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Increased levels of neutrophil extracellular trap remnants in the circulation of patients with small vessel vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic antibodies during remission

Running title: NET remnants in ANCA-associated vasculitis

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Abstract

Objectives:

Neutrophil extracellular traps (NETs) have been visualized at the site of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) lesions. Increased levels of NET remnants in the circulation have been reported in some AAV patients with active disease. The aim of the present study was to analyze NET remnants in a larger cohort of AAV patients with different degrees of disease activity and to elucidate possible factors responsible for remnant variation.

Methods:

Levels of NET remnants in the circulation of healthy controls (HCs) ($n=31$) and AAV patients ($n=93$) were determined with ELISA. NET remnants were then correlated to ANCA levels, spontaneous and induced cell death (NETosis/necrosis) in vitro, neutrophil count and corticosteroid therapy.

Results:

Patients with active disease showed higher levels of circulating NET remnants compared to patients in remission ($P=0.026$) and HCs ($P=0.006$). From patients sampled both at remission and active disease we found increased levels during active disease ($P=0.0010$). In remission, ANCA-negative patients had higher levels of NET remnants than ANCA-positive patients and a negative correlation was observed between NET remnants and PR3-ANCA ($r_s=-0.287$, $P=0.048$). NET remnants correlated with neutrophil count in HCs ($r_s=0.503$, $P=0.014$) but not in patients during remission. Neutrophils from patients showed an enhanced spontaneous cell death ($P=0.043$).

Conclusions: We found increased levels of circulating NET remnants in patients with active AAV. Furthermore, AAV patients exhibited an increased propensity for spontaneous cell death. NET remnant levels seem to be positively related to disease activity and neutrophil count, but inversely related to ANCA at least during remission.
Key words: Small vessel vasculitis, ANCA-associated vasculitis, neutrophils extracellular trap (NET), NET remnants, ANCA
Introduction

Vasculitides are characterized by vessel wall inflammation as the defining feature and they can affect any organ system in the body. A subgroup of vasculitides, characterized by involvement of small vessels, neutrophil-rich necrotizing inflammation and presence of anti-neutrophil cytoplasmic antibodies (ANCAs) is referred to as ANCA-associated vasculitis (AAV) (1). AAV comprises granulomatosis with polyangiitis (GPA, formerly Wegener’s granulomatosis), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome).

In AAV, ANCA are directed against one of two enzymes found in the azurophilic granules of neutrophils, proteinase 3 (PR3) or myeloperoxidase (MPO).

Dying neutrophils surrounding the walls of small vessels is a histological hallmark of AAV. Traditionally it has been assumed that these neutrophils die by necrosis, but recently neutrophil extracellular traps (NETs) have been visualized at the site of vasculitic lesions (2). NETs were described for the first time in 2004 by Brinkmann and colleagues (3) and are released as a result of a programmed cell death mechanism called NETosis. NETs can be referred to as a tangled web of chromatin (histones and DNA) decorated with about 20 different proteins (4, 5). Several of these proteins, such as histones, MPO, PR3, HMGB1 and LL37, possess proinflammatory characteristics and NETs have in vitro been shown to induce interferon (IFN)-α production in dendritic cells (5), cause epithelial and endothelial cell damage (6-8) and act as bridge between the innate and adaptive immune system (9). NETs trap and kill microbes, including bacteria, fungi and protozoa (3, 10-14) but have also been connected to various autoinflammatory or autoimmune diseases, such as preeclampsia, systemic lupus erythematosus (SLE), Felty’s syndrome (FS) and rheumatoid arthritis (RA) (15-19). NETs have also been suggested to be important in a rodent model of drug-induced vasculitis (20), implying a role of NETs in the pathogenesis of AAV as well. Further, increased levels of NETosis-derived products (or NET remnants) in the circulation have been reported in a small number of patients with active vasculitis (2). These NET remnants were defined as complexes of
nucleosomes and MPO. A nucleosome is composed of DNA wrapped around a core of eight histone proteins (two of each of the histones H2A, H2B, H3 and H4), and constitutes the repeating structural units of chromatin (21). As chromatin constitutes the backbone of NETs, and MPO is present in the NETs, we adopted the definition of NET remnants as circulating nucleosome/MPO complexes in this study.

The aim of the present study was to analyze such NET remnants in a larger cohort of AAV patients with different degrees of disease activity and to elucidate possible factors responsible for variations in circulating remnants. Levels of NET remnants were correlated to ANCA levels, spontaneous and induced cell death (NETosis/necrosis) in vitro, neutrophil count and corticosteroid therapy.

Material and methods

Study population

Peripheral whole blood was drawn from 93 AAV patients and 34 healthy controls (HCs) into EDTA-treated tubes (Terumo Europe N.V., Leuven, Belgium) (Table 1). Eleven of the patients were sampled both during active disease and during remission. All samples were collected after obtaining informed consent and the study was approved by the Regional Ethical Review Board in Linköping.

Collection of plasma samples and isolation of PMNs through Percoll density gradient centrifugation

The blood was centrifuged at 1500xg for 15 minutes. Plasma samples were stored at -70°C for subsequent measurements of NET remnants and ANCA levels. PMNs were isolated from a subgroup of the participants, by a modified version of a method already described (22). The buffy coat was removed and layered on top of a discontinuous Percoll (GE Healthcare, Uppsala, Sweden) gradient with 63% and 72% percoll. PMNs were separated from PBMCs and red blood cells by centrifugation at 490xg for 25 minutes at room temperature (RT). The interphase containing PMNs was collected.
and washed once in PBS by centrifugation at 300xg for 5 minutes. Remaining red blood cells were lysed through brief hypotonic lysis with double-distilled water at 4°C and the cells were then centrifuged at 300xg for 5 minutes at 4°C. The hypotonic lysis and the subsequent washing step were repeated once. The PMNs were then resuspended in RPMI 1640 medium (Gibco, Paisley, UK) containing 0.5% fetal bovine serum (FBS, PAA Laboratories GmbH, Köln, Germany) and kept on ice until use. Viability of the cells was confirmed by trypan blue dye exclusion (viability ≥95%).

**NET remnant ELISA**

A 96 well Nunc MaxiSorp immunoplate was coated with a monoclonal mouse anti-nucleosome antibody (B6.SLE-1, 0.5 µg/ml) at 4°C over night. Blocking solution was added for 30 minutes at RT before incubation with standards (plasmapheresis sample from a specific patient) and plasma samples for 1.5 h at RT. Standards and samples were run in duplicates. As standard curve, a 7 point dilution series with 2-fold dilutions of the standard, ranging from 1000 to 15.6 arbitrary units (a.u.), was used. The plate was then incubated with first a rabbit anti-human MPO antibody (1:500, DAKO, Carpinteria, CA) for 1 h at RT and then with an alkaline phosphatase-conjugated swine anti-rabbit antibody (1:500, DAKO) for another hour at RT. A substrate for alkaline phosphatase (4-Nitrophenyl phosphate disodium salt hexahydrate, Sigma-Aldrich) was then added and the plate was read at 405 nm using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). Logarithmic (log) transformation (X=Log(X)) was applied to the standard curve and all samples were interpolated from the standard curve using the sigmoidal dose-response (variable slope) equation (Supplementary Fig. 1).

**MPO- and PR3-ANCA ELISA**

PR3- and MPO-ANCA titers in plasma samples from AAV patients in remission were determined with the Wieslab Capture PR3-ANCA test kit (cut off values; neg<4 IU/ml, borderline 4-6 IU/ml and positive >6 IU/ml) and Wieslab MPO-ANCA test kit (cut off values; negative <6 IU/ml, borderline 6-8
IU/ml and positive >8 IU/ml, respectively, according to the manufacturer’s protocols (EuroDiagnostica, Malmö, Sweden).

*In vitro* cell death assay for quantification of NETosis/necrosis

5x10^4 PMNs were seeded onto a 0.001% poly-L-lysine (Sigma-Aldrich)-coated 96-well plate (Sarstedt, Nümbrecht, Germany) and allowed to settle for 1 h at 37°C in 5% CO₂. The cells were then stimulated with 2 ng/ml or 8 ng/ml tumor necrosis factor (TNF)-α (Sigma-Aldrich), 10 nM phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich), 1% TritonX-100 (Sigma-Aldrich) or left unstimulated for 4h at 37°C in 5% CO₂. DNA was measured hourly with the cell-impermeable dye Sytox Green (2.5 µg/mL, Molecular Probes, Inc., Eugene, OR, USA) at 523 nm using an FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT, USA).

Visualization of NETs

4x10^5 PMNs were seeded onto 0.001% poly-L-lysine (Sigma-Aldrich) coated coverslips (Harvard Apparatus, Holliston, MA, USA) and allowed to settle for 1 h at 37°C in 5% CO₂. The cells were then stimulated with 10 nM PMA or left unstimulated for 4 h at 37°C in 5% CO₂ before fixation with 4% formaldehyde solution (Sigma-Aldrich) for 30 minutes at RT. All the following incubations were also conducted at RT. After fixation, the coverslips were washed with PBS and then incubated in blocking buffer (PBS + 2% BSA) for 30 minutes. Thereafter, a goat anti-human elastase antibody was added (1:50, Merck Millipore, Billerica, MA, USA) and allowed to bind for 1 h. The coverslips were then washed again before incubation with a rabbit anti-goat antibody conjugated with Alexa 488 (1:2000, Molecular Probes) for another hour. After another washing step, the coverslips were mounted onto a slide with a small drop of ProLong Gold anti-fade Mounting Media containing DAPI (Molecular Probes). The coverslips were left for 24 h to cure before examination in a Carl Zeiss Axiovert 200M microscopy (Carl Zeiss Microlmaging GmbH, Göttingen, Germany).
Statistics

Data were analyzed using GraphPad Prism, version 6.02 (GraphPad Software, Inc., San Diego, CA, USA) or SPSS statistical software package (version 22.0; IBM, Chicago, IL, USA). Data that passed D’Agostino and Pearson omnibus normality test was considered to have a Gaussian distribution. The data obtained from the measurement of NET remnants possessed a log-normal distribution and we therefore log-transformed the data before further analyses. The results from the analyses with NET remnants are presented as back-transformed data to the original scale. When comparing the level of NET remnants between more than two groups with independent observations, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test as post test was used, as the data had a Gaussian distribution. Kruskal-Wallis test followed by Dunn’s multiple comparison test was used for the comparison of neutrophil count between more than two groups with independent observations, as the data had a non-Gaussian distribution. Unpaired t test was used in all comparisons between two groups with independent observations, except in the analysis comparing the levels of NET remnants in active patients with different organ involvement (in which Mann-Whitney U test was used as there were only 6 patients in one of the groups), as these data followed a Gaussian distribution. The matched observation analysis was performed with Wilcoxon matched-pairs signed rank test as the data had a non-Gaussian distribution. For correlations analyses, Spearman’s rank correlation coefficient was calculated. Data with a Gaussian distribution is presented as mean (95% CI) or, when data was back transformed from logarithmic scale, geometric mean and 95% CI, whereas data with a non-Gaussian distribution is presented as median and interquartile range (IQR). P-values <0.05 were considered statistically significant in all analyses.

Results
Patients with active AAV have increased levels of NET remnants in the circulation.

Patients with active AAV (n=24) showed higher levels of circulating NET remnants compared to both patients in remission (n=69) and HCs (n=31) (geometric mean, active 561 a.u. (95% CI: 437-729 a.u.) vs remission 424 a.u. (95% CI: 382-470 a.u.), P=0.026, vs HCs 378 a.u. (95% CI: 324-446 a.u.), P=0.006) (Fig. 1A). The difference between patients in remission and HCs was not significant. For 11 patients, samples taken during active disease were compared to samples taken when the disease was in remission (these 11 remission samples were not included in the 69 remission samples above). In 4 cases the remission sample was taken before a relapse, and in 7 cases the active sample preceded the remission sample. In all cases NET remnants were higher in the active samples (geometric mean 493 a.u. (95% CI: 339-735 a.u.)) compared to the remission sample (259 a.u. (95% CI: 204-331 a.u.), P=0.001) (Fig. 1B). There was no significant correlation between BVAS (r_s=0.029, P=0.893) or CRP (r_s=0.312, P=0.138) and NET remnants in patients with active disease (n=24), but when comparing the levels of NET remnants in those with disease limited to the ENT-area and/or nervous system (n=6) (median 334 a.u.) to those with a more widely distributed disease (n=18) (median 649 a.u.) there was a significant difference (P=0.0383) (Supplementary Table 1).

Relation between ANCA and circulating NET remnants

At the time of sampling, 46 patients in remission were ANCA-positive. Based on the specificity at diagnosis, 33 of 48 PR3-ANCA patients were positive and 13 of 27 MPO-ANCA patients were positive (Table 1). For 2 patients diagnosed with MPO-ANCA and for 1 patient diagnosed with PR3-ANCA, the ELISA at sampling yielded a borderline result and their results were excluded from the comparison between ANCA-negative and ANCA-positive samples. As shown in Fig. 2A, ANCA negative patients at sampling (n=26) showed higher levels of NET remnants in the circulation (geometric mean 474 a.u. (95% CI: 401-564 a.u.) compared to ANCA positive patients at sampling (n=46) (366 a.u. (95% CI: 321-421 a.u.), P=0.0206). This finding was statistically significant in patients who were PR3-ANCA positive at diagnosis (Fig. 2B), but could not be seen in the MPO-ANCA subset (Fig. 2C). A negative
correlation could be seen between the levels of NET remnants and the levels of PR3-ANCA ($r_s=-0.287$, $P=0.048$) (Fig. 2D) whereas no significant correlation could be observed between NET remnants and MPO-ANCA ($r_s=-0.078$, $P=0.701$) (Fig. 2E). In active disease there was a negative correlation between NET remnants and MPO-ANCA ($n=8$) ($r_s=-0.77$, $P=0.0295$) but not between NET remnants and PR3-ANCA ($n=16$) ($r_s=0.007$, $P=0.9803$) (Supplementary Table 1).

**AAV patients show increased spontaneous NETosis/necrosis**

To further determine possible sources explaining the differences in circulating NET remnants we measured *in vitro* cell death. As described in the methods section we used a 96-well plate Sytox Green assay, and measured both the spontaneous cell death, death induced by TNF-α and by PMA. Unstimulated neutrophils from AAV patients ($n=23$) showed an increased cell death after 4 h of incubation (mean 5.38% (95% CI: 4.14-6.62%)) compared to neutrophils from HCs ($n=17$) (mean 3.90% (95% CI: 3.10-4.70%), $P=0.043$) (Fig. 3A). This was found also when the analysis was limited to patients in stable remission, removing 5 patients with active disease (open triangles, Fig. 3A). The results are expressed as percentage of total DNA (lysis with 1% Triton X-100) and reflect the cells dying during the 4 h. To elucidate the mechanism accounting for the spontaneous cell death, dying cells were visualized using fluorescence microscopy. As shown in Fig. 4A-F, NETs could be seen in the untreated samples from both AAV patients and healthy controls. The spontaneously formed NETs in both HCs and AAV patients had similar appearance as those induced by PMA (Fig 4G-I). Stimulation of the neutrophils with a low (2 ng/ml; Fig. 3B) or high (8 ng/ml; Fig. 3C) dose of TNF-α attenuated the difference in cell death (mean AAV patients 6.56% (95% CI: 5.06-8.05%) vs HCs 5.28% (95% CI: 3.74-5.81%), $P=0.217$, and mean AAV patients 6.81% (95% CI: 5.01-8.60%) vs HCs 5.58 (95% CI: 3.35-7.81%), $P=0.370$, respectively). A moderate dose of PMA (10 nM) induced massive cell death after 4 h, with no statistical difference between AAV patients and HCs (mean 89% (95% CI: 84-94%) vs 93% (95% CI: 83-102%), respectively, $P=0.461$) (Fig. 3D). Patients with high levels of spontaneous cell
death tended to have high levels of circulating NET remnants but overall the correlation was not statistically significant ($r_s=0.2727, P=0.2080$).

**Neutrophil count and NET remnants in the circulation**

Both AAV patients with active disease ($n=18$) and patients in remission ($n=66$) showed a higher neutrophil count than HCs ($n=23$) (median active $5.5\times10^9$/liter (IQR 4.1-7.8$\times10^9$/l) and remission $5.2\times10^9$/l (IQR 3.8-7.0$\times10^9$/l) vs HCs $3.3\times10^9$/l (IQR 3.1-4.2$\times10^9$/l) ($p<0.001$). In this analysis, remission samples from patients that also had samples taken during active disease were not included. There was a significant correlation between neutrophil count and the levels of NET remnants in HCs ($r_s=0.503, P=0.014$) and a similar correlation was also observed in patients with active disease ($r_s=0.468, P=0.050$). No significant correlation between these parameters could be seen for patients in remission ($r_s=0.182, P=0.118$).

**Corticosteroid therapy, NET remnants and spontaneous cell death**

At the day of sampling, 51 of 80 patients in remission were on oral corticosteroid therapy (mainly prednisolone). No statistical difference in the levels of NET remnants was observed between treated ($n=51$) and untreated ($n=28$) patients in remission (geometric mean 412 a.u. (95% CI: 363-468 a.u.) vs 366 a.u (95% CI: 307-437 a.u.), respectively, $P=0.256$). In a similar fashion there was no significant difference between patients sampled before onset of induction therapy for active disease ($n=16$) and patients sampled after ($n=8$) (median before 605 a.u. vs after 342 a.u., $P=0.233$) (Supplementary Table 1).

Among those patients in remission examined in the spontaneous cell death assay ($n=17$), 10 were treated with corticosteroids. Treated patients exhibited similar propensity as the untreated patients ($n=7$) to undergo NETosis/necrosis (mean 5.95% (95% CI: 4.00-7.90%) vs 5.50% (95% CI: 2.40-8.60%), respectively, $P=0.776$).
Discussion

In this study we show that the levels of NET remnants are increased in the circulation of AAV patients with active disease. In patients sampled both at remission and active disease we found increased levels when the disease was active. This confirms and extends the findings previously published by Kessenbrock et al. (2), supporting a role of NETs in AAV. NETosis has been described also in SLE (15) and rheumatoid arthritis (19), but we could not observe a significant correlation with CRP in active disease, suggesting that NET remnants and CRP at least partly indicate activity in different pathogenic pathways. Interestingly, in a recent study measuring calprotectin (present in NETs), increased levels of calprotectin in the circulation during both active disease and remission could be seen (23).

We adopted the definition of NET remnants as complexes of nucleosomes and MPO, as suggested by Kessenbrock et al. The mixing of the nuclear components with the content of azurophilic granulae is a critical step in NETosis, and provides the basis for the formation of complexes between nucleosomes and MPO. NETs are disintegrated and partly degraded by DNases (24), which most likely is the reason for the release of MPO/nucleosome complexes into the circulation. When MPO is released into the circulation by exocytosis, there is a strong tendency to form complexes with ceruloplasmin (25, 26), indicating that MPO not normally form complexes with nucleosomes.

When analyzing factors possibly contributing to the variation of NET remnants in remission we found lower levels in ANCA positive patients along with an inverse correlation with circulating levels of PR3-ANCA. This result is somewhat counterintuitive as isolated IgG from AAV patients can induce NETosis in vitro (2). NETosis is dependent on oxidative burst, i.e. production of reactive oxygen species (ROS) (27), and ANCA-induced ROS is considered to be an essential mechanism in the pathogenesis of AAV (28). However, there are differences in epitope specificity between patients (29, 30), and ANCAs in remission may be less pathogenic (26) and consequently induce less NET
formation. A possible explanation for the inverse relationship between PR3-ANCA and NET remnants during remission is that ANCA through the formation of immune complexes enhance the clearance of the remnants. We have previously reported on such an inverse relationship between ANCA and PR3/alpha-1-antitrypsin complexes in the circulation (31). The number of anti-MPO positive patients during remission was low (especially at higher levels), which makes the analysis of the relationship between MPO-ANCA and NET remnants difficult to interpret. Interestingly there was a negative correlation between NET remnants and MPO-ANCA in patients with active disease, even though treatment and disease extension are factors that most probably blur the correlation in active patients.

We have previously reported on natural autoantibodies (NAAs) with specificity for MPO and PR3 (32). As clearance of apoptotic cells and cellular debris by natural antibodies is thought to be important for maintaining homeostasis (33, 34), our present findings imply a physiological role for at least some antibodies with ANCA specificity. A recently published study shows that anti-histone antibodies (histone is another major NET component) could ameliorate experimental glomerulonephritis (35). A hypothetical model for the dual role of ANCA with respect to circulating NET remnants is depicted in Figure 5.

In this study we describe that neutrophils from AAV patients show enhanced spontaneous NETosis/necrosis in vitro. Whether this is a primary event and related to pathogenesis in AAV cannot be discerned from the present study. We have previously shown that neutrophils from AAV patients are less prone to undergo natural cell death, apoptosis (36), suggesting that these neutrophils possibly are more prone to undergo other forms of cell death. TNF-α treatment attenuated the difference in spontaneous cell death between HCs and AAV patients, mainly by increasing the cell death in HCs, suggesting that circulating cytokines in the patients may have induced the difference in the first place. AAV patients have been shown to have increased levels of several cytokines (37-39),
Although only certain cytokines yet have been shown to induce NETosis (including TNF-α) (10, 16, 40).

We found circulating NET remnants to be strongly correlated to neutrophil count in healthy controls, while the correlation was less obvious in AAV patients. A reason for this could be that other factors such as the propensity for NETosis and altered clearance disturbs the correlation between neutrophil count and circulating remnants in patients.

Corticosteroids have profound effects on neutrophil counts and neutrophil biology (41). We have recently shown that also small doses given during remission in AAV give strong effects on intracellular levels of transcription factors and miRNAs (42). Subsequently it was important to assure that the major findings in the present study were not caused by the therapy, and our data show that ongoing low-dose prednisolone treatment was neither related to circulating NET remnants nor the spontaneous cell death in vitro. Members in our group have also reported that ROS production in neutrophils are not affected by corticosteroid therapy (43).

In conclusion we found increased levels of circulating NET remnants in patients with active AAV. Furthermore, AAV patients exhibited an increased propensity for spontaneous cell death. NET remnant levels seem to be positively related to disease activity and neutrophil count, but inversely related to ANCA at least during remission. The latter suggests that more NETosis is taking place than indicated by the present assay for NET remnants, emphasizing a complexity between production and clearance of NETs. As NETs possess proinflammatory properties, our findings suggest a potentially beneficial role for some of the antibodies with ANCA specificity. The many factors affecting the levels of NET remnants probably reduce the utility of NET remnants as biomarkers, but this study constitutes a foundation for future larger studies with sequential sampling and long-time follow-up to evaluate such a capacity.
Key messages

NET remnants are increased in the circulation of patients with active AAV.

NET remnants seem to be negatively related to ANCA in AAV at least during remission.

Spontaneous NETosis/necrosis in vitro is increased in AAV patients.

Acknowledgement

The anti-nucleosome antibody B6.SLE-1 was a kind gift from Dr. Christopher Sjöwall at Linköping University. We also like to thank Marianne Petersson, Lotta Martinsson and Ingrid Göransson at Linköping University Hospital for their help during blood sample collection.

Conflict of interest

No conflicts of interest.

Funding

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References

**Table 1: Study population**

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\(^{a}\)Eleven patients were sampled both during active disease and remission. HCs, healthy controls; AAV, ANCA-associated vasculitis; IQR, interquartile range; PR3, proteinase 3; MPO, myeloperoxidase; WBC, white blood cell count; na, not available.
Figure 1. Circulating NET remnants. (A) Levels of NET remnants in plasma from 31 HCs, 69 AAV patients in remission and 24 AAV patients with active disease. Patients with active disease showed increased levels of NET remnants compared to patients in remission and HCs. (B) For 11 patients, samples taken during remission were compared to samples taken when the disease was active (these 11 remission samples were not included in the 69 remission samples in A). Also here, NET remnants were increased during active disease. Bars indicate geometric mean and 95% CI. *$P<0.05$, **$P<0.01$. 
Figure 2. Circulating NET remnants and ANCA levels. AAV patients in remission who at sampling were ANCA-negative (n=26) had higher levels of NET remnants in the circulation compared to ANCA-positive patients (n=46; A). This difference was mainly due to the patients with PR3-ANCA specificity at diagnosis (n=33 positive/14 negative; B), and not to the MPO-ANCA group (n=13/12; C). A significant negative correlation was observed between NET remnants and PR3-ANCA levels (D) but not for MPO-ANCA levels (E). Bars indicate geometric mean and 95% CI. *P<0.05.
Figure 3. *In vitro* neutrophil cell death (NETosis or necrosis). AAV patients exhibited an increased spontaneous cell death (A), which was still significant after removal of five patients with active disease at sampling (open triangles). The results are expressed as percentage of total DNA (lysis with 1% Triton X-100) and reflect the increase in cell death during 4 hours. After stimulation with a low (2ng/ml; B) or a high (8ng/ml; C) dose of TNF-α or a moderate dose of PMA (10nM; D) no significant differences between patients and controls were found. Bars indicate mean value and 95% CI. *P*<0.05.
Figure 4. Visualization of NETs. After incubation of neutrophils for 4h at 37°C with or without 10 mM PMA, NETs were visualized by immunofluorescence microscopy using a 100x objective. DNA, the backbone of NETs, was labeled with DAPI (blue), and elastase, a granule protein within the NETS, was labeled with an Alexa Fluor 488-conjugated antibody (green). Neutrophils from both patients (4A-C) and HCs (4D-F) spontaneously produced NETs, resembling PMA-induced NETs (4G-I). Double labeled NETs are indicated by arrows in the merged images (C, F and I). This figure is not intended for quantification of NET formation; rather, the images shown were selected to demonstrate that neutrophils are capable of producing NETs spontaneously.
Figure 5. A hypothetical model for a dual role of ANCA on NET remnants. NETs are degraded by DNases (23) into smaller breakdown products (NET remnants), containing nucleosomes, HMGB1, ANCA-antigens and other proinflammatory molecules (2, 4, 5) (A). The remnants facilitate their own clearance by opsonisation through the induction of “natural” autoantibodies of which some have ANCA specificity (B). Under unfavorable circumstances, some ANCA (red) reacting with PR3 and MPO on the surface of neutrophils are produced (C), leading to ROS production, more NETs and vasculitic lesions (2, 40) (D). Infections (14), drugs (20) and possibly epigenetic events (41) trigger such events by increasing NET formation (E). B, B cell; Th, T helper cell; DC, dendritic cell.
Supplemental Table: Characteristics of AAV patients with active disease

<table>
<thead>
<tr>
<th>Patient ID</th>
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<th>NET remnants (a.u.)</th>
<th>Sample at Diagnosis/Relapse</th>
<th>ANCA Subtype/level (IU/ml)</th>
<th>Prednisolone (mg/day)</th>
<th>Days from start of induction therapy</th>
<th>BVAS</th>
<th>CRP</th>
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G=general; E=Ear, Nose and Throat; L=lung; K=kidney; N=nervous system; M=mucocutaneous; S=skin; NET=neutrophil extracellular trap; PR3=proteinase 3; MPO=myeloperoxidase; BVAS=Birmingham vasculitis activity score; CRP=C-reactive protein
Supplemental Figure 1. NET remnant ELISA standard curve. For levels of NET remnant quantitative
detection, a standard curve consisting of a 7 point dilution series with 2-fold dilutions, ranging from
1000 to 15.6 arbitrary units (a.u.), of the standard (plasma sample obtained from an plasma
exchange performed on a patient with active AAV) was used. Logarithmic (log) transformation
(X=Log(X)) was applied to the standard curve and the samples were interpolated from the standard
curve using the sigmoidal dose-response (variable slope) equation. The figure shows three replicates
of the standard curve to demonstrate the consistency of the assay. A standard curve was applied to
each plate when measuring NET remnants.