RESEARCH LETTER

# Impact of neutrophil extracellular trap formation on thromboembolic events and prognosis in patients with newly-diagnosed lymphoproliferative disorders

Anna Hoppe, Ewelina Wojtasińska, Joanna Rupa-Matysek

Department of Hematology and Bone Marrow Transplantation, Poznan University of Medical Sciences, Poznań, Poland

**Introduction** Neutrophil extracellular trap (NET) formation (NETosis) is a type of innate immune system response, in which an activated neutrophil releases its nucleus content, forming an extracellular trap that allows for sequestration of microbes in the bloodstream, their inactivation, and elimination.[1,2] The activity of this process can be measured by circulating free DNA, nucleosomes, neutrophil elastase, myeloperoxidase activity, and plasma concentration of citrullinated histone H3 (CH3).[2,3] The role of NETosis has also been confirmed in various pathologic conditions, such as autoimmune diseases,[2,3] preeclampsia, metastasis promotion, and cancer-associated thrombosis (CAT) in solid tumors.[2,4] Although lymphoproliferative disorders (LDs), including both Hodgkin (HL) and non-Hodgkin lymphoma (NHL) and multiple myeloma (MM), carry one of the highest risks of CAT,[5] its pathogenesis remains unclear,[6,7] and data on NETosis role in CAT in patients with LDs are scarce.

**Patients and methods** To evaluate the ability of neutrophils to form NETs in patients with various LDs determined based on CH3 levels, and to assess their impact on diagnosis, disease stage, CAT development, and outcomes, we prospectively analyzed 65 previously untreated patients with LD qualified for systemic treatment in our outpatient department in the years 2019–2021. The study inclusion criteria were age of at least 18 years, an Eastern Cooperative Oncology Group score of maximum 2, and a diagnosis of LD: MM, HL, or NHL that qualified the patients for a systemic treatment. Key exclusion criteria were any other malignancy treated within the previous 2 years, any thromboembolic event (TE) within 3 months, and preceding chronic anticoagulation. The control group consisted of 55 healthy participants aged 19 to 63 years.

The study met the criteria of the Helsinki Declaration and was approved by the local Bioethics Committee (696/19).

Before the treatment initiation, we measured height, body weight, and body mass index, and conducted laboratory tests including complete blood count (CBC) and screening coagulation parameters, such as activated partial thromboplastin time, prothrombin time / international normalized ratio, and D-dimer levels. We also evaluated the levels of selected biochemical parameters, such as C-reactive protein (CRP), creatinine, albumin, lactate dehydrogenase (LDH), and β2-microglobulin (β2m). For every participant, an additional sample of 10 ml of peripheral blood was collected (into vacuum tubes containing ethylenediaminetetraacetic acid as an anticoagulant). The samples were stored at –70 °C to –80 °C and then used to determine the concentrations of CH3. The levels of CH3 were determined using a commercial enzyme-linked immunosorbent assay (Shanghai Sunred Biological Technology Co., Shanghai, China).

Venous thromboembolic event (VTE) risk assessment was performed before chemotherapy initiation based on the Khorana score for lymphoma patients[8] and the IMPEDE-VTE and SAVED scores for myeloma patients.[4] VTE prophylaxis options and its duration depended on physician's discretion. In the myeloma group, the options of pharmacologic VTE prophylaxis included acetylsalicylic acid (81–325 mg once daily) and low-molecular-weight heparin (LMWH) (enoxaparin 40 mg daily or an equivalent). In the lymphoma group, direct oral anticoagulants (eg, apixaban 2.5 mg twice daily) or

Correspondence to:
Anna Hoppe, MD,
Department of Hematology and Bone Marrow Transplantation, Poznan University of Medical Sciences, ul. Szamarzewskiego 84, 60-569 Poznań, Poland,
phone: +48 61 854 95 71,
email: anna.kawka@skpp.edu.pl
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**TABLE 1** Patient characteristics

| Parameter | Myeloma (n = 35) | Lymphoma (n = 30) | *P* value |
|---|---|---|---|
| Age at diagnosis, y | 64 (55.6–67.6) | 50.3 (38.5–65.8) | 0.008 |
| Men, n (%) | 45.7 (16) | 56.7 (17) | 0.38 |
| Body weight, kg | 70 (58–79) | 70 (62–79) | 0.71 |
| Height, cm | 164 (162–173) | 170 (162–178) | 0.17 |
| BMI, kg/m$^2$ | 24 (21–28) | 24.5 (21.3–26.9) | 0.87 |
| WBC, G/l | 6.7 (4.7–8.6) | 6.6 (4.7–9.8) | 0.63 |
| LYMPH, G/l | 1.8 (1.3–2.42) | 1.1 (0.89–1.56) | <0.001 |
| HGB, g/dl | 10.2(9.3–12.5) | 13.1 (11.7–13.6) | <0.001 |
| PLT, G/l | 260 (199–346) | 302.5 (219–353) | 0.3 |
| APTT, s | 30.3 (26.5–33.1) | 30.8 (28.8–32.5) | 0.81 |
| PT, s | 13 (11.9–13.6) | 12.6 (11.7–13.5) | 0.34 |
| D-Dimer, µg/l | 1819 (1428–4579) | 727.7 (286–1715) | 0.1 |
| CRP, mg/l | 4 (4–10) | 5.45 (4–26) | 0.2 |
| Albumin, g/l | 33 (27–37) | 37.5 (34–40.6) | 0.002 |
| LDH, IU/l | 187 (141–235) | 216 (179–325) | 0.02 |
| Serum creatinine, µmol/l | 81 (63–172) | 69.5 (57–82) | 0.02 |
| CH3, pg/ml | 905.6 (664.7–1710.4) | 952 (749.4–3050) | 0.37 |
| OS, mo | 54.0 (37–75) | 32 (11–47) | 0.002 |
| Time to relapse, mo | 2.1 (0.6–9.1) | 10.8 (2.4–20.8) | 0.1 |
| Time to VTE, d | 53 (24–691) | 196 (133–259) | 0.1 |

Data are presented as median and interquartile range unless indicated otherwise.

SI conversion factors: to convert hemoglobin to g/l multiply by 10.

Abbreviations: APTT, activated partial thromboplastin time; BMI, body mass index; CH3, citrullinated histone H3; CRP, C-reactive protein; HGB, hemoglobin; LDH, lactate dehydrogenase; LYMPH, absolute lymphocyte count; OS, overall survival; PLT, platelets; PT, prothrombin time; VTE, venous thromboembolic event; WBC, white blood cells

LMWH (enoxaparin 40 mg daily or an equivalent) were used. The patients were observed for symptoms of CAT for at least 2 years. Clinically suspected TE was confirmed by ultrasound examination for deep vein thrombosis (DVT) or computed tomography angiography for pulmonary embolism (PE) according to standard diagnostic procedures.

**Statistical analysis** Due to non-normal distribution of variables, we used nonparametric tests and we present the data as medians and interquartile ranges (IQRs). Statistical comparisons were performed using the $\chi^2$ test with the Yates correction and the maximum likelihood $\chi^2$ tests when required for categorical variables, the Mann–Whitney test for continuous variables, and the Kruskal–Wallis test for the comparison of more than 2 groups. To compare the survival distribution, we the used the log-rank test.

Correlations between the variables were assessed using the Spearman correlation coefficient. A *P* value below 0.05 was assumed significant. All statistical analyses were performed with StatSoft Statistica package version 13.0 (StatSoft, Kraków, Poland).

**Results** Of the 65 patients with LDs, 30 had lymphoma (14 HL and 16 NHL), and 35 had MM. The group characteristics are presented in TABLE 1. Median (IQR) observation time was 741 (543–850) days.

**NETosis activity** The median (IQR) pre-chemotherapy values of CH3 were significantly higher in the study group (947.98 [730.9–2028.1] pg/ml) than in the control group (793.3 [624.6–888.1] pg/ml; *P* <0.001). Before therapy, the NET level did not correlate with D-dimers, a market of excessive clotting (*P* = 0.91), or with CRP, a marker of inflammation (*P* = 0.43).

**Myeloma vs lymphoma** The patients in the lymphoma group were younger than those with MM (51 vs 61 years; *P* = 0.008), had lower absolute lymphocyte count (1.1 vs 1.8 G/l; *P* <0.001), higher hemoglobin levels (13.1 vs 10.2 g/dl; *P* <0.001), higher serum LDH levels (216 vs 187 IU/l; *P* = 0.02), and lower serum creatinine levels (69.5 vs 81 µmol/l; *P* = 0.02). There was no significant difference in CH3 concentrations between the lymphoma and myeloma patients (952 vs 906 pg/ml; *P* = 0.37), as well as in the lymphoma subgroups (NHL vs HL: 993 vs 925 mg/ml; *P* = 0.64).

**Tumor burden** In the entire cohort, a positive correlation of CH3 with serum β2m level (R = 0.59; *P* <0.001) was found. For lymphoma patients, NET activity was similar in the patients with early (I–II) and advanced (III–IV) stage of the disease (900.3 vs 1015.5 pg/ml; *P* = 0.3), as well as in the group with and without the presence of B symptoms (924.2 vs 998.3 pg/ml; *P* = 0.64).

In the MM group, we found negative correlations between CH3 concentration and body weight (R = –0.35; *P* = 0.045) and hemoglobin level (R = –0.33; *P* = 0.049), and a positive correlation with β2m level (R = 0.57; *P* <0.001). The myeloma patients with International Scoring System (ISS)-3 were found to have significantly higher CH3 concentrations than patients with ISS-1 (1035.3 vs 705.7 pg/ml; *P* = 0.04).

**Venous thromboembolism** A total of 20 patients with lymphoma (67%) and 34 individuals with myeloma (97%) received VTE prophylaxis, including prophylactic doses of LMWH in 42 cases (65%), acetylsalicylic acid in 10 cases (15%; only MM), and 1 patient with lymphoma received a direct oral anticoagulant. No impact of thromboprophylaxis (*P* = 0.38) and the type of VTE prophylaxis (*P* = 0.08) on VTE development was found. After a median (IQR) follow-up of 741 (27–1367) days, 9 patients (14%) developed 11 TEs, including 1 case of isolated PE, 2 patients presented simultaneous DVT and PE, whereas 6 patients developed isolated DVT, 2 of them had recurrent episodes. A total of 9 cases (82%) of VTEs were observed in myeloma patients, and 1 patient with NHL developed DVT/PE.

In the VTE group, higher platelet count (358 vs 260 G/l in the non-VTE group; $P$ = 0.04) was found. However, anthropometric measurements, other CBC, coagulation, and biochemical parameters were comparable for both the VTE and non-VTE groups. There was no significant difference in the levels of NETs considering the VTE status (890 vs 951 pg/ml; $P$ = 0.72). Neither a high Khorana score (≥3) nor the IMPEDE-VTE and SAVED scores (for MM patients) were predictive of VTE development in our study cohort. Further details about the VTE and non-VTE groups are available in Supplementary material (*Tables S1* and *S2*).

**Clinical outcomes** In the whole study group, 19 patients had recurrences during the median (IQR) of 137 (43–374) days, and 18 patients died. None of the deaths were related to VTE. No difference in the NET activity was found for the patients who relapsed and those in remission during follow-up (948 vs 947; $P$ = 0.54). The CH3 activity was similar in the patients who died and in the survivors (1038 vs 943; $P$ = 0.3). In a log-rank analysis of the probability of survival (0.53; $P$ = 0.6), progression-free survival (1.45; $P$ = 0.15), and VTE-free survival rates (1.11; $P$ = 0.27), no differences were found in the patients with prechemotherapy CH3 activity equal to or below the 25th percentile and those with mean platelet volume above the 25th percentile.

**Discussion** CAT is a major complication in malignant neoplasm patients, increasing their morbidity and mortality.[9] Thus, identification of high-VTE risk patients is crucial for proper thromboprophylaxis. Various prognostic tools, such as the Khorana and ThroLy scores have been proposed for VTE risk assessment in cancer patients.[4,8,10] However, in some populations, they were not accurate enough for predicting VTE.[6,10] In our study, neither the Khorana score nor the IMPEDE-VTE or SAVED scores succeeded in identifying the patients at a high risk of VTE. A better understanding of CAT mechanisms and identification of novel markers of VTE could help create better prognostic tools.

One of the proposed mechanisms of CAT is NETosis, a type of innate immune response mechanism involving formation of a fibrous net capable of sequestrating bacteria and enhancing neutrophil effectiveness in the elimination of microbes.[2] Furthermore, NETs do not only trap pathogens, but they also bind platelets and erythrocytes leading to thrombus formation. NETs increase procoagulant plasma activity by activating factor XII, binding von Willebrand factor, and blocking tissue factor pathway inhibitor and platelet activation.[1] In our study, we attempted to investigate the NETosis phenomenon in the patients with newly-diagnosed lymphoproliferative disorders. No impact of NETosis activity, measured by plasma CH3 levels, on VTE development was found, which may suggest different pathogenesis of CAT in lymphoproliferative malignancies. Mauracher et al[11] explained that higher levels of CH3 are associated with increased VTE in patients with solid tumors, but in the lymphoma population an inverse correlation between NET activity and VTE was found. Interestingly, myeloma patients had lower CH3 levels than individuals with other malignancies.[11] Our study showed a higher platelet count in the patients who developed VTE, which may confirm previous observations on the major role of platelet activation in CAT development in lymphoid malignancies.[7]

We also found a positive correlation between β2m and CH3 levels in the myeloma group. β2m is a well-known marker of tumor burden, and it correlates with reduced survival in myeloma patients.[12] Moreover, CH3 negatively correlated with hemoglobin levels. Furthermore, the patients with ISS-3 stage had significantly higher CH3 levels than those in the ISS-1 group. These observations may suggest increased NETosis activity in advanced stages of myeloma with possible impact of NETs on the disease progression. However, this requires further investigation. Our findings correspond with previous studies of Li et al,[13] who reported NET upregulation in the presence of myeloma cells. The same authors confirmed that a decrease in NET formation by peptidylarginine deiminase 4 inhibition delays the progression of MM in a murine model. Furthermore, Nie et al[14] showed higher NET activity in patients with advanced diffused large B-cell lymphoma when compared with patients at its early stages.

In conclusion, our preliminary data may suggest a link between NETosis and MM progression, although this requires further investigation. Larger prospective cohort studies are warranted to assess the role of NETosis in CAT in LD. Thrombosis remains a significant complication in patients with lymphoproliferative neoplasms even in the thromboprophylaxis era; thus, future research is needed for a better understanding of the mechanisms leading to CAT and improvement in patient risk stratification.

## SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

## ARTICLE INFORMATION

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