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ORIGINAL RESEARCH

Activation of complement component 3 is associated with airways disease and pulmonary emphysema in alpha-1 antitrypsin deficiency

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ABSTRACT

Introduction Alpha-1 antitrypsin (AAT) deficiency (AATD) is associated with early onset emphysema. The aim of this study was to investigate whether AAT binding to plasma constituents could regulate their activation, and in AATD, exploit this binding event to better understand the condition and uncover novel biomarkers of therapeutic efficacy.

Methods To isolate AAT linker proteins, plasma samples were separated by size exclusion chromatography, followed by co-immunoprecipitation. AAT binding proteins were identified by mass spectrometry. Complement turnover and activation was determined by ELISA measurement of C3, C3a and C3d levels in plasma of healthy controls (n=15), AATD (n=51), non-AATD patients with obstructive airway disease (n=10) and AATD patients post AAT augmentation therapy (n=5).

Results Direct binding of complement C3 to AAT was identified in vivo and in vitro. Compared with healthy controls, a breakdown product of C3, C3d, was increased in AATD (0.04 µg/mL vs 1.96 µg/mL, p=0.0002), with a significant correlation between radiographic pulmonary emphysema and plasma levels of C3d (R^2=0.37, p=0.001). In vivo, AAT augmentation therapy significantly reduced plasma levels of C3d in comparison to patients not receiving AAT therapy (0.15 µg/mL vs 2.18 µg/mL, respectively, p=0.001).

Discussion Results highlight the immune-modulatory impact of AAT on the complement system, involving an important potential role for complement activation in disease pathogenesis in AATD. The association between plasma C3d levels and pulmonary disease severity, that decrease in response to AAT augmentation therapy, supports the exploration of C3d as a candidate biomarker of therapeutic efficacy in AATD.

Key messages

What is the key question?

► We questioned whether alpha-1 antitrypsin (AAT) binding to plasma constituents could regulate their activation, and in AAT deficiency (AATD), exploit this binding event to gain further insight into the pathogenesis of emphysema.

What is the bottom line?

► AAT binds directly to complement component C3, and in AATD, elevated levels of the complement activation product C3d were detected in plasma and airway samples, and correlated with airway obstruction and radiographic pulmonary emphysema.

Why read on?

► Intravenous AAT augmentation therapy normalised plasma AAT levels *in vivo*, and concomitantly reduced circulating levels of C3d, indicating that C3d may serve as a determinant of therapeutic efficacy for emerging therapies in AATD.

INTRODUCTION

Alpha-1 antitrypsin (AAT) deficiency (AATD) is an autosomal codominant inherited condition that leads to premature development of lung disease including bibasal panacinar emphysema and bronchiectasis, with diagnosis of fixed airway obstruction often made at a young age (<40 years). The WHO, American Thoracic Society and European Respiratory Society advocate targeted testing for AATD in all individuals with chronic obstructive pulmonary disease (COPD), non-responsive asthma, cryptogenic liver disease and first-degree relatives.[1]

AATD is characterised by circulating levels of AAT below the protective threshold of 11 µmol/L as a result of a mutation to the SERPINA1 gene. The most common variants of this disease are the Z (Glu342Lys) and S (Glu264Val) mutations caused by the substitution of glutamic acid for lysine or valine at positions 342 and 264, respectively. The Z mutation causes the most severe plasma deficiency and occurs in more than 95% of individuals with AATD.[2] Frequent acute pulmonary exacerbations are characterised by excessive bronchial inflammation and increased protease burden in the airways, leading to gradual decline in gas transfer in affected individuals.[3] The paradigm of a protease:antiprotease imbalance in the pathogenesis of AATD emphysema involves unopposed action of serine proteases released from neutrophils including cathepsin G and proteinase 3, but primarily neutrophil elastase (NE).[4] Infusion of plasma purified AAT protein (augmentation therapy; 60 mg/kg/week) has proven therapeutic benefit,[5 6] that serves to increase the level of AAT in both serum and lungs of affected

Table 1 Characteristics of ZZ-AATD and COPD patients employed in figures 3 and 4

	ZZ-AATD	COPD
Number of subjects	56 (n=51 plasma donors, n=5 BAL/IS donors)	15 (n=10 plasma donors, n=5 BAL/IS donors)
Age, years (mean±SD)	50.29±14.4	70.86±6.71
Gender (male/female)	31/25	8/7
BMI	26.78±5.2	31.01±4.28
FEV1 (% predicted)	77.2±33.6	57.3±23.32
FVC (% predicted)	105.7±23.9	87±5.29
FEV1/FVC (% predicted)	58.7±20.66	50±16.94
DLCO (% predicted)	65.56±24.9	36±10.86

AATD, alpha-1 antitrypsin deficiency; BAL, bronchoalveolar lavage; BMI, body mass index; COPD, chronic obstructive pulmonary disease; DLCO, Diffusion capacity for carbon monoxide; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity; IS, induced sputum.

Table 2 Characteristics of ZZ-AATD patients not receiving AAT augmentation therapy and those on augmentation therapy employed in figure 5

	ZZ-AATD (-aug)	ZZ-AATD (+aug)
Number of subjects	11	5
Age, years (mean±SD)	60.2±8.7	62.2±6.0
BMI	25.2±3.9	26.3±1.8
FEV1 (% predicted)	42.5±14.7	35.8±8.6
FVC (% predicted)	83.6±10.3	87.6±12.8
FEV1/FVC (% predicted)	42.02±9.4	33.6±10.9
DLCO (% predicted)	43.3±14.4	39.2±16.4

AAT, alpha-1 antitrypsin; AATD, AAT deficiency; +aug, receiving augmentation therapy; -aug, no augmentation therapy; BMI, body mass index; DLCO, Diffusion capacity for carbon monoxide; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity.

individuals,[7] with double AAT dosing (120 mg/kg/week) shown to enhance clinical benefits in AATD.[8]

New insights into the diverse functions of AAT have demonstrated that AAT possesses unique anti-inflammatory properties independent of anti-protease function, affecting several cell types through the modulation of inflammation caused by leukotriene B_4,[9] tumour necrosis factor alpha,[10] CXCL-8,[11] interferon gamma[12] and interleukin-1β.[13] Mechanisms of immune-regulation include direct binding of AAT to either receptors or antagonists, as demonstrated by AAT binding to CXCL-8 thereby controlling neutrophil migration.[11] The full range of AAT binding partners and consequential immune-regulatory effect is an area that is particularly important for AATD patients, and promotes better understanding of the mechanisms of action of AAT augmentation therapy in altering disease progression in AATD. Accordingly, the aim of this study was to investigate the spectrum of proteins that interact with AAT, and to determine the implications for AATD following the novel discovery that AAT binds and regulates activation of complement component 3.

MATERIALS AND METHODS

Study population

AAT phenotype was determined by isoelectric focusing with immunofixation with the Hydragel-18 AAT Isofocusing kit (Sebia, Evry, France). AATD individuals with a confirmed ZZ phenotype were recruited from the Irish National AATD Registry (table 1). Pulmonary function testing was performed in all participants according to American Thoracic Society standard. High Resolution CT images of AATD subjects were obtained on a Siemens 16-slice scanner. A modified version of Bhalla's scoring system to determine severity of emphysema and bronchiectasis was applied to each scan as previously described.[14] AAT levels were determined by routine nephelometry, and AAT sufficient patients with COPD were recruited from the general respiratory clinic in Beaumont Hospital (n=15) (table 1). All subjects were free from pulmonary exacerbation in the 6 weeks prior to enrolment to the study. Healthy non-smoking control subjects (n=59) were recruited (forced expiratory volume in one second (FEV1) 99.56%±7.9% predicted, mean age 30.38 years±5.01). AATD patients on augmentation therapy were receiving plasma purified AAT from CSL Behring (Zemaira), given intravenously at a dosage of 60 mg/kg body weight weekly (table 2). For isolation of plasma blood samples were collected in lithium heparin or ethylene diaminetetraacetic acid (EDTA) bottles (Sarstedt-Monovette) and centrifuged at 350 x g for 5 min at room temperature. Plasma was aliquoted for immediate use or stored at −80°C. All participants provided written informed consent, which was approved by the Beaumont Hospital Institutional Review Board (reference 13/92). Induced sputum (IS) and bronchoalveolar lavage (BAL) was obtained and processed as previously described.[15]

Fast protein liquid chromatography

Size exclusion permeation chromatography was performed on freshly drawn HC (n=6) plasma using a Superdex 200 GL 10/300 column (GE Healthcare) with the AKTA Prime plus (GE Healthcare) Fast Protein Liquid Chromatography system, following calibration with known molecular weight standards. Ultraviolet absorbance at 280 nm was measured using PrimeView 5.0 software. Eluted fractions corresponding to the protein elution profile at 280 nm were visualised by SDS-PAGE. Samples were pooled from high molecular mass fractions 21 to 24 and were incubated with AAT-Select (GE Healthcare). Following incubation for 1 hour with gentle rocking, the resin was pelleted by centrifugation and unbound supernatant removed. Following washing in 1 mL phosphate buffered saline (PBS) (x6), candidate binding partners were eluted by sequential 500 mM magnesium chloride ($MgCl_2$) or 2 M $MgCl_2$ elution steps. The supernatants were acetone precipitated for mass spectrometry analysis (in-solution) and SDS-PAGE analysis of the purified protein profile.

SDS-PAGE and Western blot analysis

Samples were resolved on 10% or 12.5% (w/v) SDS-PAGE and transferred to polyvinylidene fluoride membranes by Western blotting. Efficient transfer was verified by Ponceau S staining of membranes, followed by blocking in PBS-Tween containing 3% (w/v) non-fat dried milk and 1% (w/v) bovine serum albumin. The following primary antibodies were used; rabbit polyclonal anti-AAT (Abcam), rabbit anti-C3 (Novus Biologicals) and rabbit monoclonal anti-C3d antibody (E28-P, Abcam). Relative secondary antibodies were all horseradish peroxidase (HRP)–linked anti-goat or anti-rabbit. Immunoreactive protein bands were visualised with Immobilon Western Chemiluminescent HRP substrate solution (Merck Millipore) using a ChemiDoc MP System (Bio-Rad). Densitometry was carried out using Image Lab (Bio-Rad).

Mass spectrometry analysis

Protein samples (10 µg) were reduced and alkylated using dithiothreitol (DTT) and iodoacetamide before being enzymatically digested with Lys-C mass spectrometry grade (Promega) at a ratio of 1:100 protein:enzyme for 6 hour at 37°C followed by sequencing grade modified trypsin (Promega) at a ratio of 1:100 protein:enzyme overnight at 37°C. The reaction was stopped using trifluoroacetic acid and the peptides generated were separated using a 3 hour reverse-phase chromatography (RPC) separation into a LTQ Orbitrap XL (Thermo Fisher Scientific) operated in a top 10 data-dependent mode. Survey MS scans were acquired in the Orbitrap over the 400 to 1200 m/z range using 30 000 resolution and collision-induced dissociated MS/MS was carried out in the linear ion trap. In-gel digestion was carried out by excising each lane and dissection into bands. Each band was destained, reduced and alkylated before being digested overnight with 12.5 ng of trypsin per band. Peptides generated were extracted from the gel slices before being evaporated and suspended in 2% (v/v) acetonitrile/0.1% (v/v) formic acid for liquid chromatography MS analysis over a 30 min RPC gradient using the same MS conditions as above. The MS data generated from both the in-gel digestion and in-solution digestion were searched against the human subset of the UniProt/Swiss-Prot protein FASTA database using SEQUEST search algorithm using the following parameters: (1) peptide mass tolerance was set to 20 ppm, (2) MS/MS mass tolerance was set to 0.6 Da, (3) carbamidomethylation set as a fixed modification on Cys amino acids, (4) oxidation of methionines was set as variable modification, (5) up to two missed cleavages were allowed. The following filters were used for peptide identification in SEQUEST: for charge state +1, Xcorr >1.5, for charge state +2, >2.0 and for charge state +3, >2.5.

Enzyme-linked immunosorbent assay

A quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique was used to measure levels of C3 (Abcam), C3a (Quidel) and C3d (Cusabio) in plasma as per the manufacturer's instructions. Qualitative function of the complement classic, alternative and lectin pathways were determined as a percentage of the reference population using the Complement System Screen (Wieslab, Euro Diagnostica, Sweden). Complement turnover and activation, expressed as a percentage, were determined from the plasma concentration using the molar ratio of C3a (9 kDa) and C3d (35 kDa) to total C3 (190 kDa), respectively. In AATD subjects receiving augmentation therapy, plasma AAT and C3d levels were measured 48 hours after the intravenous plasma purified AAT infusion.

In vitro experiments of C3 binding to AAT

For affinity chromatography, purified goat polyclonal anti-AAT antibody (Abcam) or isotype control antibody (Abcam), were coupled to 1 mL HiTrap *N*-hydroxysuccinimide-activated columns (GE Healthcare Life Sciences). The plasma (1 mL) was passed over the prepared columns and incubated at 37°C for 20 min, cooled to 4°C and washed with 10 bed volumes of ice-cold Buffer B (prepared as per the manufacturers' instructions). Bound proteins were eluted in 2 mL of buffer B containing 1 M sodium chloride (NaCl) and concentrated to 100 µl using a Centricon 10 filter unit (Amicon). Total AAT and co-eluted C3 were assessed by Western immunoassay from the eluted fractions. Separately, 30 µg Complement C3 (Sigma) was coupled to HiTrap column beads, as previously described.[16] Deactivated control beads (containing no protein) or C3-beads were incubated with 10 µg of purified AAT (Athens Research) for 1 hour then washed with PBS. Bound AAT was detected by Western blotting. In a subset of experiments, AAT was coupled to microsphere polystyrene beads (Polybeads, Polysciences Europe GmbH, Germany) overnight, as previously described.[17] Control (no protein) or AAT-coated beads were then incubated with several dilutions (5 µg, 2.5 µg, 1.25 µg, 0.675 µg) of native complement C3 (Abcam). C3 binding to AAT-coated beads was measured with rabbit anti-C3 fluorescein isothiocyanate (FITC)-labelled antibody (Abcam) or a non-specific IgG_1 FITC control (IsoAb) (Thermo Fisher Scientific) using a FACScalibur flow cytometer (Becton Dickinson). Binding of glycosylated human-AAT (h-AAT) or recombinant AAT (r-AAT, Prospec, USA) to C3-coated Polybeads was quantified using goat anti-AAT FITC-labelled antibody (Abcam Ab19170, 1:5000) with an IgG_1 isotype FITC antibody used as a control antibody (Santa Cruz Biotechnology). Ten thousand events per reaction were quantified. Analysis of fluorescence was carried out using FlowJo software and data represented as median fluorescent intensity.

Factor H and I incubation with AAT

C3b (8 µg, Merck Chemicals) was incubated with Factor H (0.8 µg, Cambridge Bioscience) and Factor I (4 µg, Cambridge Bioscience) as previously described,[18] with increasing concentrations of AAT (8, 40 or 80 µg) for 15 min at 37°C. Samples were separated on a NuPage bis-tris 4% to 12% gel (Invitrogen), Western blotted and probed for C3d using the rabbit monoclonal C3d antibody. Purified C3d (Merck Chemicals) was used as a positive control.

Data analysis

All results are expressed as mean±SEM or±SD of biological replicates or independent experiments as stated in the figure legends. Student's t-test was used where distribution was normal (as assessed by the D'Agostino-Pearson normality test) and when comparisons were being made between two groups. Analysis of variance (ANOVA) was used to test comparisons between multiple groups. The Mann-Whitney U test was employed were data was not normally distributed. Linear and multiple regression modelling was employed to examine the significance of any relationship within the clinical data or between the clinical data and the ELISAs performed in this study. Adjustment was performed for covariates that included age and gender and relationships were reported using the standardised coefficient (β). Statistical significance was determined as a two-tailed p value <0.05. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, USA) or Stata MP V.13.0 (Texas, USA).

RESULTS

Plasma AAT is associated with complement C3 *in vivo*

To examine AAT for an interaction with circulating plasma proteins, plasma from healthy control donors (n=6) were individually chromatographed by conventional size exclusion chromatography in an attempt to identify associated or linker proteins. Figure 1A–C shows that after filtration through a Superdex 200 GL column, AAT eluted in a protein complex at a molecular mass of approximately 320 kDa (fraction 21 o 24). Area under the curve analyses of the plasma elution profile measured by densitometry of AAT immunobands determined that ~13% of AAT associated in a protein complex, with the remainder eluting at the much lower predicted molecular mass of 52 kDa (fraction 31 to 33) (figure 1E). To evaluate the nature of the interaction between AAT and other proteins, repeat chromatography was

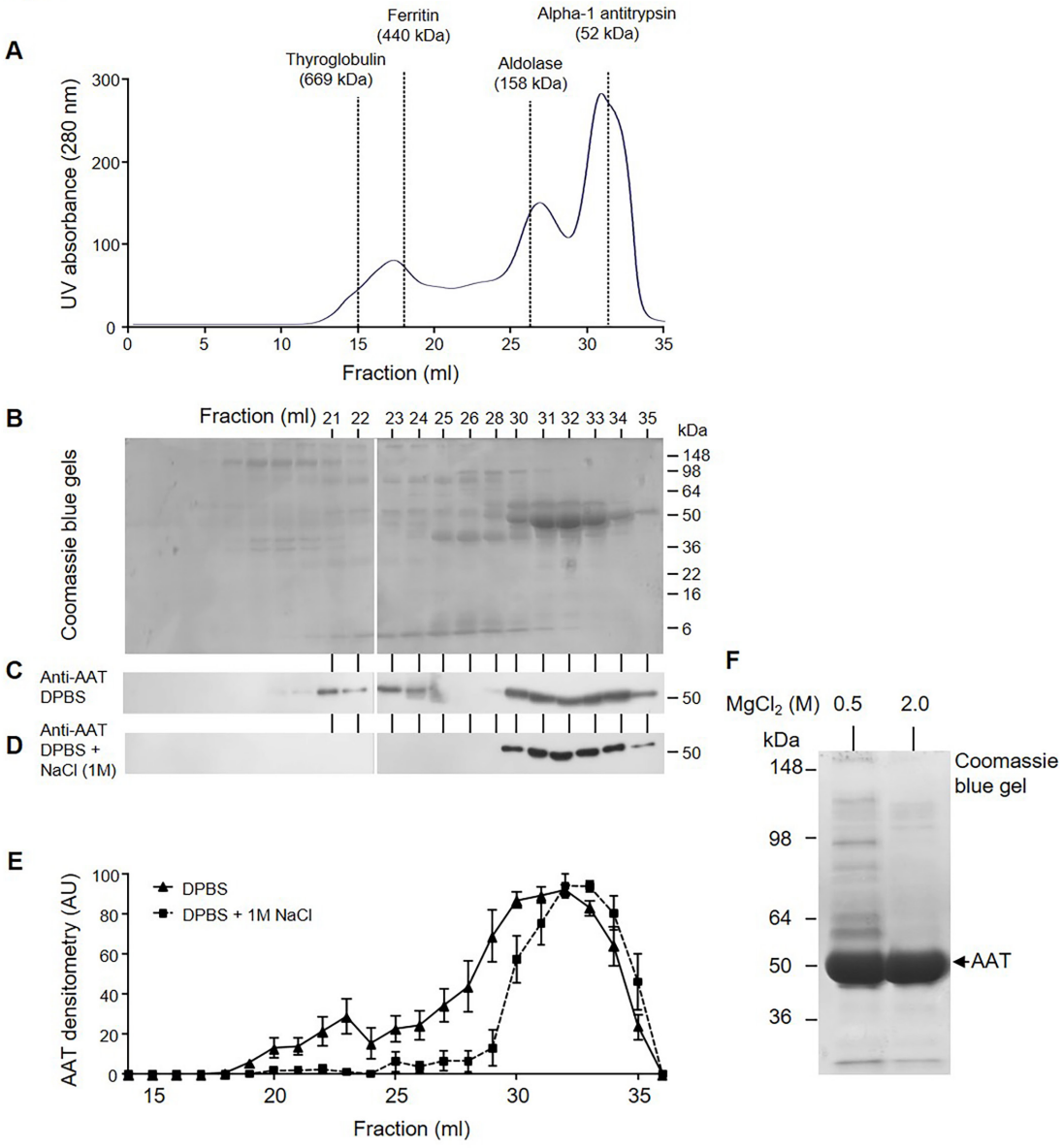

Figure 1 AAT is organised in a high molecular weight complex in plasma. (A) The protein elution chromatogram of healthy control plasma (n=6 biological repeats) was recorded at 280 nm employing the AKTA Prime$^+$ FPLC system employing a size exclusion Superdex 200 GL 10/300 column equilibrated in PBS. (B) The protein profile of fractions eluted over 14 to 35 mL (1 mL fractions) was visualised on Coomassie blue stained gels (12.5%). (C) Western blot analysis of the corresponding fractions probed with goat polyclonal anti-AAT antibody (1:5000 dilution), demonstrating AAT immunobands at two distinct molecular weight ranges. (D) Western blot analysis of HC plasma separated in PBS with 1 M NaCl demonstrating a single elution range for AAT. (E) Densitometry of AAT immunobands following repeated size exclusion chromatography in the presence or absence of 1 M NaCl (n=6 biological replicates). AAT elutes at a molecular mass of approximately 320 kDa (fraction 21 to 24) and 52 kDa (fractions 31 to 33). Each point represents mean±SEM results in B-D were run on two gels separated by a white line. (F) Purification of AAT from fraction 23 of size exclusion chromatography using AAT select resin and elution with 0.5 M or 2 M $MgCl_2$. Co-eluted AAT binding partners were visualised on Coommassie blue stained gels in the 0.5 M sample. AAT,alpha-1 antitrypsin; UV, ultraviolet.

performed in PBS plus 1M NaCl so as to disrupt electrostatic interactions between AAT and linker proteins (figure 1D and E). By Western blot analyses, the visualised profile for AAT in column buffer with high ionic strength demonstrated a single peak at the predicted molecular mass of 52 kDa (figure 1D). These results confirm that a portion of AAT is present in plasma in an electrostatic, yet reversible, high molecular mass protein complex. Subsequently, we exploited the properties of chromatographic Alpha-1 Select Resin to co-immunoprecipitate AAT and bound proteins from a pool of fractions 21 to 24, which were then visualised by Coomassie blue staining of SDS gels. AAT was eluted from the resin with 0.5 M and 2 M $MgCl_2$, with the majority of AAT interacting proteins visible in the 0.5 M $MgCl_2$ fraction (figure 1F). Mass spectrometry analyses of in-solution digests was employed to identify binding partners to AAT, with results presented in order of confidence score based on statistical analysis and the number of peptides detected (table 3). STRING V.9.0 was employed to determine previously known associations with AAT, and cluster analyses (stage 4) to group proteins according to function (online supplementary figure 1). Binding of AAT to complement C3 was highlighted as novel. Given the central role of the complement system in regulating

Table 3 Identified AAT binding partners

AAT binding partner	P value	Confidence score	Peptide count
Complement C3	8.88×10^{15}	370.28	37
Complement C4-A	1.00×10^{30}	340.29	34
Fibronectin	8.88×10^{15}	300.28	30
Apolipoprotein B-100	3.44×10^{14}	250.22	25
ITIH4	1.00×10^{30}	200.33	20
Serum albumin	1.55×10^{14}	190.27	19
Apolipoprotein A-I	6.71×10^{10}	140.30	14
Fibrinogen alpha chain	1.55×10^{14}	140.30	14
Prothrombin	5.55×10^{15}	110.24	11
Ig mu chain C region	1.19×10^{11}	90.20	9

Two-stage purification of AAT from plasma using size exclusion chromatography and co-immunoprecipitation (n=6), followed by identification of AAT binding partners by MS. The 10 highest scoring proteins identified as AAT binding partners are listed in descending order of confidence score and peptide count.
AAT, alpha-1 antitrypsin; ITIH, inter-alpha-trypsin inhibitor heavy chain.

the innate and adaptive immune response, the discovery of a possible direct interaction between AAT and C3 was relevant and was therefore further validated.

AAT directly binds complement C3 *in vitro*

In the initial gel filtration and Alpha-1 Select Resin co-immunoprecipitation experiments, AAT was extracted from plasma together with a number of other proteins, raising the question as to whether the binding of AAT to C3 was direct or through an intermediate protein or protein complex. To determine direct binding of AAT to C3, studies were performed with plasma purified, or recombinant proteins, using specific and isotype control antibodies immobilised on beads. In initial experiments, immobilised Gt-anti-AAT antibody was used to extract AAT from whole plasma and AAT C3 binding was assessed by Western blotting. Results show co-immunoprecipitation of C3 with AAT, with levels of bound C3 significantly higher on the anti-AAT beads compared with the isotype control beads (2.1 vs 1.0 AU, p=0.01) (figure 2A). To determine whether AAT attached directly to C3, C3 coated HiTrap NHS activated beads were incubated with AAT (10 µg), while beads with no C3 served as a control. Reactions were analysed by Western blotting and demonstrated significantly increased binding of AAT to C3-coated beads (2.7 vs 1.2 AU, p=0.03) (figure 2B). The role of AAT glycosylation patterns affecting protein-protein interactions has been previously documented.[19] To evaluate whether the binding interaction of C3 with AAT was glycan dependent, we performed flow cytometry analyses of C3 coated microsphere polystyrene beads exposed to h-AAT or non-glycosylated r-AAT. A significant 50% decrease in the binding of r-AAT to C3-beads compared with glycosylated h-AAT was observed (1.5 vs 1.0 AU, p=0.03) (figure 2C), indicating the involvement of AAT glycans for optimal AAT:C3 binding. Reciprocal flow cytometry experiments employing h-AAT-coated beads exposed to C3 identified a significant dose response in binding events (p trend=0.002) (figure 2D). Moreover, in competitive inhibition assays pre-incubation of AAT with C3 (concentration ratio 10:1) resulted in a significant reduction in C3 binding to AAT-coated beads (2.9 vs 0.83 AU, p=0.001) (figure 2D, histogram and bar). Collectively, this set of experiments confirms AAT and C3 binding, and raised the question of whether AATD may predispose patients to dysregulated complement activation.

Altered complement activation in AATD

To investigate complement activation in AATD, informed consent was obtained from 51 individuals recruited from the Irish National Alpha-1 Antitrypsin Deficiency Targeted Detection Programme. AATD individuals were homozygous for the Z-allele with a mean plasma AAT concentration of 0.27 g/L. No abnormality of the classic or alternative pathway of complement activation was observed. The lectin pathway was reduced in 2/13 (15.4%), which is similar to the general population (<30%) (figure 3A). Total plasma C3 was not elevated in ZZ-AATD compared with HC (556.1 vs 696.2 µg/mL, p=0.21), and did not change following correction for age and gender in a multiple regression analysis (β=−0.06, p=0.49) (figure 3B). C3a is a direct product of C3 cleavage, determined by C3a-desarg levels. When analysed with respect to the presence of airway obstruction, there was no difference observed between the HC group and ZZ-AATD individuals with FEV1/FVC (forced vital capacity) >0.7 (figure 3C). In contrast however, a significant difference was observed in plasma levels of C3a in ZZ-AATD individuals with FEV1/FVC <0.7 compared with HC samples (459.5 vs 270.2 ng/mL, respectively, p=0.02) (figure 3C), which remained significant following correction for age and gender (β=0.43, p<0.0001). An estimate of complement turnover can be determined by the molar ratio of plasma C3a to total C3 (C3a:C3%).[20] When HCs were compared with ZZ-AATD subjects with airflow obstruction, complement turnover was observed to be significantly increased by 76% (C3a:C3 1.0% vs 1.76%, p=0.03) (figure 3D).

A more specific indicator of complement activation is production of C3d, a cleavage product of C3.[21] By use of a specific ELISA for a neo-epitope exposed only on C3d, significantly increased C3d concentration was detected in ZZ-AATD plasma compared with HC (1.89 vs 0.04 µg/mL, p=0.0002) and AAT sufficient COPD plasma samples) (figure 4A). Moreover, individuals with ZZ-AATD and airflow obstruction had a higher molar ratio of complement activation (C3d:C3%) compared with those without airflow obstruction (3.15% vs 1.38%, p=0.01) (figure 4B), which remained significant following correction for age and gender (β=−0.38, p=0.039). Additionally, ELISA analysis of C3d levels in airway samples, revealed significantly increased levels of C3d in AATD BAL and IS compared with HC (34.6 vs 0 µg/mL, p=0.004) or COPD samples (34.6 vs 0.76 µg/mL p=0.005) (figure 4D). No relationship was detected between C3d:C3% and CT-determined bronchiectasis (online supplementary figure 1 online supplementary figure 2), but in contrast, a significant correlation between radiographic emphysema and C3d:C3% (R^2=0.37, 95% CI 0.15 to 0.54, p=0.001) was observed (figure 4D), which remained significant following correction for age and gender (β=0.53, p<0.001). The combined findings of very low levels of C3d in AAT sufficient COPD samples, and a relationship between radiographic pulmonary emphysema in ZZ-AATD and complement activation, led to the concept that C3d production was due to a lack of an appropriate antiprotease protective screen, which was next explored.

The antiprotease AAT modulates C3d production *in vitro* and *in vivo*

Factor H and I are an integral part of the production of C3d through C3b cleavage. Factor I is a serine protease that AAT could potentially inhibit and thereby regulate C3d production. By Western blot analyses it was confirmed that incubation of C3b (8 µg) with Factor H (0.8 µg) and Factor I (4 µg) caused increased levels of C3d production, however, C3b cleavage by Factor I

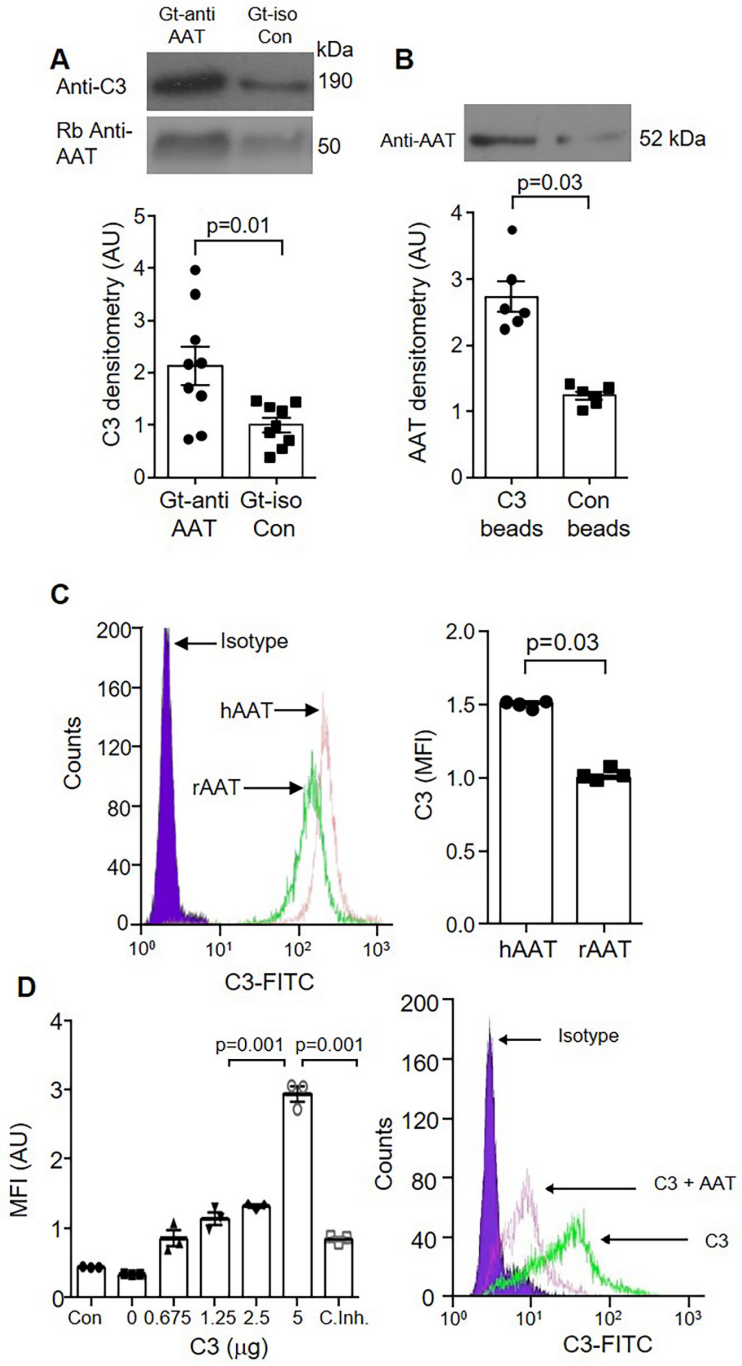

Figure 2 AAT binds complement component C3. (A) Goat polyclonal anti-AAT antibody (Abcam) or isotype control antibody were coupled to HiTrap NHS-activated HP columns (GE Healthcare). Increased co-immunoprecipitation of C3 with AAT from HC plasma on the anti-AAT column compared with isotype control column by Western blot analysis (upper panel). Results are expressed as relative densitometry units (DU) (2.1 vs 1.0 AU, p=0.01, Student's t-test). (B) Complement C3 bound NHS activated beads or control beads were incubated with purified AAT (10 µg) for 1 hour. Western blot densitometry analysis demonstrates a significant increase in AAT binding to C3-beads compared with control reactions (n=3 independent experiments, (2.7 vs 1.2 AU, p=0.03, Mann-Whitney U test). (C) Analysis of the ability of exogenous C3 to bind plasma purified human AAT (h-AAT) compared with recombinant non-glycosylated AAT (r-AAT) was examined by flow cytometry. h-AAT-coated 10 µm microsphere polystyrene beads displayed a significant increase in C3 binding and mean fluorescence intensity units (MFI) (1.5 vs 1.0 AU, p=0.03, Mann-Whitney U test). (D) Dose response relationship of C3 binding to AAT-beads by flow cytometry analysis using an anti-C3c FITC labelled antibody (p trend=0.002). Bar graph and histogram: pre-incubation of 5 µg C3 with AAT (50 µg) resulted in competitive inhibition (C.Inh.) of C3 binding events to the AAT-labelled beads (2.9 vs 0.83 AU, p=0.001, n=3 independent experiments, ANOVA). All data are displayed as mean±SEM. AAT,alpha-1 antitrypsin, ANOVA, analysis of variance.

was unperturbed by the addition of 8 to 80 µg AAT (figure 5A). As AATD neutrophils release increased levels of primary granules containing NE,[9] and in vitro NE has been shown to cleave complement,[22 23] we next assessed the production of C3d by NE in whole plasma. By Western blot analyses and by use of a monoclonal antibody to C3d, formation of C3d in HC or AAT sufficient COPD plasma on addition of exogenous NE (338 nM) was not observed (figure 5B). In contrast however, addition

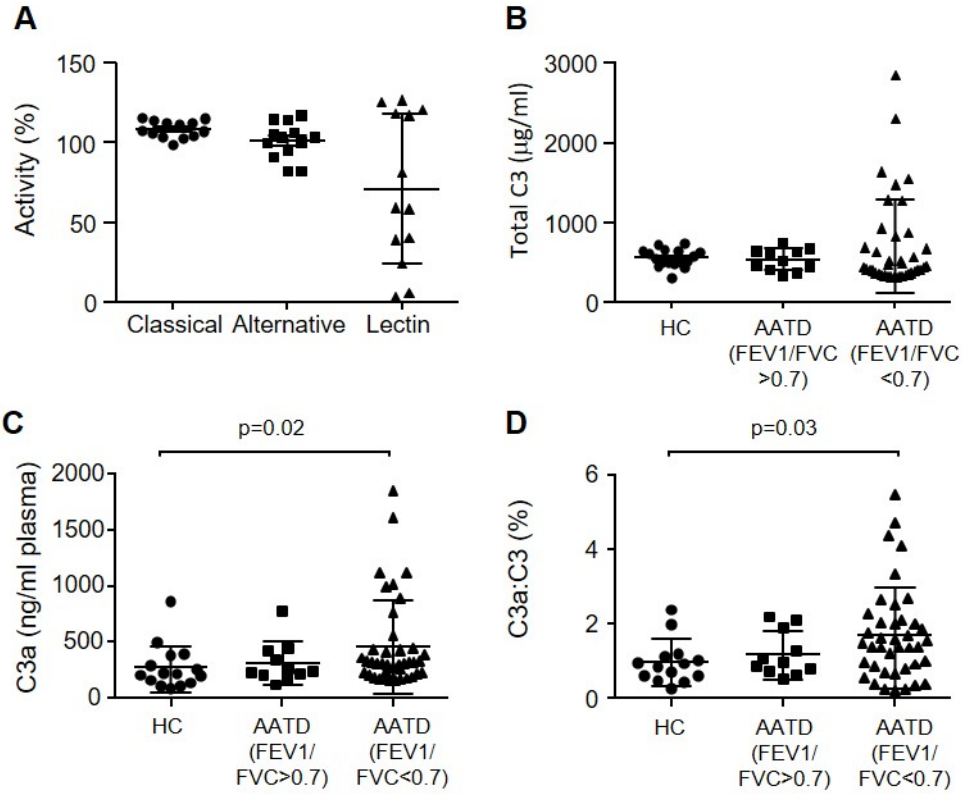

Figure 3 No significant difference in C3 plasma levels in ZZ-AATD. (A) Qualitative determination of the classical, alternative and lectin (MBL) complement pathways in serum of ZZ-AATD (ZZ) individuals (n=13) was performed using the complement system screen (Weislab). (B) No significant difference in C3 plasma levels between HC (n=17), ZZ-AATD individuals with an FEV1/FVC >0.7 (n=11) or ZZ-AATD individuals with an FEV1/FVC <0.7 (n=40) (556.1 vs 696.2 µg/mL, p=0.21, ANOVA). All data are displayed as mean±SD. (C) Increased C3a plasma levels (459.5 vs 270.2 ng/mL, p=0.02) and (D) molar ratio of C3 complement turnover, expressed as the percentage C3a over total (C3a:C3%), in ZZ-AATD individuals with an FEV1/FVC <0.7 (n=40) compared with HC (n=14) (C3a:C3 1.0% vs 1.76%, p=0.03, Mann–Whitney U test). AAT,alpha-1 antitrypsin, AATD, AAT deficiency; ANOVA, analysis of variance; FVC, forced vital capacity; FEV1, forced expiratory volume in one second.

of exogenous NE to ZZ-AATD plasma caused increased C3d production (figure 5B), suggesting that NE may play a role in the observed increased plasma levels of C3d in ZZ-AATD in vivo.

Next we examined the effect of restoration of humoral protective AAT levels on C3d production in vivo in study subjects receiving AAT augmentation therapy infusions (60 mg/kg of patient body weight). Plasma was isolated from HC, ZZ-AATD patients with obstructive disease (FEV1 of 42.5%±14.7% predicted) and ZZ-AATD patients on augmentation therapy (FEV1 35.8%±8.6% predicted) (table 2). Two days post infusion, the circulating plasma levels of AAT were significantly increased, in comparison to levels in patients not receiving AAT treatment (25 µM and 5 µM, respectively, p<0.001), with levels restored to those of HC (figure 5C). To investigate whether AAT augmentation therapy corrected the dysregulated pattern of C3d production in ZZ-AATD, the C3d profile of ZZ-AATD plasma was assessed by ELISA analysis (figure 5D). A significant decrease in C3d plasma levels was recorded in ZZ-AATD patients receiving AAT augmentation therapy compared with those not on therapy (0.15 vs 2.18 µg/mL, respectively, p=0.001). Collectively, these results illustrate that weekly AAT infusions modulate dysregulated complement activation and C3d production in vivo, which is clearly related to the levels of AAT in plasma.

DISCUSSION

The results of this study establish the interaction between AAT and complement C3, and demonstrate that by-products of C3 cleavage are elevated in plasma of individuals with AATD. Collectively, our findings indicate that deficiency of AAT results in a diminished capacity to inhibit processing of C3 to C3d, supporting a potential role for dysregulated protease-mediated complement activation in AATD. AAT does not inhibit the regulated proteolysis of C3 by Factor H and I in vitro, however AATD plasma does exhibit decreased buffering capacity against the dysregulated proteolysis of complement by exogenous NE. In vivo, in ZZ-AATD, once the concentration of AAT is raised post augmentation therapy, infused AAT functions to disrupt C3 activation, thereby decreasing C3d plasma levels.

Protein-protein interactions are an immediate lead into biological function and in this study we analysed AAT biological interactions by conventional chromatography. Importantly, the early chromatographic elution peak of AAT at a high molecular mass was not attributable to the presence of covalently bound proteases or circulating polymerised AAT, but rather to AAT present in a high molecular weight complex with proteins involved in the complement system. The validity of these results is supported by the identification of previously characterised binding partners to AAT, including fibrinogen, apolipoprotein B-100[24] and immunoglobulin light chains.[25] Of interest, as AAT is an acute phase protein and can reach higher concentrations particularly during inflammation, its ability to bind proteins may have important implications for the regulation of inflammation. Indeed, alterations in binding events through increased sialylation of AAT during resolution of inflammation associated

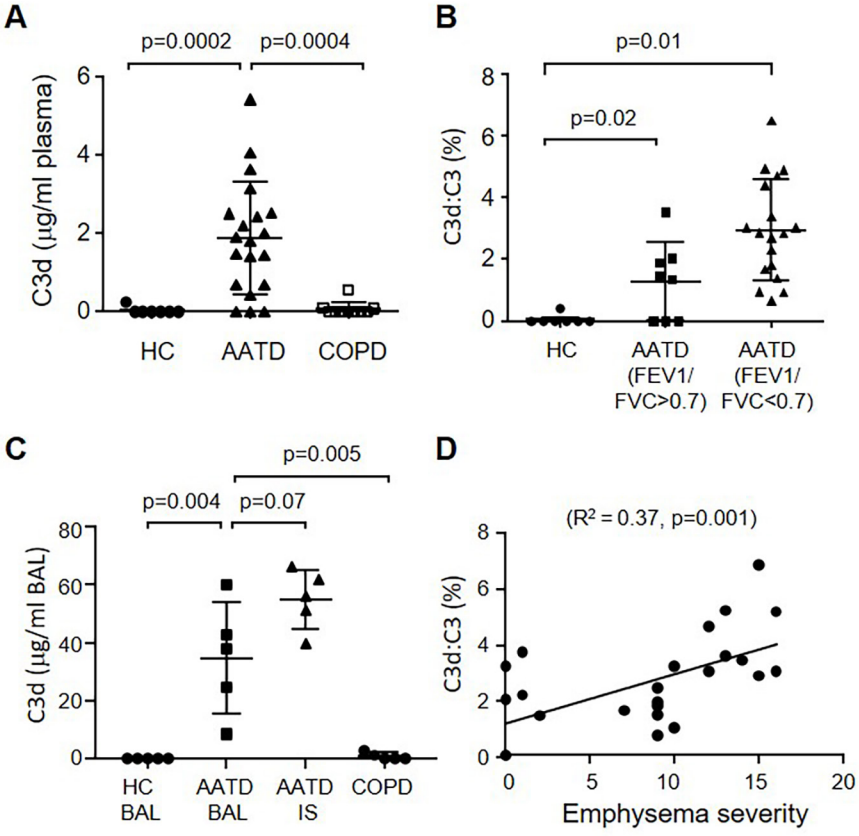

Figure 4 C3 activation products in ZZ-AATD plasma and airway samples are significantly increased. (A) C3d levels were measured in plasma of HC (n=7), ZZ-AATD (n=20) and COPD patients (n=10) by ELISA. The mean C3d concentration was increased in ZZ individuals compared with HC (2.0 vs 0.04 µg/mL, p=0.0002) or COPD samples (1.89 vs 0.07 µg/mL, p=0.0004), Mann-Whitney U test). (B) The molar ratio of complement activation, expressed as the percentage C3d over total (C3d:C3%) was increased in ZZ-AATD individuals with an FEV1/FVC >0.7 (n=8) compared with those with an FEV1/FVC <0.7 (n=18) (3.15% vs 1.38%, p=0.01) and healthy controls (n=7) (3.15% vs 0.01%, p=0.02, ANOVA). (C) C3d levels were measured in BAL or induced sputum (IS) of HC, ZZ-AATD or COPD patients by ELISA. The mean C3d concentration was increased in AATD BAL and is individuals compared with HC or COPD samples (n=5 patients per group, 34.6 vs 0 µg/mL, p=0.0044 and 34.6 vs 0.76 µg/mL p=0.005, respectively, Mann-Whitney U test). (D) Complement activation correlates with the degree of radiographic emphysema (r=0.61, r^2=0.37, 95% CI 0.15 to 0.54, p=0.001). All data are displayed as mean±SD. AAT,alpha-1 antitrypsin, AATD, AAT deficiency; ANOVA, analysis of variance; BAL, bronchoalveolarlavage; COPD, chronic obstructive pulmonary disease; FVC,forced vital capacity; FEV1, forced expiratory volume in one second.

with community acquired pneumonia, increases AAT's ability to modulate the immune response induced by CXCL8.[19] The association of AAT with C3, which is the point of convergence of the classic, alternative and lectin pathways of the complement system, in plasma is of particular interest given the relative abundance of both proteins and their central roles in innate immune defence. Recent studies have indicated that fluid phase covalent protein complexes can develop between AAT and C3b, the major cleavage fragment of C3.[26] In the presence of excess complement activation within plasma, AAT may provide important functional redundancy to less abundant complement control proteins as a non-specific humoral inhibitor of C3 activation. To understand this further, we assessed complement activation in AATD. Complement activation (C3d:C3%) and C3d levels were elevated in ZZ-AATD individuals compared with healthy controls and non-AATD COPD patients, indicating that this process may reflect a potential role for the complement system in the immunopathology of AATD. In concurrence, our findings revealed that C3 activation correlates with radiographic emphysema in AATD individuals, a finding that remained statistically significant following correction for covariates including age and gender.[27] It is known that C3 can be cleaved to activate complement by neutrophil-derived proteases including NE,[28] however, little is known about the interplay between AAT, neutrophil proteases and the complement system. Of particular relevance to this study, proteolytically active NE has been detected localised to the plasma membrane surfaces of circulating immune cells including neutrophils,[9] suggesting that one source of dysregulated protease mediated C3d production may originate in part from the surface of this circulating cell. Consequently, the increased C3d observed in AATD could be due to the absence of the direct binding of AAT to C3 or to the lack of AAT-serine protease inhibition, or a combination of both.

While C3d and C4d deposition has been noted to occur in lung allografts after transplantation[29] and targeting the C3d receptor CR2 can ameliorate lung injury in a mouse model of ischemia reperfusion injury,[30] the role of C3d in AATD is unknown. However, a potential role for the complement system is emerging in the pathogenesis of emphysema and COPD. In this regard, animal studies indicate that complement activation is a central effector mechanism for lung inflammation following cigarette smoke exposure, possibly through C3aR mediated signalling.[31] Moreover, as a costimulatory immune adjuvant, C3d bridges innate and adaptive immunity by augmenting Fc gamma receptor signalling in monocytes and dendritic cells,[32] and increased C3d levels in AATD may have important implications

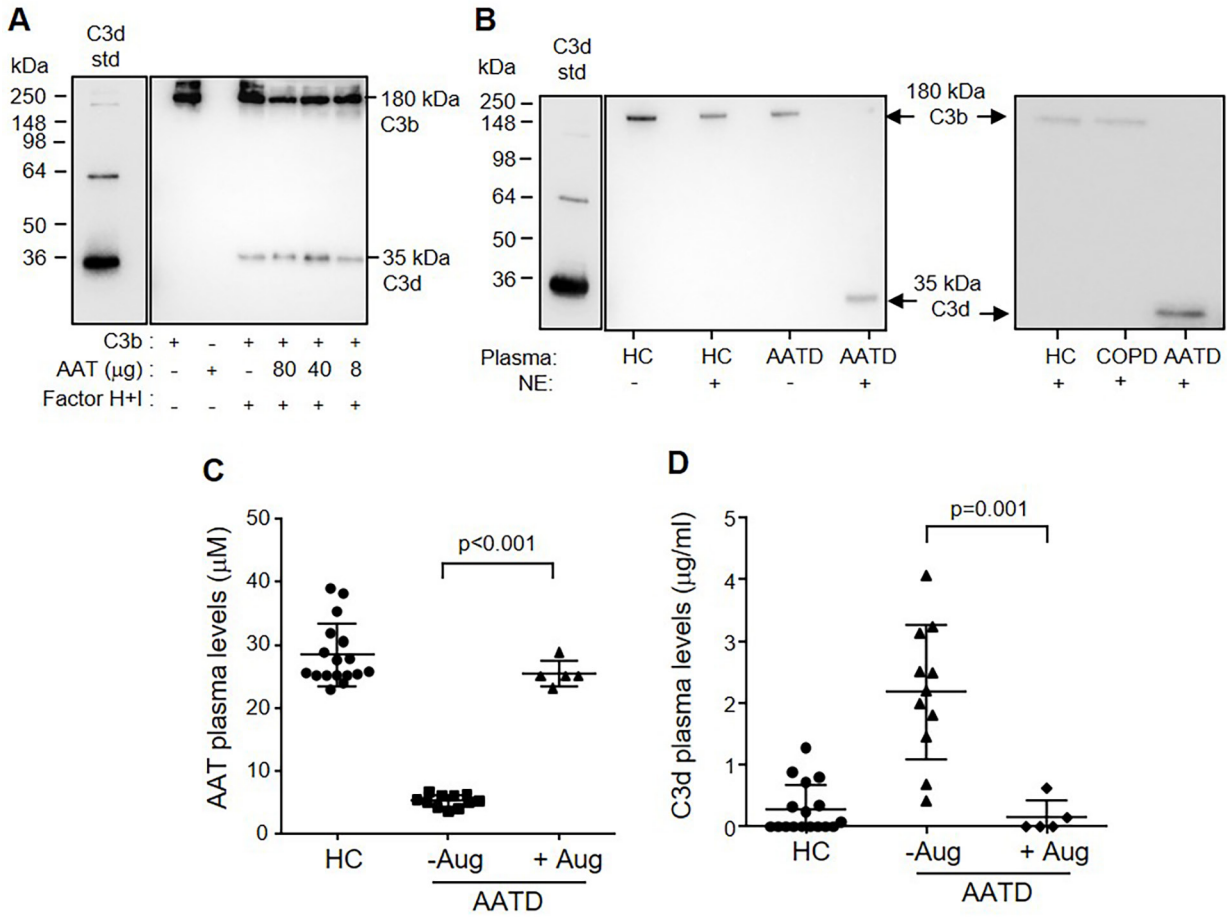

Figure 5 In vitro and in vivo impact of AAT on C3d production. (A) C3b (8 µg) incubated with factor H (0.8 µg) and factor I (4 µg), in the absence or presence of AAT (8, 40 or 80 µg) was assessed by Western blot analyses using a C3d rabbit monoclonal antibody which recognises C3b (180 kDa) and C3d (33 kDa). AAT had no effect on C3d production. (B) NE (338 nM) was added to 1% (v/v) HC, COPD or ZZ-AATD plasma and samples were electrophoresed under non-reducing conditions and Western blotted for C3d. An increase in C3d production by exogenous NE in ZZ-AATD plasma was observed. Western blots in panels A and B, are representative images of n=5 separate experiments. (C) Increased plasma levels of AAT in AATD patients receiving AAT augmentation therapy (+aug, n=5) compared with patients not receiving treatment (-aug) (25 µM and 5 µM, respectively, p<0.0001, n=11, Mann-Whitney U test). (D) ELISA analysis for levels of C3d in plasma isolated from ZZ-AATD +aug. Results in µg/ml demonstrate significantly reduced levels of C3d in patients receiving AAT-augmentation therapy (+aug) (n=5) compared with those not receiving treatment (-aug) (n=11) (0.15 vs 2.18 µg/mL, p=0.001, Mann-Whitney U test), but similar to levels in HC control (n=17) plasma samples (0.15 vs 0.27 µg/mL, p=0.62, Mann-Whitney U test). All data are displayed as mean±SD. AAT,alpha-1 antitrypsin, AATD, AAT deficiency; COPD, chronicobstructive pulmonary disease; NE, neutrophil elastase; +aug,receiving augmentation therapy; -aug, no augmentation therapy.

for the amplification of auto-inflammatory processes in AATD. Of note, the lower inflammatory threshold in AATD increases an individuals' risk of a variety of auto-inflammatory conditions, the best described being ANCA-associated vasculitis and in particular granulomatosis with polyangiitis.[33 34] A study of AATD-associated panniculitis revealed C3 deposition in pathological samples of affected areas,[35] and evidence exists to support the use of augmentation therapy not only in the treatment of granulomatosis with polyangiitis (GPA) vasculitis,[36] but also panniculitis in AATD.[37]

Existing biomarkers for AATD lung disease include desmosine and isodesmosine, well-studied lung elastin degradation products that are elevated in COPD[38] and significantly reduced post double dose AAT augmentation therapy.[8] A further biomarker includes the plasma degradation product of fibrinogen (Aα-Val360), which is also increased in AATD, linked to airflow obstruction severity, and reduced post AAT augmentation therapy.[39] The current study has uncovered C3d as a potential novel and specific biomarker for AATD lung disease. Nonetheless, C3d possesses a relatively short half-life of 4 hours, and this should be taken into account when considering its use as a biomarker in clinical practice.[40] A limitation of the present study is the recruitment of a relatively small number of AATD patients and the absence of a validation cohort. Regardless of this however, the results reveal that plasma levels of C3d are reduced in AATD patients receiving AAT augmentation therapy, compared with untreated patients, with levels of C3d reduced to that recorded in healthy control individuals.

In summary, developing new treatments for AATD necessitates the discovery of new and relevant biomarkers for patient stratification and for monitoring drug efficacy. Our findings reveal increased C3d levels occurring specifically in an AATD population that correlates with the severity of pulmonary emphysema, but not in AAT sufficient subjects with COPD. Complement activation may be a novel mechanism for disease pathogenesis in AATD and represent a clinically relevant biomarker for evaluating new alternative therapies for AATD in the future.

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