# Neutrophil Extracellular Traps Participate in the Development of Gastric Cancer Associated Thrombosis

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**Research Article**

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# Abstract

**Background:** Development of venous thromboembolism (VTE) is associated with high mortalities among gastric cancer (GC) patients. Neutrophil extracellular traps (NETs) have been reported to correlated with prothrombotic state in some diseases. We hypothesize that NETs participate in the development of GC-associated thrombosis.

**Methods:** The level of NETs in blood and tissue samples of patients were analyzed by ELISA, flow cytometry and immunofluorescence (IF). NETs generation and hypercoagulation of platelets and endothelial cells (ECs) *in vitro* were observed by IF. NETs procoagulant activity (PCA) was performed by fibrin formation and thrombin-antithrombin complex (TAT) assays. Thrombosis *in vivo* was measured in murine model induced by flow stenosis in the inferior vena cave (IVC).

**Results:** NETs were likely to form in blood and tissue samples of GC patients compared with healthy individuals. *In vitro* studies that GC cells and their conditioned medium (CM), but not gastric mucosal epithelial cell can stimulate NETs releasing from neutrophils. In addition, NETs induced hypercoagulable state of platelets by up-regulating the expression of phosphatidylserine (PS) and P-selectin on the cells. Furhter, NETs stimulated adhesion of normal platelets on glass surfaces. Similarly, NETs triggered the conversion of ECs to hypercoagulable phenotypes by down-regulating the expression of their intercellular tight junctions but up-regulating that of tissue factor (TF). Treatment of normal platelets or ECs with NETs augmented the level of plasma fibrin formation and TAT complex. Meanwhile, in the models of IVC stenosis, tumor-bearing mice showed stronger ability to form thrombi and NETs were abundantly accumulated in the thrombi compared with control mice. Notably, combination of DNase-1, activated protein C (APC) and Sivelestat markedly abolished the PCA of NETs.

**Conclusions:** Our findings demonstrate that GC-induced NETs strongly increase the risks of VTE development both *in vitro* and *in vivo*. NETs are potential therapeutic targets in the prevention and treatment of VTE in GC patients.


# Background

Gastric cancer (GC) is one of the most prevalent gastrointestinal tumors and the third most fatal cancer world[1, 2]. Meanwhile, venous thromboembolism (VTE) is a common complication in GC patients, relative to the healthy individuals[3–5]. Notably, VTE is associated with high mortalities of GC patients[6, 7]. Several factors such as tumor stage and increased concentration of D-dimer contribute to the development of VTE in GC patients[8, 9]. However, the molecular mechanism underlying procoagulant activity (PCA) in GC patients are poorly understood. Uncovering molecular targets associated with VTE in GC patients can help in the development of appropriate therapy, which can improve the clinical outcomes of these patients.

Neutrophil extracellular traps (NETs) are web-like structures composed of filamentous DNA and antimicrobial proteins such as citrullinated histone-3 (citH3), neutrophil elastase (NE), myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9) and Cathepsin G (CatG)[10, 11]. They result from interaction between neutrophils and bacterial or other stimulating factors[12, 13]. Overall, they protect the host from pathogens related damages. Besides the prime protective functions, undesirable effects of NETs in autoimmune diseases have been reported[14–16]. Recent researches have linked NETs to the development of metastasis and cancer-associated thrombosis[17, 18]. Particularly, NETs results in arterial and venous thrombosis, both mediated by neutrophils[19]. Furthermore, using mouse models, it was shown that thrombosis in breast cancer tissues was closely linked to formation of NETs[20]. This underlines the probable relationship between NETs and cancer-related thrombosis.

A recent study revealed that priming of metastatic pancreatic cancer cells with platelets stimulates neutrophils to release NETs, which promote thrombosis in both static and dynamic state[21]. Additionally, we firstly reported that platelets derived from GC can stimulate neutrophils to release NETs. Meanwhile, NETs enhance PCA in GC patients, which positively correlate with expression of thrombin-antithrombin (TAT) complex and level of serum D-dimers[22]. Moreover, both human and animal studies suggest that enhanced thrombosis may result from over-expression of activated platelets[23, 24]. Nevertheless, little is known about the interaction between NETs and platelets activation in GC patients. Meanwhile, the injury of venous ECs in

cancer patients is also closely related to venous thrombosis[25, 26]. Interestingly, cytotoxicity of NETs against ECs enhances PCA in oral squamous cancer, even in patients with obstructive jaundice and inflammatory bowel disease[27–29]. Even so, the potential mechanism underlying ECs injury in GC patients is poorly understood.

Our central hypothesis is that GC-induced NETs participate in the VTE responses by platelets activation and endothelium injury. Therefore, we first explored the complex relationship between NETs formation and platelets activation as well as ECs injury. Further, we showed the effect of NETs on thrombosis in IVC stenosis mice models. In general, our results may offer that NETs are potential therapeutic targets in the prevention and treatment of VTE in GC patients.

# Methods

### Patients and tissue samples

Sixty-three patients newly diagnosed with primary GC and thirteen healthy donors (HD) attending the Second Affiliated Hospital of Harbin Medical University between October 2019 and April 2021 were enrolled in this study. GC diagnoses were performed based on pathological examinations. Pathologic tumor-node-metastasis (TNM) stage and histological classification of GC were performed according to the 7$^{th}$ American Joint Committee on Cancer (AJCC) guidelines[30]. Underage (<18 years), pregnant, those on anti-tumor or anticoagulant treatment before surgical treatment or patients with underlying complications such as endocrine system, cardiovascular, hematological system, infectious and other cancers were all excluded from the study. We extracted tumor and adjacent normal tissues from GC patients who consented to this study in writing. Protocol for this study was approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University (No. KY2016-032).

### Isolation of human plasma, platelets and neutrophils

Fresh whole venous bloods were collected into tubes containing 3.2% sodium citrate using 21-gauge needles. The patients underwent overnight fasting before blood collecting. The blood was centrifuged at 150×g for 20 min at room temperature (RT) to obtain platelet rich plasma (PRP), within 1 h of collection. Clean top PRP layer was collected in to a new tube, diluted with platelet wash buffer (TBD, Tianjin, China) and centrifuged at 460×g for 20 min at RT to obtain clean platelets and platelet free plasma (PFP)[31]. The platelets were resuspended in pre-warmed modified Tyrode's buffer (Solarbio, Beijing, China). Neutrophils were isolated based on the density gradient centrifugation, using the whole blood neutrophil isolation kit (TBD, Tianjin, China). After lysing erythrocytes based on the red blood cell lysis buffer (TBD, Tianjin, China), the purity (>96%) and viability (> 96%) of neutrophils were assessed using Wright-Giemsa staining and Trypan blue staining, respectively.

### Cell lines and conditioned medium (CM)

Human metastatic gastric cancer KATO-Ⅲ and MKN-45 cell lines, Human primary gastric cancer AGS cell line, Human gastric mucosal epithelial cells (GES-1), Human umbilical cord endothelial cells (HUVECs) and Mouse forestomach squamous carcinoma (MFC) cell line were purchased from PROCELL (Wuhan, China). All cell lines were characterized using short tandem repeat (STR) profiling. The GES-1, AGS, MKN-45 and MFC were cultured in RPMI1640 (Gibco, USA), KATO-Ⅲ were cultured in IMDM (Gibco, USA), HUVECs were cultured in DME/F12 (HyClone, USA). All medium were supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin solution (Beyotime, Beijing, China). Incubation was performed at 37 °C under 5% $CO_2$ in humidified environment. To prepare CM, the cells were cultured to 90% confluence in media supplemented with 10% FBS, washed three times using 1×PBS and re-cultured for 48 h in media without FBS. The supernatant was centrifuged at 1,500× g for 10 min at 4 °C to remove cell debris. The CM was collected and stored at -80 °C till further use.

### Animal models

Male wild-type (WT) C57BL/6 mice (7-9 weeks old weighing 20-26g) were purchased from Animal experimental center of Harbin Medical University and all procedures were approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Harbin medical University (No.KY2016-032). The animals were individually housed and maintained under standard conditions (12-hour light/dark cycle, 22 ℃ ± 1 ℃) and provided a conventional laboratory diet and unrestricted supply of drinking water. In the subcutaneous tumor models, mice were injected with MFC cells ($2x10^7$cells/ml) subcutaneously into the right axillary, the tumor was allowed to 1000$mm^3$. Thereafter, the murine model of deep vein thrombosis (DVT) was performed as previously described[32]. The mice were anesthetized by intraperitoneal injection of 2,2,2-Tribromoethanol (Sigma, USA), the intestines were turned out to expose the IVC after entering the abdominal cavity through the median abdominal incision. The IVC was carefully separated below the left renal vein plane. After 5-0 (1mm) suture passed through the IVC, 3-0 (2mm) suture was placed at the parallel part of the IVC as the blocking line. The IVC was ligated and 3-0 suture was carefully extracted. This procedure has been shown to decrease vascular lumen by about 90%. The other branches of IVC were ligated to the level of iliac vein. Thereafter, the abdominal incision was closed, mice were sacrificed after 6 or 48 h and thrombi formed in the IVC were harvested. The IVC stenosis mice in the treatment groups were injected with DNAse-1 (50μg/mouse, Roche) intraperitoneally every 12 h until the time of sacrifice.

### Quantification of plasma NETs marker

Plasma cell-free DNA (cf-DNA), MPO-DNA and citH3-DNA complex were quantified using capture ELISA as previously described [22,27]. The quantification of cf-DNA was performed by the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, USA). For detection of NETs complexes, MPO ELISA kit (Jianglaibio, Shanghai, China), and citH3 ELISA kit (Jianglaibio, Shanghai, China) were combined with Quant-iT PicoGreen dsDNA assay kit (Invitrogen) respectively to detect MPO-DNA and citH3-DNA.

### Fibrin formation and TAT complex assay

Fibrin formation of platelets and ECs were detected by turbidity as previously described[33]. Platelets and ECs monolayers were stimulated by NETs with or without DNase-1, APC and Sivelestat treatment alone or together for 4 h, and then 150 μl PFP from HD were co-cultured with them at 37℃ for 2 min, followed by the addition of 50 μl of prewarmed 25 mmol/l $CaCl_2$. The fibrin formation was detected by measuring OD at 405 nm. For detection of TAT complex level, Human and Mouse TAT complex ELISA kit (Jingkbio, Shanghai, China) were performed as previously described[34].

### Flow cytometry

Circulating NETs were measured using flow cytometry. Here, whole blood from HD and GC patients was first diluted with 1×PBS and incubated in the dark at RT for 30min with FITC-conjugated-citH3 (eBioscience, USA) and PE-conjugated-MPO (eBioscience, USA) antibodies. For the assessment of PS and P-selectin expression on platelets, platelets ($2×10^6$ cells) isolated from blood of HD and GC patients were incubated with FITC-conjugated-lactadherin (Haematologic, EssexJunction, VT), APC-conjugated CD62P (Biolegend, USA) and Percp-conjugated-CD41 (Biolegend, USA) antibodies.

### Immunofluorescence imaging of NETs

Tumor and paratumor tissues of GC patients were first cultured overnight at 4℃ with primary rabbit anti-Histone H3 (1:500, ab5103, Cambridge, UK) and mouse anti-MPO (1:500, ab90810, Cambridge, UK) antibodies, washed three times with PBS before further incubation for 1h at RT with Alexa Fluor 594 conjugated goat anti-rabbit (1:200, proteintech, China) and Alexa Fluor 488 conjugated goat anti-mouse (1:200, proteintech, China) secondary antibodies. Thereafter, the tissues were stained for 5min at RT in the dark with 4',6-diamidino-2-phenylindole (DAPI) and anti-fade mounting medium (Solarbio, Beijing, China). Thrombi in the IVC of tumor models or control mice were stained with primary rabbit anti-histone H3(1:500, ab5103, Cambridge, UK) and rat anti-Ly6G (1:500, Novus, USA), and then it was incubated with the with Alexa Fluor 594 conjugated goat anti-rabbit (1:200, proteintech, China) and Alexa Fluor 488 conjugated goat anti-rat (1:200, proteintech, China) secondary antibodies as previously described[35]. The tissues images were captured using a confocal microscope.

Neutrophils ($5\times10^5$ cells) isolated from healthy individuals were seeded and incubated in glass-based poly-L-lysine-coated 24-well plate for 1h at 37℃ under 5% $CO_2$ chamber. Thereafter, cell suspension of KATO-Ⅲ, MKN-45, AGS, GES-1 cells ($2\times10^5$ cells) or CM from GC cells were co-cultured with neutrophils for 4 h at 37℃ under 5% $CO_2$. To detect and quantify NETs, the samples of CM group were incubated with primary rabbit anti-Histone H3 and mouse anti-MPO antibodies and thereafter fluorescent secondary antibodies. The samples of cell-cell contact groups, NETs were stained with Sytox® Green (Solelybio, Beijing, China) for 10 min in the dark at RT. All experiments were analyzed by confocal microscope.

### Preparation of cell-free NETs

Cell-free NETs were isolated from neutrophils of GC patients as previously described, but with slight modifications[36]. Briefly, neutrophils ($1\times10^7$ cells/ml) were cultured for 4 h at 37 °C under 5% $CO_2$ in media supplemented with 500 nM PMA (HY-18739, MCE, USA). The supernatant was abandoned, then ice-cold 1×PBS were added to wash down the cell layer of neutrophils to obtain the NETs medium and centrifuged at 1,500×g for 10 min at 4 °C to remove cell debris. Thereafter, 1.5ml of the supernatant (sterile DNA-protein complex) was centrifuged at 15,000×g for 15min at 4℃. The resultant pellets were suspended in ice-cold 1×PBS and quantified using spectrophotometry. The medium containing the NETs was stored at -80℃ for subsequent experiments.

### Platelet activation and adhesion assay

Platelets activation and adhesion assays were performed as previously described[21]. Briefly, glass-based wells of 24 well plate were coated with cell-free NETs after overnight incubation with corresponding medium at 4 ℃ in a humidified chamber. For controls, 1% of denatured bovine serum albumin (dBSA) was used. Platelets suspension ($1\times10^7$ cells/ml) was then seeded in the wells, cultured for 1 h at 37 ℃ under 5% $CO_2$, fixed for 15 min at RT with 4% paraformaldehyde and washed three times using 1×PBS before 20 min permeabilization using 0.1% Triton-X 100. The platelets were then incubated for 30 min with Alexa Flour 594 conjugated phalloidin primary antibodies (1:300, ThermoFisher, Waltham, USA). To assess PS and P-selectin expression, the platelets were stimulated by NETs for 1 h, and the cells were firstly stained with FITC-conjugated-lactadherin (Haematologic, EssexJunction, VT) for 30min, and then stained with primary rabbit anti-P-selectin (1:200, Proteinteck, China), and mouse anti-CD41 (1:500, Novus, USA) antibodies, imaging were observed and photographed using confocal microscopy.

### HUVECs stimulation assay

HUVECs were incubated for 4 h in 24 well plates with cell-free NETs or PBS. The cells were fixed in 4% paraformaldehyde for 15 min at RT, washed three times using 1×PBS and thereafter blocked for 1h using 10% goat serum with 1% BSA solution in PBS. For detection of TF expression, ECs were incubated overnight at 4℃ with rabbit anti-TF (1:500, ab228968, Cambridge, UK) and mouse anti-CD31 (1:500, ab9498, Cambridge, UK) primary antibodies. Then cells were washed with PBS and re-incubated for 1 h at RT with Alexa Fluor 594 conjugated (Proteinteck, China) goat anti-rabbit and Alexa Fluor 488 (Proteinteck, China) conjugated goat anti-mouse secondary antibodies. For detection of intercellular junctions of cells, incubated overnight at 4℃ with rabbit anti-VE-cadherin (1:500, ab33168, Cambridge, UK) primary antibodies followed the Alexa Fluor 488 (Proteinteck, China) conjugated goat anti-rabbit secondary antibodies and further incubated with Alexa Flour 594 conjugated phalloidin primary antibodies (1:300, ThermoFisher, Waltham, USA). They were then stained with 4’,6-diamidino-2-phenylindole (DAPI) and fixed with mounting medium (Solarbio, Beijing, China) for 5min at RT in the dark. The cells were then observed and photographed using a confocal microscope. The photos were analyzed using Image J software.

### Inhibition assay

For inhibition assays, platelets and HUVECs were co-cultured with cell-free NETs for 1h at 37 ℃ in humidifier chamber in the presence of DNAse-1 (100 U/mL, Roche, Swiss), APC (100nM, HY-P1918, MCE, USA) and Sivelestat (100nM, HY-17443, MCE,

USA) alone or together. DNAse-1 cleaves NETs-DNA whereas APC and Sivelestat disrupts histone and NE functions, respectively.

### Statistical analysis

Comparisons between groups were performed using Student's t-tests, paired t-tests and analysis of variance (ANOVA). Continuous data was expressed as mean ± standard deviation (SD). All analyses were performed using GraphPad Prism software, V. 8.0. P< 0.05 was considered statistically significant.

## Results

### GC patients display greater NETs formation

The level of plasma cf-DNA, citH3-DNA and MPO-DNA complexes between GC patients (n=63) and healthy donors (HD, n=13), which reflects the concentration of NETs, were measured using capture ELISA. In general, the levels of NETs marker were significantly higher in patients with stage □/□/□ GC, relative to those with HD (Fig. 1A-C). There was also a significant difference in preoperative and postoperative plasma NETs marker levels in GC patients (Fig. 1D-F). Moreover, the levels of NETs marker positively correlated with that of serum D-dimer. This suggests that NETs are associated with hypercoagulation and VTE development in GC patients (Table 1). The level of NETs (MPO+/citH3+ neutrophils) in circulation in GC patients and HD were measured using flow cytometry. We found circulating NETs were higher in blood of patients with either of GC stages (II, III, and IV), relative to HD counterparts (Fig. 1G, H). Furthermore, based on MPO and citH3 levels, immunofluorescence staining revealed that NETs were significantly higher in tumor microenvironment relative to paratumor tissue of the same patients (Fig. 1I, J).

### GC cells stimulates formation of NETs by neutrophils

To assess whether gastric cancer cells can directly stimulate NETs formation, we analyzed the expression of NETs in a co-culture of GC cell lines and neutrophils. Immunofluorescence analysis revealed that compared with GES-1, the rate of NETs formation was significantly high in GC cells (Fig. 2A). However, formation of NETs was greater in metastatic than the non-metastatic GC cell line (Fig. 2B). Moreover, we measured the NETs formation when normal neutrophils were co-cultured with CM from KATO-□, MKN-45, AGS and GES-1. Immunofluorescence analysis further revealed that CM of KATO-□ and MKN-45 exerted greater neutrophils activation for NETs formation, relative to CM of AGS cells. However, the CM of GES-1 had no effect on NETs formation (Fig. 2C, D). Overall, these findings demonstrate that GC cells stimulate NETs formation through both intercellular contact and non-contact mechanisms.

### NETs contribute to hypercoagulation of platelets

To examine the effect of NETs on platelets activation, we measured the levels of PS and P-selectin expression on these cells. Flow cytometry revealed that compared to HD, the expression of PS and P-selectin was significantly high on platelets of patients with GC (Fig. 3A-D). In addition, platelets isolated from HD were co-cultured with NETs medium or PBS before analyzing PS and P-selectin expression. We found NETs stimulates PS and P-selectin expression on platelets by confocal microscope (Fig. 3E-G). Flow cytometry also demonstrated this hypercoagulable phenotype of platelets which was stimulated by NETs (Fig. 3H, I).

In inhibition assay, we added DNase-1, APC and Sivelestat alone or together to cleave DNA, histone and NE, which were the most functional factors of NETs. At the highest concentration of NETs (0.5ug/ml), flow cytometry revealed that DNase-1, APC, Sivelestat or a combination of the three inhibited 60.2%, 47.1%, 41.9% and 83.2%, respectively, of PS expression (Fig. 3H) and 55.2%, 47.0%, 41.0% and 91.76%, respectively, of P-selectin expression on platelets (Fig. 3I).

### NETs promote platelets adhesion and prothrombotic state

Previous studies have shown that NETs promote thrombosis in murine late-stage breast cancer models and deep vein thrombosis models[18, 37]. However, whether NETs derived from GC neutrophils have ability to stimulate platelets adhesion under static condition was unknown. In order to determine the effect of NETs on platelets adhesion, platelets isolated from HD were further seeded in NETs-coated wells to measure the effects of NETs on sticking of platelets on blood vessels. Confocal microscopy revealed that NETs enhanced sticking of platelets on to glass slides (Fig. 4A, B), indicating that NETs induce development of thrombosis. Moreover, results revealed that fibrin formation and TAT complex level were obviously increased when control plasma were co-cultured with platelets activated by NETs (Fig. 4C, D).

Inhibition assay revealed that digestion of NETs-DNA using DNase-1 modulated adhesion of platelets on glass surfaces. Even so, few platelets still adhered to the NETs-coated well pretreated with DNase-1 (Fig. 4A). This suggests that other protein components other than NETs-DNA participate in the adhesion of platelets. Further, NETs were treated with DNase-1, APC, Sivelestat or a combination of the three inhibited 67.1%, 56.6%, 38.9% and 91.8% of platelets adhesion (Fig. 4B). The degree of platelets adhesion in the combination group were comparable to that of control. Further, we found DNase-1, APC, Sivelestat or a combination of the three reduced 81.1%, 73.9%, 64.3% and 90.7%, respectively, of fibrin formation (Fig. 4C) and inhibited 78.9%, 57.4%, 51.2% and 91.9%, respectively, of TAT complex level (Fig. 4D) at the highest concentration of NETs. Taken together, these findings demonstrate that NETs play a role in the development of thrombosis.

### NETs drive hypercoagulation of ECs

To detected the effect of NETs on ECs thrombogenicity, HUVECs were co-cultured with NETs medium. Confocal microscopy revealed that NETs destroyed the normal intercellular junctions between ECs (Fig. 5A, C). Moreover, NETs treatment up-regulated the expression of tissue factor (TF) on surface membrane of ECs (Fig. 5B, D). In addition, plasma fibrin formation and TAT complex level were significantly increased when control plasma were incubated with ECs monolayers activated by NETs (Fig. 5E, F). Further inhibition assays were performed to assess the effect of NETs on ECs after pretreatment with DNase-1, APC, Sivelestat or a combination of the three. We found NETs treatment after incubation with DNase-1, APC, Sivelestat or all the three returned 47.5%, 36.3%, 33.4% and 86.5%, respectively, of VE expression on ECs (Fig. 5C) whereas similar treatment inhibited 60.4%, 44.8%, 38.6% and 95.5%, respectively, of TF expression (Fig. 5D). Meanwhile, we found NETs treatment after incubation with above inhibitors inhibited 59.7%, 54.4%, 54.0% and 91.5%, respectively, of fibrin formation level (Fig. 5E) and also inhibited 51.2%, 35.6%, 25.9% and 84.3%, respectively, of TAT complex level (Fig. 5F). Taken together, these findings suggest that NETs promote hypercoagulation of ECs, thus inhibiting NETs function can protect against venous injury.

### NETs promote the formation of thrombi in IVC flow restriction models of tumor-bearing mice.

Based on these findings in vitro and pivotal role of NETs in thrombosis, we hypothesized that GC-induced NETs can also promote thrombosis in vivo. Here, in mice IVC flow stenosis models, we found tumor-bearing mice demonstrate more capacity to form thrombi and showed heavier weight and longer length of thrombi compared to control mice (Fig. 6A-D). In the 6 h models, three of 9 of control mice showed thrombi, whereas seven of 9 of tumor-bearing mice formed thrombi. In the 48 h models, all mice demonstrated thrombi in IVC. In addition, confocal imagines of thrombi formed in the tumor-bearing mice after 48h IVC stenosis showed that NETs were significantly accumulated compared to control mice (Fig. 6E-H). The thrombi of control mice included some neutrophils (Ly6G+) but wasn't activated to form NETs (Fig. 6G). Furthermore, tumor-bearing mice showed higher fibrin formation and TAT complex level compared to control mice (Fig. 6I, J).

In inhibition assay, we infused DNase-1 in mice immediately after IVC stenosis and examined thrombosis after 6 or 48h of surgery. We found treatment with DNase-1 significantly inhibited the thrombi formation in IVC stenosis models of tumor-bearing mice (Fig. 6A-D). Further, fibrin formation and TAT complex level of tumor-bearing mice were significantly decreased by DNase-1 treatment (Fig. 6I, J). These data potent suggest that NETs play a role in thrombosis in vivo which was induced by GC and inhibit NETs by DNase-1 have a protective effect on thrombosis in this mice model.

## Discussion

Inflammation is one of the hallmarks of cancer. Meanwhile, neutrophils are among the most important immune cells implicated for promoting tumor progression[38, 39]. On the other hand, NETs participate in cancer progression by promoting proliferation, invasion, metastasis and angiogenesis of cancer cells as well as thrombosis in numerous tumor types[40–42]. A recent study using mice models through $Jak^{2V617F}$ knock in revealed that most myeloproliferative neoplasms (MPN) display NETs formation and deep vein thrombosis[43]. Our previous studies revealed that NETs promote migration and metastasis of GC cells both in vitro and in vivo through epithelial mesenchymal transition[44]. Intriguingly, inhibition of NETs promotes apoptosis and inhibits the invasion of GC cells by regulating the expression of Bcl-2, Bax and NF-κB proteins[45]. Our initial studies revealed that NETs released by neutrophils in GC patients promoted conversion of thrombin and fibrin[22]. Accordingly, we hypothesized that NETs promote thrombosis in GC patients. We then investigated the interactions between GC cells, neutrophils, platelets and ECs, with a keen focus on their role in cancer-associated thrombosis.

In this study, we found the level of plasma NETs marker and citH3 positive neutrophils were significantly higher in GC patients relative to HD. Also, the expression of NETs decreased significantly after resection of GC tissues. Furthermore, we found that neutrophils infiltration and NETs formation were up-regulated in tumor tissues, relative to adjacent paratumor tissues of the same GC patient. Moreover, the levels of serum D-dimer positively correlated with tumor TNM stage, consistent with previous findings[46]. These findings suggest that expression of NETs promotes GC development and thrombosis in the same group of patients.

Recent studies have shown that hepatocellular cancer and hypoxic CM stimulate production of NETs by neutrophils[42]. However, the relationship between GC cells and neutrophils is poorly understood. Our experiments demonstrated that both metastasis and non-metastatic GC cancer cells directly stimulates production of NETs from neutrophils, contrary to GES-1. Further, analyses revealed that CM of GC cells, but not that of GES-1, also induce formation of neutrophils-related NETs. This suggests that NETs formation is also mediated by factors secreted by GC cells. Inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) as well as damage associated molecular patterns (DAMPs), all over-expressed tumor microenvironments, stimulates neutrophils to release NETs[47, 48]. Even though IL-8 was the most over-expressed cytokine in GC patients, whether or not it is the main mediator of NETs formation and subsequent underlying mechanism in GC cancer remain to be validated.

A recent study showed that NETs promotes thrombosis by activating and promoting adhesion of platelets in venous walls of pancreatic cancer patients[21]. Activated platelets display expression of PS on their surface membranes[49]. Meanwhile, P-selectin expression on the surface membrane of platelets is also associated with thrombosis[50]. In this study, we found that compared to HD, PS and P-selectin expression on platelets were significantly high in GC patients, particularly those with □/□ GC. Moreover, NETs treatment upregulated PS and P-selectin expression on platelets. Also, NETs stimulated adhesion of normal platelets on glass slides. However, even though DNAse-1 treatment modulated this phenomenon, some platelets still adhered to the glass slides, suggesting that other secretory factors participated in the adhesion property.

Previous studies have shown that histone in NETs promote thrombosis in colorectal cancer patients[51]. NE is another most abundant protein that binds NETs. Even though the potent protein stimulates tumor progression both in vitro and in vivo, mechanisms underlying NE-mediated cancer-associated thrombosis remain to be clarified. A recent study on DVT using mice models showed that NE deficiency or NE inhibition alone does not completely inhibit DVT[52]. In this study, we found hypercoagulation of platelets was not completely mediated by NETs-DNA, but also by other secretory proteins in the NETs such as histone and NE. Consequently, DNase-1 treatment of NETs had no complete effect on hypercoagulation of platelets. However, a combination of DNase-1, APC and Sivelestat treatment almost completely inhibited hypercoagulation of platelets. Although Sivelestat didn't show a strong anti-hypercoagulation effect like that of DNase-1, it nonetheless modulated activation and adhesion of platelets. This demonstrated that histones and NE also participate in activation and adhesion of platelets.

DVT can be triggered by injury to vascular ECs. Previous studies have shown that under certain malignancies, NETs can induce dysfunction and apoptosis of ECs[33]. In patients with chronic pancreatic disease and pancreatic cancer, NETs exert their cytotoxic against ECs via intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression[25]. Recent studies have shown that treatment of ECs with NETs derived from patients with colorectal cancer promotes and fasten production of fibrin and corresponding coagulation[51]. In this study, we found that NETs treatment inhibited secretion of intercellular junctions in ECs and promoted hypercoagulation of platelets by up-regulating TF expression. Moreover, NETs treatment up-regulated the expression of PS on ECs. On the other hand, ECs activated by NETs significantly increase the level of TAT complexes and fibrin generation in plasma of HD. Given that a combination of DNase-1, APC and Sivelestat treatment completely inhibited hypercoagulation, the process is regulated through numerous mechanisms. In general, these findings strongly suggest that NETs contribute to GC-associated thrombosis.

In the late stage of murine mammary tumor models, thrombus was found in the venous of lung and NETs was accumulated in it, indicating that cancers induced NETs contribute to the cancer-associated thrombosis[18]. Here, we demonstrated that GC-bearing mice have more ability to form thrombi than control mice and NETs was abundantly present in the thrombi of tumor-bearing mice IVC stenosis models, most of this response can be blocked with DNase-1 treatment, which was similar with previous researches. The DVT models by flow restriction of IVC may result a hypoxia microenvironment to recruit neutrophils and stimulate NETs releasing. In addition, cancer cells often secrete more inflammatory factors, which aggravate the recruitment of neutrophils to form NETs under a hypoxic condition[48]. Therefore, neutrophils exposed to two major triggers of NETs releasing, tumor hypoxia environment and IVC flow restriction, which then participate in the development of thrombi in GC.

# Conclusions

Our findings demonstrate that GC cells can directly induce NETs formation, which in turn strongly increases the risks of VTE development both *in vitro* and *in vivo*. In addition, we found that not only NETs-DNA, but also histone and NE participate in the development of cancer-associated thrombosis. Accordingly, NETs are potential therapeutic target against VTE in GC patients.

# Abbreviations

VTE: venous thromboembolism; GC: gastric cancer; NETs: Neutrophil extracellular traps; IF: immunofluorescence; PCA: procoagulant activity; TAT: thrombin-antithrombin complex; ECs: endothelial cells; IVC: inferior vena cave; CM: conditioned medium; PS: phosphatidylserine; TF: tissue factor; APC: activated protein C; citH3: citrullinated histone-3; NE: neutrophil elastase; MPO: myeloperoxidase; MMP-9: matrix metalloproteinase-9; CatG: Cathepsin G; HD: healthy donors; TNM: tumor-node-metastasis; AJCC: American Joint Committee on Cancer; RT: room temperature; PRP: platelet rich plasma; PFP: platelet free plasma; GES-1: Human gastric mucosal epithelial cells; HUVECs: Human umbilical cord endothelial cells; MFC: Mouse forestomach squamous carcinoma cells; FBS: fetal bovine serum; dBSA: denatured bovine serum albumin; WT: wild-type; DVT: deep vein thrombosis; cf-DNA: cell-free DNA; DAPI: 4’,6-diamidino-2-phenylindole; MPN: myeloproliferative neoplasms; IL: interleukin; TNF-α: tumor necrosis factor-alpha; DAMPs: damage associated molecular patterns; ICAM-1: intercellular cell adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1;

# Declarations

### Ethics approval and consent to participate

We extracted blood and tissue samples from GC patients who consented to this study in writing. Protocol for this study including clinical patients and animals were approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University (KY2016-032).

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.


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### Authors' contributions

XMZ and JCL designed the study, completed the experiments and drafted the manuscript. SFY and JQJ collected the patient clinical data and part of experiments. LZ, CJL and CYC participated in the animal experiments. HY and AGZ performed the statistical analysis. All authors read and approved the final manuscript.


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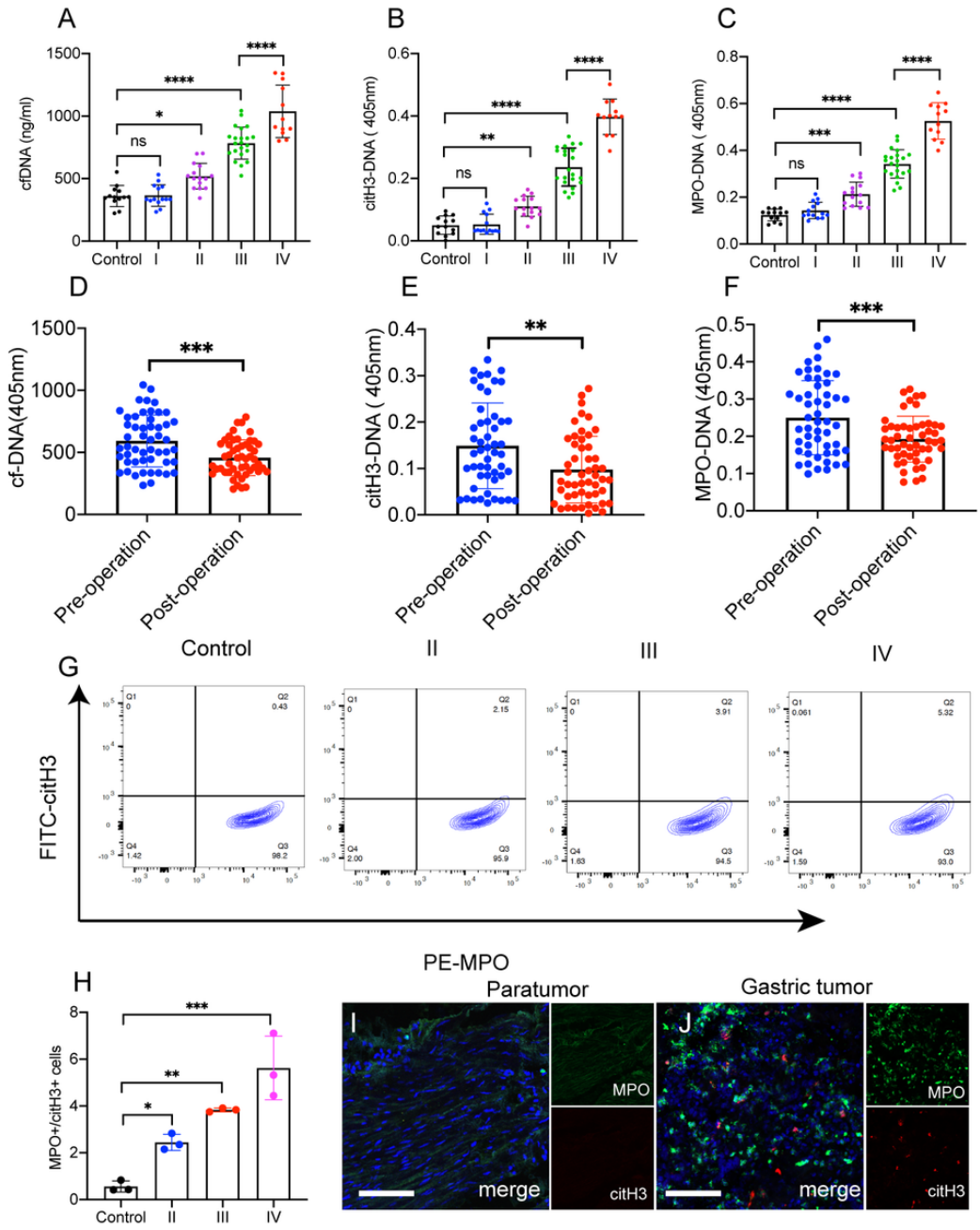
# Tables

**Table 1** The main clinical and laboratory features of 13 healthy subjects and 63 patients diagnosed with GC.

| Characteristics | Control (n=13) | Stage I (n=14) | Stage II (n=15) | Stage III (n=22) | Stage IV (n=12) |
|---|---|---|---|---|---|
| Male (%) | 61.5% | 50% | 53.3% | 72.7% | 66.6% |
| Age(years) | 58.46±5.43 | 57.0±10.07 | 63.07±9.12 | 60.32±11.54 | 63.64±9.68 |
| Erythrocytes($\times10^{12}$/L) | 4.70±0.57 | 4.46±0.32 | 4.12±0.83 | 4.13±0.69 | 4.03±0.81 |
| WBC ($\times10^{9}$/L) | 6.87±2.86 | 6.57±2.04 | 8.60±2.54* | 8.58±4.66* | 10.60±5.25** |
| Neutrophil($\times10^{9}$/L) | 4.36±3.0 | 4.62±2.54 | 6.36±2.82* | 6.75±4.92* | 8.53±5.46*** |
| Hb (g/L) | 140.84±20.31 | 137.23±10.76 | 121.29±26.65 | 121.23±29.72 | 111±33.23* |
| PLT ($\times10^{9}$) | 259.15±59.0 | 242.69±52.19 | 293.29±83.74 | 250.64±104.63 | 302.0±156.9 |
| ALB(g/L) | 44.84±3.27 | 43.33±4.84 | 38.06±6.29 | 37.39±9.23 | 35.59±4.08* |
| PT (s) | 11.24±1.03 | 12.66±4.16 | 12.42±2.03 | 12.05±0.86 | 12.24±1.57 |
| APTT (s) | 35.06±2.71 | 36.03±7.54 | 34.52±3.46 | 33.49±3.81 | 35.08±4.68 |
| D-dimer (mg/L) | 53.30±48.16 | 238.46±265.28*** | 280.21±269.55*** | 291.23±262.88*** | 950.0±743.72**** |
| Fibrinogen (mg/L) | 2.46±0.43 | 2.69±0.58 | 3.67±0.98* | 3.19±0.61* | 4.68±2.61** |

WBC, white blood cells; Hb, hemoglobin; PLTs, platelets. ALB, albumin; PT, prothrombin time; APTT, activated partial thromboplastin time. Data are presented as numbers (percentages) or the median ± SD. *P□0.05, **P□0.01, ***P□0.001, ****P□0.0001 vs. healthy control.

# Figures




## Figure 1

NETs were accumulated in samples of GC patients. A Plasma levels of NETs marker cf-DNA, B citH3-DNA and C MPO-DNA in GC and HD were measured by ELISA. HD, n=13; stage I, n=14; II, n=15; III, n=22; IV, n=12. Comparison of D cf-DNA, E citH3-DNA and F MPO-DNA in plasma of patients with GC pre-opreation and post-operation by ELISA. n=51 G, H Activated neutrophils in circulating environment of GC patients and HD were measured by flow cytometry with APC-MPO and FITC-citH3 staining. I NETs accumulation were detected by confocal microscopy with MPO and citH3 staining in paratumor and J tumor samples from same GC patient. Red-citH3, Green-MPO, Blue-DAPI. Scale bars: 25um. All values are mean±SD. Statistics, one-way ANOVA. ns=no significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

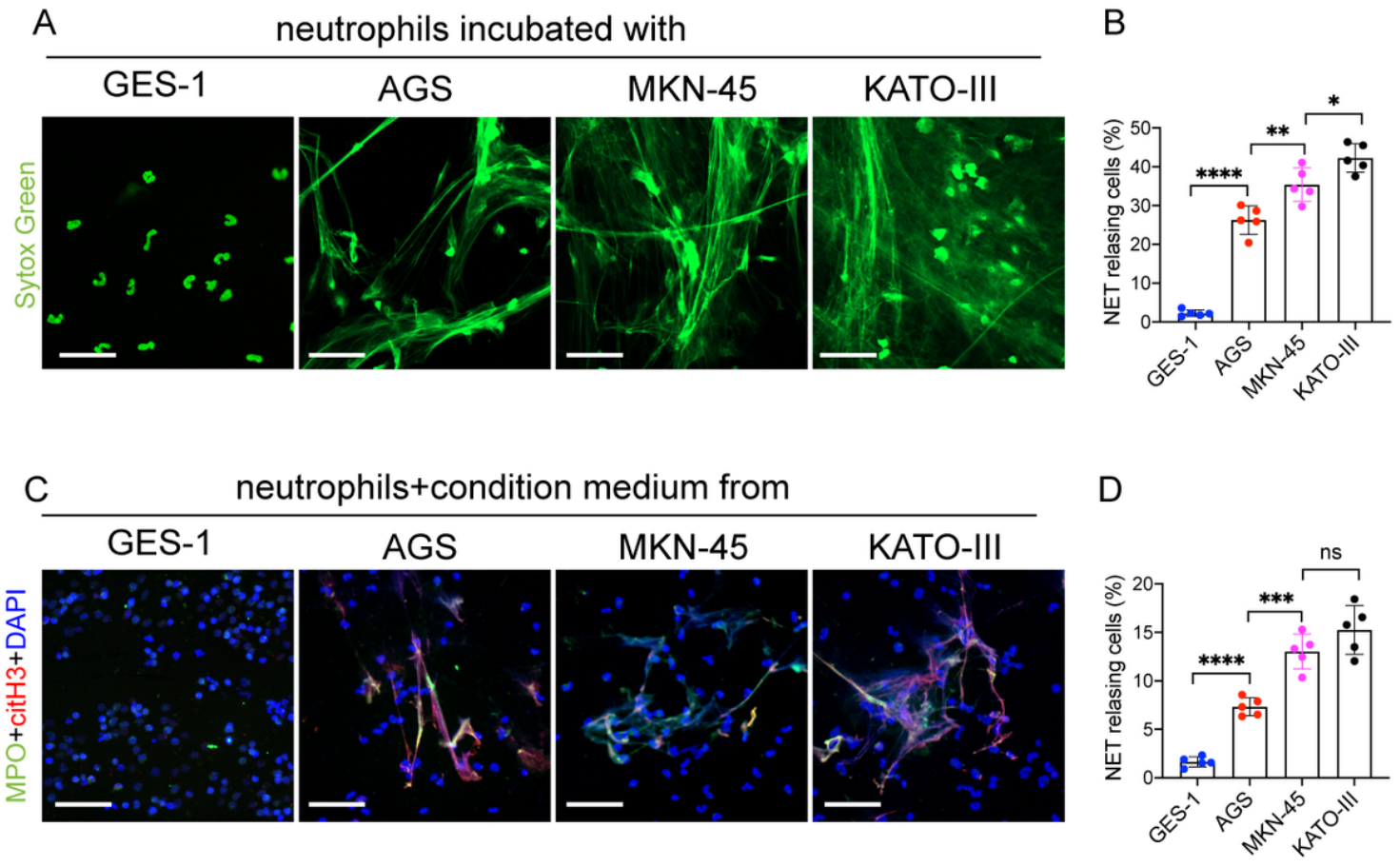
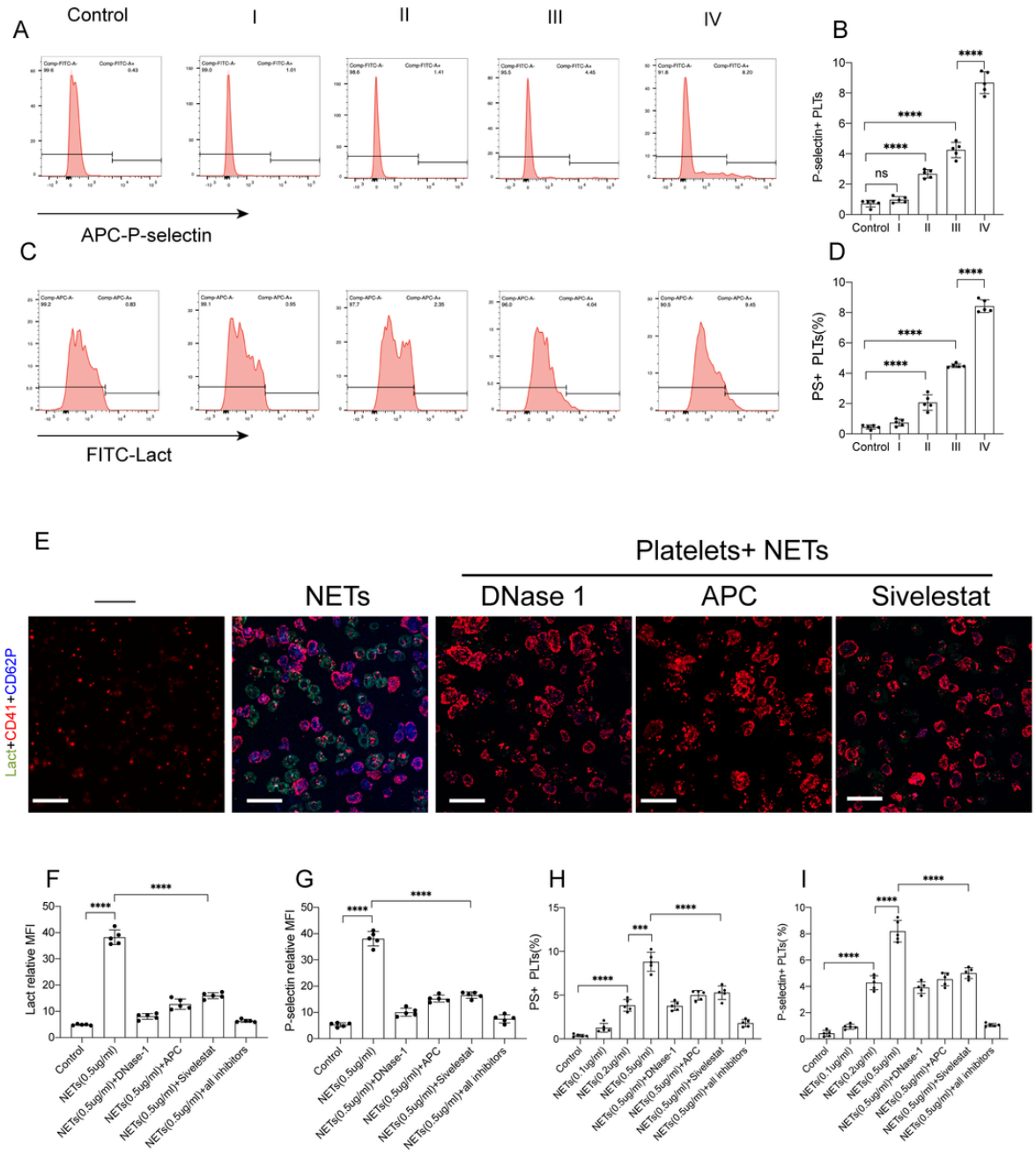



**Figure 2**

GC cells can stimulate neutrophils to form NETs. A, B Control neutrophils were co-cultured with normal gastric mucosal epithelial cells (GES-1) or GC cells (AGS, MKN-45 and KATO-3) and NETs formation were measured by confocal microscopy with cell-impermeable Sytox-Green staining. C, D Control neutrophils were co-cultured with CM from GES-1 or GC cells, and stained with MPO and citH3. Red-citH3, Green-MPO, Blue-DAPI. Scale bars: 20um. All values are mean±SD. Statistics, one-way ANOVA. ns=no significant, *P□0.05, **P□0.01,***P□0.001, ****P□0.0001.




## Figure 3

NETs contribute to hypercoagulation of platelets. A, B The level of PS exposure and C, D P-selectin expression on platelets of each stage GC patients and healthy individuals were detected by flow cytometry. E-G PS exposure and P-selectin expression were measured when isolated platelets were co-cultured with NETs or in the present of DNase-1, APC and Sivelestat alone or together by confocal microscopy, and H, I which were detected by flow cytometry. Red-platelets, Green-Lactadherin, Blue-P-selectin. Scale bars:10um. All values are mean±SD. Statistics, one-way ANOVA. ns=no significant, *P 0.05, ***P 0.001, ****P 0.0001.

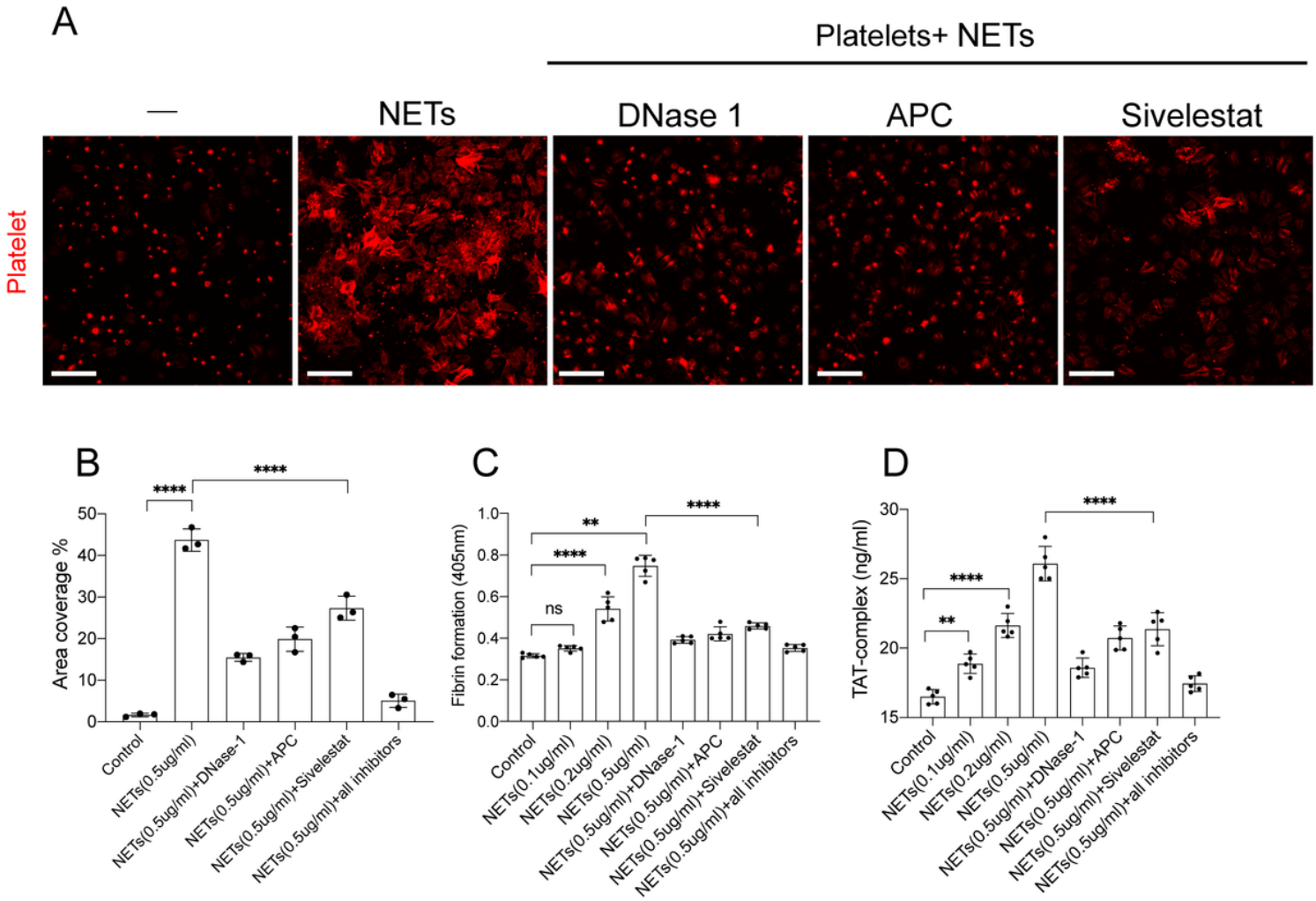
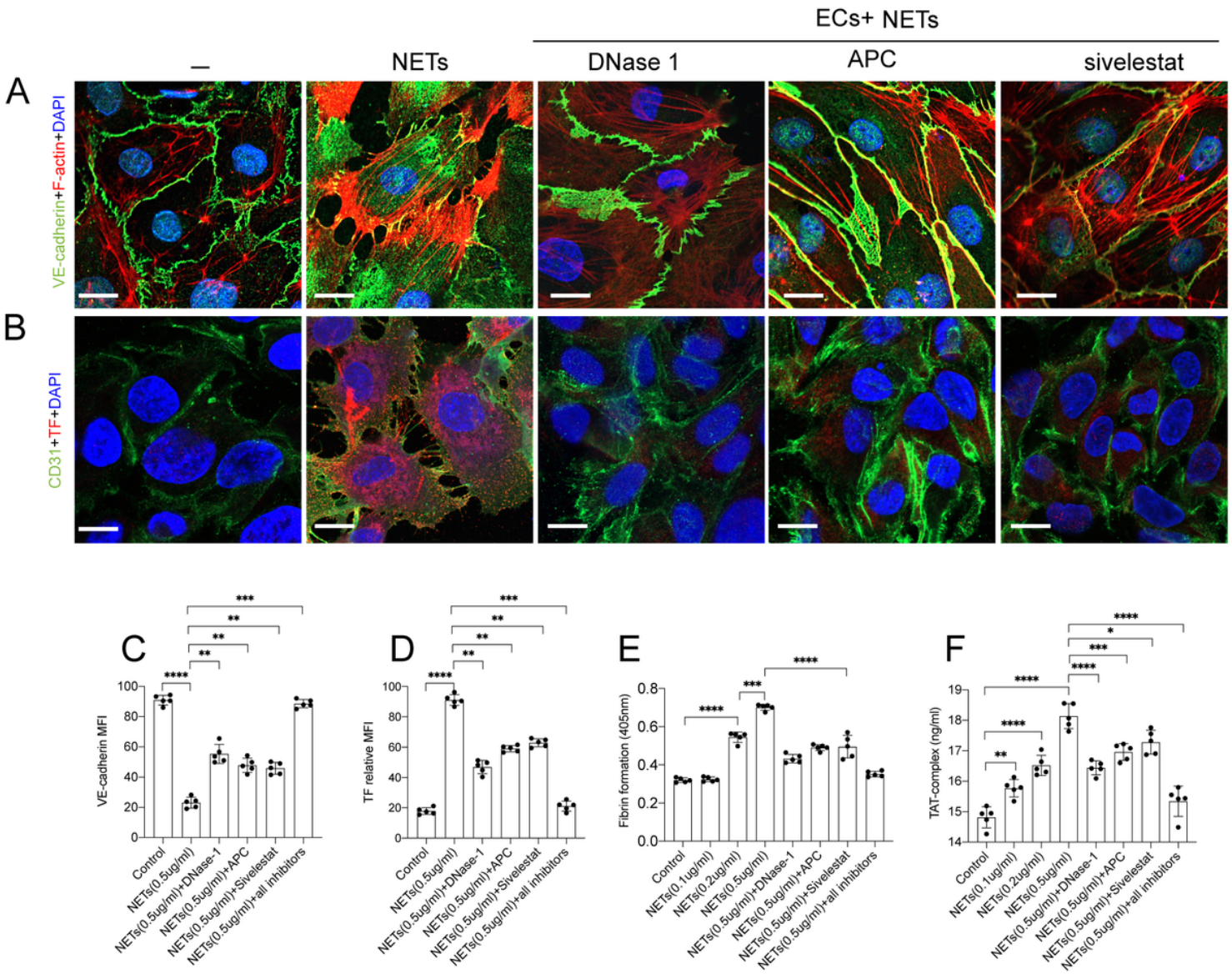



**Figure 4**

NETs promote platelets adhesion and prothrombotic state. A Isolated platelets were incubated with glass slide which was coated with 1%dBSA or NETs or NETs pretreated with DNase-1, APC and Sivelestat alone or together, followed the F-actin components of platelets with 594-Phalloidin staining. Red-platelets. B Detection of percentage area coverage of platelets adhesion were analyzed by Image J software. C Isolated platelets were co-cultured with different concentration of NETs for 30 min with or without DNase-1, APC and Sivelestat treatment alone or together, then the plasma fibrin formation and D TAT complex level of activated platelets were analyzed. A Scale bars:10um. All values are mean±SD. Statistics, one-way ANOVA. ns=no significant, *P□0.05, **P□0.01,***P□0.001, ****P□0.0001.




## Figure 5

NETs drive hypercoagulation of ECs. HUVECs were co-cultured with NETs or PBS in the presence of DNase-I, APC, Sivelestat alone or together for 4 h and analyzed by confocal microscopy. A The intercellular junctions of ECs were stained with VE-cadherin and Phalloidin. Red-Phalloidin, Green-VE, Blue-DAPI. B ECs activation were stained with CD31 and TF. Red-TF, Green-CD31, Blue-DAPI. C VE expression and D TF expression on ECs were detected by confocal microscopy and analyzed by Image-J software (the expression is indicated as mean fluorescence intensity [MFI]). E HUVECs monolayers were stimulated by various concentration of NETs for 4h, followed by determination of plasma fibrin formation and F TAT complex level by ELISA. A, B Scale bars:10um. All values are mean±SD. Statistics, one-way ANOVA. ns=no significant, *P□0.05, **P□0.01,***P□0.001, ****P□0.0001.

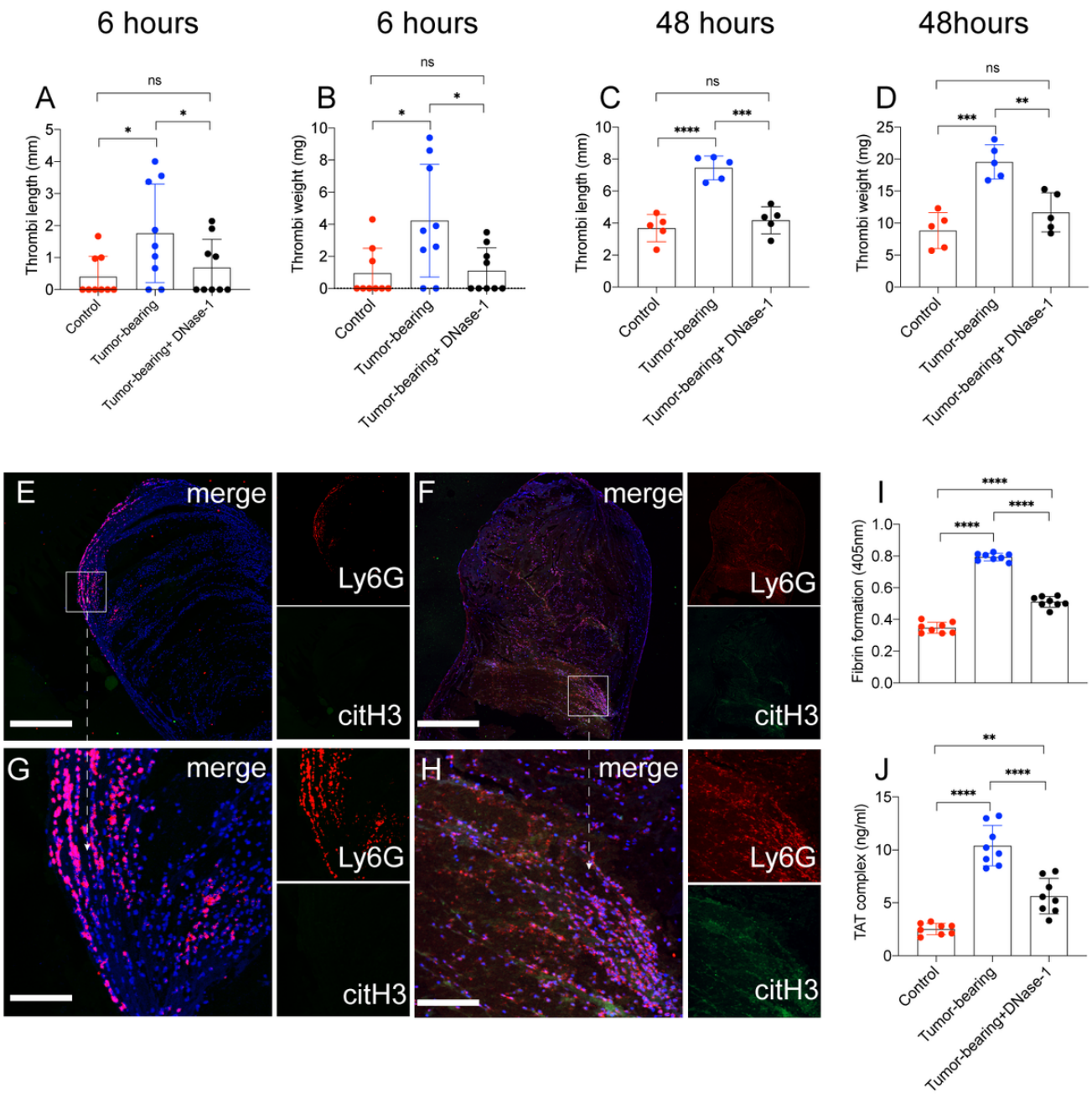



**Figure 6**

Tumor-bearing mice showed more ability to form thrombi by IVC flow restriction. A The values for weight and B length of thrombi present in control, tumor-bearing mice or DNase-1(50μg/mouse) infused tumor-bearing mice at the time of 6 h or C, D 48 h after surgery. 6 h models, n=9; 48 h models, n=5. E Confocal imaging of thrombi derived from control mice and F tumor-bearing mice with Ly6G and citH3 staining. G Enlarged part of thrombi derived from control mice and H tumor-bearing mice. Red-Ly6G, Green-citH3, Blue-DAPI. I The plasma fibrin formation and J TAT complex level of control, tumor-bearing mice or DNase-1 infused tumor-bearing mice were detected by ELISA. Each group mice, n=8. E, F Scale bar: 200um. G, H Scale bar: 20um. All values are mean±SD. Statistics, one-way ANOVA. ns=no significant, *P<0.05, **P<0.01,***P<0.001, ****P<0.0001.