EXTENDED REPORT

Tissue factor expression in neutrophil extracellular traps and neutrophil derived microparticles in antineutrophil cytoplasmic antibody associated vasculitis may promote thromboinflammation and the thrombophilic state associated with the disease

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ABSTRACT

Objectives Antineutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is characterised by neutrophil activation. An elevated prevalence of venous thromboembolic events has been reported in AAV. Because of the critical role of neutrophils in inflammation associated thrombosis, we asked whether neutrophil tissue factor (TF) may be implicated in the thrombotic diathesis in AAV.

Methods Neutrophils from four patients and sera from 17 patients with ANCA associated vasculitis with active disease and remission were studied. TF expression was assessed by immunoblotting and confocal microscopy. Circulating DNA levels were evaluated. TF expressing microparticles (MPs) were measured by flow cytometry and thrombin–antithrombin complex levels by ELISA.

Results Peripheral blood neutrophils from four patients with active disease expressed elevated TF levels and released TF expressing neutrophil extracellular traps (NETs) and MPs. TF positive NETs were released by neutrophils isolated from the bronchoalveolar lavage and nasal biopsy specimens. Elevated levels of circulating DNA and TF expressing neutrophil derived MPs were further observed in sera from patients with active disease. Induction of remission attenuated the aforementioned effects. Control neutrophils treated with sera from patients with active disease released TF bearing NETs and MPs which were abolished after IgG depletion. Treatment of control neutrophils with isolated IgG from sera from patients with active disease also resulted in the release of TF bearing NETs. TF implication in MP dependent thrombin generation was demonstrated by antibody neutralisation studies.

Conclusions Expression of TF in NETs and neutrophil derived MPs proposes a novel mechanism for the induction of thrombosis and inflammation in active AAV.

INTRODUCTION

Antineutrophil cytoplasmic autoantibody (ANCA) associated vasculitides (AAV) are a group of systemic autoimmune disorders characterised by inflammation and necrosis of small to medium sized vessel walls and presence of ANCA directed either to proteinase 3 (PR3) or myeloperoxidase (MPO).1 Systemic inflammation and constitutional symptoms and signs accompany AAV, while renal and pulmonary involvement dominates the clinical manifestation of these disorders.2–7

Clinical and experimental evidence suggests a critical role for neutrophils and their interaction with ANCA in the pathogenesis of AAV. Primed neutrophils activated with ANCA produce reactive oxygen species, undergo degranulation and release neutrophil extracellular traps (NETs).3 NET deposition in the renal parenchyma of patients with ANCA associated small vessel vasculitis has also been described.8 Moreover, it has been reported recently that NET structure facilitates the uptake of neutrophil proteins from myeloid dendritic cells, resulting in ANCA generation.9

Systemic inflammation has been previously correlated with an increased incidence of venous thromboembolism (VTE) in patients with granulomatosis with polyangiitis, formerly known as Wegener’s granulomatosis, and microscopic polyangiitis.6–9 This incidence is significantly elevated in patients with active disease, increasing approximately to 6.7/100 person years compared with 1.8/100 person years in patients in remission and with 0.3/100 person years in a control population.9,10 However, the pathway involved in inflammation dependent VTE in patients with active AAV has not been elucidated.

Tissue factor (TF) is the major initiator of physiological coagulation and a principal trigger of venous thrombosis. Intravascular exposure of TF, circulating in the form of TF expressed in blood cells or bound to microparticles (MPs), is the critical event in the activation of the coagulation cascade, leading to TF/factor VII complex formation, factor X activation and the subsequent generation of thrombin.11 In addition to their role in thrombosis, these proteases exert signalling through protease activated receptors (PARs) and have a
significant implication in endothelial and platelet activation and/or induction of inflammation.11

Recent studies in several in vivo models of thrombosis describe a critical role for neutrophils in VTE, through NET release and TF dependent activation of the coagulation cascade.12–16 Additionally, TF has been identified in NETs by two separate groups, further reinforcing the role of neutrophils in thromboinflammation.16 17

To test whether neutrophil derived TF could provide a link between inflammation and thrombogenicity in AAV, localisation of TF in NETs and neutrophil derived MPs from affected patients was studied. Moreover, we investigated the involvement of the inflammatory environment and ANCA in this process.

MATERIALS AND METHODS

Patients
Peripheral blood polymorphonuclear cells (PMNs) were isolated from four patients with active disease (three PR3-ANCA positive and one MPO-ANCA positive). Bronchoalveolar lavage fluid (BALF) PMNs and nasal biopsy specimens were obtained from two of these patients. PMNs were also isolated from six healthy individuals, which served as controls. Additionally, serum from eight healthy individuals (control), seven patients with rheumatoid arthritis (RA) with high disease activity (see online supplementary table S1), 17 patients with AAV during active disease and 14 of these during remission was collected for the purposes of the study. Moreover, renal biopsy specimens were obtained from three of these patients with renal involvement. Diagnosis was made according to the criteria adapted from the American College of Rheumatology and the 1994 Chapel Hill disease definitions.18 19 Assessment of disease activity was performed according to the modified Birmingham Vasculitis Activity Score (BVAS).20 Clinical data are provided in table 1 and in the online supplementary table S1. No VTE events were diagnosed at the time of blood collection and none of the patients or control subjects was under treatment with antplatelet agents or anticoagulants. All patients with AAV and RA were negative for antiphospholipid antibodies. Informed consent was obtained from each volunteer. The study protocol was in accordance with the Helsinki Declaration and all procedures were approved by the local ethics committee (Scientific Committee of the University Hospital of Alexandroupolis, Greece).

Stimulation and inhibition studies
PMNs were incubated in the presence of 6% serum from control subjects or patients with AAV (native, MP depleted or IgG depleted) for 1 or 3 h, as previously described.17

MP isolation and characterisation
MPs were isolated, characterised and calculated as previously described.21

IgG isolation
IgG isolation was performed using protein G coated beads (Merck Millipore, Massachusetts, USA) in a magnetic field, according to the manufacturer’s instructions.

Serum DNA quantification
Serum was diluted 1/10 v:v in phosphate buffered saline (PBS). Diluted serum was mixed with 50 μL of propidium iodide (1 μg/mL in PBS) to label circulating DNA. Fluorescence was recorded in a fluorometer (TEACAN, Switzerland) at a 536 nm excitation with a 617 nm filter set. DNA concentrations were calculated according to a DNA standard curve (0.1 ng/mL to μg/mL range). Autofluorescence was determined with PBS mixed with 1 μg/mL of propidium iodide.

Immunofluorescence
Expression of TF, MPO, elastase and high mobility group box-1 (HMGB-1) in NETs was performed by immunofluorescence, as previously described.17 Staining for LC3B and p62/SQSTM1 was used to detect autophagy induction. To assess the formation of acidified autophagosomes, staining with LysoTracker was performed.

Immunoblotting and thrombin–antithrombin complex ELISA assay
Western blotting and thrombin–antithrombin (TAT) complex ELISA assay were performed as previously described.22 23

RESULTS

Release of TF expressing NETs
PMNs isolated from four patients with active disease before treatment initiation (day 0) released TF expressing NETs after 3 h of incubation, as demonstrated by localisation of TF in extracellular structures stained with 4’,6-diamidino-2-phenylindole (DAPI) and elastase (figure 1A; see also online supplementary figure S1). Localisation of MPO and the inflammatory mediator HMGB-1 in NETs was also identified (see online supplementary figure S2). Additionally, PMNs isolated from BALF generated TF expressing NETs (figure 1B). In a previous study, we have shown localisation of TF inside autophagosomes, prior to delivery to NETs.17 For this reason, TF co-localisation with LC3B was further evaluated after 1 h of incubation (figure 1A). Co-localisation of LC3B with p62/SQSTM1 was also demonstrated (see online supplementary figure S3).

To evaluate the effect of treatment initiation on NET release and TF expression, PMNs were isolated at days 4 and 15, after treatment initiation with pulsed corticosteroids. Significant attenuation of NET release was observed at day 4 in three of these patients, while the percentage of NET releasing cells was comparable with the control group on day 15 (figure 1C).
Based on the recently proposed correlation between circulating nucleosomes, neutrophil activation and deep vein thrombosis (DVT), DNA content in sera was measured as an indirect marker of in vivo NET formation. DNA levels followed a similar pattern of reduction to that observed in the percentage of NET releasing neutrophils (figure 1D). Additionally, reduction in circulating neutrophil derived MPs that expressed TF was observed after treatment initiation (figure 1E). Of interest, the percentage of NET releasing PMNs (figure 1C), circulating DNA levels (figure 1D) and neutrophil derived MPs (figure 1E, F) remained elevated in a patient with high disease activity who did not survive. Finally, increased levels of TF expression were detected by immunoblot in cell lysates from neutrophils isolated on day 0 compared with days 4 and 15 (figure 1G). Expression of TF in PMNs isolated from BALF (41% and 29% of total cells, respectively) was also demonstrated by immunoblotting. Representative data are shown in (A), (B), (G) and (H).

**In vitro release of TF expressing NETs**

To investigate the involvement of the inflammatory environment of AAV in the formation of TF expressing NETs, control PMNs were treated with MP depleted sera collected from patients with active disease and on remission. Increased percentage of NET releasing cells and enhanced TF expression were observed in cells treated with sera collected during acute disease compared with remission (figure 2C, D). Moreover, cells treated with serum from RA patients released NETs that had minimal expression of TF (see online supplementary figure S4). Additionally, significant elevation of cell free DNA content was observed in sera from patients with active disease compared with control sera and sera from patients in remission (figure 2E), indicating a correlation between circulating DNA and in vivo NET formation. Serum DNA levels were significantly correlated with BVAS (r=0.523, p=0.004).

**Neutrophil derived TF bearing MPs**

We next accessed the presence of neutrophil derived MPs expressing TF in sera during active disease and remission. Increased numbers of TF bearing neutrophil derived MPs were detected in samples obtained during active disease compared with remission, identified by flow cytometry as Annexin V+/CD66b+/TF+ events (figure 3A). We further identified TF expression in CD66b+/...
CD62P− events, to confirm that these MPs were not platelet–neutrophil mixed type MPs and exclude the interference of platelet derived TF in measurements (see online supplementary figure S5A). Moreover, the numbers of neutrophil derived MPs in AAV were elevated compared with controls and with the RA group, irrespective of AAV disease activity (figure 3B). Expression of TF in endothelial MPs followed a similar pattern during disease course. However, induction of remission did not have a significant effect on TF expression in platelet derived MPs (see online supplementary figure 5B, C). Additionally, a significant correlation

Figure 2  In situ tissue factor (TF) expressing neutrophil extracellular traps (NETs) and after in vitro stimulation of polymorphonuclear cells (PMNs) with sera from patients with antineutrophil cytoplasmic antibody associated vasculitis (AAV). (A) In situ intravascular deposition in a nasal biopsy specimen and (B) glomerular deposition of TF expressing NETs in a renal biopsy specimen, visualised as extracellular structures by staining with elastase (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue). Localisation of TF (green) in NETs. Original magnification 600×. Representative data from two independent experiments in (A) and three in (B) are presented. (C) In vitro activation of control PMNs with sera from AAV patients induces the release of NETs expressing TF. NET formation by control PMNs treated with serum from AAV patients with high disease activity (AAV serum) or during remission (AAV serum remission). PMNs were incubated for 3 h. Localisation of TF (green) on NETs was assessed by confocal microscopy (z stack analysis, 0.3 μm per plane). DNA (blue) is labelled with DAPI. Original magnification 1000×. Scale bar represents 5 μM. Two out of 17 representative independent experiments are shown. (D) Percentage of NET releasing control neutrophils treated with serum from AAV patients (n=17). Control (ctr) represents PMNs treated with sera from control subjects. (E) Circulating DNA levels in sera from control subjects (ctr) or patients with AAV with active disease (n=17) or remission (n=14). Data presented as mean±SD (*p<0.005).
was found between total and TF bearing neutrophil derived MPs with BVAS ($r=0.566$, $p=0.001$ and $r=0.496$, $p=0.006$, respectively).

**Effect of IgG and TNF blockage**

To evaluate the role of ANCA and tumour necrosis factor (TNF) in our experimental model, IgG depletion and TNF blockage with etanercept in MP depleted sera from four patients with active disease and IgG class ANCA directed to PR-3 were performed. IgG depletion resulted in a reduced production of total and TF bearing neutrophil derived MP counts. Serum pretreatment with etanercept had a similar effect (figure 4A, B). Additionally, TF expression in PMNs treated with serum from patients with active disease was attenuated by immunoglobulin depletion or TNF blockage (figure 4D, F).

**Figure 3**  Identification of tissue factor (TF) bearing neutrophil microparticles (MPs) in sera from patients with antineutrophil cytoplasmic antibody associated vasculitis (AAV). (A) MPs were initially identified by FSC-SSC analysis, compared with 3 μm latex beads (arrow). Gated events (R1) were further analysed for expression of Annexin V-CD66b. Neutrophil derived MPs were further identified as double positive events (R2). Expression of TF was measured in R2. Representative data from analysis of MPs isolated from control serum, serum from patients with AAV during high disease activity (AAV active) and in remission (AAV remission). (B) Levels of TF bearing and total neutrophil MPs (polymorphonuclear cell (PMN) MPs) in sera from control (ctr) subjects ($n=8$), patients with active AAV ($n=17$) or in remission ($n=14$), or patients with rheumatoid arthritis (RA) ($n=7$). Data presented as mean±SD (*$p<0.05$, **$p<0.005$, Student’s $t$ test).
Figure 4. Effect of IgG depletion and tumour necrosis factor (TNF) blockage in the in vitro activation of control polymorphonuclear cells (PMNs) with sera from patients with antineutrophil cytoplasmic antibody associated vasculitis (AAV) with increased disease activity. Control PMNs were treated with serum from control subjects (ctr), serum from patients with active AAV before (AAV serum) or after IgG depletion (IgG (−)) or preincubated with etanercept (aTNF), and levels of tissue factor (TF) bearing (A) and total (B) neutrophil derived (PMN microparticles (MPs)) were measured using flow cytometry. (C) Effect of TF blockage on MP dependent thrombin generation. MPs were isolated from culture supernatants from control neutrophils incubated with medium, control serum (ctr) or serum from patients with active disease (AAV serum). MPs were further incubated with MP depleted serum from control subjects and thrombin–antithrombin (TAT) complex levels were measured by ELISA. TF neutralising antibody (AAV serum–aTF) inhibited MP dependent thrombin generation. Data extracted from four independent experiments and presented as mean±SD are shown in (A)–(C) (*p<0.05, Student’s t test). (D) Effect of IgG depletion in neutrophil extracellular trap (NET) release from control PMNs incubated with serum from patients with active AAV. TF (green) and LC3 (red) staining were analysed by confocal microscopy (z stack analysis, 0.3 μm per plane). DNA (blue) is labelled with 4′,6-diamidino-2-phenylindole (DAPI). Original magnification 1000×. Scale bar represents 5 μm. (E) Percentage of NET releasing neutrophils described in (D). Data extracted from four independent experiments and presented as mean±SD (*p<0.05, Student’s t test). (F) Effect of IgG depletion or treatment with etanercept in the ability of sera from AAV patients to induce TF expression in control PMNs. One out of four independent experiments is shown. All sera used for stimulations were MP depleted.
It has been reported previously that MPs released by primed neutrophils on activation with ANCA are able to activate the coagulation system. To investigate whether TF expression is responsible for this effect, PMNs were incubated with MP depleted sera from patients with active AAV. Released MPs incubated with MP depleted control serum and TAT complex levels were measured. Increased TAT complex levels were generated in serum incubated with MPs released by PMNs treated with sera from patients with AAV. Treatment with anti-TF antibody significantly attenuated this effect, suggesting a key role for TF in MP dependent thrombin generation (figure 4C). Moreover, IgG depletion significantly inhibited the percentage of NET releasing neutrophils (figure 4E) while TNF blockage had minimal effect (data not shown). Additionally, TF expression in neutrophils treated with serum from patients with active disease was attenuated by immunoglobulin depletion or TNF blockage (figure 4F).

Finally, treatment of TNF-α primed PMNs with either isolated ANCA IgG or anti-MPO polyclonal antibody resulted in increased DNA levels in culture supernatants (figure 5A, B) and elevated DNA levels in culture supernatants (figure 5C). Moreover, TF expressing MPs (figure 5D) were also observed in TNF-α primed PMNs treated with anti-MPO antibody.

**DISCUSSION**

This study provides evidence for the role of neutrophils in the hypercoagulability of AAV through generation and extracellular delivery of TF expressed in NETs and MPs. Moreover, autophagy was shown to mediate the delivery of TF to NETs. The correlation between disease activity and TF production further reinforces the potential role of neutrophil derived TF in the observed thrombogenicity during active disease.

Several autoimmune and/or inflammatory disorders, including systemic lupus erythematosus, inflammatory bowel disease and polymyositis/dermatomyositis, are linked to increased risk for the development of VTE. Endothelial dysfunction and disequilibrium between inducers and inhibitors of coagulation and fibrinolysis pathways due to proinflammatory mediators have been proposed to contribute to hypercoagulability in these syndromes. The increased incidence of VTE in patients with AAV has been previously reported. Moreover, this risk is significantly elevated during periods of high disease activity, suggesting the involvement of inflammatory mediators and/or immune cells, including neutrophils, in this process. Detection of elevated D-dimer and TAT complex levels in these patients supports the activation of the coagulation cascade. However, the incidence of VTE is not correlated with the prevalence of antiphospholipid antibodies or mutations in thrombosis associated factors. Even though the presence of antiplasminogen antibodies affecting in vitro fibrinolysis has been previously implicated in the hypercoagulability of AAV and an interplay between endothelial cells and ANCA or activated neutrophils in arterial vessels has been associated with disease pathogenesis, the involvement of the extrinsic coagulation system in AAV related thrombogenicity has not been completely elucidated.

Even though the involvement of NETs in the infliction of endothelial injury has been previously suggested in AAV, their role in the thrombogenic tendency that characterises active disease has not been extensively studied. Recently, Nakazawa et al demonstrated the presence of NETs in a thrombus from a patient with microscopic polyangiitis and DVT. Herein, we showed that peripheral PMNs isolated from patients with AAV during active disease released elevated numbers of NETs that expressed TF. Deposition of TF expressing NETs in the vasculature of nasal biopsy specimens and glomeruli and release of such structures from PMNs isolated from BALF were also demonstrated. Moreover, circulating DNA levels were used as an indirect method to study in vivo NET formation. Even though this approach is limited by the fact that other cell populations, including damaged endothelium, could contribute to increased DNA levels. DNA levels were found to correlate with disease activity and the ability of serum derived from patients to induce NET formation. Additionally, the involvement of the inflammatory environment in this process was confirmed by in vitro stimulation studies. The direct effect of ANCA in TF expression and release of TF expressing NETs was demonstrated using IgG depleted serum and isolated IgG fraction from patients with active disease in the in vitro experimental procedure.

Even though a randomised, placebo controlled clinical trial has demonstrated minimal effect of anti-TNF treatment in patients with granulomatosis with polyangiitis, priming with TNF-α is required for ANCA induced NET release. In our experimental model, serum treatment with etanercept had no effect on the percentage of NET releasing PMNs. This observation could be attributed to the presence of other proinflammatory mediators implicated in neutrophil priming. Granulocyte colony-stimulating factor and C5a could be proposed as candidate mediators, considering that they have been implicated both in neutrophil priming and AAV pathogenesis.

Our findings are in accordance with several lines of evidence that propose a key role for neutrophils in VTE, either through NET release or through TF expression. In vivo experimental models of sepsis and DVT suggest the critical implication of NETs in thrombosis either through platelet entrapment and activation or through activation of the coagulation cascade. The association between elevated levels of circulating nucleosomes and elastase-α1–antitrypsin complexes and high risk for DVT in humans also reinforces the proposed role for neutrophils in thrombogenesis. Additionally, TF production by neutrophils attached to injured endothelium has been detected as the initiatory event in thrombus formation in a DVT model. Furthermore, TF expression has been identified in NETs released by both murine and human neutrophils. In human neutrophils, we identified the localisation of TF in autolysosomes in neutrophils from patients with sepsis, prior to delivery to NETs, which was confirmed in the present study.

The contribution of circulating TF bearing MPS derived from monocytes, platelets or endothelial cells in coagulation and thrombosis is well characterised in several prothrombotic disease models. Elevated levels of circulating neutrophil MPs have been previously identified in patients with vasculitis and end stage renal disease. Increased levels of neutrophil MPs, which were able to induce thrombin generation, were also observed in paediatric patients with AAV. Another study in a paediatric population further associated TF bearing MPs with thrombin generation, using in vitro TF blockade. However, the cell origin of TF bearing MPS was not defined. Herein, we demonstrated the expression of functional TF in neutrophil MPs, which was correlated with disease activity.

In addition to their essential role in coagulation, thrombin, factor Xa and TF:factor VIIa complex exert signalling through PARs. PAR-1 and PAR-2 are prominently expressed on epithelial, mesangial and endothelial cells in the kidney. Signalling of thrombin and factor Xa through PAR-1 and PAR-2, respectively, has been previously implicated in the development of crescentic glomerulonephritis in animal models by promoting fibrin deposition and glomerular mononuclear cell
Figure 5  In vitro stimulation of polymorphonuclear cells (PMNs) with disease associated stimuli. Control PMNs, resting or primed with recombinant tumour necrosis factor (TNF-α), were treated for 3 h with isolated IgG fraction (100 μg/mL) from healthy individuals (HI IgG, n=6) or from patients with antineutrophil cytoplasmic antibody associated vasculitis (AAV IgG, n=6) or polyclonal antiminperoxidase antibody (aMPO).

(A) Localisation of tissue factor (TF; green) in neutrophil extracellular traps (NETs) (z stack analysis, 0.3 μm per plane). Original magnification 600×.

(B) Percentage of NET releasing neutrophils. (C) DNA levels in culture supernatants. (D) Evaluation of total and TF expressing neutrophil derived microparticles (MPs) in neutrophils treated with polyclonal anti-MPO antibody. Neutrophil derived MPs were identified as Annexin V+/CD66b+ events. TF expression was further evaluated in these MPs. Data presented as mean±SD (*p<0.05; ns, non-significant). Representative data of six independent experiments are shown.
infiltration. PAR-2 mediated augmentation of renal plasminogen activator inhibitor expression and inhibition of matrix metalloproteinase-9 activity have been proposed to contribute to glomerular injury. Moreover, studies in human and animal models demonstrated the involvement of extrinsic coagulation system and PAR signalling in the inflammatory process that leads to lung injury. It is conceivable that the glomerular or alveolar deposition of TF expressing NETs may have a significant role in amplification and progression of renal or lung injury in AAV, which are severe and even fatal disease manifestations. Our data regarding TF expression in NETs in glomeruli and NETs released by BALF derived neutrophils further support this hypothesis.

Even though a clear correlation between active disease state and neutrophil TF was demonstrated in the present study, the number of enrolled patients is a limitation for drawing of conclusions regarding the role of the detection of neutrophil derived TF as a biomarker for the characterisation of patients with an increased risk for VTE. Prospective studies in larger cohorts are needed to evaluate this issue.

In conclusion, this study described a novel mechanism for the hypercoagulability and infliction of tissue injury in AAV (see online supplementary figure S6). Neutrophil activation by inflammatory mediators and ANCA induces expression of TF and release of TF expressing NETs and MPs. It is proposed that the subsequent activation of the extrinsic coagulation cascade may have a significant pathogenic role in hypercoagulability that characterises active AAV. Additionally, signalling through PARs and resulting activation of endothelial, epithelial and/or mesangial cells could be an alternative pathway for the involvement of neutrophil derived TF in the pathogenesis of AAV. However, the role of other NET constituents, including histones and MPs derived from other cell populations, like endothelial cells and platelets, in the thromboinflammatory response of AAV cannot be ignored and needs to be further evaluated.

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**Contributors**

Study conception and design: KK, DTB, DV, KR and IM. Acquisition of the data: KK, AC, EA, PS, AG, SA, LN, AGia, MK and IM. Patient follow-up and clinical sample acquisition: DV, DBT, PS, MF, PSI, KR and IM. Analysis and interpretation of the data: KK, DV, DBT, KR and IM. Writing of the paper: KK, KR and IM. KR and IM share senior authorship.

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**Ethics approval**

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**REFERENCES**


Basic and translational research


Tissue factor expression in neutrophil extracellular traps and neutrophil derived microparticles in antineutrophil cytoplasmic antibody associated vasculitis may promote thromboinflammation and the thrombophilic state associated with the disease

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