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Protective alpha1-antitrypsin effects in autoimmune vasculitis are compromised by methionine oxidation

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Abstract

**Background.** Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) are life-threatening systemic autoimmune conditions. ANCA directed against proteinase 3 (PR3) or myeloperoxidase (MPO) bind their cell surface-presented antigen, activate neutrophils and cause vasculitis. An imbalance between PR3 and its major inhibitor α1-antitrypsin (AAT) was proposed to underlie PR3- but not MPO-AAV. We measured AAT and PR3 in healthies and AAV patients and studied protective AAT effects pertaining to PR3- and MPO-ANCA.

**Methods.** Plasma and blood neutrophils were assessed for PR3 and AAT. Wild-type, mutant, and oxidation-resistant AAT species were produced to characterize AAT-PR3 interactions by flow cytometry, immunoblotting, FRET assays, and surface plasmon resonance measurements. Neutrophil activation was measured using the ferricytochrome C assay and AAT methionine-oxidation by Parallel Reaction Monitoring.

**Results.** We found significantly increased PR3 and AAT pools in both PR3- and MPO-AAV patients, however, only in PR3-AAV did the PR3 pool correlate with ANCA titer, inflammatory response and disease severity. Mechanistically, AAT prevented PR3 from binding to CD177, thereby reducing neutrophil surface antigen for ligation by PR3-ANCA. Active PR3-AAV patients showed critical methionine-oxidation in plasma AAT that was recapitulated by ANCA-activated neutrophils. The protective PR3-related AAT effects were compromised by methionine-oxidation in the AAT reactive center loop but preserved when two critical methionines were substituted by valine and leucine.

**Conclusion.** Pathogenic differences between PR3- and MPO-AAV are related to AAT regulation of membrane-PR3, attenuating neutrophil activation by PR3- rather than MPO-ANCA. Oxidation-resistant AAT could serve as adjunctive therapy in PR3-AAV.
Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) comprises a group of life-threatening systemic autoimmune diseases, most frequently affecting the lungs and kidneys.(1) The majority of patients with the clinical syndrome of granulomatosis with polyangiitis (GPA) have ANCA to proteinase 3 (PR3), whereas most patients with microscopic polyangiitis (MPA) have ANCA directed against myeloperoxidase (MPO). The ANCA autoantigens are exclusively expressed by myeloid cells, with highest amounts in neutrophils. Mechanistically, ANCA binding to their cell surface-presented target autoantigens initiates neutrophil activation and subsequent vascular inflammation and injury.(2) PR3 has a typical bimodal cell membrane pattern (mPR3) with distinct mPR3\textsuperscript{low} and mPR3\textsuperscript{high} neutrophil subsets.(3) The percentage of the mPR3\textsuperscript{high} subset varies between 0 and 100% in the population, is genetically determined, and stable in each individual.(4-6) mPR3\textsuperscript{high} neutrophils respond more vigorously to PR3-ANCA induced activation(7) and a large mPR3\textsuperscript{high} neutrophil subset represents a risk factor for AAV and worse clinical outcomes.(4, 6, 8) The bimodal mPR3 pattern is a consequence of the epigenetically controlled, subset-restricted expression of CD177(9) that functions as a high-affinity PR3 receptor yielding distinct CD177\textsuperscript{neg}/mPR3\textsuperscript{low} and CD177\textsuperscript{pos}/mPR3\textsuperscript{high} neutrophils.(10, 11) While the neutrophil-activating effect of both PR3- and MPO-ANCA have been established \textit{in vitro}, the pathogenicity of only MPO-ANCA was confirmed in animal models, as convincing PR3-ANCA models are lacking.(12-18)

The two clinical syndromes GPA and MPA, and the two ANCA specificities to PR3 and MPO, result in distinct clinical characteristics but the differences in underlying pathogenic mechanisms remain less clearly defined.(19, 20) PR3, the autoantigen in PR3-AAV, is a member of the neutrophil serine protease (NSP) family with pathogenic
implications in both AAV entities. (21) (22-24) α1-antitrypsin (AAT) is the major PR3 inhibitor and recent genome-wide association studies (GWAS) found strong GPA and PR3-AAV associations with SNPs near or within the genes for PR3 (PRTN3) and AAT (SERPINA1). (25, 26) These genetic associations were not seen in MPO-AAV patients suggesting that PR3- and MPO-AAV are genetically distinct diseases. PRTN3 SNPs from the AAV GWAS provided an eQTL affecting neutrophil PR3 transcription (26) and a pQTL that associated with increased plasma PR3 levels in healthy controls (HC). (27) The GWAS-identified SERPINA1 SNP in PR3-AAV is in linkage disequilibrium with the SERPINA1 null (z) allele that leads to lower plasma AAT. (26-28) Based on these findings, mechanistic concepts regarding disease pathogenesis were proposed suggesting roles for increased PR3 and/or reduced AAT in PR3-, but not in MPO-AAV. (25-27)

Because systematic studies investigating variations in levels of PR3 and AAT in AAV are lacking, we prospectively evaluated PR3 and AAT in HC and AAV patients and explored clinical correlations. Our patient-related data led us to experimentally characterize protective AAT effects targeting mPR3 on neutrophils thereby reducing neutrophil activation by PR3-ANCA. Importantly, we also identified critical disease-modifiers of the PR3-AAT interaction that diminished the protective AAT effects and tested an AAT variant that resisted this loss-of-function modification.
Results

Plasma PR3, AAT and the PR3:AAT ratio are significantly increased in active PR3- and MPO-AAV patients

We recruited 50 HC and 114 AAV patients (37 active and 39 remission PR3-ANCA patients and 22 active and 16 remission MPO-ANCA patients) from three hospitals between January 2020 and July 2021 for assessment of PR3 and AAT. A flow diagram outlining the study design and the participating patients is depicted in Figure 1. HC and patient characteristics are listed in Supplemental Table 1. Plasma PR3 and AAT levels were significantly increased in both active PR3- and MPO-AAV patients compared to HC and both values normalized with disease remission (Figure 2A and B). Interestingly, only one remission AAV patient showed strongly decreased AAT values, suggesting the presence of a homozygous SERPINA1 z-allele. Increased PR3 exceeded increased AAT leading on average to a 3.3-fold higher plasma PR3:AAT molar ratio in active disease (Figure 2C). Plasma PR3 levels correlated with CRP in PR3- but not in MPO-AAV (Figure 2D). Plasma AAT levels correlated with the inflammation marker CRP in PR3- and MPO-AAV (Supplemental Figure S1A).

Plasma, neutrophil, and total PR3 blood pools are increased in active AAV patients and correlate with inflammation, vascular injury, and PR3 autoimmunity in PR3- but not MPO-AAV

We measured PR3 protein in lysates from blood neutrophils by ELISA and found that neutrophils from active PR3-AAV patients contained 1.7-fold and from active MPO-AAV 2.0-fold more PR3 compared to HC (Figure 2E). PR3 immunoblots showed similar PR3 band pattern in PR3-AAV patients and HC indicating that AAV-associated glycosylation did not differ between patients and HC (Figure 2F). PR3 transcription is
silenced in mature blood neutrophils but is reactivated in active AAV. (29-31) We observed that PR3, but not AAT transcription, was significantly increased in neutrophils from active PR3-AAV patients compared to HC and remission patients (Supplemental Figure S1B and C).

We calculated the PR3 antigen pool in plasma (PR3 concentration x estimated plasma volume), in neutrophils (PR3 neutrophil content x blood neutrophil count), and, taken together, the total PR3 blood pool. The neutrophil PR3 pool was approximately 100-times larger compared to the plasma PR3 pool, but both pools increased significantly in active AAV and normalized with remission (Supplemental Figure S1D and E). Hence, the total PR3 blood pool was strongly increased in active AAV (3.8-fold in PR3- and 3.2-fold in MPO-AAV) (Figure 2G). We observed significant positive correlations of the total PR3 blood pool with clinical markers of systemic inflammation (CRP), anemia (hemoglobin), and kidney injury (creatinine, erythrocyturia) in PR3- but not in MPO-AAV patients (Table 1). Similar correlations were seen for the plasma and the neutrophil PR3 pool individually. The plasma PR3 pool, but not the total PR3 blood and neutrophil pool correlated with autoimmunity (PR3-ANCA titer). The AAT plasma pool was also increased in patients with both ANCA specificities (Figure 2H).

**PR3 is increased on the cell membrane of neutrophils from PR3-AAV patients**

Because PR3-ANCA binding to PR3 on the neutrophil membrane (mPR3) initiates neutrophil activation, we assessed mPR3 on isolated neutrophils from HC and AAV patients. A representative flow cytometry histogram illustrates the typical bimodal mPR3 staining (Figure 2I). We analyzed our cohort with respect to both the portion of mPR3<sup>high</sup> neutrophils and to the amount of mPR3 (as assessed by the mean fluorescence intensity, MFI), respectively. Compared with HC, the portion of mPR3<sup>high</sup>
neutrophils was increased in both active and remission PR3-AAV patients with a similar trend for MPO-AAV supporting the notion that this AAV risk parameter is genetically determined (Figure 2J).(5, 6, 32) In contrast, mPR3 amounts were significantly increased in active but not remission PR3-AAV patients indicating that inflammatory diseases elevate the amount of PR3 on the neutrophil surface without changing the percentage of mPR3<sup>high</sup> neutrophils (Figure 2K). Parallel assessment of mCD177 in the HC and patient groups showed the same pattern (Supplemental Figure S1F and G). In HC, the mPR3 amount showed an inverse correlation with plasma AAT that was not observed in AAV patients (Figure 2L). These observations led us hypothesize that extracellular AAT reduced mPR3, but that this effect was compromised in AAV despite increased AAT levels.

**Recombinant wild-type, but not mutant AAT binds and neutralizes proteolytically active PR3, and releases PR3 from the neutrophil membrane in a reversible manner.**

To test our hypothesis, we produced recombinant wild-type (wt)-AAT and, as an important control protein, a mutant form (mut-AAT) that we had generated recently(33) containing amino acid substitutions in the reactive center loop (Figure 3A). As expected, wt- but not mut-AAT bound purified PR3 and PR3 in cell-free supernatants (cf-SN) from activated neutrophils as indicated by the formation of the typical 72-kDa PR3:AAT complex in anti-PR3 immunoblots (Figure 3B). Consequently, wt- but not mut-AAT abrogated the PR3-specific proteolytic FRET activity in cf-SN (Figure 3C). Importantly, neutrophil incubation with increasing wt-AAT concentrations dose-dependently reduced mPR3 on neutrophils as determined by flow cytometry (Figure 3D). At higher concentrations, mut-AAT reduced the MFI somewhat without affecting the typical bimodal mPR3 pattern underscoring the importance of an appropriate
control protein. Wt-AAT did not reduce surface levels of CD11b, CD18 or CD66b (Figure 3E). Moreover, wt-AAT equally reduced mPR3 when neutrophils were incubated on fibronectin or on glomerular microvascular endothelial cells (gMVEC) (Figure 3F).

Neutrophils function not only in blood at high AAT concentrations, but also in inflamed tissues where AAT concentrations are significantly lower. We therefore assessed whether the mPR3 reduction by AAT was reversible under inflammatory conditions. Neutrophils were incubated with wt-AAT to reduce mPR3, washed to remove AAT, and then primed with TNFα. We observed a strong reappearance of mPR3 over time in the presence of mut-AAT that was again suppressed by the wt-AAT (Figure 3G). Two other natural inhibitors of NSPs, namely serpinA3 and the chelonianin SLPI did neither neutralize proteolytic PR3 activity nor reduced mPR3 underscoring the importance of AAT in regulating mPR3 (Figure 3H and I). These findings establish that AAT reduced mPR3 on the surface of neutrophils in a dose-dependent and reversible manner.

**AAT removes mPR3 from the neutrophil surface by preventing PR3 binding to CD177 and thereby reducing PR3-ANCA induced neutrophil activation**

Given the importance of mPR3 for PR3-ANCA binding and subsequent neutrophil activation, we explored the molecular mechanism by which AAT reduces mPR3. To exclude the possibility that AAT merely prevented antibody binding to PR3 by steric hindrance, mPR3 reduction by wt-AAT was confirmed using three additional monoclonal antibodies (mabs) to PR3, including clones MCPR3-2 and WGM-2 that recognize two different PR3 epitopes (Figure 4A).(34, 35) We then performed a mPR3 shedding assay after incubation of surface-biotinylated neutrophils with buffer, wt-, and mut-AAT, respectively. We collected the cf-SNs and performed a streptavidin-based
pulldown of biotinylated proteins from these cf-SNs followed by anti-PR3 immunoblotting. We found significantly more biotinylated mPR3 in the supernatants from wt-AAT-treated neutrophils, indicating that PR3 was indeed released from the neutrophil surface (Figure 4B).

The bimodal neutrophil mPR3 pattern is caused by subset-restricted expression of the PR3 receptor CD177 yielding distinct CD177_{neg}/mPR3_{low} and CD177_{pos}/mPR3_{high} neutrophils.\(^{(9, 10)}\) Flow cytometry after double-staining for PR3 and CD177 revealed that wt-AAT treatment reduced mPR3 particularly on the CD177_{pos}/mPR3_{high} subset (Figure 4C). We hypothesized that AAT competes with CD177 for PR3 binding and performed surface plasmon resonance measurements (SPR) (Figure 4D). We observed that PR3, but not AAT, bound with high affinity to immobilized CD177 (Kd 20 x 10^{-9}M). PR3 preincubation with wt- but not with mut-AAT prevented PR3 binding to immobilized CD177. Importantly, CD177-bound PR3 dissociated from CD177 when wt- but not mut-AAT was used as soluble analyte.

These data indicate that AAT reduced mPR3 on the surface of CD177_{pos}/mPR3_{high} neutrophils by complexing PR3 thereby preventing PR3 binding to CD177.

*Disease-associated modification of the AAT:PR3 interaction and its pathologic consequences*

We next explored disease-associated factors that modify mPR3, AAT, and possibly their interaction. We first compared the susceptibility of HC and active AAV patient neutrophils to mPR3 reduction by AAT. Wt-AAT decreased mPR3 on neutrophils from HC, active, and remission PR3-AAV patients to a similar degree, excluding intrinsic neutrophil factors reducing the mPR3-lowering AAT effect (Figure 5A). We then determined the consequence of AAT-mediated mPR3 reduction for ANCA-induced neutrophil activation by measuring NADPH oxidase-dependent neutrophil respiratory
burst - a robust activation indicator. When HC neutrophils were incubated with wt-AAT prior to the ANCA stimulation, superoxide production was significantly reduced in response to PR3- but not MPO-ANCA (Figure 5B).

We next assessed the effect of AAV patient plasma on mPR3 using HC neutrophils. To exclude quantitative AAT effects, we selected the few plasmas from active PR3-AAV patients and HC with similar AAT levels (Figure 5C). mPR3 depletion was less efficient when using active PR3-AAV patient plasma, whereas active MPO-AAV or remission AAV, and HC plasma all showed similar effects (Figure 5D). We reasoned that either inflammatory mediators in active PR3-AAV patient plasma led to higher mPR3 levels, or that AAT protein is modified, affecting its capacity to bind and consequently deplete PR3 from the neutrophil membrane, or that both occurred. Spiking HC plasma with inflammatory mediators at picomolar concentrations, as found in active PR3-AAV patients(36-38), increased mPR3 and this effect was further accelerated by PR3- but not MPO-ANCA (Figure 5E). These findings show that disease-characteristic inflammatory mediators counteract the mPR3-lowering AAT effect thereby increasing mPR3 in the presence of identical AAT concentrations.

AAT oxidation, specifically oxidation of the two surface-exposed methionine (M) residues M351 and M358 in the reactive AAT center loop (Figure 6A), is a well-established mechanism that can reduce the inhibitory AAT activity towards NSPs at inflammatory sites.(39) (40). Conceivably, oxidative stress in AAV could lead to AAT oxidation and could therefore impair mPR3 depletion by AAT. To determine whether this modification occurs in vivo, patient plasma was assessed by quantitative targeted mass spectrometry, namely Parallel Reaction Monitoring (PRM). We assessed unmodified and oxidized variants of peptides covering critical M351 and M358, and the unexposed M385 that is inaccessible to oxygen radicals as a control. We observed significantly increased M351 and M358 double-oxidation in plasma from active PR3-
AAV patients that decreased with remission and was not seen for M385 (Figure 6B). These data indicate that this site-specific modification of AAT indeed occurs in active PR3-AAV patients in vivo. Moreover, the level of M351/M358 double-oxidation correlated with CRP, an indicator of systemic inflammation (Figure 6C). Conceivably, oxidative AAT modification is even more relevant in close proximity of ANCA-activated neutrophils. This notion is supported by our observation that M351/M358 double-oxidation of wt-AAT strongly increased in the presence of ANCA-activated neutrophils and N-chlorosuccinimide (NCS)(41) that served as positive control (Figure 6D). Again, oxidation of M385 was not affected. The estimated ratio between oxidized and unmodified M351/M358 was much higher after in vitro exposure of AAT to activated neutrophils than in plasma samples from active PR3-AAV patients (Supplemental Figure S2).

In contrast to unmodified wt-AAT, chemically oxidized wt-AAT (ox-AAT) did not form complexes with purified PR3, did not abrogate proteolytic PR3 activity (Supplemental Figure S3), and did not compete with CD177 for PR3 binding by SPR (Figure 7A). Importantly, ox-AAT, produced from recombinant wt-AAT or commercially available Respreeza™, a drug that is repetitively given to patients with AAT-deficiency, did neither diminish mPR3 nor neutrophil activation by PR3-ANCA (Figure 7B and C).

Finally, we produced a recombinant AAT containing valine and leucine substitutions (VL-AAT) for the critical, oxidation-susceptible methionine residues in the reactive center loop (M351V/M358L).(42) This oxidation-resistant VL-AAT preserved its capacity to reduce proteolytic PR3 activity, mPR3 on neutrophils, and respiratory burst in response to PR3-ANCA even in the presence of NCS oxidant (Figure 7D-F). Respiratory burst inhibition was dose-dependent over a range between 0.1 and 10 µM (Supplemental Figure S4). These findings show that critical methionine-oxidation in the AAT center loop, found in active PR3-AAV patients, compromises protective AAT
effects, and that protection was preserved with oxidation-resistant VL-AAT, even when exposed to strong oxidative stress.
Discussion

We explored alterations in the PR3 autoantigen and its major inhibitor AAT in AAV to define their roles in disease pathogenesis. We report several novel findings. First, PR3 and AAT were increased in active AAV patients. This finding pertains to both PR3- and MPO-AAV. However, only in PR3-AAV did increased PR3 correlate with markers of systemic inflammation, kidney injury, and autoimmunity. Second, AAT diminished the binding of PR3 to the CD177 receptor on the neutrophil surface thereby reducing neutrophil activation in the presence of PR3- but not MPO-ANCA. Third, disease modifiers, including inflammatory mediators and oxidative AAT modification, reduced neutrophil mPR3 depletion by AAT in active PR3-AAV. Substitution of critical methionine residues in the reactive AAT center loop preserved the protective AAT effects even when exposed to oxidants.

Associations between PR3-AAV and SNPs near or within the \textit{PRTN3} gene were reported from different patient cohorts.(25, 26) Some of these \textit{PRTN3} SNPs provided an eQTL affecting neutrophil PR3 transcription\(\text{\textit{(26)}}\) and reactivated PR3 transcription was reported in active AAV patients.\(\text{\textit{(31, 43)}}\) In addition, some of the \textit{PRTN3} SNPs from the AAV GWAS provided a pQTL that associated with increased plasma PR3 in HC.\(\text{\textit{(27)}}\) However, whether active AAV patients harbor increased neutrophil PR3 protein was not investigated in these studies. We provide novel information, namely that the PR3 protein content of a single neutrophil has approximately doubled in both active PR3- and MPO-AAV. The total PR3 blood pool, but also the individual neutrophil and plasma PR3 pools, correlated with clinical inflammation, and kidney injury markers in the former, but not the latter. Increased PR3 protein may be linked to the PR3-AAV pathogenesis by various effects. For example, more mPR3 on the neutrophil surface leads to more PR3-ANCA binding resulting in stronger neutrophil activation. Increased
neutrophil PR3 elevates caspase 1-independent IL-1β generation, cleavage of various extracellular substrates, and vascular damage.(23, 24) Plasma PR3 correlated with the PR3-ANCA titer conceivably by providing more autoantigen for the autoimmune response, particularly in individuals with a susceptible HLA-DP(25, 26) background.

Plasma AAT is the major PR3 inhibitor and is lowest in homozygous carriers of the z-allele. Significant PR3-AAV associations with SNPs in the SERPINA1 gene in linkage disequilibrium with the z-allele, a low level mutant, were found in GWAS suggesting a link between AAT levels and disease risk.(25, 26) However, the mere presence of AAT deficiency did not increase the prevalence of PR3-ANCA and PR3-AAV as observed in patients with AAT deficiency-related chronic obstructive lung disease supporting the importance of the HLA context.(44) Our data might seem paradoxical at first sight, as plasma AAT levels were found to be increased in the vast majority of active PR3- and MPO-AAV patients with only one remission PR3-AAV patient having low AAT plasma levels. AAT is mainly produced by the liver and levels increase with the acute-phase inflammatory response. Accordingly, we expected to observe increased AAT plasma concentrations in active AAV patients and positive correlations between the systemic inflammation marker CRP and AAT as described by others.(45) However, what was less expected was the increase of PR3 in plasma that exceeded the increase in plasma AAT on a molar ratio. Plasma AAT was still in excess compared to plasma PR3. Nevertheless, this subtle imbalance favoring PR3 has implications for the local neutrophil environment when cells release PR3 and other NSPs into their surroundings. This assumption is supported by a study on AAT and neutrophil elastase that measured and modeled AAT- and neutrophil elastase-dependent quantum proteolytic events and areas around neutrophils in human serum.(46)
PR3-ANCA binding to PR3 on the neutrophil cell membrane and the subsequent neutrophil activation are central to the PR3-AAV disease concept. We confirm that the mPR3\textsuperscript{high} neutrophil percentage and the mPR3 amount are increased in PR3-AAV, and that the latter, but not the former depends on disease activity.(4, 8, 32) The high-affinity PR3 receptor CD177 followed the same membrane expression pattern as mPR3. Triggered by the observation that plasma AAT correlated inversely with mPR3 in HC but not in our AAV patient cohorts, we performed mechanistic studies to test the hypothesis that AAT depletes mPR3 and that disease-modifiers counteract this effect. We found that increasing AAT concentrations progressively reduced the neutrophil mPR3 amount. The effect was reversible under inflammatory conditions when AAT was removed, mimicking emigration from the plasma to extravascular inflammatory sites. We provide novel mechanistic evidence that AAT binding to PR3 prevented PR3 binding to the CD177 neutrophil surface receptor. Moreover, CD177-bound PR3 dissociated from CD177 in the presence of AAT. Critically, this effect specifically protected against PR3- but not against MPO-ANCA induced neutrophil activation, clearly separating the pathogenesis of the two conditions. However, we observed that the inverse correlation between mPR3 and AAT seen in HC was compromised in both active and remission AAV patients. The fact that some remission PR3- and MPO-AAV patients, despite a BVAS of zero, still had slightly elevated CRP levels (Supplemental Table 1) and persistent low-titer ANCAs suggest some residual inflammation that may explain this observation (Supplemental Figure S6).

We observed that plasma from active PR3-AAV patients caused less mPR3 depletion compared to plasma from HC and MPO-AAV patients despite having similar AAT concentrations. Disease-associated cytokines, together with ANCA, contributed to this effect. In addition, we identified AAT oxidation as an AAV-related modifier that weakened the mPR3-lowering AAT effect. Oxidative inactivation of the AAT
antiproteinase activity in the respiratory tract of smokers was implicated in emphysema.\(^{(47, 48)}\) AAT harbors nine methionines with M351 and M358 located in the reactive center loop and responsible for NSP binding. M351 and M358 are exposed on the surface of the molecule and are therefore susceptible to methionine sulfoxide oxidation resulting in AAT inactivation.\(^{(40)}\) Activated neutrophils are an important ROS source and we reasoned that AAV, a neutrophil-mediated inflammatory condition, leads to oxidation-induced AAT inactivation. We found significantly increased M351 and M358 double-oxidation in plasma AAT of active PR3-AAV patients. The level of oxidation correlated with the inflammation marker CRP suggesting that the observed modification was caused by systemic inflammation. This notion is further supported by the fact that ANCA-activated neutrophils caused strong M351 and M358 double-oxidation \textit{in vitro}.

We provide firm evidence that critical methionine oxidation is responsible for the reduced capacity of oxidized AAT to neutralize proteolytic PR3 activity, neutrophil mPR3, and diminished neutrophil activation by PR3-ANCA. An AAT species containing valine and leucine substitutions for the critical, oxidation-susceptible methionine residues in the reactive center loop (M351V/M358L, VL-AAT) preserved all these protective effects even when exposed to a strong oxidant. Our findings are consistent with recent studies showing that mice expressing high levels of the M351V/M358L-substituted AAT variant, were protected against an excess of oxidants \textit{in vivo}.\(^{(42)}\) A schematic illustrating key findings of protective AAT effects in the AAV context is shown in Figure 8. Our data support the notion that quantitative AAT aspects do not sufficiently reflect the complex AAV disease situation and that additional qualitative AAT aspects and the balance with PR3 are important. In addition to oxidative modification, AAT polymerization occurs in patients with AAT deficiency alleles leading to proinflammatory AAT properties that concern both PR3- and MPO-AAV.\(^{(49)}\) This
state-of-affairs may explain why AAV patients with a z- or s-allele showed more intra-alveolar hemorrhage independent of the ANCA subtype.\(^{(50)}\)

In summary, our data indicate an increased PR3 pool in active AAV patients that correlates with important clinical disease features. AAT exerts concentration-dependent protective anti-proteinase effects in PR3-AAV that are compromised by oxidative modifications. An oxidation-resistant AAT preserved protection despite oxidative stress and could possibly be explored as an adjunct for current AAV therapeutic strategies.
Methods

Patients and healthy human individuals

Patients with AAV, based on Chapel Hill Consensus Conference criteria (51), were recruited in the Department of Nephrology and Medical Intensive Care, Charité Universitätsmedizin Berlin, Department of Nephrology, Helios Klinikum Berlin-Buch, Berlin, and Department of Nephrology, Endocrinology and Diabetology, Ernst von Bergmann Klinikum, Potsdam, all Germany. Disease activity was assessed by the Birmingham Vasculitis Activity Score Version 3 (BVAS) (52) and patients were grouped according to ANCA specificity (PR3-ANCA versus MPO-ANCA) and disease activity (active versus remission). Between January 2020 and July 2021, a total of 50 HC and 114 ANCA-associated vasculitis (AAV) patients were recruited. All patients were ANCA positive and had generalized disease. At the time of blood sampling, 11/37 active PR3-AAV patients were treatment naïve, 25/37 had received steroids for less than 10 days, and 12/37 had received the first dose of iv cyclophosphamide or rituximab. The numbers for active MPO-AAV patients were 10/22, 10/22, and 3/22, respectively. For remission patients, 21/39 PR3-AAV were on steroids and 20/39 on azathioprine or rituximab maintenance. The numbers for remission MPO-AAV patients were 11/16 and 5/16, respectively. Clinical and routine laboratory data were collected, and samples were assessed based on material availability. 7/114 AAV patients had total leukocyte but no neutrophil counts. For these patients, neutrophil numbers were calculated from the measured total leukocyte count using the average neutrophil percentage derived from the corresponding disease group. Population characteristics together with clinical and laboratory information are summarized in Supplemental Table 1.
Preparation of neutrophils, neutrophil lysates, and plasma from human blood samples

Venous heparinized blood samples were divided for neutrophil isolation and for plasma preparation. Neutrophils were isolated using density-gradient centrifugation as previously described.(6) For preparation of neutrophil lysates, 1x10^6 neutrophils were resuspended in 15 µl lysis buffer supplemented with protease inhibitors (1% NP40, 0.1 mM Quercetin, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 5 mM Iodoacetamid, 20 mM NaF, 1 mM PMSF, 0.2 mM Na3VO4, followed by centrifugation at 18,000g for 10 min at RT. Cell-free supernatants were stored at -80°C until analyzed. Plasma was collected after blood centrifugation at 1,250g for 10 min at RT and stored at -80°C.

Preparation of human IgG

Normal- and ANCA-IgG were prepared from HC and patients with active MPO- and PR3-ANCA disease using a High-Trap-protein-G column in an Äkta-FPLC system (Cytiva Europe GmbH, Freiburg, Germany).

PR3 and AAT mRNA expression in isolated neutrophils

Total mRNA was extracted from 5x10^6 neutrophils using 500 µl QIAzol and the RNeasy Purification kit (Qiagen). RNA was treated with deoxyribonuclease I (Qiagen) and cDNA was prepared using hexanucleotide primers and RevertAid First Strand cDNA Synthesis Kit following the manufacturers protocol (Thermo Fisher Scientific). For quantitative RT-PCR TaqMan technology (Thermo Fisher Scientific) was used with oligonucleotides and probes for human PR3 (for primer 5'-TGTCACCGTGGTCACCTTCTT-3', rev primer 5'-CCCCAGATCACGAAGGAGTCTAT-3', 6-FAM 5'-TTGCACTTTTCGTCCTGCCTCCG-
human AAT (for primer 5’- TGGATTTGGTCAAGGAGCTT-3’, rev primer 5’-GTCCTCTTCTCGGTGTCCT3’-3’), human 18S (for primer 5’-ACATCCAAGGAAGGCAGCAG-3’; rev primer 5’-TTTTCGTCACTACCTCCCCG-3’; 6-Fam 5’-CGCGCAAATTACCCACTCCCGAC-3’); TaqMan Fast Universal PCR Master Mix and Fast SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific). Each sample was measured in duplicate and expression levels were normalized to 18S expression. Quantification was performed using an Applied Biosystems 7500 Sequence detector and data were analyzed using SDS 7500 software and ΔΔCt comparative analysis as described by Applied Biosystems (Applied Biosystems, Thermo Fisher Scientific).

Expression and purification of recombinant α1-antitrypsin species

Wt-(human M1 [V213]) and mut-AAT (A355D/I356P/P357D/M358S) were produced from plasmid pTT5 (National Research Council (NRC), Canada) which carried the reading frames of an Igk chain secretion signal and a C-terminal His tag. Introduction of the cDNA modification of mut-AAT was described previously.(33) In addition, an oxidant-resistant AAT variant was produced with amino acid substitutions replacing M351 with valine (V) and M358 with leucine (L). The AVL-AAT plasmid was a kind gift from Dr. Ron Crystal (Ithaca, NY, USA) and was previously described.(42) We amplified a c-terminal AVL-AAT part containing the oxidation-resistant methionine substitutions in the reactive center loop (M351V/M358L) using the forward primer- EcoRV 5’-CTAGACGATATCATCACCAAGTTCCTGGAAA-3’ and the reverse primer- AgeI 5’-TTACTAACCUGCCTTTTGCCTCGGATTGACGA-3’ and subcloned this c381 bp product into the aforementioned wt-AAT (human M1 [V213]) using the restriction enzymes EcoRI and AgeI. The pTT5 AAT plasmid with the substitutions [V213, V351, L358] was confirmed by sequencing. The recombinant AAT proteins were expressed
in human embryonic kidney (HEK) 293-6E cells (NRC, Canada) by transient transfection as described.(53) Briefly, cells were cultured in suspension in Freestyle 293 Expression Medium (Invitrogen) supplemented with 0.1% Pluronic F-68 (Gibco, Thermo Fisher Scientific), 25 μg/ml geneticin G-418 (Roche), and 0.5% Bacto TC Lactalbumin Hydrolysate (BD Biosciences) and purified from the culture supernatant by passage over a Ni-sepharose column (HisTrap HP, GE Healthcare).

Chemical and neutrophil-induced AAT oxidation

For chemical oxidation, 400 µl of 8 μM wt-, mut-, or VL-AAT were incubated with 1.6 mM N-chlorosuccinimidem (NCS) oxidant (Aldrich) in 0.1 M Tris, pH 8.0 for 20 min at RT. After 10 min at RT, the reaction was quenched by adding 9.6 mM L-methionine. Zeba-spin columns (Thermo Fisher Scientific) were used for buffer exchange to PBS. Protein integrity was checked by gel electrophoresis and Coomassie staining.

For neutrophil-induced AAT oxidation, 0.25 mM AAT was incubated in buffer containing 2x10^6 neutrophils. Neutrophils were then left untreated or were primed with 2 ng/ml TNFα prior to adding 75 μg/ml PR3- or MPO-ANCA IgG, respectively. After 30 min, the reaction was stopped, and samples were analyzed using parallel reaction monitoring to detect methionine oxidation.

Assessment of methionine oxidation using Parallel Reaction Monitoring

For sample preparation, AAT in vitro assay samples were resolved in lysis buffer (final concentration: 1% sodium deoxycholate, 10 mM dithiothreitol, 40 mM chloroacetamide, 1 mM EDTA (all Sigma-Aldrich), 150 mM NaCl, 50 mM Tris-HCl, pH 8) and heated for 10 min at 95°C. 100 ng Trypsin (Promega) and LysC (Wako, Osaka, Japan) were added to each sample and digested for 5 hours at 37°C. For human plasma samples, a 1:10 dilution (in water) was prepared and 10 µl were used for
digestion. 2x lysis buffer (2% sodium deoxycholate, 20 mM dithiothreitol, 80 mM chloroacetamide, 2 mM EDTA, 300 mM NaCl, 100 mM Tris-HCl, ph 8.0) was added to each sample, followed by incubation at 95°C for 10 min. Digest was performed with 1 µg Trypsin and LysC for 5 hours at 37°C. The digestion was stopped by acidifying each sample to pH < 2.5 with 10% formic acid. After centrifugation to pellet insoluble material (14,000 rpm, 10 min) the peptides were extracted and desalted using stage tip protocol.(54)

For Parallel Reaction Monitoring (PRM), peptides for targeted analyses were selected based on methionine containing sequence and on abundance and scores (unmodified AAT control peptides) in human plasma shot-gun proteome data. Charge state, retention time and optimal collision energy for each peptide was defined with AAT in vitro samples and retention time window was set to 20 min for analytical runs. Peptide samples were separated on a reversed-phase column (20 cm fritless silica microcolumns with an inner diameter of 75 µm, packed with ReproSil-Pur C18-AQ 1.9 µm resin (Dr. Maisch GmbH) using a 98 min gradient with a 250 nl/min flow rate of increasing Buffer B concentration (from 2% to 60%) on a High Performance Liquid Chromatography (HPLC) system (ThermoScientific) analyzed on a Q-Exactive HFx (Thermo Fisher Scientific). The PRM settings were: 30,000 resolution; 2e5 AGC target; 1.6 m/z isolation window; 60 ms maximum ion injection time. Data analyses was carried out using Skyline software package.(55) Peaks were manually selected based on retention time and dot product and the total intensity value of the correct peak area was extracted for peptide quantitation. The median intensity values of 7 unmodified AAT control peptides were used for normalization of the methionine containing peptide pairs (unmodified and methionine oxidized). Peptide used in PRM analyses provided as Supplemental Table 2.
SDS-PAGE and immunoblotting analysis

Immunoblotting was performed as described before(23) using mab to human PR3 (EPR6277, Abcam), mab rabbit anti-AAT (EPR9090-71, Abcam), polyclonal rabbit anti-actin (13E5, Cell Signaling Europe) together with corresponding secondary antibodies and visualized by enhanced chemiluminescence (Thermo Fisher Scientific).

Assessment of PR3-, NE-, and CatG-specific proteolytic activity by FRET

For assessing specific proteolytic activity for PR3, NE, and CatG 100 µl of cell-free supernatants (cf-SN) plus 50 µl HBSS buffer were incubated with 20 µM selective FRET substrates as described.(24) The selective FRET substrates for human NSPs were: PR3: 2-Abz-VAD-(nor)V-ADYQ-EDA-Dnp; NE: 2-Abz-APEEI MRRQ-EDADnp; and CatG: 2-Abz-EPFWEDQ-EDA-Dnp. Fluorescence was monitored over 45 min using a plate reader (excitation 320 nm, emission 420 nm, SpectraMax i3x, Molecular Devices). The proteolytic activity is reported as percent of control.

Measurement of superoxide release

Superoxide was measured using SOD-inhibitable ferricytochrome C reduction as described.(32) Briefly, 7.5x10⁶ neutrophils were pretreated with 5 ug/ml cytochalasin B for 15 min on ice, primed with 2 ng/ml TNFα and incubated with buffer, wt-AAT, mut-AAT, or VL-AAT as indicated. After 15 min, normal, PR3-, or MPO-ANCA IgG (each 75 µg/ml) or isotype control, mab to PR3 (clone 43), or a mab to MPO (Acris) each 5 µg/ml was added. Experiments were performed in 96-well plates at 37°C for up to 60 min, and sample absorption with and without 300 U/ml SOD was measured at 550 nm in a microplate reader (Molecular Devices).
Measuring mPR3 on the neutrophil membrane by flow cytometry

$1 \times 10^6$ neutrophils were incubated in 100 µl HBSS without or with wt-AAT, mut-AAT, VL- AAT, or in 100 µl diluted plasma from HC or AAV patients as indicated. When indicated samples were stimulated with 2 ng/ml TNF$\alpha$, a cytokine cocktail from 40 pg/ml TNF$\alpha$, 30 pg/ml IL-6, 65 pg/ml GM-CSF, or 5 µg/ml mabs to PR3 (clone 40) or MPO (Acris). Incubation was performed either in suspension conditions in tubes, on fibronectin (FN)-coated 24-well plates (Sarstedt), or on a confluent glomerular microvascular endothelial cell (gMVEC, Cell Systems) monolayer for 30 min at 37°C. Membrane-PR3 (mPR3) was assessed by flow cytometry using the following mabs to PR3: clone 43-8-3 and 81-3-3, clone WGM-2-FITC (Abcam), clone MCPR3-2 (Thermo Fisher Scientific) with corresponding FITC conjugated secondary anti-mouse IgG (Agilent). Double-staining for mPR3 and mCD177 was performed with Alexa647 conjugated mab anti-PR3 (clone 43-8-3) and Alexa488 labelled mab anti-CD177 (Biolegend). Additional surface proteins were stained with FITC-conjugated anti-CD11b, anti-CD18, or anti-CD66b mabs (all Beckmann Coulter). Cells were analyzed using a BD FACS Calibur or a BD FACS CANTO II (BD Biosciences) and FlowJo software (TreeStar).

Biotinylation of cell surface proteins

$5 \times 10^6$ neutrophils were stimulated with 2 ng/ml TNF$\alpha$ (30 min, 37°C) and incubated with cell impermeable EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) for 60 min on ice to biotinylate cell surface proteins. After incubation with 0.25 µM wt- or mut-AAT for 30 min, supernatants were collected and biotinylated proteins were pulled-
down with Dynabeads MyOne StrepAvidin T1 beads (Thermo Fisher Scientific), followed by electrophoresis and immunoblotting for PR3.

Assessment of plasma and neutrophil PR3 by ELISA

PR3 in human plasma and neutrophil lysates was assessed using a commercial PR3 ELISA (Elabscience Biotechnology Inc.) following manufactures instructions. Measurements detect both free and AAT-complexed PR3 (Supplemental Figure S5). The absorbance was determined at 450 nm in a plate reader (Molecular Devices). Neutrophil PR3 levels were normalized for µg neutrophil protein. The neutrophil PR3 pool was calculated from the PR3 amount per neutrophil multiplied with the number of circulating blood neutrophils in an estimated blood volume of 5 L. HC were imputed to have normal values of five neutrophils/nl. The plasma PR3 pool was calculated from plasma volume x PR3 plasma levels. Plasma volume was calculated using the following equations: \((23.7 \times \text{height (cm)} + 9.0 \times \text{bodyweight (kg)} - 1709) \times \frac{(100 - (\text{hematocrit} \times 0.91))}{57.23}\) for men and \((0.5 \times \text{height (cm)} + 8.4 \times \text{weight (kg)} - 4811) \times \frac{(100 - (\text{hematocrit} \times 0.91))}{61.78}\) for women.(56)

Assessment of plasma AAT by nephelometry

Plasma samples from HC and AAV patients were analyzed for AAT protein by nephelometry on an Atellica Neph 630 platform (Siemens).

Surface Plasmon Resonance

Experiments were performed on a Biacore™ T200 instrument (GE Healthcare) using standard amine chemistry for coupling CD177 to the sensor chip (CM5, Cytiva Lifesciences). For kinetic measurements 5 µg/ml CD177 in sodium acetate pH 4.5 was used with an immobilization level of approximately 770 counts. For wash-experiments
30 µg/ml CD177 in sodium acetate pH 4.5 was injected for 420 seconds, resulting in an immobilization level of around 5800 counts. PR3 and AAT dilutions as soluble analytes were prepared in 10 mM HEPES, 150 mM sodium chloride and 0.05% Tween20 (Sigma-Aldrich) running buffer. The flow rate was 30 µl/min and the assay temperature 25°C.

Statistics

Results are given as means. Comparisons were made using ANOVA with Tukey post-hoc analysis and Student’s-t test as indicated using GraphPad Prism8 software. Differences were considered significant at p<0.05 (*), or p<0.01 (**).

Study approval

The study was approved by the local ethic committee (Charité-Universitätsmedizin Berlin, Germany, EA4/025/18), and patients as well as healthy controls (HC) gave written, informed consent.
Author contributions

These authors contributed equally: Max Ebert and Uwe Jerke. M.E. collected and analyzed patient samples. U.J. performed and analyzed mechanistic studies. R.K. and A.D.S. conceived and designed the study. R.K., U.J., M.E., A.S., D.J., A.D.S., M.K., L.K., K.U.E. and P.M. planned and guided experiments. M.E. collected human samples. M.E., U.J., M.K. analyzed human samples and performed experiments. R.K., A.S., L.K., M.B., and S.E. cared for the patients. R.K. wrote and all authors revised the manuscript and approved the final version of the manuscript.

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References


Figure 1.

Healthy controls (HC) and anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) patients were recruited for assessment of PR3 and alpha1-antitrypsin (AAT).
Figure 2. PR3 and AAT are both increased in patients with active PR3- and MPO-AAV and normalize with remission. Plasma from healthy controls (HC), active (act) and remission (rem) PR3- and MPO-AAV patients were analyzed for (A) plasma PR3 (n=50, 37, 39, 22, 16 from left to right) and (B) plasma AAT (n=50, 36, 39, 22, 16 from left to right). The normal AAT concentration
between 16.2-30.2 µmol/L (28) is indicated. (C) The plasma PR3 to AAT molar ratio was calculated (n=50, 36, 39, 22, 16 from left to right). (D) Plasma PR3 correlation with plasma CRP in PR3- and MPO-AAV is illustrated. (E) Neutrophil PR3 was assessed in HC and active PR3- and MPO-AAV patients by ELISA (n=50, 37, 39, 22, 15 from left to right), and (F) by immunoblotting in randomly selected HC and PR3-AAV patients. Densitometry measurements of single PR3 bands normalized to β-actin loading control are indicated. (G) The total PR3 blood pool (n=50, 37, 39, 22, 15 from left to right) and (H) plasma AAT pool (n=50, 36, 39, 22, 16 from left to right) was calculated in HC and AAV patients. Neutrophils from HC and AAV patients were stained with an anti-PR3 mab and analyzed by flow cytometry. (I) A typical histogram illustrates the bimodal staining pattern with distinct mPR3 low and high neutrophil subsets. (J) The percentage of mPR3 high neutrophils is depicted (n=50, 36, 37, 20, 13 from left to right). (K) The mPR3 amount is given as expression index of the mean MFI (MFI E1, n=50, 36, 37, 20, 13 from left to right). (L) Spearman correlation between mPR3 amount and plasma AAT is illustrated. HC are depicted as red circles (r=-0.32, p=0.02), all PR3-AAV patients as black triangles (r=0.24, p=0.04) and all MPO-AAV patients as grey squares (r=0.23, p=0.21). Individual results are depicted, and the mean is indicated. One-way ANOVA was performed with Tukey post-hoc testing. * is p<0.05, ** is p<0.01.
Figure 3. AAT dose-dependently and reversibly reduces mPR3 on suspension and adherent neutrophils. (A) Wild-type (wt) and mutant (mut) AAT with substitutions in the reactive center loop was produced. (B) Wt- or mut-AAT was incubated with purified PR3 or cell-free supernatants (cf-SN) from ionophore A23187-activated neutrophils and assessed by immunoblotting using an anti-PR3.
mab. Wt- but not mut-AAT formed the typical 72-kDa AAT:PR3 complex. A typical of 3 experiments is shown. (C) Incubation of cf-SN from ionophore A23187-activated neutrophils with 0.25 µM wt- but not mut-AAT neutralized the proteolytical PR3 activity using a PR3-specific FRET substrate (n=5/group). (D) TNFα-priming increased mPR3 on isolated neutrophils as shown in a typical flow cytometry histogram. Incubation of 1x10^6 primed neutrophils with the indicated wt- but not mut-AAT concentrations for 30 min decreased mPR3 staining in a dose-dependent manner. A typical experiment together with the corresponding statistics is depicted (n=6/group). (E) Incubation of primed neutrophils with 0.25 µM wt-AAT did not decrease neutrophil surface staining for CD11b, CD18, and CD66b by flow cytometry (n=3/group). (F) Adding 0.25 µM wt- but not mut-AAT decreased mPR3 on primed neutrophils in suspension, adherent to fibronectin, and adherent to a glomerular microvascular endothelial cell monolayer, respectively (n=3/group). (G) Isolated neutrophils were incubated with 0.25 µM wt- or mut-AAT for 30 min (pre), followed by washing to remove AAT (wash). In the presence of newly added 0.25 µM mut- but not wt-AAT, mPR3 reoccurred on the surface of primed neutrophils over a 45 min time period (n=3/group). Effect of wt-AAT, serpinA3, and SLPI on (H) proteolytic activity of PR3, NE, and CatG using specific FRET substrates, and on (I) neutrophil mPR3 by flow cytometry. Individual results and the means ± s.e.m. are given. For statistical analysis, one-way ANOVA was performed with Tukey post-hoc testing. * is p<0.05, ** is p<0.01.
Figure 4. AAT reduces mPR3 on the neutrophil surface by competing with CD177 for PR3 binding, and subsequently ameliorates neutrophil activation by PR3- but not MPO-ANCA. (A) Isolated neutrophils were primed with TNFα, incubated with 0.25 µM wt- or mut-AAT for 30 min, and mPR3 was stained using 4 different anti-PR3 mAbs (n=3/group). By flow cytometry, all antibodies detected decreased mPR3 after neutrophil incubation with wt-AAT. Note that clone MCPR3-2 and WGM-2 recognize two different PR3 epitopes. (B) Surface proteins of isolated neutrophils were biotinylated prior to incubation with 0.25 µM wt- or mut-AAT. Supernatants were collected after 30 min and biotinylated proteins were pulled-down with streptavidin followed by electrophoresis and immunoblotting with an anti-PR3 mAb. A typical immunoblot and the corresponding statistics of three independent experiments are given. (C) Primed isolated neutrophils were incubated with 0.25 µM wt- or mut-AAT for 30 min followed by double-staining with mAbs to PR3 and CD177. A typical flow
continued Figure 4.

cytometry experiment together with the corresponding statistics, separately for the distinct CD177\textsuperscript{neg} and CD177\textsuperscript{pos} subsets, is given (n=9/group). (D) The experimental set-up together with the corresponding surface plasmon resonance sensorgrams are depicted. CD177 was immobilized to the sensor chip and the soluble analytes are indicated. High affinity CD177 complex formation is observed with PR3, but not with wt- or mut-AAT (left panel). Preincubating PR3 with wt- but not mut-AAT prevented PR3 from binding to immobilized CD177 (middle panel). Applying wt-AAT after the CD177:PR3 complex had formed, displaced PR3 from CD177 (right panel). Individual results and the mean ± s.e.m. are given. **A, B** One-way ANOVA and **C** repeated measures one-way ANOVA was performed with Tukey post-hoc testing. * is p<0.05, ** is p<0.01.
Figure 5. Disease-related modifiers of the mPR3-lowering AAT effect in the plasma of PR3-AAV patients. (A) Neutrophils were isolated from HC, active and remission PR3-AAV patients (n=5/group). Primed neutrophils were incubated with buffer or 0.25 µM wt- or mut-AAT for 30 min. By flow cytometry, wt-AAT significantly reduced mPR3 on neutrophils of all groups. (B) Primed neutrophils were incubated with 0.25 µM wt- or mut-AAT for 30 min prior to the stimulation with human ANCA-IgG. Superoxide release was measured using the ferricytochrome C reduction assay (n=4 independent experiments, each using two different ANCA preparations). (C) Plasma from HC and AAV patients, selected for similar AAT concentrations, was diluted to achieve AAT of 0.25 µM and showed similar AAT band optical densities in AAT immunoblots. (D) HC neutrophils were incubated for 30 min in buffer or diluted plasma from HC or patient neutrophils as indicated. mPR3 was assessed by flow cytometry. The mPR3 reduction by plasma was significantly less with plasma from 8 different active PR3-AAV patients (n=4 independent experiments) (E) HC neutrophils were incubated in HC plasma as in c, or in HC plasma supplemented with a cytokine cocktail and mabs to PR3 or MPO as indicated (n=6/group). Individual results and the mean ± s.e.m. are given. For statistical analysis the following test were performed, A, D One-way ANOVA and B, E Repeated measures one-way ANOVA within each group was performed with Tukey post-hoc testing respectively. * is p<0.05, ** is p<0.01.
Figure 6. AAT methionine oxidation at position M351 and M358 is increased in plasma of active PR3-AAV patients and in the presence of ANCA-activated neutrophils in vitro. (A) The AAT structure is depicted to illustrate the surface-exposed methionine (M) at position 351 and 358 in the reactive center loop (in red), and the non-exposed M385. The structure is colored according to its secondary structure elements (helix, light magenta; β strands, light blue; loops, gray). Structural figure based on Protein Data Bank (PDB) entry 1ATU and were prepared with PyMOL (Schrödinger) software. (B) Plasma from HC, active and remission PR3-AAV patients (n=9/group) was analyzed for AAT site-specific methionine oxidation by Parallel Reaction Monitoring (PRM). M351/M358 double-oxidation was significantly increased in plasma AAT from active PR3-AAV patients, whereas M385 oxidization was not changed. Scatter plot (top) shows AAT protein normalized log2 intensities of oxidized peptides.
continued Figure 6.

For each group individual HC and patient data points are shown as circles, and the mean is indicated as a red line. The heatmap (bottom) displays the median normalization scores of the intensity data.

(C) M351/M358 double-oxidation of plasma AAT correlated with the inflammation marker CRP in plasma from PR3-AAV patients (n=18). (D) purified wt-AAT was exposed to resting HC neutrophils, or TNFα-primed neutrophils stimulated with PR3- or MPO-ANCA IgG, respectively. After 30 min the reaction was stopped, and AAT was analyzed for methionine oxidation by PRM (n=3 independent experiments/group). AAT exposure to ANCA-activated neutrophils resulted in strong increase in M351/M358 double-oxidation, whereas M385 was not significantly affected. Intensities (log2) of oxidized peptides are normalized to AAT protein abundance and shown as individual data points for each treatment in the scatter plot with the red line indicating the mean value per group (top) and as heat maps of median normalization scores (bottom). For B and D One-way ANOVA was performed with Tukey post-hoc testing respectively. * is p<0.05, ** is p<0.01.
Figure 7. Methionine oxidation at position M351 and M358 abolishes AAT binding to PR3 and AAT-mediated protection from PR3-ANCA mediated neutrophil activation. (A) wt-AAT was exposed to the oxidant N-chlorosuccinimide (NCS). NCS-treated wt-AAT (ox-AAT) showed strongly reduced competition with CD177 for PR3 binding by SPR. At 0.25 µM, (B) oxidized wt- and EMA-approved Respreeza™ (Resp)-AAT caused no mPR3 reduction on neutrophils by flow cytometry (n=3/group), and (C) oxidized wt-AAT less PR3-ANCA induced neutrophil activation by superoxide release (n=3 independent experiments, using four different ANCA preparations). (D-F) AAT with valine and leucine substitutions for M351 and M358 (VL-AAT) was produced. At 0.25 µM oxidation-resistant VL-AAT (VL) exposed to NCS reduced (D) PR3 FRET activity (n=5/group), (E) mPR3 on primed neutrophils (n=5/group), and (F) PR3-ANCA induced superoxide release (n=4 independent experiments, each using two different ANCA preparations). Individual results and the mean ± s.e.m. are given. For statistical analysis the following test were performed B, D, E one-way ANOVA and C, F repeated measures one-way ANOVA within each group was performed with Tukey post-hoc testing respectively. * is p<0.05, ** is p<0.01.
**Figure 8.** Antiproteinase-related AAT effects are protective in PR3- but not MPO-AAV.

(1) Binding of PR3- (right side) and MPO-ANCA (left side) to their target antigens on the cell membrane activates neutrophils. (2) AAT decreases mPR3 and thereby protecting from neutrophil activation by PR3- but not MPO-ANCA. (3) Oxidative AAT modification compromises the protective anti-proteinase AAT effect in PR3-ANCA vasculitis.
Table 1

Table 1. Correlation of the total PR3 blood pool, plasma PR3 pool and neutrophil PR3 pool with prespecified clinical parameters

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Pearson correlation coefficient r is given and p-values (for each pool) were adjusted for multiple correlations using the Bonferroni correction.