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ORIGINAL RESEARCH

Neutrophil Extracellular Traps Enhance Procoagulant Activity and Predict Poor Prognosis in Patients With Metastatic Breast Cancer

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Objective: Neutrophil extracellular traps (NETs) are associated with poor prognosis and an increased risk of venous thromboembolism (VTE) in metastatic breast cancer (MBC). This study aims to determine whether NETs promote hypercoagulability and if NETs and plasma hypercoagulability markers are biomarkers of survival in MBC.

Methods: Circulating levels of neutrophil extracellular trap (NET) markers and hypercoagulability markers (TAT, fibrinogen, and D-dimer) were assessed in 112 MBC patients before treatment, compared to 55 healthy controls. Stratified by NET levels and plasma TAT, fibrinogen, and D-dimer, the correlation with overall survival was analyzed. The NET procoagulant activity was evaluated using fibrin and purified coagulation complex production assays, and by measuring coagulation time (CT).

Results: MBC patients exhibited significantly elevated plasma NET levels compared to healthy controls (all $P<0.05$), circulating MPO-DNA and NE-DNA levels were positively correlated with plasma TAT, fibrinogen, D-dimer, CT, FVIIIa, and platelet (PLT) counts. Additionally, we observed a significant increase in NETs formation in control neutrophils exposed to MBC plasma compared to those exposed to control plasma. NETs from MBC neutrophils significantly increased the potency of control plasma to generate thrombin and fibrin, effects that were notably attenuated by DNase I. Plasma TAT and D-dimer levels were significantly higher in MBC patients who died within three years post-recruitment compared to those who survived beyond three year. Plasma TAT and D-dimer were inversely correlated with survival. High plasma levels of MPO-DNA were associated with significantly worse overall survival (HR: 2.445, 95% CI: 1.255–4.762, $P=0.007$). MBC patients with both high D-dimer and high MPO-DNA had significantly reduced survival (HR: 2.450, 95% CI: 1.332–4.488, $P=0.002$).

Conclusion: Our results highlight the increased release of NETs in MBC patients and reveal that NET formation enhances hypercoagulability and cancer progression. Targeting NETs may be a potential therapeutic strategy to inhibit MBC progression and mitigate thrombotic complications in MBC.

Keywords: neutrophil extracellular traps, procoagulant activity, hypercoagulability, metastatic breast cancer, prognosis

Introduction

It is well-established that numerous malignant diseases are associated with a hypercoagulable state.[1] Cancer-associated venous thromboembolism (VTE) is particularly prevalent in cancer patients and represents a leading cause of mortality.[2] Specifically, women with breast cancer exhibit a 3- to 4-fold increased risk of developing VTE compared to their cancer-free counterparts of the same age.[3] Moreover, the prognosis for cancer patients who develop VTE is significantly worse than for those without VTE, suggesting that VTE serves as a surrogate marker for malignancy.[4] Studies have shown that plasma concentration of the thrombin-antithrombin III (TAT) and D-dimer levels in early breast cancer patients are

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significantly higher than those in healthy controls.[5] Circulating fibrinogen, which can be converted to fibrin by thrombin, is crucial for distinguishing breast cancer from benign breast conditions[6] and increases as the disease progresses.[7]

Neutrophil extracellular traps (NETs) have been linked to the formation of VTE and may also play a significant role in cancer-associated thrombosis.[8,9] NETs consist of fibers of decondensed chromatin (DNA and histones) coated with antimicrobial proteins such as myeloperoxidase (MPO), cathepsin G, and neutrophil elastase (NE). These fibers are released by neutrophils in response to extracellular pathogens, including bacteria, protozoa, and fungi.[10] The chromatin fibers form a network that traps pathogens, preventing their dissemination within the host and facilitating their elimination due to their antimicrobial properties.

The involvement of NETs in thrombosis was demonstrated when citrullinated histone H3 (CitH3) was identified in the thrombi of patients with VTE, and it was shown that dissociating NETs could promote thrombus lysis.[11] During the process of NETosis, a network of chromatin is extruded through the membranes of activated neutrophils, which can trap and activate platelets and coagulation factors, potentially initiating thrombosis.[12,13] Indeed, fuchs and colleagues have shown that NETs provide a scaffold for the activation and aggregation of platelets and red blood cells, which contribute to the formation of the thrombus's red portion.[14] This data is further supported by experiments in which mice injected with DNase-I, a DNA-cleaving nuclease, showed degradation of NETs and a lack of platelet aggregate formation.[14] This suggests that NETs form an essential pro-thrombotic substrate necessary for thrombosis.

NETs can support a pro-tumoral role, being implicated in tumor growth, metastasis, and the awakening of dormant cancer cells. This involvement may explain why NETs are considered markers of poor prognosis in cancer patients, particularly those with terminal disease.[15] Studies have shown that plasma from treatment-naive patients with lung or upper gastrointestinal (GI) adenocarcinoma exhibits elevated NETs expression (measured as myeloperoxidase, MPO) compared to plasma from healthy individuals. This elevation in NETs was more pronounced in patients with advanced lung cancer compared to those with less advanced diagnoses.[16] Similarly, higher NETs expression has been observed in patients with early-stage head and neck cancer compared to healthy control participants of the same age.[17] In breast cancer, Rivera-Franco MM et al[18] demonstrated that NETs levels increase in proportion to the disease stage, with higher levels of neutrophil elastase- deoxyribonucleic acid (NE-DNA) complexes observed in regional and metastatic disease. However, the number of cases included in these studies is relatively small, and the complex relationship between plasma NETs, procoagulant activity, and prognosis in metastatic breast cancer (MBC) patients requires further investigation.

In this study, we hypothesized that NET formation by peripheral neutrophils is associated with a hypercoagulable state in MBC patients. To test this hypothesis, we evaluated NET production by circulating neutrophils, determined the frequency of NET-producing neutrophils, measured coagulation time, and assessed thrombin/fibrin formation. We also analyzed the correlations between NET marker levels and the hypercoagulable state. Furthermore, we investigated the association between NETs and serum markers of coagulation function with prognosis in MBC patients. Our results have the potential to inform future drug development efforts targeting these coagulation mechanisms in MBC patients.

Materials And methods

Patients and Study Design

A prospective cohort of MBC patients (n=112) was recruited at Guangxi Medical University Cancer Hospital between July 2017 and June 2019, with follow-up extending until July 2023. All patients had either a new diagnosis of metastatic disease or new evidence of clinical or radiological disease progression. Patients receiving anticoagulation therapy or those with a known non-breast cancer diagnosis within the last five years were excluded. The study utilized patient files, including routine laboratory results and medical histories. Additionally, a control group of 55 healthy, age-matched individuals was included.

Blood Sampling

Peripheral blood samples were collected using 0.109 mol/L sodium citrate (Becton Dickinson, San Jose, CA, USA). Plasma was separated by centrifuging whole blood at 1550 ×g for 15 minutes, then aliquoted and stored at −80°C. Control plasma from healthy adults was also included in the analysis.

For TAT, fibrinogen, and D-dimer analysis, citrate tubes were centrifuged within 60 minutes of blood collection using a precooled centrifuge at 4°C for 20 minutes at 2500 g. Plasma was transferred to a clear tube, leaving the cell pellet behind, and centrifuged again at 4°C for 20 minutes at 2500 g. The resulting platelet-poor plasma was aliquoted into cryovials and stored at −80°C until analysis, with the final 0.5 mL in the clear tube discarded.

All analyses of whole blood and plasma samples were conducted by analysts trained in good clinical practice (GCP) who were blinded to patient outcome data.

TAT, Fibrinogen, and D-Dimer Analysis

For TAT detection, 2 mL of sodium citrate anticoagulated whole blood was centrifuged at 3000 rpm at room temperature for 15 minutes (Universal, Germany). After centrifugation, the serum was inspected for hemolysis. If hemolysis was present, the sample was discarded and a new blood sample was collected. If the serum appeared cloudy, it was sealed and subjected to high-speed centrifugation (F2-MC Beckman, USA) at 12000 rpm for 30 minutes. Post-centrifugation, the serum was separated into two layers, and the lower clear serum was used for analysis. Clear and impurity-free serum was analyzed using a fully automatic chemiluminescence immunoanalyzer (Wondfo Shine i2900, China).

Plasma fibrinogen and D-dimer levels were measured using an automatic hemostatic detection system (SYSMEX CA-7000, Japan) in the clinical laboratory of the Cancer Hospital of Guangxi Medical University. All detection reagents were original, instrument-compatible, and used within their validity period. Instruments were regularly calibrated by the manufacturer, and all testing procedures followed the standard operating procedures (SOP) of the instruments.

Measurement of NET Formation

As markers for NETs, circulating myeloperoxidase-deoxyribonucleic acid (MPO-DNA) complexes, nucleosomes, and NE were measured in patient plasma. Using enzyme-linked immunosorbent assay (ELISA), plasma levels of MPO-DNA complexes, neutrophil elastase- deoxyribonucleic acid (NE-DNA) complexes, and CitH3 were analyzed as previously described.[19,20]

Immunofluorescence

To further visualize NETs formation, we employed immunofluorescence microscopy as described in previous research. Neutrophils, both stimulated and unstimulated, were fixed with 4% paraformaldehyde and blocked using 2% BSA. The fixed and blocked samples were stained overnight with a mouse anti-MPO monoclonal antibody (1:100, Wanleibio, Shenyang, China) and then visualized with a secondary antibody (1:100, AF488-rabbit anti-mouse) using fluorescence microscopy (Leica, DM400B, Germany). DAPI (100 ng/mL) was used for DNA counterstaining. NET formation was quantified by calculating the percentage of neutrophils producing NETs. All experiments were performed in triplicate.

Procoagulant Activity And fibrin Formation Assays

We isolated neutrophils from both control and MBC patients and treated them with 25 nM PMA for 4 hours, after which NETs were collected as described previously. To assess thrombin generation, we used a TAT approach as outlined by Stakos et al (2015). TAT was measured in control plasma mixed with NETs (each 20%) following the manufacturer's instructions (BlueGene, Shanghai, China). In inhibition assays, NETs were treated with either 100 U/mL DNase I or 40 μg/mL anti-tissue factor (TF) at 37°C for 20 minutes before being mixed with plasma samples. After a 3-minute incubation, samples were mixed with 100 μL warmed $CaCl_2$ (25 mm) and the time to fibrin strand formation was recorded.

To further assess the procoagulant role of NETs, we performed a fibrin generation test as previously described.[21] Fibrin (clot) formation was monitored by measuring the optical density at 405 nm using a Spectramax microplate reader at 37°C for 1 hour. A one-stage recalcification approach was used to assess coagulation time via a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany).[22]

Statistical Analysis

GraphPad Prism v15.0 and SPSS v26.0 were utilized for all statistical analyses. Continuous variables were expressed as means ± standard deviation (SD). Student's t-tests were employed for comparing quantitative values, while multiple comparisons were conducted using the least significant difference (LSD) method. The Kruskal–Wallis test was used to assess ordered variables. For variables not conforming to a normal distribution, medians with interquartile ranges (IQR) were reported. Spearman's rank correlation was employed to explore relationships between specific continuous variables.

Overall survival (OS) was defined as the time from study entry to death or last follow-up (with a minimum follow-up of 65 months), up to July 2023. Survival differences between patient groups were assessed using log-rank (Mantel-Cox) regression. A significance threshold of $P < 0.05$ was applied throughout the study.

Results

Patient Characteristics

Of the 112 MBC patients recruited, the mean age was 44 years (range 33–68). Among them, 47.3% were estrogen receptor (ER) positive and Her2 negative, 22.3% were ER positive and Her2 positive, 16.1% were ER negative and Her2 positive, and 14.3% were triple negative. Additionally, 19.6% had bone metastases only. By the 3-year follow-up mark, 36 patients had died. At the final follow-up in July 2023, a total of 43 patients had died, with a median survival of 28 months (range 7–67 months). We also enrolled 55 healthy controls. Table 1 summarizes the patient characteristics. The average age of the MBC patients was similar to that of the control group.

Table 1 Characteristics of Metastatic Breast Cancer Patients at Diagnosis

Characteristic	MBC Cohort (N = 112, %)	Healthy Controls (N = 55)
Age at baseline–years		
Mean	44	42
Range	33–68	30–57
Hypertension		
No	104 (92.9)	
Yes	8 (7.1)	
Diabetes		
No	106 (94.6)	
Yes	6 (5.4)	
Hyperlipidemia		
No	94 (83.9)	
Yes	18 (16.1)	
Molecular subtype		
ER+, Her2−	53 (47.3)	
ER+, Her2+	25 (22.3)	
ER−, Her2+	18 (16.1)	
Triple negative	16 (14.3)	
Metastatic sites		
Bone only	22 (19.6)	
Locally advanced	10 (8.9)	
Single visceral site		
Liver	30 (26.8)	
Lung	51 (45.5)	
Brain	5 (4.5)	
Other	2 (1.8)	
Multiple visceral sites (≥2)		
No	49 (43.7)	
Yes	63 (56.3)	

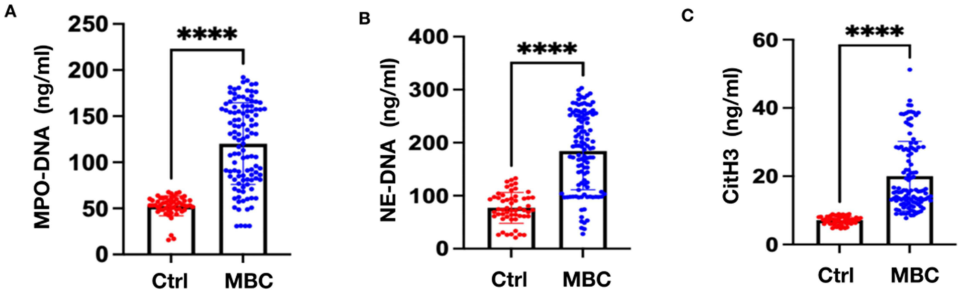

Figure 1 MBC patients exhibit increased circulating NETs. The expression of NETs markers MPO-DNA (**A**), NE-DNA (**B**), and CitH3 (**C**) in plasma of MBC patients (n=112) and healthy control patients (n=55) were examined by flow cytometry and these NETs markers were significantly elevated in MBC patients. **** P<0.0001.

MBC Patients Exhibit Increased Circulating NETs and Associated With Increased Coagulation Activity

NET formation in the plasma of MBC patients was measured. Plasma levels of MPO-DNA complexes, CitH3, and NE-DNA were significantly higher in MBC patients compared to controls (all $P < 0.05$) (Figure 1A–C). Additionally, circulating MPO-DNA and NE-DNA levels were positively correlated with plasma TAT, fibrinogen, D-dimer, CT, FVIIIa, and platelet (PLT) counts (Table 2). These findings suggest that NETs are associated with increased coagulation activity and a heightened risk of VTE in MBC patients.

MBC Plasma Primes Neutrophils to Form NETs

To further investigate whether the circulating environment of MBC induces NETs release from neutrophils, we examined the effect of MBC patient plasma on NETs formation. This was identified by immunofluorescence microscopy through the colocalization of DAPI and MPO, as well as CitH3 ex vivo. We observed a significant increase in NETs formation in control neutrophils exposed to MBC plasma compared to those exposed to control plasma (Figure 2A).

Following stimulation with MBC plasma, control neutrophils exhibited a significant increase in NETs formation (measured by MPO-DNA quantification) compared to baseline levels. However, this increase was not significant when compared to control neutrophils stimulated with plasma from healthy individuals (Figure 2B). The frequency of NET-releasing neutrophils was also higher when control neutrophils were treated with MBC patient plasma than when treated with CTR plasma, confirming that circulating factors in MBC promote neutrophil NET release (Figure 2C).

Procoagulant Activity of NETs Derived From patients with MBC

To investigate the procoagulant activity of NETs in MBC patients, we evaluated plasma thrombin levels and fibrinogen potency in control plasma treated with NETs. TAT levels increased significantly after the addition of NETs isolated from MBC patients to control plasma compared to baseline (Figure 3A). However, TAT levels were not significantly elevated after incubation with NETs released by neutrophils from healthy individuals (Figure 3A). Similarly, NETs from MBC

Table 2 Correlation Between NETs Markers and Hypercoagulable State in MBC

Variables	MPO-DNA (ng/mL)		NE-DNA (ng/mL)	
	r^2	*p*	r^2	*p*
TAT (pg/mL)	0.631	<0.001	0.616	<0.001
Fibrinogen (g/l)	0.335	0.004	0.325	<0.001
D-dimers (ng/mL)	0.588	<0.001	0.624	<0.001
CT (s)	0.393	<0.001	0.295	0.002
FVIIIa (U/mL)	0.557	<0.001	0.435	<0.001
PLT (10^9/L)	0.290	0.002	0.213	0.024

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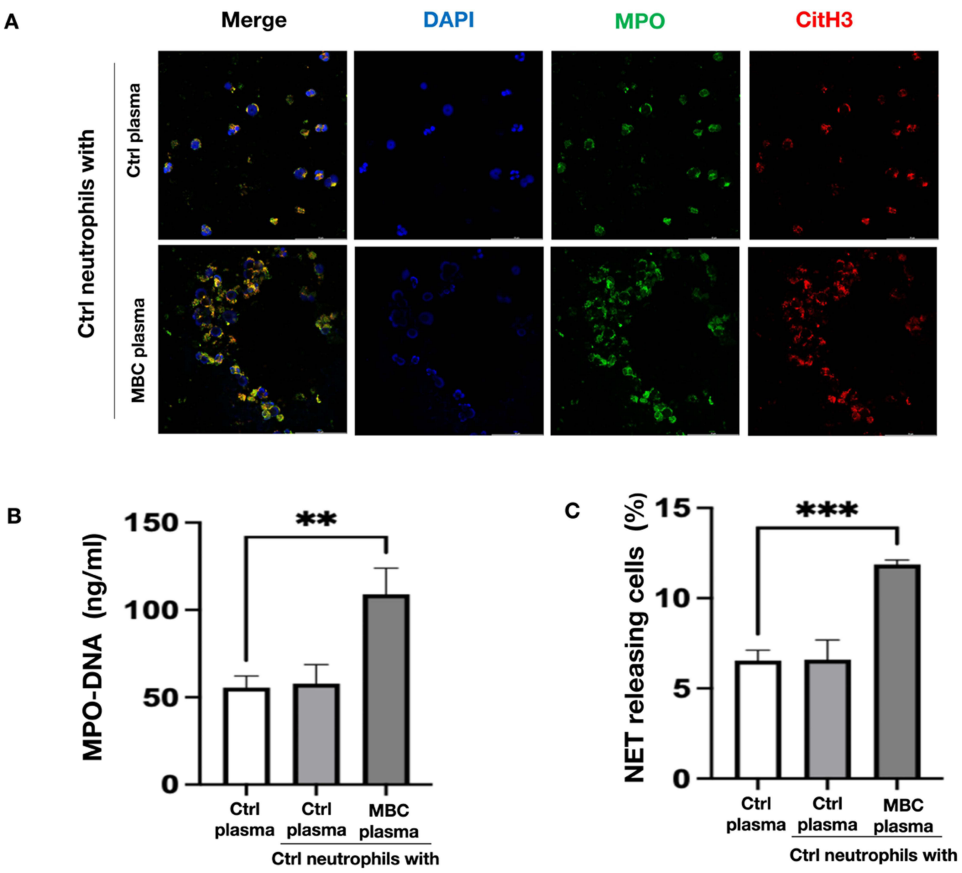

Figure 2 MBC plasma primes neutrophils to form NETs. (**A**) Confocal results showed a significant increase in NETs formation in control neutrophils exposed to MBC plasma compared to those exposed to control plasma. (**B**) Flow cytometry results showed that control neutrophils exhibited a significant increase in MPO-DNA quantification following stimulation with MBC plasma. (**C**) The frequency of NET-releasing neutrophils was also higher when control neutrophils were treated with MBC patient plasma than when treated with control plasma. ** P<0.01, *** P<0.001.

patient neutrophils significantly increased fibrinogen production in control plasma (Figure 3B). In contrast, NETs from control neutrophils had an insignificant effect on fibrinogen formation.

Furthermore, coagulation time was markedly reduced for NETs isolated from MBC patients compared to controls, while there was no significant difference in coagulation time when comparing control NETs (Figure 3C). To confirm that the procoagulant effects of neutrophils are dependent on NET formation, we conducted inhibition experiments using DNase I. The results showed that DNase I significantly reduced the procoagulant effect of NETs released by neutrophils from MBC patients (Figure 3D-F).

More NETs Formation and Hypercoagulability Are correlated With cancer-specific Outcomes

Plasma TAT levels were significantly higher in MBC patients who died within three years post-recruitment compared to those who survived beyond three years (median 571.24 vs 492.51 pg/mL, $P<0.0001$, Figure 4A), while D-dimer levels showed a similar trend (median 381.94 vs 288.10 ng/mL, $P<0.0001$, Figure 4C). When dichotomized into low (<571.24 pg/mL) and high (≥571.24pg/mL) groups base on the median, high TAT levels were associated with significantly reduced survival (HR: 1.987, 95% CI: 1.059–3.730, $P=0.026$, Figure 4B). High D-dimer levels were also associated with significantly reduced survival when divided into low (<381.94 ng/mL) and high (≥381.94 ng/mL) groups (HR: 2.908, 95% CI:1.533–5.516, $P=0.001$, Figure 4D). Fibrinogen levels, however, were not associated with reduced survival.

Plasma MPO-DNA levels were significantly higher in MBC patients who died within three years post-recruitment compared to those who survived beyond three years (median 162.91 vs 83.06 ng/mL, $P<0.0001$, Figure 4E). We also

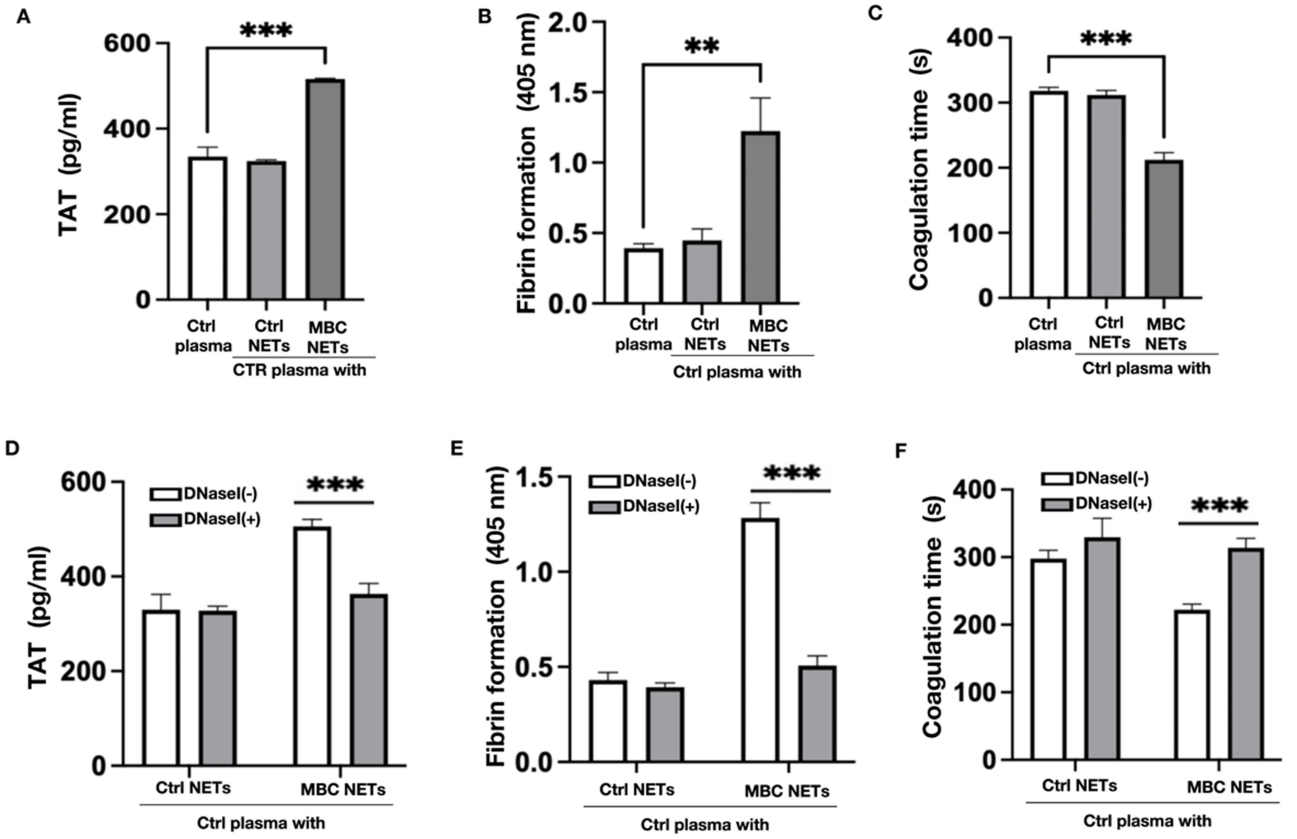

Figure 3 Procoagulant activity of NETs released by neutrophils derived from patients with MBC. (**A**) TAT levels increased significantly after the addition of NETs isolated from MBC patients to control plasma compared to baseline. (**B**) NETs from MBC patient neutrophils significantly increased fibrinogen production in control plasma. (**C**) coagulation time was markedly reduced for NETs isolated from MBC patients compared to controls, while there was no significant difference in coagulation time when comparing control NETs. (**D–F**) DNase I significantly reduced the effect of NETs released by neutrophils derived from patients with MBC on control plasma generation of thrombin and fibrin. ** P<0.01, *** P<0.001.

analyzed the OS of MBC patients with preoperative plasma MPO-DNA levels above or below the median. High plasma MPO-DNA levels were linked to markedly worse OS (HR: 2.445, 95% CI: 1.255–4.762, *P*=0.007, Figure 4F) in univariate analysis. Additionally, MBC patients with both high D-dimer and high MPO-DNA levels had significantly reduced survival according to Log rank testing (HR: 2.450, 95% CI: 1.332–4.488, *P*=0.002, Figure 5).

Discussion

Hypercoagulability significantly increases morbidity and mortality in cancer patients.[23,24] Activated neutrophils are pivotal in cancer-associated thrombosis in animal models.[25] This study aimed to investigate coagulation activity and NETs levels in MBC patients at diagnosis and their correlation with prognosis. We hypothesized that the interplay between NETs and plasma coagulation amplifies metastasis transmission, signifies more aggressive disease, and correlates with reduced survival in MBC patients.

Our results demonstrated that MBC patients exhibited significantly higher NETs levels and clotting activity compared to age-matched healthy controls. Furthermore, NETs formation was positively correlated with TAT, fibrinogen, and D-dimer levels. In vitro studies revealed that plasma from MBC patients could induce NETs release from neutrophils of healthy controls. Additionally, neutrophils from MBC patients induced greater procoagulant NETs formation compared to those from healthy controls.

We also found that NETs increased fibrin and D-dimer production in MBC patients. Degradation of NETs by DNase I prolonged coagulation time in plasma from stage IV patients and reduced D-dimer and fibrin production, indicating that

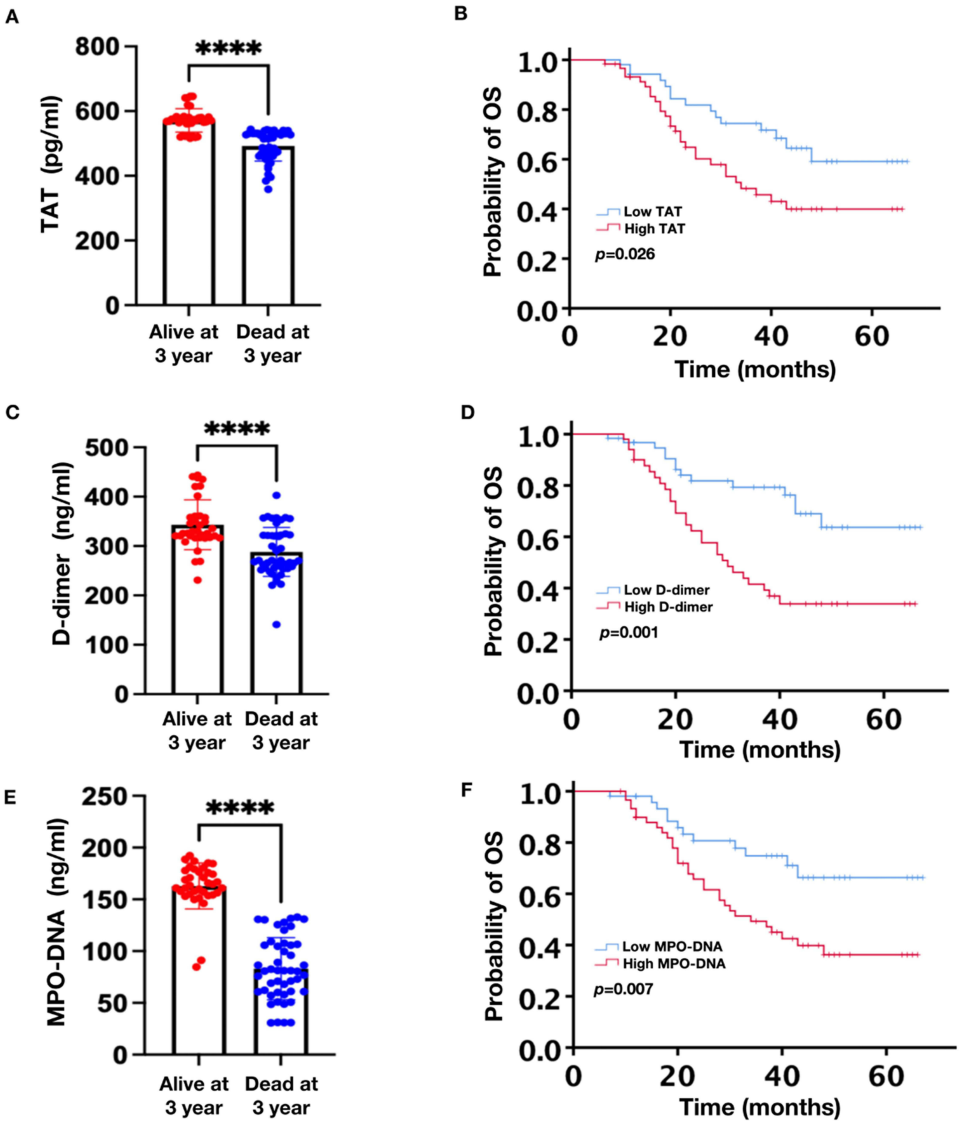

Figure 4 Coagulation plasma markers TAT and D-dimer, and NETs marker MPO-DNA are associated with poorer survival in metastatic breast cancer. Coagulation markers TAT and D-dimer were quantified by immunoassay in metastatic breast cancer (MBC) patient plasma samples. (**A**) TAT, (**C**) D-dimer, (**E**) MPO-DNA: concentrations compared to survival groups, ****p<0.0001. (**B**) TAT, (**D**) D-dimer, (**F**) MPO-DNA: log-rank (Mantel–Cox) tests comparing survival in months from study entry in these coagulation and NETs markers dichotomised around the median values (TAT 571.24pg/mL, D-dimer 381.945ng/mL, MPO-DNA 162.91 ng/mL) were carried out.

NETs are crucial regulators of hypercoagulability in MBC patients. Prognostic analysis revealed that elevated NETs levels and clotting activity were associated with poor outcomes in MBC patients.

The intricate relationship between NETs and tumors has garnered significant attention in recent years. Studies have shown that NETs are not merely an immune mechanism of neutrophils but also facilitate tumor metastasis through various strategies. The primary mechanisms include: recruitment of neutrophils to metastatic sites to form NETs and contribute to pre-metastatic niche formation;[26] promotion of epithelial-to-mesenchymal transition (EMT), enabling tumor cell migration and invasion;[27] adherence to and trapping of circulating tumor cells to promote their colonization and proliferation;[28] and enhancement of tumor cell mitochondrial function, altering their energy metabolism and awakening dormant micrometastatic tumor cells.[29]

The correlation between NETs and cancer prognosis has been substantiated in the literature. Zhao et al developed a prognostic nomogram model to predict breast cancer patient outcomes by analyzing data from a public database.[30] Their results suggest that high-risk scores correlate with poor clinical outcomes. Similarly, Rivera-Franco et al[18] demonstrated that NETs levels increase proportionally with disease stage, observing higher NE-DNA complex levels in regional and metastatic

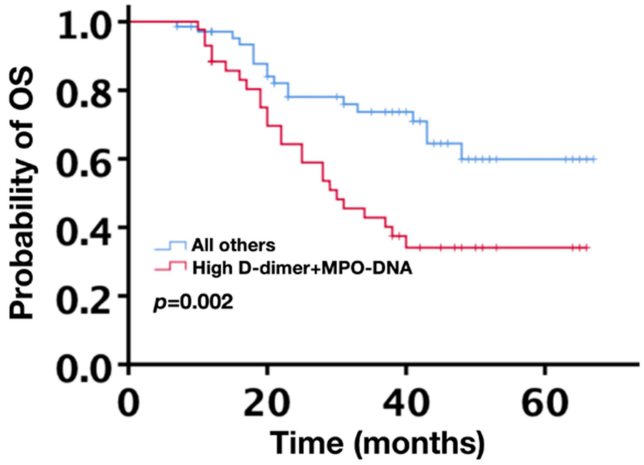

Figure 5 The combined presence of a high D-dimer and circulating MPO-DNA is associated with reduced survival in metastatic breast cancer. Survival in patients with high MPO-DNA (≥162.91 ng/mL) and high D-dimer (≥381.94ng/mL) (red line, n=43) was compared to all other patients (blue line, n=69). A log-rank (Mantel–Cox) test comparing survival in months from study entry in the shown NETs/coagulation marker groups was performed.

diseases. This aligns with the proposed mechanism wherein cancer progression and metastasis might result from NETs formation. Our findings are consistent with these studies, indicating significantly higher NETs levels in MBC patients compared to age-matched healthy controls, and associating these elevated levels with poor prognosis. To our knowledge, this is the first report linking plasma NETs levels with survival in metastatic breast cancer. Improving long-term survival for MBC remains a significant challenge. NETs may serve as a prognostic marker for MBC and could be a potential target for therapeutic intervention in patients with high NETs levels.

It is well established that many malignant diseases are associated with a hypercoagulable state. The activation of the coagulation system and platelets leads to an increased risk of thromboembolic events in cancer patients compared to the general population.[31] The mechanisms underlying the thrombotic diathesis in malignant disease are not entirely clear and are likely multifactorial. NETs are closely linked to thrombosis in several malignant tumors. Yang et al observed that NETs released from cancer patients increased levels of TAT in vitro and enhanced the ability of control plasma to generate fibrin, an effect that was mitigated by DNase I treatment.[32] Additionally, NETs have been observed in thrombosis from cancer patients in several reports.[33,34]

One study demonstrated the presence of NETs in coronary, cerebral, and pulmonary microthrombi in patients with various cancer types, indicated by positive intracellular staining for H3Cit and co-localization between H3Cit and extracellular DNA (eDNA).[35] The same study found that circulating H3Cit levels positively correlated with TAT and soluble P-selectin levels, suggesting a link between NETs and coagulation activation in cancer patients.[35] Similarly, Oklu et al[36] observed abundant histone-DNA complexes in thrombi from cancer patients. Elevated plasma levels of circulating nucleosomes and eDNA were also noted compared to healthy controls, although they did not correlate with TAT levels. Unfortunately, no NET-specific assays were used in this study, limiting the conclusions from the plasma analyses. Guy et al[37] observed higher levels of MPO-DNA in patients with myeloproliferative neoplasms (MPN) with a history of thrombosis compared to those without. Another study demonstrated that pancreatic cancer cells induced NETs, contributing to a high VTE risk in patients.[32] Increased circulating NETs in gastric cancer patients have also been found to enhance procoagulant activity.[14]

The procoagulant role of NETs in patients with breast cancer has not been previously reported. Our results demonstrate that NETs' procoagulant activity is markedly increased in MBC patients relative to healthy controls. These findings are consistent with previous clinical studies in other cancer patients and suggest that neutrophils and NETs play a significant role in cancer-associated thrombosis. This may be one of the mechanisms by which NETs influence the prognosis of cancer patients.

The mechanism of NETs' procoagulant activity has been verified in several studies. NET fibers can bind platelets indirectly or directly, promoting aggregation.[38] In murine pancreatic adenocarcinoma, NETs promoted hypercoagulability by inducing TF release and stimulating platelets.[39] Moreover, NETs bind factor XII, which can undergo activation after

contacting NET-entrapped pathogens, thereby stimulating fibrin formation and thrombus stabilization. Our results also show that NETs increased fibrin and thrombin production in MBC patients. Furthermore, the degradation of NETs by DNase I prolonged prothrombin time and attenuated D-dimer and fibrin generation in plasma from MBC patients, indicating that NETs are important regulators of hypercoagulability in MBC patients.

Recent work suggests that targeting NETs can mitigate hypercoagulability, reduce cancer metastasis, and prevent organ dysfunction in patients.[40–42] Investigating whether DNase I can be safely implemented as a therapeutic anticoagulant in MBC patients with high NET levels warrants further investigation. Previous studies have demonstrated that fibrinogen plays a crucial role in the occurrence and development of malignant tumors. Fibrinogen forms a growth factor container associated with tumor cells by combining with VEGF, TGF-β, and FGF, thereby regulating the proliferation, angiogenesis, apoptosis, and metastasis of tumor cells. Angelidakis et al[40] showed that fibrinogen acts as a bridge connecting platelets and circulating junction proteins on tumor cells, thus enhancing tumor cell adhesion. Additionally, endothelial cell adhesion molecule-1 binds to fibrinogen, resulting in the stable fixation of circulating tumor cells on endothelial cells. Fibrin can also form a stable skeletal structure in the tumor extracellular matrix, which facilitates the migration of endothelial cells and the spread of tumor cells. Other studies have indicated that tumor cells can produce endogenous fibrinogen, which, when combined with fibroblast growth factor, leads to the proliferation of endothelial cells and the increase of new blood vessels.[43,44] Moreover, under the action of thrombin, circulating fibrinogen is converted into a fibrin matrix that, together with platelets, forms a protective structure around the tumor cells. This structure protects tumor cells from natural killer cell-mediated elimination. These findings underscore the multifaceted role of fibrinogen in tumor progression, highlighting its involvement in cellular adhesion, vascularization, and immune evasion, which collectively contribute to the malignancy of cancer cells.

D-dimer is a biomarker that reflects a pre-thrombotic state or ongoing thrombosis. It serves as a sensitive indicator for diagnosing fresh thrombi and disseminated intravascular coagulation (DIC) and is effective in differentiating primary from secondary fibrinolysis.[45] Elevated D-dimer levels indicate the simultaneous activation of both the coagulation and fibrinolytic systems, reflecting increased thrombin production and heightened fibrinolytic activity, which are critical for diagnosing thrombotic disorders.[46] Clinically, D-dimer is frequently used as a specific marker to evaluate hypercoagulability and hyperfibrinolysis. In healthy individuals, D-dimer levels are typically low or undetectable However, when there is an imbalance in the anticoagulation and fibrinolytic systems or the formation of activated thrombi within blood vessels, the degradation products of fibrin increase, leading to elevated D-dimer levels. This elevation is often observed in patients with malignant tumors due to the activation of their coagulation and fibrinolytic systems. The presence of increased D-dimer levels in cancer patients underscores its importance as a marker of hypercoagulability and thrombotic risk in malignancies.

In our study, elevated plasma fibrin and D-dimer levels in MBC patients were associated with poor prognosis. The combination of high NETs and high D-dimer levels correlated with increased metastatic potential and worse outcomes. This connection between plasma coagulation, NETs, and survival suggests that targeting the coagulation system therapeutically could potentially improve breast cancer outcomes. In vivo, breast cancer xenograft models have demonstrated a reduction in tumor growth and metastasis when treated with anti-tissue factor antibodies.[47,48] Additionally, the direct thrombin inhibitor dabigatran has shown efficacy in reducing tumor growth and liver micrometastases in a breast cancer murine model, indicating a potential anti-cancer effect of the direct oral anticoagulant (DOAC) class of drugs.[49]

Our study has some limitations that need to be addressed. First, it was a retrospective study from a single institution, the number of study patients included was small, especially of those who developed VTE. In addition, this study is also limited by the relatively short follow-up period. Finally, although we confirmed the association between NETs and coagulation function and their predictive value in MBC prognosis, accurately detecting and quantifying NETs remains challenging, and the practice lacks standardization, thus limiting its clinical application.

Conclusion

Our results highlight the increased release of NETs in MBC patients and reveal that NET formation enhances hypercoagulability and cancer progression. Targeting NETs may be a potential therapeutic strategy to inhibit MBC progression and mitigate thrombotic complications in MBC.

Statement of Ethics

This retrospective study was approved by the ethics committee Review Board of the Guangxi Medical University Cancer Hospital (protocol code KY2024148) and in conformity to the Declaration of Helsinki. Written informed consent was obtained from all participants.

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This paper has been uploaded to ResearchSquare as a preprint: https://www.researchsquare.com/article/rs-4796055/v1.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare for this work.

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