoV-2 infection while at the same time permitting us to sample the patients' plasma before and after the insult.

To fulfill the aims of this chapter the following objectives were set.

- To determine the prevalence and severity of SARS-CoV-2 infection in the ZZ AATD population by accessing the national AATD registry and surveying all ZZ- AATD patients.
- To investigate the inflammatory response of ZZ-AATD patients to SARS-CoV-2 infection by evaluating people with the infection during its acute phase and compare this to a bacterial like response in healthy individuals receiving intravenous lipopolysaccharide (LPS).
- To compare the IL-6 –AAT pathway mechanism in both MM and ZZ-AATD individuals who received non-replicating viral vector SARS-CoV-2 vaccination, by timing and measuring the levels of IL-6 and AAT in plasma.
- To demonstrate the presence of ER stress in ZZ-AATD individuals compared to MM individuals following vaccination by isolating and processing monocytes pre and post vaccination, for protein analysis.

5.3 Results

5.3.1 SARS-CoV-2 infection in the ZZ AATD population

Out of the 184 ZZ individuals invited to participate, 114 responded (63%). 64% of all respondents reported cocooning during the pandemic for an extended period of time (Figure 5.1 , panel A). Out of the 114 people with ZZ AATD contacted during this study, 12 tested positive for SARS-CoV-2 over the course of the pandemic (2 years), 4 of whom were admitted to hospital. Only 1 of these individuals presented to Beaumont hospital and was available to provide a blood sample. Plasma was collected from this patient and AAT levels measured by nephelometry. Isoelectric focusing with immunoblotting was performed on the patient's plasma sample with specific focus on the glycosylation pattern of his AAT. The AAT level was markedly raised for a ZZ individual at 0.89 g/L which is within the MZ range (0.6 g/L- 1.51 g/L) but nowhere near the levels noted in MM individuals with SARS-CoV-2 infection noted in chapter 4. Isoelectric focusing and immunoblotting demonstrated an abnormal glycosylation pattern and a smear pattern often seen in the setting of acute inflammatory processes (Figure 5.1 Panel B). This glycosylation pattern and increased plasma AAT levels were similar to that seen in ZZ AATD patients with severe panniculitis (Figure 5.1 Panel C), although in the case of the patient with SARS-CoV-2 infection the AAT levels were higher perhaps reflecting the effect of viral-induced IL-6 cytokinemia.

In conclusion, despite mounting a significant AAT response to SARS-CoV-2 this patient was not able to mount an AAT response equivalent to that of the MM patients with SARS-CoV-2 infection in chapter 4. This raised the question of whether this patient was making the same amount of AAT as the MM patients but was unable to secrete it perhaps setting in motion a pro-inflammatory intracellular ER stress response.

Unfortunately, as this patient was not admitted under our service, we could not get access to other biologic materials. This was a study limitation and in order to further answer this question we turned our attention to a situation where we could predict a SARS-CoV-2 type response and get pre and post inflammatory stimulus bloods and cells for

evaluation. The best means of doing this was via access to MM and ZZ individuals receiving vaccination for SARS-CoV-2 infection, bearing in mind that the stimulus, while similar, would be much less in intensity.

Figure 5.1

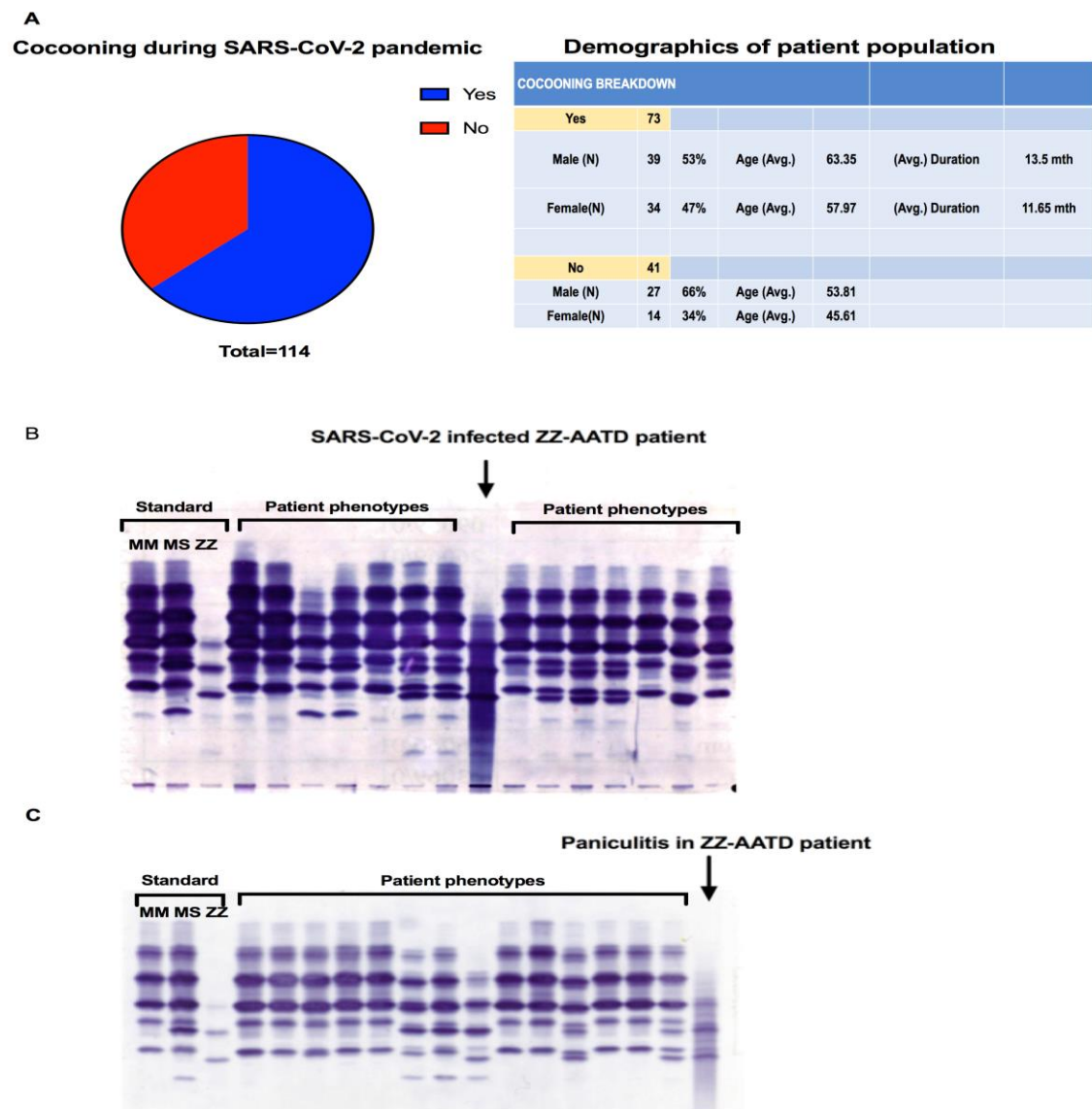


Figure 5.1

SARS-CoV-2 infection in the ZZ AATD population

- A.** Pie chart representation of those from the national AATD registry, who cocooned for a prolonged period of time during the pandemic. Further break down of the demographics is provided on the table to the right of the pie chart. **B.** Representative IEF gel of a ZZ-AATD individual with an acute SARS-CoV-2 infection. Significant hyperglycosylation is evident by the smearing of the Z AAT glycoforms on the gel. **C.** Representative IEF gel of a ZZ-AATD individual with an acute panniculitis. Hyperglycosylation is noticeable by the smearing of the Z AAT glycoforms on the gel

5.3.2 IL-6-AAT response in ZZ AATD individuals stimulated with a non-replicating viral vector SARS-CoV-2 based vaccine

Due to the lack of ZZ AATD patients testing positive for SARS-CoV-2 and presenting to hospital, the response to the AstraZeneca (Az) vaccine, a non-replicating viral vector SARS-CoV-2 vaccine which would provide a stimulus characteristic of SARS-CoV-2 albeit with expectedly less inflammation was evaluated. In addition, a healthy control population receiving the vaccine was compared to a cohort of healthy controls (n=12) who received an IV infusion of 2ng/kg of LPS (samples provided by Newcastle University), imitating a bacterial sepsis to determine whether the inflammatory response to the vector-based vaccine was virus specific. These groups were then compared with the previous patient groups with severe SARS-CoV-2 ARDS (as outlined in chapter 4) (Figure 5.2 Panel A $P < 0.0001$).

The patients who received an IV infusion of 2ng/kg of LPS, were sampled prior to and 24h after infusion. Their plasma was analyzed in the RCSI/Beaumont Centre for both IL-6 and AAT production post infusion. As we can see in Figure 5.2 panel B there is a significant increase in AAT production post LPS infusion (1.221g/L \pm 0.13 to 1.38g/L \pm 0.19, $P=0.0027$) as well as in IL-6 (0.033pg/ml to 0.13 pg/ml $P=0.0264$). As a surrogate bacterial sepsis model this is a useful comparison for the vector-based vaccine experiments, which act as surrogate viral infection model.

In the vaccine cohort, plasma was taken from MM (n=10) and ZZ (n=10) patients 24h prior to vaccination and 24, 48 and 72h post vaccine. In MM individuals there was a significant increase in AAT at 48h from 1.46 g/L \pm 0.45 to 1.68 g/L \pm 0.45($P < 0.0001$) while in the ZZ-AATD cohort there was no significant change at 48 h (pre-0.17 g/L \pm 0.10, 48hrs 0.19 g/L \pm 0.11(NS= 0.798) despite similar increases in IL-6 production in plasma from 0h to 24 h in both MM (1.14 \pm 0.63 pg/ml to 5.76 \pm 3.7, $P=0.0241$) and ZZ-AATD 1.436 \pm 0.22pg/ml to 6.5 \pm 4.3pg/ml, $P=0.0433$) individuals (Figure 5.2 Panel C, Panel D).

This was further reflected in the glycosylation pattern in both MM individuals and ZZ individuals who were vaccinated. MM individuals demonstrated an inflammatory profile with increased M0,M1 bands at 24h while ZZ patients have no discernible difference in glycosylation between the pre and 24, 48 and 72 h post time points (Figure 5.2 Panel E). This raised the question of whether retained intracellular misfolded Z-AAT in the ER post vaccination could result in ER stress? To evaluate this, circulating monocytes were harvested and purified from plasma and investigated for ER stress. Previous work has shown that ZZ monocytes retain Z protein in the ER, resulting in ER stress and express a pro inflammatory phenotype when stimulated (199).

Figure 5.2

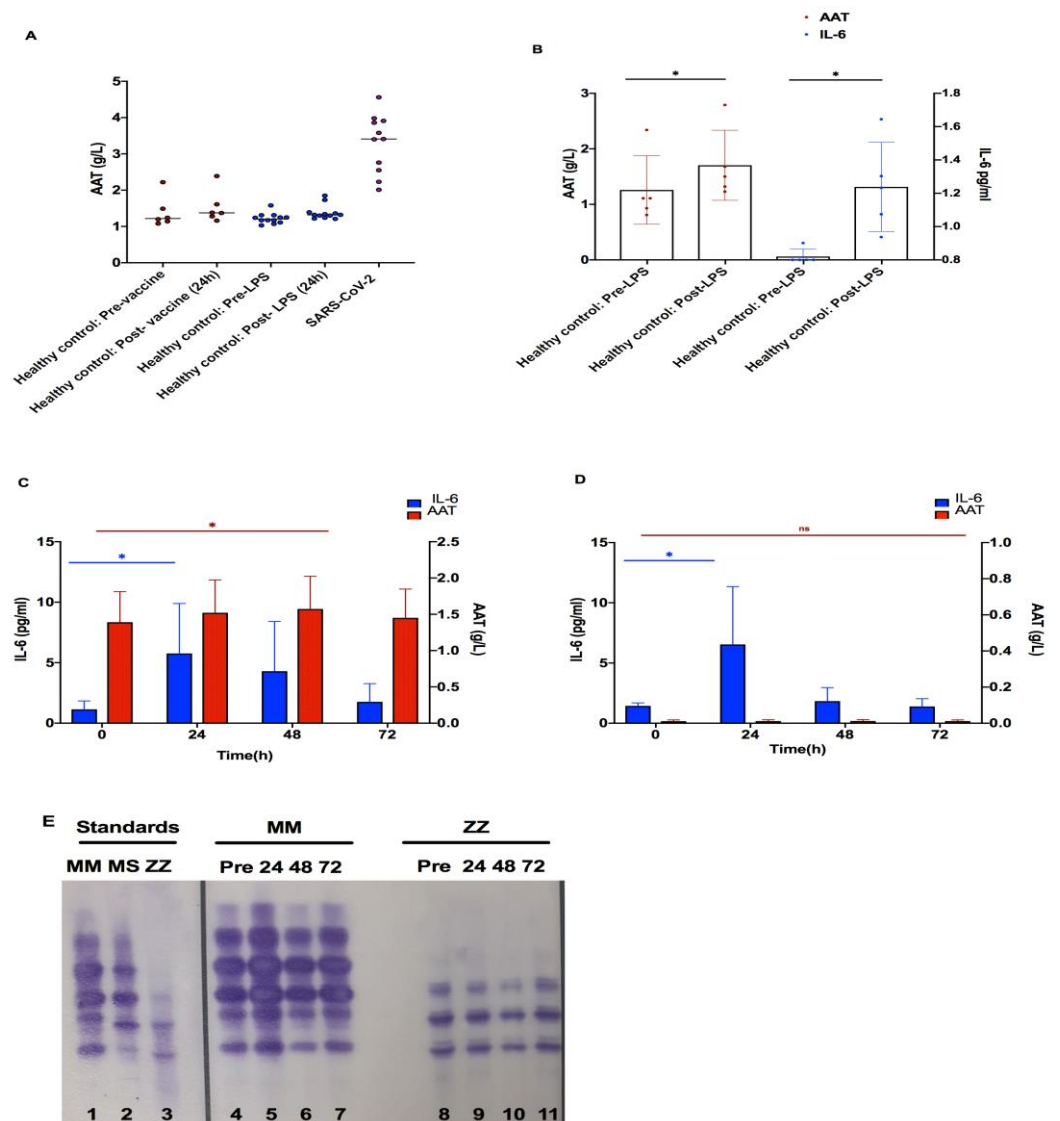


Figure 5.2 IL6-AAT response in ZZ AATD individuals stimulated with a non-replicating viral vector SARS-CoV-2 based vaccine

A. AAT levels in HC patients treated with either a SARS-CoV-2 vector based vaccine or IV LPS (24h post treatment) compared to AAT levels of Severe SARS-CoV-2 $P < 0.001$) One way ANOVA Tukey's multiple comparisons test (* signifies $P < 0.05$). **B.** A significant increase in AAT production post LPS infusion ($P = 0.0027$) as well as an increase in IL-6 ($P = 0.0264$, Paired T test) (* signifies $P < 0.05$). **C.** A significant increase in AAT levels was seen post vaccine in MM individuals post vaccine at 48h ($P < 0.0001$) (Red). There was a significant increase in IL-6 levels in plasma at 24 h in MM individuals post vaccine at 24h ($P = 0.0241$, paired T test) (Blue3) (* signifies $P < 0.05$). **D.** There was no significant change in AAT levels in the ZZ-AATD cohort at 48h post vaccine ($NS = 0.798$) (Red). A significant increase in IL-6 levels was seen in ZZ AATD individuals at 24 h ($P = 0.0433$) (Paired T-test) (Blue) (* signifies $P < 0.05$). **E.** Representative IEF gel reflecting the appropriate AAT inflammatory response in MM individuals post vaccination, noting the increased glycosylation at 24h in MM in comparison to the ZZ AATD individuals with no noticeable hyperglycosylation throughout the 3 post vaccination timepoints

5.3.3 ER stress in monocytes isolated from ZZ-AATD individuals vaccinated with non-replicating viral vector SARS-CoV-2 vaccine.

IL-6 levels peaked in both MM and ZZ individuals at 24 hours post vaccine while secreted AAT levels in MM individuals, peaked at 48hr post vaccine and fell at 72 h (Figure 5.2 Panel C, D). (in keeping with our *in vitro* HepG2 cell data in Chapter 3). AAT levels in plasma did not change in ZZ individuals at any time point post-vaccine (Fig 5.2 Panel B). This reflects intracellular AAT retention in ZZ individuals and can potentially result in increased ER stress at these time points.

Resting monocytes from ZZ individuals showed increased GRP57 and GRP78 on Western analysis compared to MM monocytes. Both these parameters increased 48 h post vaccination in ZZ monocytes but not in MM monocytes (representative blots shown In Figure 5.3 Panel A). This is consistent with ER stress. This ER stress was transient and not present at 72h post vaccination as evidenced by Western analysis probing for GRP-78 and phosphorylated-IkBa, at this timepoint both of which demonstrated a resolution of ER stress at 72h post vaccine (Figure 5.3 panel B)

Figure 5.3

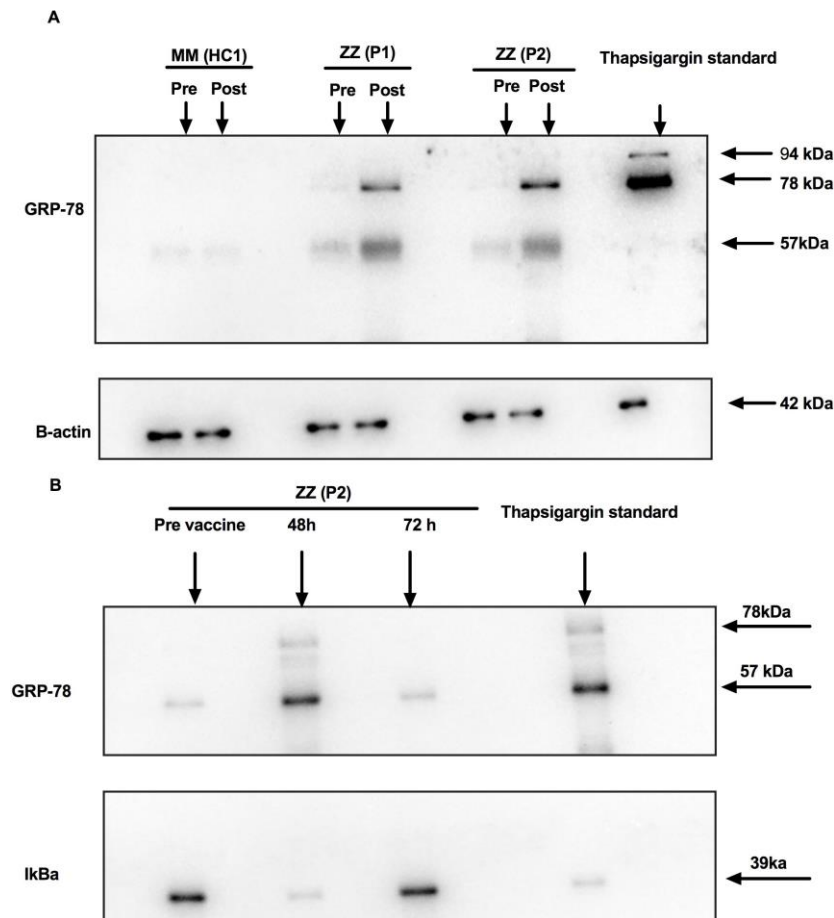


Figure 5.3

Western blot analysis of ER stress in monocytes from subjects vaccinated with a SARS-CoV-2 vector based vaccine.

A. Representative western blot analysis probing for GRP-78 (and subsequent chaperone proteins GRP 57 and GRP 94): Monocytes collected prior to vaccination and 48 hours post vaccination are aligned side by side. (From left to right) Healthy control MM-AAT individual (HC1) pre and 48 hours post vaccine followed by two ZZ individuals (P1 and P2) pre and 48 hours post vaccine. The standard is thapsigargin cultured MM monocytes. The representative image corrected for B-actin as seen in the western below GRP-78 (Figure 5.3, panel A) demonstrates increased ER stress at 48 h. **B.** Samples from P2 in Figure 5.3 panel A, were used to look at pre, 48h and 72h timepoints for GRP-78 and Phosphorylated-IκBa and determined that the ER stress was transient, resolving at 72h

5.4 Discussion

This chapter demonstrates that in the largest ZZ AATD population studied to date (n=114), a large number of individuals were infected with SARS-CoV-2 (n=12) despite cocooning and of these, 4 patients had an outcome sufficiently severe to necessitate presentation to hospital. This suggests SARS-CoV-2 infection is common in ZZ AATD and potentially has a more severe clinical impact on ZZ AATD patients compared to the general population, although this figure is now influenced by the advent of more infectious but less virulent forms of SARS-CoV-2. Other centers have attempted to perform similar studies such as Schneider C.V & Strnad who utilized the UK biobank which has recruited and genotyped >500,000 participants. Only 141 of those who were genotyped were ZZ AATD and only 3 of whom were positive for SARS-CoV-2 infection. This low number of ZZ individuals was noted by the researchers, who concluded that this limited the usefulness of the data pertaining to ZZ individuals. (200). In the present study we had access to 227 ZZ AATD individuals enrolled on the Irish national AATD registry, as per the design of our study we followed these patients up over time, in particular over the first and second wave of the pandemic prior to advent of vaccination. Eligible participants were contacted via phone with a maximum of three staggered phone calls made in order to recruit. The participants were invited to complete a customized Google form survey, focusing on SARS-CoV-2 infection rates, exacerbation frequency and cocooning habits. To encourage participation the survey response modality was flexible allowing them to respond via phone, email or post. Out of the 184 invited to participate, 114 individuals responded. Unlike other studies of this kind the review of outcomes of those who tested positive were not limited to SARS-CoV-2 related deaths but also to severity as pertaining to hospital and intensive care unit admission during the pandemic.

The single ZZ AATD individual who presented to our hospital with SARS-CoV-2 had significant hyperglycosylated AAT indicative of inflammation and a plasma AAT level (0.89g/L) within range of an MZ patient, higher

than that of a severe acute exacerbation of panniculitis, but as noted in the previous chapter this was significantly lower than the AAT response observed in otherwise healthy MM individuals with an acute SARS-CoV-2 infection. Not only is the ZZ-AATD individual unable to secrete the amount of AAT necessary to mount the appropriate anti-inflammatory response but the retained misfolded Z AAT may be pro-inflammatory by increasing ER stress as shown in ZZ monocytes post vaccination. Using the AZ vaccine as a surrogate for SARS-CoV-2 infection we were able to observe the appropriate IL-6 expression, increased AAT secretion and AAT hyperglycosylation in MM individuals at 24, 48 and 72 h post vaccine administration, while those with ZZ-AATD clearly demonstrate, minimal AAT secretion despite similar IL-6 expression to MMs, causing intracellular ZAAT retention and ER stress. This ER stress was transient following vaccination but may be more prolonged in the setting of a chronic inflammatory or severe acute stimulus.

Although both Z and M AAT are produced mainly in hepatocytes the possibility of evaluating intracellular retention and stress responses in the liver are not feasible. The closest surrogate *in vivo* model to liver cells for AAT production are circulating monocytes. The monocytes isolated from peripheral blood when lysed and probed for GRP-78 and GRP-57 revealed significant ER stress 48 hours post vaccination in ZZ-AATD. In comparison, ER stress is not present pre or post vaccination in MM individuals. However, the ER stress caused by an acute stimulus i.e. the vaccine in ZZ individuals is only temporary, resolving at 72h, unlike an ongoing inflammatory stimulus such as SARS-CoV-2 infection. The LPS response in MM individuals is similar to their AZ vaccine response in magnitude both in IL-6 and AAT responses and although an LPS challenge was not performed in ZZ individuals, it is likely that the ZZ response to LPS would mirror that seen in AZ vaccinations.

In conclusion, by observing the SARS-CoV-2 infection trends in the ZZ-AATD population and using AZ vaccines as a surrogate for SARS-CoV-2 infection we were able to demonstrate that while the anti-inflammatory response of ZZ-AATD individuals is increased *in vivo* in response to

SARS-CoV-2 infection and minimally to AZ vaccination it is inadequate and may actually be pro-inflammatory.

Chapter 6: General Discussion

6.1 General discussion

In this thesis the utility of several *in vitro* models of AAT production, secretion and retention in Z and M cell lines was tested at rest and in response to stimuli. HEK-293 cells transfected with both M and Z constructs and HepG2 cells as well as a HepG2 cell line, CRISPR-edited to produce Z AAT were assessed by ELISA, rt-PCR and Western blot analysis. These four *in vitro* cell models were broadly able to recapitulate what occurs *in vivo*. However, there were advantages and disadvantages with each cell line. HEK-293 cells do not normally produce AAT and therefore had to be transduced with M and Z constructs. These cells may not respond to the same stimuli as natural AAT producing cells and the levels of AAT secreted and retained may owe much to the relative transfection efficiencies. They did show AAT retention in the Z transfected cells compared to the M transfected cells and while they did respond to gentamicin the levels of AAT secreted were low and it is unclear whether this is what would occur naturally with AAT producing cells. This reiterated the need for a more natural AAT producing cell line, specifically a liver cell line. In this regard, while HepG2 cells are liver cells and should be closer in their responses to *in vivo* liver cells, there has been, up until now, no ZZ AATD HepG2 cell lines. This study utilized such a cell line for the first time and showed that broadly it reproduced what might be expected from a Z cell line in secretion and retention of AAT. In light of the data surrounding the AAT levels required *in vivo* in SARS-CoV-2 infection or similar inflammatory conditions the response of our *in vitro* models to a similar inflammatory insult was evaluated. The M AAT producing HEPG2 cell line was able to mount a significant response to SARS-CoV-2 plasma demonstrating the amount of AAT that would be required in an inflammatory response, the subsequent and equally significant decrease in AAT production in response to tocilizumab suggests that IL-6 is the key cytokine driving AAT in SARS-CoV-2 plasma. Interestingly IL-6 also increases ST6GAL1 production which increases the sialylation of AAT modulating its effect on neutrophil

chemotaxis as described in chapter 4 and 5 (19). This study was the first to determine the IL-6 response of this modified Z liver cell line in secretion and retention of AAT and should be a valuable resource for future research in this area. While both M and Z HEPG2 cell lines demonstrated a significant AAT response on exposure to IL-6 or SARS-CoV-2 plasma, it was evident that there was less extracellular AAT secretion and more intracellular retention in the Z cells compared to M cells recapitulating their role as a useful model in AATD but also providing an opportunity to evaluate other intracellular responses specifically ER stress in cells as close as possible to *in vivo* liver cells. With this as background the response of these M and Z AAT cell lines to gentamicin was extremely important as an indicator of future potential efficacy. The results were somewhat disappointing as although both M and Z cell lines responded to gentamicin by increasing AAT secretion, the levels of AAT secreted were quite small in the Z cell lines and was associated with increased intracellular retention and inflammation.

These results suggested a re-evaluation of the current concepts regarding potential treatments of ZZ AATD. The dogma employed by most pharmaceutical companies to show efficacy in pharmaceutical trials is that the plasma AAT level should be raised above 11 μ M, and that this would be sufficient to protect the lung. Our *in vitro* studies were not producing elevations in that range, but we were also acutely aware of data showing that even people with mild forms of AATD with levels significantly in excess of 11 μ M had increased risk of lung disease in the setting of an inflammatory insult such as smoking. The 11 μ M AAT level target is stationary and fails to account for the need to increase AAT levels in the setting of infection or inflammation. The advent of the SARS-CoV-2 pandemic further challenged this theory. Otherwise healthy AAT replete individuals mounted a significant acute AAT response of 50-70 μ M in plasma in response to SARS-CoV-2 infection but even then, there was evidence of active NE in the airways suggesting that an actual protective threshold in a ZZ individual is a moving target based on the degree of inflammation. In this study, SARS-CoV-2-infected patients exhibited

evidence of body compartment-specific, protease–antiprotease balances and imbalances . Patients with severe SARS-CoV-2 infection produced an appropriate acute phase AAT response in plasma. AAT in plasma was active against NE and highly sialylated important for regulating neutrophil chemotaxis. However, this was not mirrored in the lung, with the protease–anti-protease balance varying by compartment (airways versus alveolus) with AAT in the airways being mostly cleaved or complexed with NE, and with active free NE detectable in the airways. This finding suggested that the AAT acute phase response in SARS-CoV-2 ARDS was insufficient to protect the airways from NE-induced inflammation damage in the setting of severe inflammation

(Figure 6.1). AAT was not the only anti-protease affected. SLPI, the major locally produced antiprotease in airways, was also cleaved in the airways of people with SARS-CoV-2. There was also reduced SLPI RNA expression in superficial airway epithelia of acutely infected SARS-CoV-2 lungs, further diminishing the airway antiprotease protective shield resulting in unopposed NE damage of the lung. This raised a number of concerns; would people with AATD be of significantly increased risk from SARS-CoV-2 infection and would the protective AAT response to SARS-CoV-2 infection be impaired by the use of newer medications directly aimed at inhibiting IL-6?

Looking first at the latter point we specifically evaluated tocilizumab, a monoclonal antibody directed against the IL-6R, which was increasingly being used as a therapeutic in SARS-CoV-2 infection, without significant evidence of efficacy. Tocilizumab does not discriminate between classical or trans-IL-6 signalling and, therefore, blocks the many anti-inflammatory effects of IL-6, including AAT induction.(173) We had previously shown *in vitro* that IL-6 in SARS-CoV-2 plasma was inhibited by pre-treatment with tocilizumab with resultant decrease in AAT induction and secretion(173). We had also shown that tocilizumab inhibited ST6GAL1 vital for sialylation of AAT(173). In the context of our data outlining the need for an acute phase AAT response to dampen airway inflammation and prevent airway damage, the use of tocilizumab was a potential cause for

concern. In concordance with the *in vitro* data, a decrease in AAT levels as well as in AAT glycosylation was shown in people with SARS-CoV-2 infection treated with tocilizumab, with a coinciding increase in their IL-6 /AAT ratio at day 28 of treatment. Such inhibition of AAT induction may worsen the pre-existing protease-anti-protease balance in the lungs. I

One potential therapy therefore is to augment the AAT in blood and lungs of people with SARS-CoV-2 infection by administration of plasma purified AAT. This approach has been attempted before in cystic fibrosis (CF) where, despite a high to normal AAT level in plasma the massive NE burden in the lung overwhelms the anti-protease protection resulting in lung damage (92). In this context a person with CF (PWCF) who acquired SARS-CoV-2 infection while an inpatient in Beaumont hospital was given plasma purified AAT on a compassionate basis with good effect (142). These data along with the compartmentalisation data paved the way for the first administration of AAT to people with severe SARS-CoV-2 in a randomised placebo control trial. In this context the RCSI/Beaumont clinical research group designed a phase 2, multicenter, randomized, double-blind, placebo-controlled trial of intravenous plasma-purified AAT(120/mg/kg, 1/52) (Prolastin, Grifols, S.A.) for moderate-to-severe ARDS secondary to SARS-CoV-2 infection monitoring its effect on pro and anti-inflammatory cytokines and clinical parameters. Following administration of a single IV infusion of AAT, plasma concentrations of pro-inflammatory cytokines such as IL-6 were significantly decreased, an effect mirrored by increases in circulating AAT levels. In addition, levels of the anti-inflammatory cytokine IL-10 and differential M0/M1 glycosylation of endogenous AAT were preserved, indicating that the decrease in inflammation observed did not come at the cost of pro-resolution mediators. Treatment with IV AAT was safe, feasible and ultimately determined to be biochemically efficacious for SARS-CoV-2 patients. This study, the first of its kind, built on the *in vivo* data from the people with SARS-CoV-2 evaluated in this thesis and the outcome measures were to a large extent predicated on this work.

According to previously published literature MM individuals with severe

SARS-CoV-2 ARDS demonstrated a blunted AAT response whilst having a major IL-6 driven cytokinemia, suggesting a distinct inflammatory phenotype, associated with altered immune-metabolism. With this in mind it was hypothesized that the ZZ population would also have a blunted, ineffectual anti-inflammatory response to SARS-CoV-2. Thus, leaving the lung even more open to inflammation and proteolytic damage than seen in an AAT replete individual.

Due to this concern the RCSI/Beaumont group launched an awareness and education campaign, with the aim to contact all ZZ individuals on the AATD registry urging them to practice cocooning and stress the importance of protective techniques such as hygiene and distancing. However, despite these measures, proportionally, the number of ZZ individuals who contracted SARS-CoV-2 was higher than the national average. In total 12 ZZ patients contracted SARS-CoV-2 of whom 4 had an infection severe enough to warrant a hospital admission, one of whom presented to our hospital. While the lack of infected ZZ patients was a study limitation, the plasma of the patient who did present to Beaumont hospital exhibited significant hyperglycosylation of AAT similar to that seen in ZZ AATD patients with severe panniculitis, with the circulating AAT concentration increased to that of the MZ AATD range. This provided an insight, that despite a significant AAT response to SARS-CoV-2 this patient was incapable of matching levels secreted by MM individuals during similar inflammatory insults with the potential for intracellular retention of ZAAT exacerbating inflammation.

Attempts to further investigate the IL-6/AAT response in AATD were aided by the national rollout of the Az vaccine. This provided a similar but less inflammatory SARS-CoV-2 vector based stimulus for ZZ individuals. A comparable IL-6 pattern in both MM and ZZ individuals was observed; a significant increase in IL-6 levels, peaking at 24 hr post vaccine. This corresponded with a significant increase in AAT levels 48 hr post vaccine in MM individuals, which was not replicated in those with ZZ AATD. Z AAT levels did not increase at any point post vaccine despite a similar surge in IL-6, suggesting possible ER retention of Z AAT as seen

previously in our *in vitro* cell models. This was confirmed by monocytes (acting as a liver cell surrogate) isolated from ZZ individuals pre- and post-vaccine. All ZZ patients demonstrated an increase in ER stress markers 48 hours post vaccine, corresponding with the peak increase in circulating AAT levels in healthy MM individuals. In comparison, ER stress is totally absent pre or post vaccination in MM individuals. The ER stress seen in ZZ individuals was however only temporary, resolving at 72h post vaccine, unlike an ongoing inflammatory stimulus such as SARS-CoV-2 infection.

By observing the SARS-CoV-2 infection trends in the ZZ AATD population and using Az vaccines as a surrogate for SARS-CoV-2 infection we were able to demonstrate that overall the vaccine is safe for ZZ AATD patients to use and that while the anti-inflammatory response of ZZ AATD individuals is increased *in vivo* in response to SARS-CoV-2 infection it is inadequate and may even be pro-inflammatory leading to ER stress.

With the exception of the lack of ZZ AATD patients presenting to our hospital with SARS-CoV-2 infection another major limitation of these studies was that the patient samples were, of necessity, collected from different centres. Beaumont hospital did not initially have facilities in place to access and process airway secretion samples. Therefore, initially, the majority of TA samples were from UNC (n=10), who treated them with 6M urea for safe handling. As a result, FRET analysis of protease activity analysis was not possible in these samples. Once Beaumont hospital was able to handle TA samples, five additional TA were collected from the ICU in Beaumont hospital which were suitable for NE activity analysis by FRET. No difference was observed on Western analysis of AAT or NE in the UNC 6M urea treated TA samples compared to the TAs from Beaumont hospital, both showing similar patterns of AAT degradation and uncomplexed NE. A suspension of bronchoscopy services during the first wave of the SARS-CoV-2 pandemic prevented us from obtaining small airways lavages. Therefore, the alveolar compartment of SARS-CoV-2 patients was evaluated in UNC by immunohistochemistry of post-mortem specimens. This was done under our advice and guidance specifically with regards

which antibodies to use (which we supplied) and which proteases and anti-proteases to evaluate. As the vast majority of patients in our ICU at the time had SARS-CoV-2 infection, we had minimal access to nsARDS samples necessitating our using samples from a prior study conducted in a different centre. However these samples were amenable to quantitative and protease activity analysis and thus provided a suitable disease comparison group. Our centre was not using tocilizumab as a therapeutic at the time of the study, so we accessed patient samples from a sister hospital in Dublin (SVH).

Despite these study limitations the data generated during the course of this research, as stated in our introduction, has contributed significantly to the body of knowledge regarding AATD, AAT's role in the inflammatory process and its potential use outside of AATD. This study has explored and evaluated the available in-vitro models, determined the target levels of secreted AAT required, especially during acute inflammatory periods such as SARS-CoV-2 and the potential downsides of increasing AATD production in ZZ AATD cells. It also suggests that while gentamicin may not be a feasible therapeutic option, the concept of getting Z AAT out of the liver and into the circulation, by other means remains a worthwhile potential therapy. The future directions and possibilities of this research are outlined further in chapter 7.

Figure 6.1

The role of AAT in SARS-CoV-2 infection

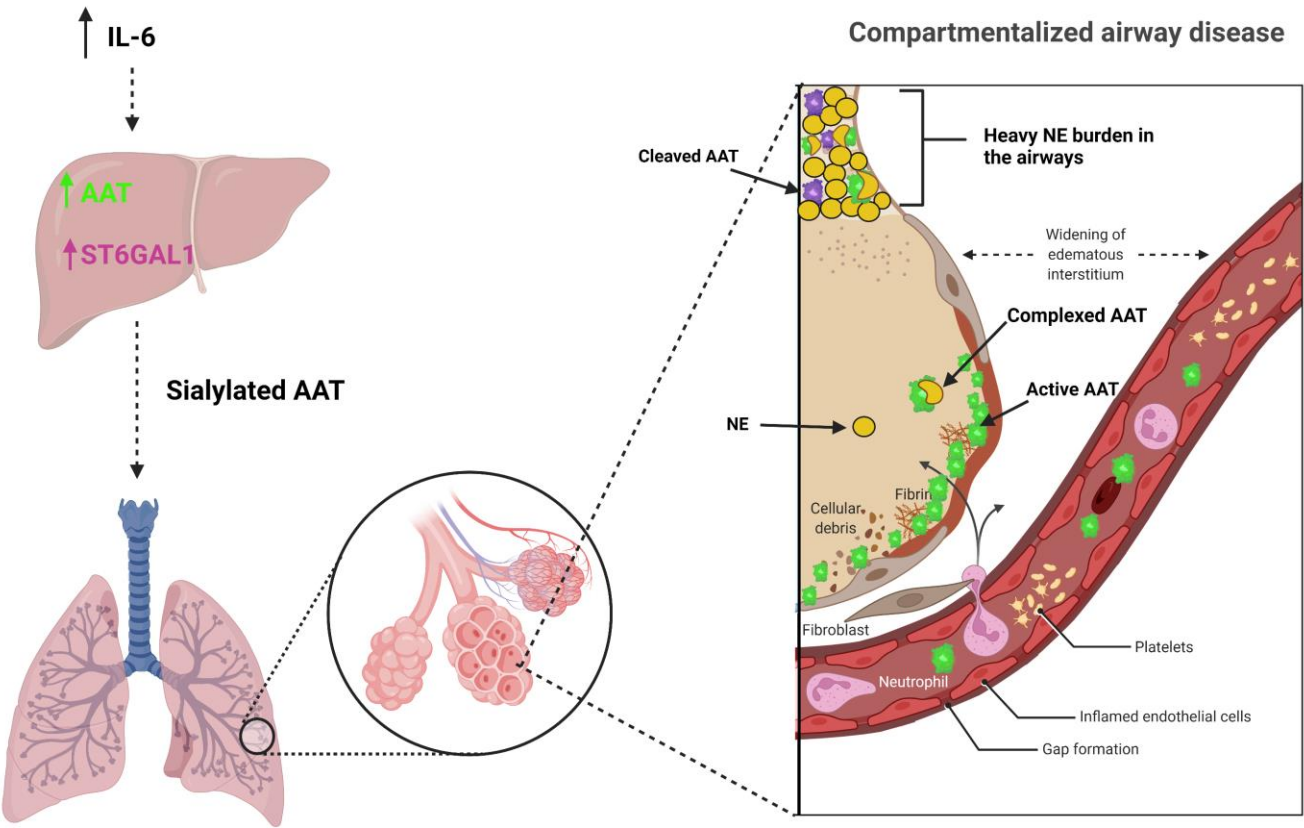


Figure 6.1

A representative diagram of the role AAT plays in SARS-CoV-2 in plasma and in the lung

Chapter 7: Future Directions

7.1 Future Directions

These data are food for thought for anyone considering therapies for AATD. Looking first at the only presently available therapy, intravenous plasma purified AAT augmentation therapy. This therapy is based on the aim of keeping the AAT level above $11\mu\text{M}$ throughout the course of therapy. It is obvious from the data shown that this does not reflect the body's needs during acute infection and that, if this threshold is to be kept as a target, it would seem important to consider administration of extra AAT during times of infection or inflammation. We have some evidence concerning this from our work with panniculitis and CF and now ARDS associated with severe SARS-CoV-2 infection. In panniculitis, of the three ZZ patients attending our center, all were initially treated with 60g/kg AAT, but it soon became evident that doses well in excess of this, (120mg/kg) were required to dampen down this inflammatory process. All three ended up on the higher dose, thus the effective dose is dependent on the degree of the inflammatory process. When this was explored in the context of SARS-CoV-2 infected MM individuals during the phase 2 trial the same dose of 120mg/kg of AAT IV was used. The data showed significantly decreased plasma concentrations of the pro-inflammatory cytokine IL-6 while increasing circulating AAT levels. In addition, sTNFR1 was also substantially decreased, while anti-inflammatory mediators remained unaffected. Unfortunately, the effect of IV AAT on airway NE activity in these patients was not explored in this study. There is a potential to investigate the anti-protease effect of IV AAT on similar patients in the future. While the phase 2 randomised control trial (RCT) delivered AAT by IV infusion, data from IHC and TA studies in SARS-CoV-2 lungs demonstrates significant compartmentalization of this disease with the majority of the inflammation localized in the upper airways. This suggests aerosol may be a more suitable mode of delivery, but at the time of the conceptualization of the RCT this was not considered feasible due to the patients being intubated with the potential risk of

aerosolizing SARS-CoV-2 within the ICU. The method of aerosol delivery of AAT has been used in one study of patients with SARS-CoV-2 infection and has shown progress in a series of studies in CF. However, this approach may lose some of the systemic anti-inflammatory effects of intravenous administration of AAT and this may be an important disadvantage.

A paradoxical way of looking at this is to say that in some cases the 11 μ M threshold may be too high to aim for, particularly if the person with AATD has no smoking history or environmental exposures and if the treatment itself is effective at getting mutant Z AAT out of the cells thereby lessening inflammation. SZ individuals who do not smoke have no increased risk for COPD. We still have to determine the risk of lung disease in non-smoking ZZs but if it is consistently present that would suggest the possibility that the protective threshold in non-smoking ZZs with no environmental risks is somewhere between 5 and 11 μ M. That would also be of major solace to groups working in the area of gene therapy for AATD. These groups have been able to show that viral vectors administered intramuscularly can achieve prolonged expression up to and beyond one year, but they are hampered by their inability to produce sufficiently high levels of AAT. A lower protective threshold perhaps with the added need to actively supplement AAT levels in plasma and lung during infections might be a reachable target. However, if the retention of AAT intracellularly is going to be a constant cause of inflammation, irrespective of the extracellular AAT level, the ideal treatment would be to get the mutant Z AAT out of cells in such quantities to protect the lung and at the same time decrease the ER stress associated with this condition. In this regard, the new approach by Vertex and others aimed not at increasing production but instead increasing secretion of modified Z protein shows some promise. This approach aims to refold the Z AAT intracellularly and chaperone the protein out of the cells. While the early results were positive with AAT levels in all treated cohorts rising by 2-3 μ M from baseline, many were disappointed with what seemed to be a fairly modest increase in AAT levels and activity.

However, in the light of the work in this thesis and other work from the lab it might be best to consider AATD not just in terms of the antigenic amount available to counteract extracellular inflammation but to also consider the inflammatory burden and how it is decreased by facilitating secretion of Z AAT out of the cells. Thus, an approach such as that used by Vertex and others which augments AAT in the extracellular space but also decreases the intracellular retention/inflammation may be most effective. Although this approach is encouraging there are certain concerns. How much will the folding correctors change the Z AAT? Will this speed up its ability to inhibit NE and will it still be able to bind IL-8, LTB4 and TNFa receptors? Will some of the secreted Z polymerize and will this act as a neutrophil chemoattractant? These questions remain to be answered. Going back to our original aim, which was to increase production and secretion of ZAAT from the intracellular compartment of cells to the external environment, we now have more data to determine whether this is a feasible effective approach. From the work presented here it would appear that the ZZ individual does try to respond to acute infection/inflammation by increasing production and secretion of AAT. The levels are quite high almost into the normal range but reflect the degree of inflammation and only occur under extreme circumstances. Even with this, the AAT response in ZZs is not sufficient to protect the lung as can be inferred from the MM SARS-CoV-2 ARDS group. The response to lesser viral-type stimuli as outlined by the vaccination responses is interesting. Here, we had the ability to evaluate MM and ZZ individuals before and after vaccination. The response in MMs is what might be expected given the IL-6 levels and the inflammatory stimuli. However, for the same inflammatory stimuli ZZ individuals were unable to secrete any AAT and gave evidence of ER stress, suggesting that even more ER stress might be present in those situations where the inflammatory stimulus is greater as in SARS-CoV-2 infection and ARDS. Therefore, at this juncture, we would not recommend a strategy such as gentamicin or ataluren in the treatment of AATD. The resultant increase in Z AAT secretion is not sufficient even for the quiescent state and the risk of over production and under-secretion is a new worry. The future in AATD

therapy lies in decreasing the intracellular gain of function and addressing the extracellular loss of function. At the moment parallel but different therapies are available; augmentation therapy for the lung and new therapies aimed at knocking out Z AAT production in the liver, but a single approach achieving both by increasing secretion of AAT from the intracellular area into the extracellular space seems most attractive.

7.2 Concluding remarks

The best strategy to provide sufficient anti-protease and anti-inflammatory protection would be a therapy such as gene editing or protein folding correction which would not only decrease liver inflammation by getting the mutant Z protein secreted and protect the lung with this secreted protein but also incorporate an acute phase response element which would respond to inflammatory stimuli in an appropriate non-inflammatory fashion.

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