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Full Length Article

Neutrophil extracellular traps induced by activated platelets contribute to procoagulant activity in patients with colorectal cancer

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A R T I C L E I N F O

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A B S T R A C T

Patients with colorectal cancer (CRC) are at increased risk of venous thrombosis, but the precise mechanisms of thrombogenesis in CRC remain largely unknown. We aimed to identify the novel role of neutrophil extracellular traps (NETs) in the induction of procoagulant activity (PCA) in CRC, and to evaluate its interactions with platelets and endothelial cells (ECs). In this study, we first showed that the levels of NETs in the peripheral blood of CRC patients were increased in parallel with cancer progression and reached significance in stage II patients compared to healthy subjects. In addition, neutrophils from CRC patients were more prone to produce NETs, resulting in shortened coagulation time, significantly increased thrombin-antithrombin (TAT) complexes and fibrin fibrils compared to healthy controls. Furthermore, platelets from CRC patients stimulated healthy neutrophils to extrude NETs, which could be inhibited by the depletion of HMGB1. Conversely, NETs from CRC patients could also induce the exposure of PS on platelets, leading to markedly enhanced PCA. Importantly, ECs were also converted to a procoagulant phenotype when exposed to NETs from CRC patients. The PCA of NETs-activated platelets or ECs could be inhibited either by the cleavage of NETs with DNase1 or the blockage of histone with activated protein C (APC). Our results reveal the complex interactions between neutrophils, platelets and ECs and their potential role in the hypercoagulable state in CRC. We propose that NETs may provide new therapeutic targets to combat the thrombotic consequences of CRC.

1. Introduction

Cancer-associated thrombosis (CAT) is the second leading cause of death among patients with cancer, and the occurrence of venous thromboembolism (VTE) is approximately 4- to 6.5-fold higher in cancer patients than patients without cancer [1,2]. Colorectal cancer (CRC) is the fifth most common cancer and also the fifth cancer-related cause of death in China [3]. The estimated risk of developing VTE in CRC patients within 2 years of diagnosis was reported to be 8.2% [4], and patients with metastatic disease have a 5- to 13-fold higher risk compared to those with localized disease [5,6]. Additionally, the development of VTE has been shown to be a significant predictor of earlier

Abbreviations: CRC, colorectal cancer; PCA, procoagulant activity; NETs, neutrophil extracellular traps; ECs, endothelial cells; ELISA, capture enzyme-linked immunosorbent assay; CT, coagulation time; TAT, thrombin-antithrombin; CAT, cancer-associated thrombosis; VTE, venous thrombosis embolism; DVT, deep vein thrombosis; HUVECs, human umbilical vein endothelial cells; PMA, phorbol myristate acetate; BSA, bovine serum albumin; EDTA, ethylenediamine-tetraacetic acid; PBS, phosphate-buffered saline; MDP, microparticle-depleted plasma; APTT, activated partial thromboplastin time; polyP, polyphosphate; G-CSF, granulocyte colony-stimulating factor; IL-8, interleukin-8; DAMPs, danger associated molecular patterns; HIF, hypoxia-inducible factors; PFP, platelet-free plasma; PRP, Platelet-rich plasma; PS, phosphatidylserine; TNM, tumor-node-metastasis; DAPI, 4,6-diamidino-2-phenylindole; NE, neutrophil elastase; Cf-DNA, cell-free DNA; MPO-DNA, myeloperoxidase-DNA; HMGB1, high-mobility group box 1; APC, activated protein C

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Table 1
Baseline characteristics of patients with colorectal cancer and healthy subjects at inclusion.

Characteristics	Controls (n = 20)	Stage I (n = 12)	Stage II (n = 15)	Stage III (n = 19)	Stage IV (n = 14)
Gender (male, %)	11 (55%)	5 (41.7%)	9 (60%)	9 (47.3%)	7 (50%)
Age (years)	61.5 ± ; 9.5	62.4 ± 10.6	63.2 ± 10.1	62.7 ± ; 10.0	59.9 ± ; 12.1
Albumin (g/l)	37.9 ± ; 5.1	34.8 ± ; 6.4	34.4 ± 6.7	37.0 ± 6.4	37.2 ± 4.9
Total cholesterol (mM)	4.0 ± 0.6	4.0 ± 1.1	3.9 ± 1.2	3.50 ± 0.9	3.5 ± 1.2
Triglycerides (mM)	0.9 ± 0.4	1.3 ± 0.3	1.4 ± 0.8	1.3 ± 0.6	1.7 ± 0.6
CEA (ng/ml)	ND	2.2 (1.1–2.3)	4.8 (3.2–19.0)[b]	4.2 (2.6–7.2)[b]	9.5 (7.7–12.7)[b,c,d]
CA199 (U/ml)	ND	8.5 (4.8–10.1)	14.8 (11.2–29.2)[b]	20.4 (11.3–47.1)[b]	64.1 (12.6–732.6)[b,c,d]
Platelet count (10^9/L)	251.5 ± 48.4	271.4 ± 95.0	264.3 ± 97.0	273.0 ± ; 89.8	303.0 ± 78.6
Erythrocyte count (10^{12}/L)	4.4 ± ; 0.5	4.2 ± 0.7	4.3 ± 0.8	4.5 ± ; 0.6	4.3 ± 0.8
Hemoglobin (g/L)	129.0 ± 19.5	119.7 ± 28.4	125.7 ± ; 31.4	123.6 ± 26.6	121.2 ± 29.9
PT (s)	11.8 ± 0.7	10.9 ± 0.8	11.3 ± 1.0	11.1 ± 0.8	11.0 ± 0.8
APTT (s)	32.2 ± 2.7	26.6 ± 3.4[a]	27.5 ± 4.1[a]	25.5 ± 2.8[a,b,c]	24.4 ± 2.5[a,b,c]
D-dimer (mg/L)	0.14 (0.07–0.18)	0.32 (0.18–1.02)[a]	0.39 (0.21–1.81)[a]	0.75 (0.29–1.21)[a,b,c]	0.77 (0.31–1.19)[a,b,c]
Fibrinogen (g/L)	3.4 ± 1.4	3.6 ± 1.1	3.7 ± 1.2	3.5 ± 1.0	3.8 ± 0.9
Current smoking, n (%)	3 (15%)	2 (16.67%)	3 (20%)	3 (15.79%)	4 (28.57%)

Data are expressed by mean ± standard deviation [SD], percentage or median (interquartile range [IQR]). PT prothrombin time, APTT activated partial thromboplastin time. ND not determined.

[a] $P < 0.05$ versus healthy controls.
[b] $P < 0.05$ versus stage I.
[c] $P < 0.05$ versus stage II.
[d] $P < 0.05$ versus stage III.

death in CRC patients [7]. Although there is an obvious prothrombotic state in CRC patients, manifesting as increased levels of hemostatic cells (platelets), coagulation factors and markers (tissue factor, fibrinogen, D-dimer and microparticles) [8], the precise mechanism of hypercoagulability is still not fully understood. Therefore, finding effective new targets and therapies to prevent or treat thrombosis in CRC patients are of particular importance.

Increased leukocyte count is a well-known predictor of CAT and is therefore included in the CAT risk assessing model (Khorana model) [9]. Recent studies have shown that neutrophils actively participate in the development of both arterial and venous thrombus, and this process is dependent on the formation of neutrophil extracellular traps (NETs) [10,11]. NETs are web-like chromatin structures decorated with cytoplasmic, granular and nuclear components of neutrophils formed through a process called NETosis [12]. Originally described as an antimicrobial response, NETs are now implicated in a lot of autoimmune and thrombotic diseases, exerting harmful effects [13]. Moreover, NETs have been regarded as significant contributors to cancer-associated pathologies such as tumor growth, invasion, angiogenesis, metastasis, relapse, and organ dysfunction in patients or preclinical models with cancer [14,15]. Recently, it has been reported that NETs are present in the surgical specimens and the respective metastatic lymph nodes of CRC patients [16]. However, whether NETs are elevated in the peripheral blood and whether NETs play a role in increased procoagulant activity (PCA) in CRC patients remain largely unknown. In addition, the levels of NETs associated with different tumor stages also need to be measured because advancing cancer stage has been shown to be a significant risk factor associated with the development of VTE [17].

Recent studies have shown that platelets are essential for NETosis to occur both in infectious diseases such as sepsis, and in noninfectious diseases such as deep vein thrombosis (DVT) through cell-cell contacts or soluble mediators [18–20]. It has also been reported that pancreatic cancer-primed platelets were able to stimulate NETs formation [21]. Moreover, studies from our group and others' have suggested that platelets are frequently activated in CRC patients, manifesting as increased phosphatidylserine (PS) positive cells and enhanced release of platelet-derived microparticles [22,23]. Nevertheless, little information exists on whether platelets induce NETs formation in CRC patients. Interestingly, it has also been suggested that NETs may induce procoagulant phenotypes in platelets, erythrocytes, and endothelial cells (ECs) [24]. Thus, we speculate that NETs could enhance PCA of CRC patients by eliciting the formation of procoagulant platelets and creating a vicious cycle between activated platelets and neutrophils. Moreover, endothelium injury commonly occurs in cancer patients and serves as the most important contributor to venous thrombosis formation. Therefore, the effect of NETs on ECs in CRC patients still needs to be elucidated.

In this study, we assessed susceptibility of neutrophils from CRC patients or healthy subjects to releasing NETs, investigated the contribution of NETs to PCA, and evaluated the complex interactions between activated platelets, NETs and ECs in CRC patients. In addition, potential correlation between NETs and coagulation parameters in CRC patients was also analyzed. The current results lead us to believe that NETs may play a pivotal role in the prothrombotic state of CRC patients, providing a novel target for prevention and intervention of VTE in CRC patients.

2. Materials and methods

2.1. Patients

In this study, we enrolled 60 newly diagnosed CRC patients and 20 healthy subjects. All patients were admitted to the First Affiliated Hospital of Harbin Medical University between Oct 2016 and Jun 2018. Patients were all diagnosed with CRC by pathological examinations through colonoscopy. Diagnoses were confirmed after surgical operation in those patients for whom it was indicated. Blood samples of diagnosed patients were collected for experiments before any operation or chemotherapy. We assessed the pathologic tumor-node-metastasis (TNM) staging and histological classification according to the 7th American Joint Committee on Cancer (AJCC). Exclusion criteria were age < 18 years, pregnancy, cardiovascular disease, diabetes, active or chronic infection, liver or renal dysfunction, other coexisting cancer, thromboembolic complications, platelets and/or blood coagulation disorders, and administration of anticoagulant and/or antiplatelet treatment. The main characteristics of the patients and healthy controls are shown in Table 1. This study was approved by the research ethics committee of Harbin Medical University, and informed consent was obtained from all participants.

2.2. Reagents

Human umbilical vein endothelial cells (HUVECs) and EC medium were from ScienCell (San Diego, CA, USA). MNase was from Thermo

Scientific (Waltham, MA, USA). Ficoll-Hypaque, poly-L-lysine, phorbol myristate acetate (PMA), bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole (DAPI), DNase 1, activated protein C (APC), ethylenediamine-tetraacetic acid (EDTA), Histopaque 1077 and 1119 were from Sigma-Aldrich (St Louis, MO, USA). Anti-human neutrophil elastase (NE, ab21595) antibody, Alexa Fluor 488-conjugated anti-rabbit secondary antibody (ab150077), anti-high mobility group box 1 (HMGB1) antibody (ab12029) and anti-P-selectin antibody (ab6632) were from Abcam (Cambridge, MA, USA). Alexa Fluor 488 or 647-conjugated lactadherin, Alexa Fluor 647-Annexin V, fluorescein-labeled FVa, FXa and fibrinogen were prepared in our laboratory. RPMI-1640 medium were obtained from Corning (Corning, NY, USA). Prothrombin, thrombin, human factors Va, VIIa, VIII, IXa, X and Xa were all from Haematologic Technologies (Burlington, VT, USA). The Chromogenix substrates S-2765 and S-2238 were from DiaPharma Group (West Chester, OH, USA). Quant-iT PicoGreen dsDNA Reagent was from Life Technologies. DNase I (15,000 U/ml, Sigma) was diluted in phosphate-buffered saline (PBS) containing 0.1% BSA to a concentration of 100 U/ml for inhibition experiments as described.

2.3. Preparation of platelets and neutrophils

Fresh whole venous blood samples were collected with a 21-gauge needle into a 5 ml tube containing 3.2% sodium citrate. Blood samples were centrifuged (200*g*, 10 min) at room temperature to obtain platelet-rich plasma (PRP). Then PRP were centrifuged (600*g*, 10 min) to isolate platelets, after centrifugation, we washed and resuspended platelets in HEPES buffer for the in vitro study. Freshly isolated neutrophils were obtained through density gradient centrifugation with Histopaque 1077 and Histopaque 1119 according to the manufacturer's instructions. Neutrophil purity (> 98%) and viability (> 98%) were determined by Wright-Giemsa staining and Trypan blue stain, respectively.

2.4. The stimulation, isolation and quantification of NETs

Purified neutrophils (1×10^6) isolated from patients with CRC or healthy controls were subsequently incubated for 3 h at 37 °C in 5% CO_2. For ex vivo studies, neutrophils were stimulated with 25 nM PMA at 37 °C in 5% CO_2 for 3 h. For in vitro studies, neutrophils from control individuals were treated with platelets from patients with CRC or healthy controls in a ratio of 1:50 for 3 h. For inhibition assays, platelets were pre-incubated with anti-HMGB1 (20 μg/mL) or anti-P-selectin (10 μg/mL) antibody for 30 min, and coincubated with neutrophils for 3 h. These PMA-stimulated neutrophils were further used to perform coagulation assays.

PMA-stimulated neutrophils were digested with 1 U/mL MNase for 15 min, and then the reaction was stopped with 5 mM EGTA. The supernatants (isolated NET structures) were collected by centrifugation at 1800 ×*g* for 10 min and stored at 4 °C until further use [25,26]. MNase is a nuclease that degrades link DNA in the junction region of nucleosomes and DNase degrades arbitrary sites of double or single-stranded DNA. The use of MNase was to isolate NET structures from neutrophils and to maximize the preservation of NET-specific DNA-protein structures, while DNase may completely degrade NET structures. Afterwards, we used Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) or a capture enzyme-linked immunosorbent assay (ELISA) to quantify cf-DNA or MPO-DNA complex respectively in the supernatant and plasma of newly diagnosed CRC patients as previously described [27].

2.5. Immunofluorescence

To further visualize NET formation, neutrophils were seeded into 24 wells (coated with poly-L-lysine) and stimulated with PMA (50 nM) at 37 °C in the presence of 5% CO_2 for 3 h. Afterwards, neutrophils were fixed with 4% paraformaldehyde, blocked with 2% BSA, and incubated with primary anti-NE antibody (1:100) overnight to visualize NET formation. Then the primary antibodies were detected with the secondary antibodies (1:100). DAPI (100 ng/mL) was used for DNA counterstaining. NETs were visualized by a fluorescence microscope (Leica, DM400B, Germany). The percentage of NETs was evaluated by counting the number of NET-releasing neutrophils out of the total number of neutrophils. Each test was performed in triplicate.

2.6. Analysis of PCA of integrated neutrophils and isolated NETs structures

One-stage recalcification time assay was used to evaluate the integrated PCA of neutrophils via a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany). Briefly, 100 μL suspensions of PMA-stimulated neutrophils were added to 20% pre-warmed microparticle-depleted plasma (MDP) from healthy controls in the presence of 3 mM calcium. In the inhibition assays, PMA-stimulated neutrophils were pretreated with DNase 1 (100 U/mL) for 20 min at 37 °C before added to plasma. After 3 min' incubation, 100 μL of preheated 25 mM $CaCl_2$ was added, and the coagulation time was recorded.

We quantified fibrin formation by turbidity as previously described [28]. We added PMA-stimulated neutrophils to re-calcified (10 mM, final) MDP (88% MDP, final) isolated from healthy donors. Fibrin formation was evaluated by turbidity at 405 nm through a SpectraMax 340PC plate reader. Each test was performed in triplicate. Thrombin and antithrombin (TAT) complex was detected by ELISA in control plasma incubated with isolated NETs structures in vitro as previously described [29]. Briefly, NET structures isolated from neutrophils were introduced in plasma at a final concentration of 20% and incubated for 10 min at 37 °C. The concentration of TAT complex was measured according to manufacturer's instructions. Inhibition assays were performed as described above.

2.7. PCA of healthy platelets stimulated with CRC NETs

Platelets from healthy subjects were stimulated with NETs derived from CRC patients for 30 min. Then PS positive platelets were analyzed and counted using a flow cytometry as previously described [22]. Coagulation time (CT), intrinsic FXa, extrinsic FXa and prothrombinase assays of platelets were also conducted as indicated [22]. For inhibition assays, NETs was pretreated with DNase I (100 U/mL) for 20 min or APC (100 nM) for 1 h before incubating with platelets.

2.8. ECs stimulation assays

HUVECs were incubated in growth media containing 20% of pooled serum obtained from healthy donors with or without NETs derived from CRC patients at room temperature for 24 h. For the inhibition assays, we cocultured NETs and HUVECs after pretreatment of NETs with DNase I (100 U/mL) for 20 min or APC (100 nM) for 1 h at 37 °C. At indicated time points, we centrifuged ECs and washed them twice with PBS before experiments. Then, we detected PS exposure by flow cytometer and performed CT and fibrin formation assays as previously described [28].

2.9. Confocal microscopy

To detect PS exposure on platelets, platelets with or without CRC-NETs were incubated with 4 nM Alexa Fluro 488-lactadherin. Platelets were washed twice to clear unbound antigen and evaluated immediately. Then, we used 488 nm emission line of a krypton-argon laser to excited the samples, and used narrow bandpass filters to restrict emission wavelength overlap. Images were obtained through Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany).

To detect the PS exposure on HUVECs cultured with NETs, cells were incubated with Alexa Fluor 488-lactadherin and Alexa Fluor 647-annexin V. In addition, ECs were incubated with FXa-EGRck-biotin (complexed to Alexa 647–streptavidin) and FVa-fluorescein-maleimide to visualize the the binding of FXa and FVa. To observe fibrin formation on ECs, cells were costained with Alexa Fluor 488-lactadherin and Alexa Fluor 647-conjugated fibrinogen. Then, we used 488 or 568 nm emission lines of a krypton-argon laser to excited the samples, and used narrow bandpass filters to restrict emission wavelength overlap. Images were captured through an LSM 510 System. A similarly labeled isotype matched control antibody was used to calculate the background signal.

2.10. Statistical analysis

Results are presented as mean ± standard deviation (SD) of at least triplicate measurements or medians with interquartile ranges (IQR). Statistical analysis was performed with Student's *t*-test, ANOVA or Mann-Whitney *U* test as appropriate. We used Pearson or Spearman correlation to evaluate the associations between NETs and coagulation parameters. All analyses were processed by GraphPad Prism software (Version 5.0) and SPSS 16.0 statistical software. $P < 0.05$ were considered to be significant in the results.

3. Results

3.1. Characteristics of patients and healthy controls

Sixty patients with CRC, including 12 at stage I, 15 at stage II, 19 at stage III, and 14 at stage IV along with 20 healthy controls were enrolled in this study. Table 1 shows the basic and clinical characteristics of CRC patients and healthy controls. CRC patients and healthy subjects showed no significant difference in age, sex and neutrophil counts. However, activated partial thromboplastin time (APTT) was significantly shorter and D-dimer levels was obviously higher in CRC patients than those in controls ($P < 0.05$ for both), suggesting a prothrombotic state in the patients with CRC.

3.2. Peripheral blood neutrophils from CRC patients are more prone to generate NETs

To assess the ability of neutrophils from CRC patients or healthy controls to generate NETs, we first observed extracellular co-localization of DNA and granule proteins by staining with DAPI and NE in neutrophils isolated from CRC patients and healthy controls (Fig. 1A-D). NETs showed an extracellular fiber-like structure protruding from neutrophils in samples from stage II, stage III and stage IV CRC patients (Fig. 1B-D). In contrast, such fibers were nearly absent in blood samples from healthy subjects (Fig. 1A) and patients with stage I CRC (data not shown). The percentage of NET releasing cells in CRC patients was higher than control subjects, and this difference reached significance in Stage II CRC patients (Fig. 1E, $P < 0.001$). In addition, we also found that the levels of cf-DNA and MPO-DNA complexes were markedly increased in CRC patients with stage II, III and IV compared with controls (Fig. 1F-G, all $P < 0.01$). These results indicate that neutrophils in CRC patients are more prone to release NETs and this tendency increases with tumor progression.

3.3. PCA of integrated neutrophils and isolated NETs derived from patients with CRC

To study the ability of PMA-stimulated neutrophils from different groups in promoting coagulation reactions, CT, TAT complexes levels and fibrin generation were evaluated in control plasma treated with equal levels of PMA-stimulated neutrophils or isolated NETs. We observed significantly shortened CT and enhanced fibrin formation after incubation of control plasma with PMA-treated neutrophils derived from stage II, III or IV patients compared with controls (Fig. 2A-B, all $P < 0.001$). Similarly, NETs released by neutrophils isolated from CRC patients with stage II, III or IV significantly increased TAT levels compared to control subjects (Fig. 2C, $P < 0.001$). These results suggested that PMA-treated neutrophils from CRC patients with stage II, III or IV contained more NET-releasing neutrophils and more isolated NETs, thus resulting in higher PCA than healthy controls. To further confirm the procoagulant role of NETs, we also performed inhibition assays with DNase I. Our results showed that treatment with DNase I significantly reduced the PCA of PMA-treated neutrophils and isolated NETs derived from CRC patients (Fig. 2A-C, all $P < 0.01$).

3.4. Platelets from CRC patients predisposes neutrophils to release NETs

To test whether activated platelets from CRC patients could induce NETosis, we performed a NET releasing experiment using control neutrophils mixed with either control platelets or CRC platelets. We found that in the presence of CRC platelets, more NETs were observed compared to control platelets (Fig. 3A-B). To further determine whether mediators or cell-cell interactions were involved in platelet-mediated NETosis in CRC, we performed inhibition assays. Immunofluorescence staining showed that anti-HMGB1 antibody significantly reduced NET formation, while anti-P-selectin antibody showed minimal effect on NETosis (Fig. 3C-D). We also found that NET-releasing neutrophils, cf-DNA and MPO-DNA complexes in the supernatants from healthy neutrophils significantly increased with the treatment of CRC platelets than with control platelets (Fig. 3E-G, all $P < 0.001$). Anti-HMGB1 antibody markedly decreased the levels of NETs markers, while anti-p-selectin antibody had no effect (Fig. 3E-G). These results indicate that NETosis may be induced, at least in part, through platelet-derived HMGB1 in CRC patients.

3.5. Increased PS exposure and PCA of platelets after NETs stimulation

To verify the potency of NETs derived from CRC patients to activated platelets, we evaluated PS exposure and PCA of control platelets after stimulation with CRC-NETs. Using flow cytometry, we found that PS exposure on platelets was significantly increased after co-culture with NETs from CRC patients (Fig. 4A-C, $P < 0.001$). Confocal microscopy images further showed greater PS exposure on platelets (arrow) and microparticles formation (arrowhead) after exposure to CRC-NETs (Fig. 4D-E). Because PS can provide a catalytic surface for the coagulation cascade, we next evaluated the PCA of platelets by CT, intrinsic FXa, extrinsic FXa and thrombin formation. We found the PCA of platelets stimulated with CRC NETs was markedly higher than those stimulated with control NETs (both $P < 0.001$, Fig. 4F-G), suggesting neutrophils from CRC produced more NETs than those from healthy controls and these increased NETs contributed to an enhanced platelet-associated PCA. Because recombinant APC could degrade extracellular histone and block its toxicity [30], we performed inhibition assays utilizing DNase I to dissolve the DNA scaffold and APC to inhibit histones. Treatment with DNase 1, APC, or both, caused a significant reduction in the PCA of CRC-NETs stimulated platelets, indicating that the DNA backbone and histones are both important for the cytotoxic effect of NETs (Fig. 4F, H, all $P < 0.001$).

3.6. NETs convert HUVECs to a procoagulant phenotype

To verify whether isolated NETs derived from CRC neutrophils have a cytotoxic effect on HUVECs, HUVECs were cultured with or without isolated NETs for 24 h. Our results showed that CRC-derived NETs induced an increase in PS exposure on ECs in a time-dependent manner compared to control-derived NETs (Fig. 5A). PS exposure on ECs was markedly inhibited by DNase I, APC, or both, suggesting NETs also exert a strong cytotoxic effect on the endothelium (Fig. 5B). We further evaluated whether PS exposure can support an elevated PCA of ECs. We used CT and fibrin formation assays to evaluate the integrated PCA of ECs. As showed in Fig. 5C-D, CRC-derived NETs induced shortened CT and massive fibrin formation of ECs. In addition, combine treatment with DNase I and APC exerted markedly stronger inhibitory effects than

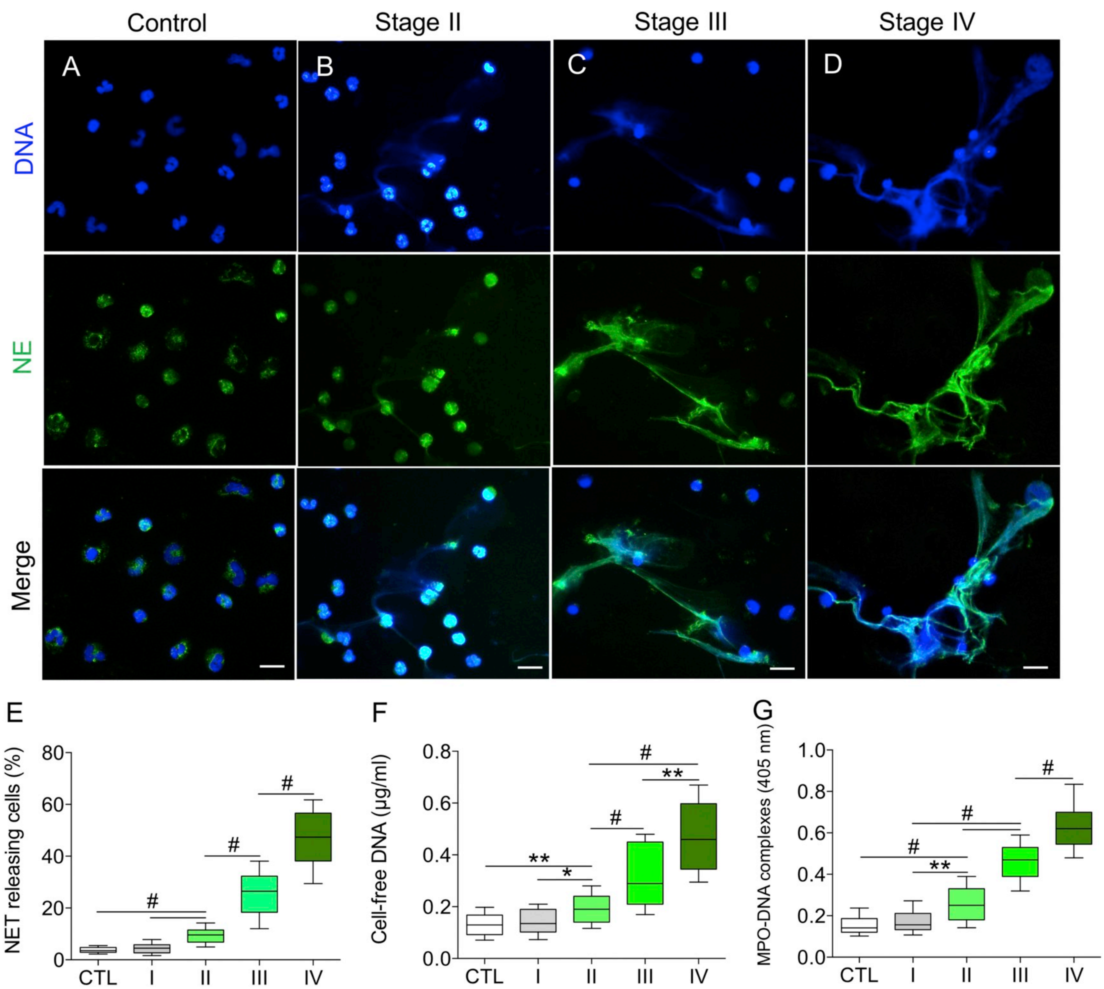

Fig. 1. Neutrophils from colorectal cancer patients are more prone to generate neutrophil extracellular traps. (A-D). Representative immunofluorescence pictures showing NETs derived from healthy subjects and CRC patients with stage II, III and IV. NETs were characterized by DNA (DAPI, blue) and neutrophil elastase (NE, green). Scale bar 20 μm. The percentage of NET-releasing neutrophils, cell free-DNA levels and MPO-DNA complexes of healthy controls and CRC patients are showing in E-G, respectively. Data are given as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). *$P < 0.05$, **$P < 0.01$, $^{\#}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DNase I or APC alone.

Using confocal microscopy, we found that after treatment of ECs with CRC-derived NETs, Alexa Fluor 488-lactadherin and Alexa Fluor 647-annexin V were costained on the filopodia and localized regions on ECs, suggesting more PS exposure on these locations (Fig. 5F). However, minimal changes were found on the margins of ECs treated with control-derived NETs (Fig. 5E). In addition, we observed an obviously colocalized region bound with FVa and FXa, indicating that CRC-NETs stimulated ECs had the ability to provide an active catalyze surface for the occurrence of coagulation reactions, most likely via exposed PS (Fig. 5G). Moreover, we also observed that fibrin networks distributed radially and densely along the filopodia of CRC-NETs treated ECs (Fig. 5H).

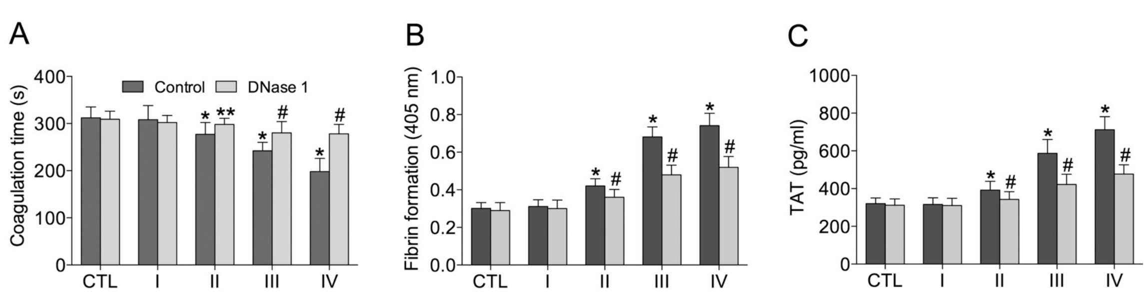

Fig. 2. NETs contribute to procoagulant activity in CRC patients. One-stage recalcification time and fibrin formation assay was performed using PMA-stimulated neutrophils from CRC patients and healthy controls. PMA-treated neutrophils from CRC patients shortened coagulation time (A) and augmented the peak turbidity of control plasma generation of fibrin (B). (C) Isolated NETs centrifuged from PMA-treated neutrophils of CRC patients increased the amount of TAT complexes in the control plasma after re-calcification. PCA of PMA-treated neutrophils and isolated NETs were in parallel with cancer progression and reached significance in stage II patients compared to healthy subjects. DNase 1 significantly reduced the effect of PMA-stimulated neutrophils or isolated NETs on CT, TAT and fibrin formation. Results are expressed as means ± standard deviation. *$P < 0.001$ vs control group, **$P < 0.01$ vs no inhibitor group, $^{\#}P < 0.001$ vs no inhibitor group.

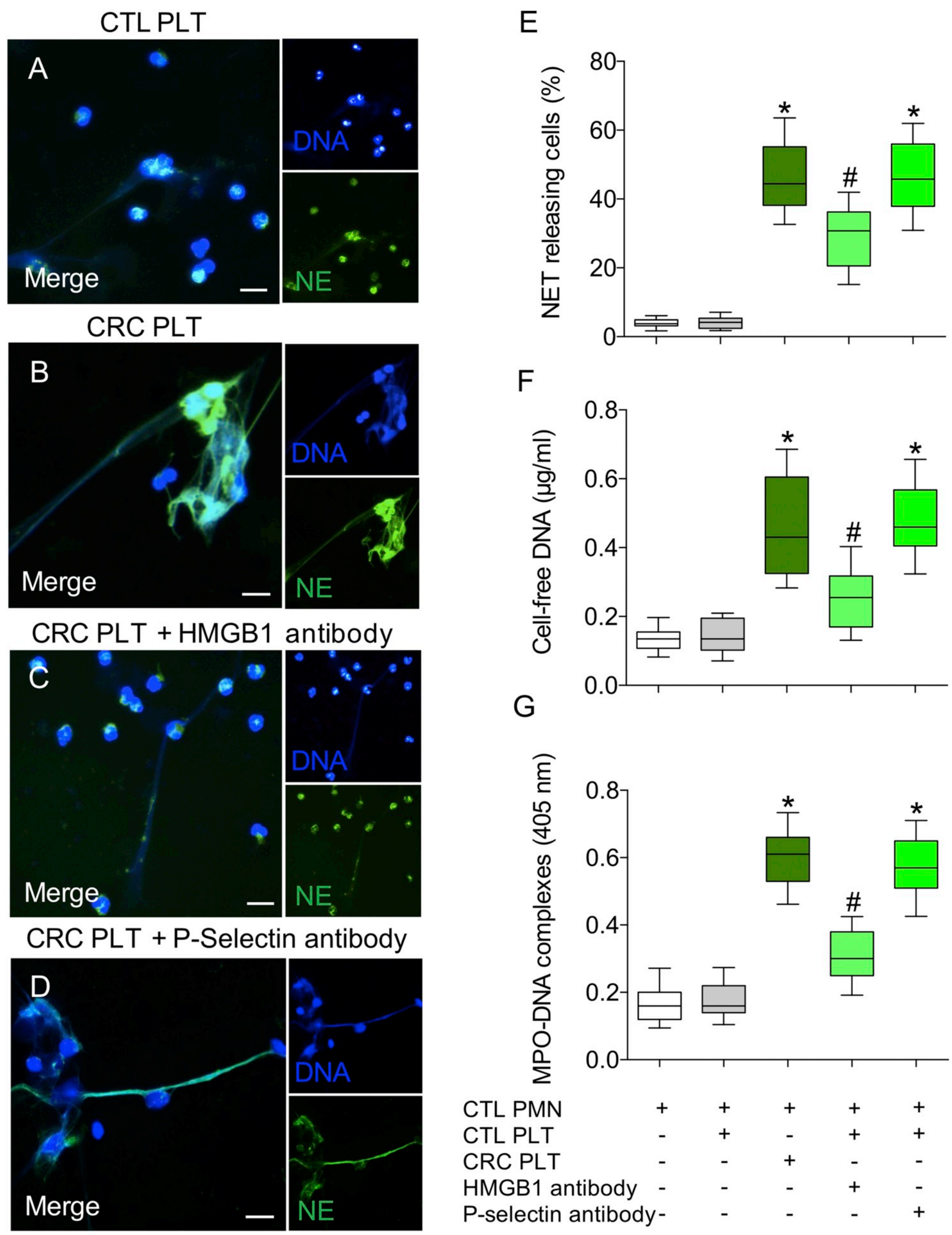

Fig. 3. Platelets from patients with CRC primes control neutrophils to release NETs. Representative immunofluorescence microphotographs showing NETs (DAPI, blue; NE, green) of control neutrophils treated by healthy platelets, CRC platelets with or without anti-HMGB1 antibody and anti-P-selectin antibody (A-D). Scale bar 20 μm. The percentage of NETs releasing cells, the levels of cell-free DNA and MPO-DNA complexes were measured for illustrating the potency of activated platelets to induce control neutrophils to generate NETs (*E*-G). Data are given as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). *P < 0.001 vs control group, #P < 0.001 vs CTL PMN + CRC PLT group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7. Correlation of NETs release and risk of VTE in patients with CRC

Circulating levels of cf-NDA are positively correlated with plasma levels of TAT (r = 0.781, P < 0.001) and D- dimer (r = 0.873, P = 0.001) (Fig. 6A-B). We also found that circulating levels of MPO-DNA complex correlate positively with the plasma levels of TAT (r = 0.579, P < 0.001) and D-dimer (r = 0.679, P < 0.001), respectively (Fig. 6C-D). These data strongly suggest that NETs contribute to coagulation activation and high risk of VTE in CRC patients.

4. Discussion

We have made several observations in our present study. First, we showed that neutrophils derived from CRC patients display an enhanced ability to release NETs in a stage-dependent manner, reaching significance in stage II CRC patients compared with heathy controls. CRC-derived NETs contribute to significantly shortened CT, markedly enhanced TAT generation and fibrin formation, which could be inhibited by DNase 1. Second, platelets from CRC patients are potential activators of NETosis. While, increased NETs also induce procoagulant platelet formation, producing a vicious cycle between activated platelets and neutrophils. Third, NETs converted ECs to a procoagulant phenotype, which could be partly inhibited by APC and DNase 1. This suggests that there may be a complex interplay among these intravascular cells that synergistically contribute to PCA in CRC. Lastly, the levels of NETs positively correlated with TAT and D-dimer in CRC patients, indicating the potential predictable role of NETs in CRC.

An increase in peripheral neutrophil counts, intratumoral neutrophils or a high neutrophil to lymphocyte ratio have been linked to poor prognosis and outcome in patients with advanced CRC [31–33], however, the exact role of neutrophils in CRC remains to be explored. In our study, we found that neutrophils derived from CRC patients were more prone to release NETs compared to those from healthy subjects. In addition, NETs formation increased in parallel with the development of TNM stage, and this increase reached statistical significance in stage II/III/IV patients compared to healthy controls. These results suggest that NETs may be involved in the progression of CRC. Moreover, NETosis was also found to be associated with the development of malignancy in gastric patients and tumor-bearing mice [34,35]. Interestingly, while one study found pro-apoptotic effects after exposure of Caco-2 cells (a

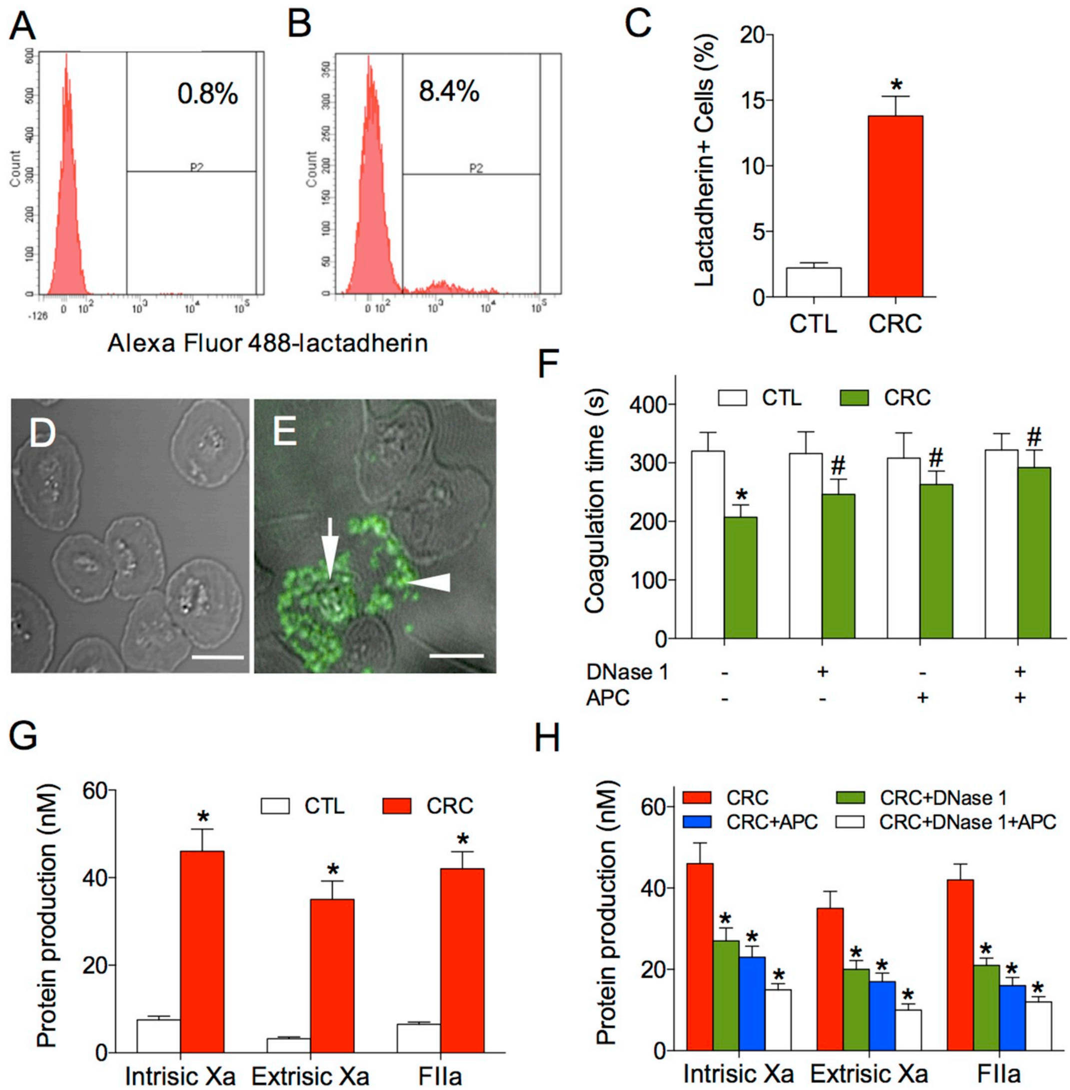

Fig. 4. NETs derived from CRC elicit phosphatidylserine exposure and promote PCA of control platelets. PS exposure on platelets treated with NETs from healthy neutrophils (A) or CRC neutrophils (B) were measured with Alexa 488-lactadherin by flow cytometry (C). Confocal microscopy images showed quiescent platelets (D) and activated platelets positive for lactadherin staining (E, arrow) with microparticles (E, arrowhead). Bars represent 5 μm. Coagulation time (F), intrinsic/extrinsic FXa and thrombin (G) as well as inhibition assays (H) were evaluated. Results are expressed as means ± standard deviation. *P < 0.001 vs control group in panels C, F, G, #P < 0.001 vs DNase 1 (−) APC (−) group, *P < 0.001 vs CRC group in panels H.

human colon cancer cell line) to NETs in vitro [16], another study reported that NETs enhanced the adhesion, migration, invasion and proliferation of cultured MC38 cells (a murine colon cancer cell line) and suggested a direct cancer-promoting role of NETs in metastatic CRC patients undergoing curative-intent liver resection and corresponding mouse models [36]. This discrepancy may be explained either by the different cell line/in vitro and in vivo environment that were studied, or by the existence of distinct subsets of tumor-associated neutrophils with pro-/anti-tumor properties. Thus, it would be very interesting to further study the precise role of NETs in CRC.

Recently, the discovery of highly thrombogenic NETs as key in vivo propagators of thrombosis sparked interest in their involvement in the pro-thrombotic state of malignancy. In the present study, our results have shown that after the stimulation of PMA, neutrophils and isolated NET structures from CRC patients contribute to significantly increased PCA manifesting as increased TAT complexes, fibrin formation and shortened CT compared to healthy controls, suggesting a procoagulant role of NETs in CRC. It has been proposed that NETs act as a scaffold for hemostatic cells, activate the contact pathway of coagulation cascade and degrade natural coagulation inhibitors [24]. Moreover, NETs have been identified as important constituents of various thrombi such as from mice models of DVT, patients with ischemic stroke or acute myocardial infarction, and abdominal aortic aneurysm patients [37–40]. Notably, it has been established that neutrophils isolated from mammary tumor-bearing mice are sensitized to release NETs, which are involved in spontaneous thrombi formation in late-stage disease [41]. Furthermore, in a spontaneous small intestinal tumor model, complement C3a resulted in NETosis and the induction of coagulation, which in turn stimulated tumorigenesis, supporting a pro-tumor role of procoagulant NETs in cancer [42]. In our studies, we have shown that NETs are procoagulant, and may therefore promote tumor growth through local thrombin generation [43]. Considering the evidence that NETs exacerbate multiple aspects of cancer-associated pathologies, which account for the absolute majority of cancer-associated deaths, we presume that NETs are very likely to play a detrimental role in the tumor biology of CRC.

Given the potential role of platelets in priming neutrophils toward NETosis in infective, non-infective, and cancer pathologies, the existence of activated platelets in CRC patients prompted us to evaluate their role in NETs formation. Host cells, including platelets, can become

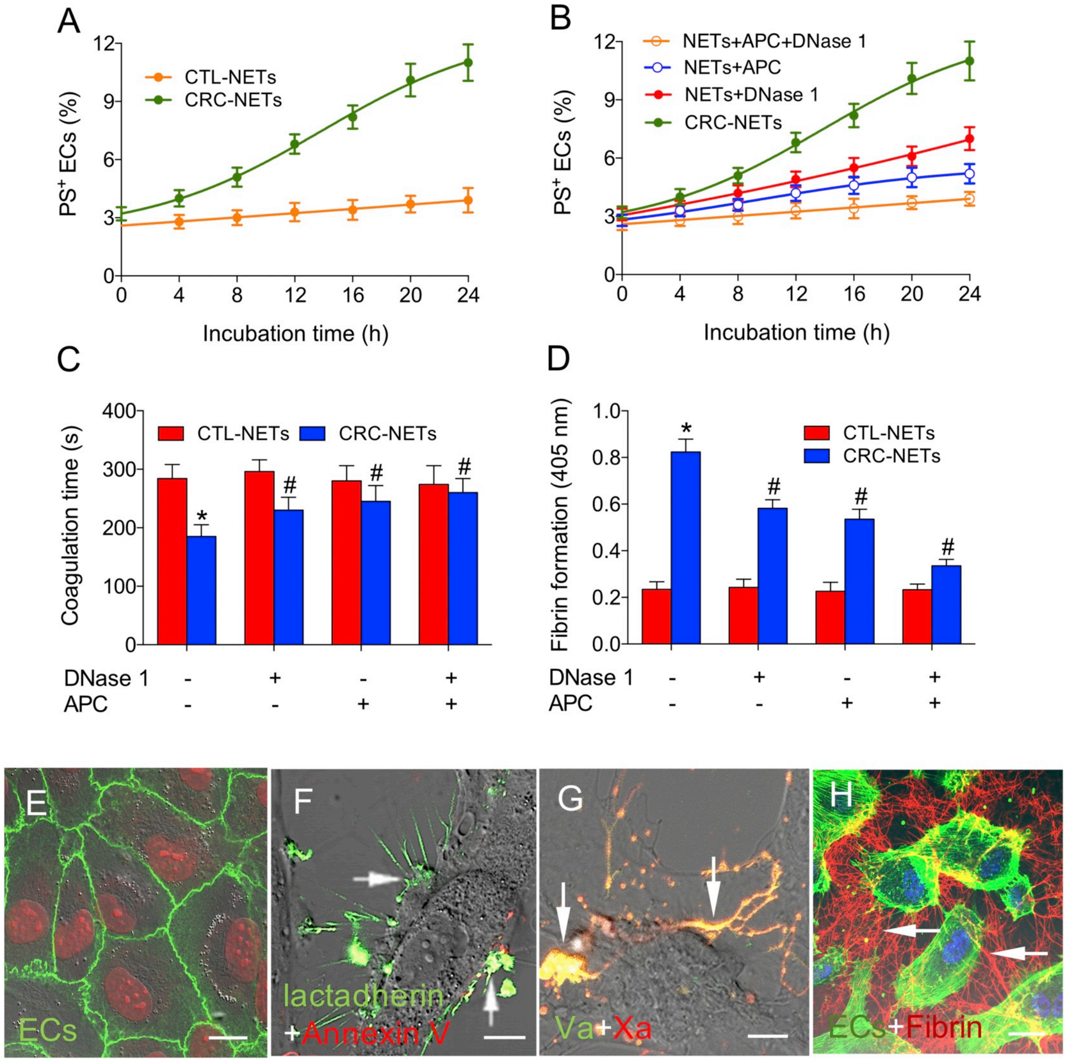

Fig. 5. NETs induce endothelial cells into procoagulant phenotype. HUVECs were incubated with isolated NETs derived from CRC patients or healthy subjects for 24 h. PS exposure on ECs treated with NETs were shown in Fig. 5A. (B) In the inhibition assays, PS exposure on ECs stimulated with NETs were assessed with or without DNase 1 or APC. Coagulation time, fibrin formation assays and inhibition assays of ECs treated with NETs were shown in Fig. 5C-D. (E, F) Representative confocal pictures showed morphology of HUVECs treated with CTL- or CRC-derived NETs. ECs retracted and exposed PS positive filopods, which costained with lactadherin (green) and annexin V (red). (G) FVa (green) and FXa (red) were costained on the filopods (arrow) and localized regions (arrow) of retracted ECs. (H) Massive fibrin fibrils distributed radially along extended filopodia (arrow), forming a dense fibrin network. Scale bar 5 mm in panels E-H. Results are expressed as means ± standard deviation. *P < 0.001 vs control group, #P < 0.001 vs DNase 1(−) APC (−) group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

'educated' by tumor cells in the tumor environment [44]. Interestingly, a recent study found that polyphosphate (polyP) released from CD68 positive mast cells induced NETosis in CRC, although polyP exists most abundantly in platelets [45]. In our study, we showed that the addition of CRC platelets markedly enhanced NETs formation in neutrophils which could be inhibited by the anti-HMGB1 antibodies, indicating the potential effects of 'educated' platelets on NETosis. Besides platelets, tumor cells can directly induce NETosis in cancer patients by secreting factors such as granulocyte colony-stimulating factor (G-CSF) and interleukin-8 (IL-8) [46,47]. Moreover, danger associated molecular patterns (DAMPs), exosomes and hypoxia-inducible factors (HIF) released by tumor cells or tumor microenvironment could also attract neutrophils and induce NETosis [48–50]. Future studies should further clarify the exact mechanisms that induce NETosis in CRC.

In our study, we also demonstrated that NETs from CRC patients trigger PS exposure on platelets and convert them to a procoagulant phenotype. Moreover, we found an increase in PMPs after stimulation of platelets with CRC-derived NETs, which may enhance PCA in collaboration with PS positive platelets. These effects are likely mediated by NET-associated proteases or cationic proteins, such as defensins and histones, rendering the NETs highly procoagulant and with a capacity to activate and aggregate platelets [51]. Additionally, NETs were found to sequester platelets and trap tumor-derived microvesicles/exosomes, enhancing their effects on thrombosis [52]. Thus, we conclude that CRC-primed platelets appear to induce NETosis, creating a proinflammatory environment and stimulating additional platelets, constituting a vicious cycle that ultimately promotes PCA and thrombosis formation.

Endothelium injury is a pivotal pathophysiologic process in thrombosis formation. In our study, we indicated that NETs from CRC patients induced PS exposure on HUVECs and elicited them to a procoagulant phenotype. Additionally, our confocal results offer visible and powerful evidence that after treatment with CRC-NETs, ECs exposed more PS and provided biological surface for the binding of prothrombinase on the filopods and localized regions. Moreover, fibrin also selectively distributed on the retracted margins of ECs, which is similar

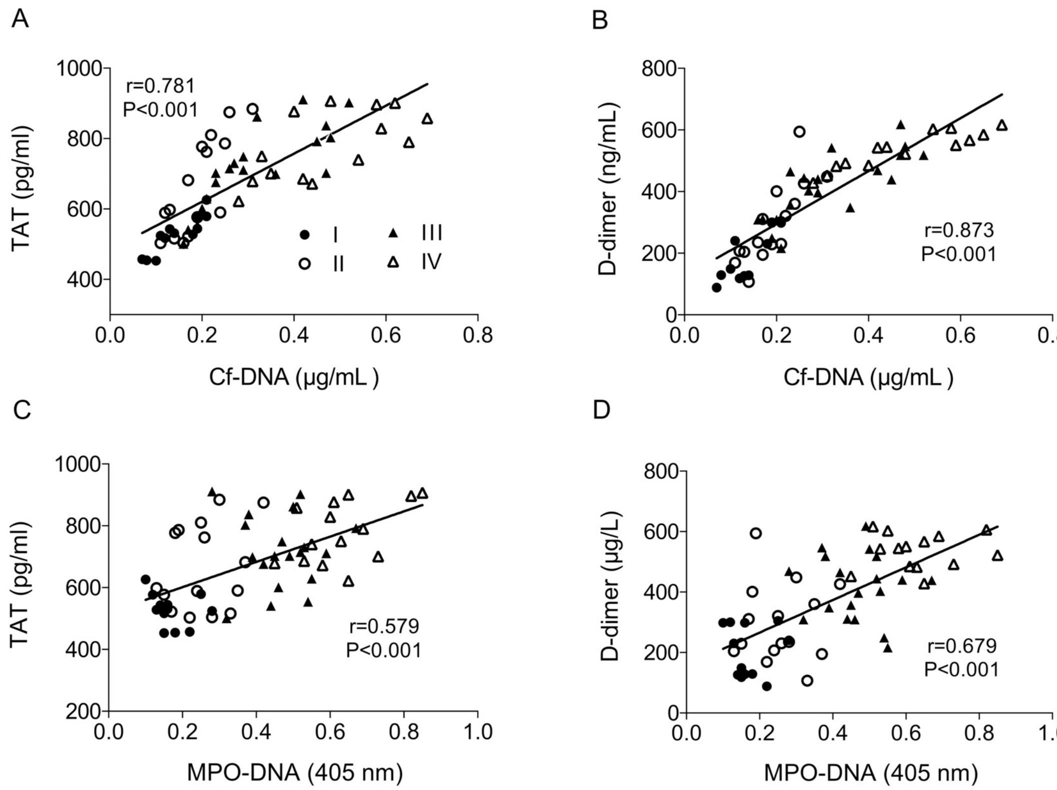

Fig. 6. Correlation between biomarkers of NETs and coagulation parameters of CRC patients. TAT complexes were strongly correlated with cell-free DNA (A) or MPO-DNA (C) in CRC patients. D-dimers were markedly correlated with cell-free DNA (B) or MPO-DNA (D) in CRC patients. r values were determined using Spearman rank correlation. $P < 0.05$ was considered significant.

to the binding of FVa and FXa. Previous study showed that NETs exert a cytotoxic role on endothelium and epithelium cell death through histones [53], while APC cleaves and detoxifies extracellular histones and was shown to inhibit PMA-induced NETosis via several intracellular signaling pathways [30,54]. Our results also showed that combine treatment with DNase I and APC protect ECs from the cytotoxic effects of NETs and decrease the resulting PCA, indicating their promising role in dampening thrombogenicity in CRC.

Our findings provide preliminary evidence that administration of DNase 1, which dissolves NETs due to their extracellular DNA content, inhibited NETs-related PCA and may thus reduce the risk of thrombogenesis in CRC patients. Consistent with these results, systemic treatment with DNase 1 abrogated the development of metastasis in a mice model of CRC [35]. Moreover, DNase 1 severely impairs the development of DVT in mice and attenuates the PCA in tumor-bearing mice [40,55]. Additionally, DNase 1 has been approved for the treatment of cystic fibrosis as a safe, low-cost, FDA-approved drug to clear extracellular DNA in the lungs [56]. Thus, using DNase 1 in CRC patients with high CAT risk may become a new therapy to prevent thrombosis. Since activated platelets and neutrophils form a mutual positive-feedback loop, treatments that interfere with this loop may represent a promising avenue for prevention or treatment of CRC-related thrombosis.

To date, there are already some standard models to evaluate and stratify the risk of CAT such as the Khorana model and the Ay model [9]. While these models represent a significant step forward, they still lack specificity and do not capture the vast majority of cancer patients that are going to develop VTE during the course of their disease, including those with CRC. An improvement in risk stratification and personalized therapy exclusively for patients with high thrombosis risk that identifies novel predictors is needed. In this study, we have shown that the biomarkers of NETs were positively correlated with the levels of procoagulant biomarkers TAT and D-dimer, suggesting a predictive role of NETs in the prothrombotic state in CRC patients. Our results may provide new predictors or standards to better define the thrombotic risk and improve current risk assessment models in CRC patients, though larger studies are needed to verify these results.

In summary, we have shown that NETs are involved in the progression of CRC and act as potential agonists in CRC-related hypercoagulability. In addition, the improved understanding of the interactions between neutrophils, platelets and ECs and their potential contribution to PCA may help explain the hypercoagulable state in CRC. Our work also indicates that closer monitoring of the levels of NETs and a deeper understanding of CRC-related thrombogenicity would be of particular importance in the fight against CAT. We propose that the rapid developments in the field of NETosis may provide new targets to both prevent and combat the thrombotic consequences of cancer.

Declaration of Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Y.Z. designed the research, performed some experiments, analyzed data, made the figures and wrote the paper; J.S. obtained funding, designed the study, performed some experiments and revised the manuscript; J.K., Y.B. and J.Z. analyzed some results and provided partial funding support; Z.D., C.W., M.Y. and X.Z. performed some experiments and analyzed some results; J.D., Y.L., H.J. and V.A.N. revised the manuscript.

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