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REVIEW ARTICLE

Potential biomarker of neutrophil extracellular traps in venous thromboembolism: a systematic review

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Background

Neutrophil extracellular traps (NETs) are a network of extracellular DNA produced by activated neutrophils to trap and disarm microbes. NETs increase the formation of thrombus by forming a network frame that activates platelets and initiates coagulation. NETs were involved in the thrombogenic process and have been reported in various animal models. However, the evidence of NETs' role in venous thromboembolism (VTE) development in humans is still scarce. This review aims to discover the relationship between NETs and VTE risk.

Methods

We performed literature search to identify relevant available articles from PubMed, Cochrane Library, MEDLINE, EMBASE, Clinical Key between October 2009 until October 2019. The inclusion criteria were: clinical trials published in English, involving humans as subjects, conducted within the past ten years, and had available and accessible full-text. In addition, Newcastle Ottawa Scale (NOS) was used to assess evidence quality.

Results

Four studies with a total of 1,430 patients, i.e. three case controls and one cohort, met our eligibility criteria. All four studies' quality was good. One study of cancer patients demonstrated that NETs increase VTE risk, two other studies demonstrated NETs correlate with deep vein thrombosis (DVT), and another study demonstrated there were increasing NETs in residual vein obstruction (RVO) and increased D-dimer. All four studies found a significant association of NETs and VTE occurrence (p=0.003; p=0.018; p<0.01; p<0.001, respectively).

Conclusions

NETs are associated with an increased VTE risk. Further studies are necessary to determine the NETs' role in VTE as a diagnostic biomarker or target of therapy

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Introduction

Venous thromboembolism (VTE), including pulmonary embolism (PE) and deep vein thrombosis (DVT), is a common cause of morbidity and premature mortality worldwide, but our knowledge in diagnosing and treating these diseases is limited.[1] A recent study estimated that in the United States, total annual VTE cases reached more than 900,000, by which more than 250,000 were fatal.[2] The average yearly attack rates of VTE related and unrelated to hospitalization were 282 and 8 per 10,000 person-years, respectively. A multicenter study in Europe involving six countries reported an estimated annual 465,715 DVT cases, 295,982 PE events, and 370,012 VTE-related deaths.[2] In Asia, the incidence of VTE ranging from 14 to 57 cases per 100,000 patient-years.[3–6] Despite advances in vascular biology and medicine, VTE's incidence has not declined in the last 25 years.[2]

The standard protocol for the diagnosis of patients with VTE is to use a D-dimer biomarker to exclude VTE diagnosis, while compression ultrasound has been the gold standard for diagnosing DVT and Computed Tomography (CT) angiography for PE diagnosis.[7,8] However, all of these diagnostic modalities have limitations such as being less specific, require a long time, uneven availability, and are quite expensive. Thus, an investigation of new diagnostic biomarkers to function as early identification of patients at low or high risk of first diagnosed and recurrent VTE is needed to enable immediate diagnosis and assessment of therapy.

Venous thromboembolism is epidemiologically related to inflammatory diseases, such as infections, cancer, and autoimmune disorders. Acute infections and autoantibody against prothrombin 11 and phospholipid are risk factors for PE and DVT.[9] The incidence of malignancy and venous thrombosis also has been described in the 19th century. More recently, VTE and mortality risk is associated with an increase in neutrophils in cancer receiving chemotherapy.[10] Neutrophils are the most numerous inflammatory cells and have recently emphasized their significance in DVT in animal studies.[11–13]

Neutrophils are the first leukocytes that came to the site of infection and neutralized phagocytic bacteria.[14] In phagosomes, microbes are killed by reactive oxygen species (ROS) and high concentrations of antimicrobial proteins. Neutrophils produce neutrophil extracellular traps (NETs) to trap and neutralize microorganisms in extracellular space. NETs are whole chromatin fiber scaffolds with antimicrobial proteins, ideal for maintaining large numbers of microbes.[15] NETs also encourage the formation of thrombus by serving as a network frame to activate platelets and initiate coagulation.[16]

Neutrophil extracellular traps productions had been studied in thrombosis models of inferior vena cava in baboons' iliac veins and Peptidyl arginine deiminase-4 (PAD4)-knockout mice. Studies revealed higher levels of plasma deoxyribonucleic acid (DNA) and NETs in venous thrombi, greater thrombus size/rate with higher von Willebrand factor (vWF) activation recruitment of platelet. Of note, histone infusion accelerates the thrombosis process while cleavage of NETs by Deoxyribonuclease 1 (DNase1) or heparin reduces the process.[17-18] The thrombogenesis role of NETs has been documented in animals with various settings of thrombosis. However, evidence of NETs' role in the risk of developing VTE in humans is still scarce. This review aims to discover whether NETs are correlated with VTE risk.

Methods

This systematic review was conducted using a predetermined protocol following "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) guidelines.[19] 1). The review was carried out in a series of steps: (i) searching the database for published articles, (ii) quality assessment for each obtained articles, (iii) extraction of data from tables and graphs, and (iv) interpretation and summary of findings. A meta-analysis of the data obtained could not be carried out because of the heterogeneity and broad diversity of study settings across the included literature. Thus, we provided the synthesis and findings of our review as a narrative summary.

Database search for identification of relevant studies

We performed a literature search to identify relevant available articles from PubMed, Clinical Key, MEDLINE, EMBASE, Cochrane Library using subject terms "neutrophil extracellular traps", "NETS", "venous thromboembolism", "VTE", "pulmonary embolism", "PE", "deep vein thrombosis", and "DVT. Search parameters were limited to a clinical trial, within the last 10 years (October 2009 to October 2019), and full-text availability. Only English language was included. Manual searches of bibliographies and references were conducted. All three authors independently conducted searches. Abstracts for all results were reviewed, and relevant studies were selected for further review. Any disagreement in screening, extraction, and analysis of data was resolved by discussion.

Review methods and study selection

We included all studies enrolling the general population whose study participants had undergone an examination of NETS or biomarker of NETS formation with VTE incidence as an outcome. This review excluded review articles, descriptive studies, non-English language articles, commentaries, letters, studies with non-human subjects, incompatibility of biomarkers with NETS formation, and studies with outcomes without VTE. Our search resulted in 108 articles, and ten more relevant articles were added from the bibliography, reference lists, and related citations of the primary articles, with a total of 128. Of the citations received, 82 duplicates were issued, leaving 46 articles, as illustrated in figure 1. All articles were screened by two independent reviewers (EPBM and IPD) based on inclusion and exclusion criteria.

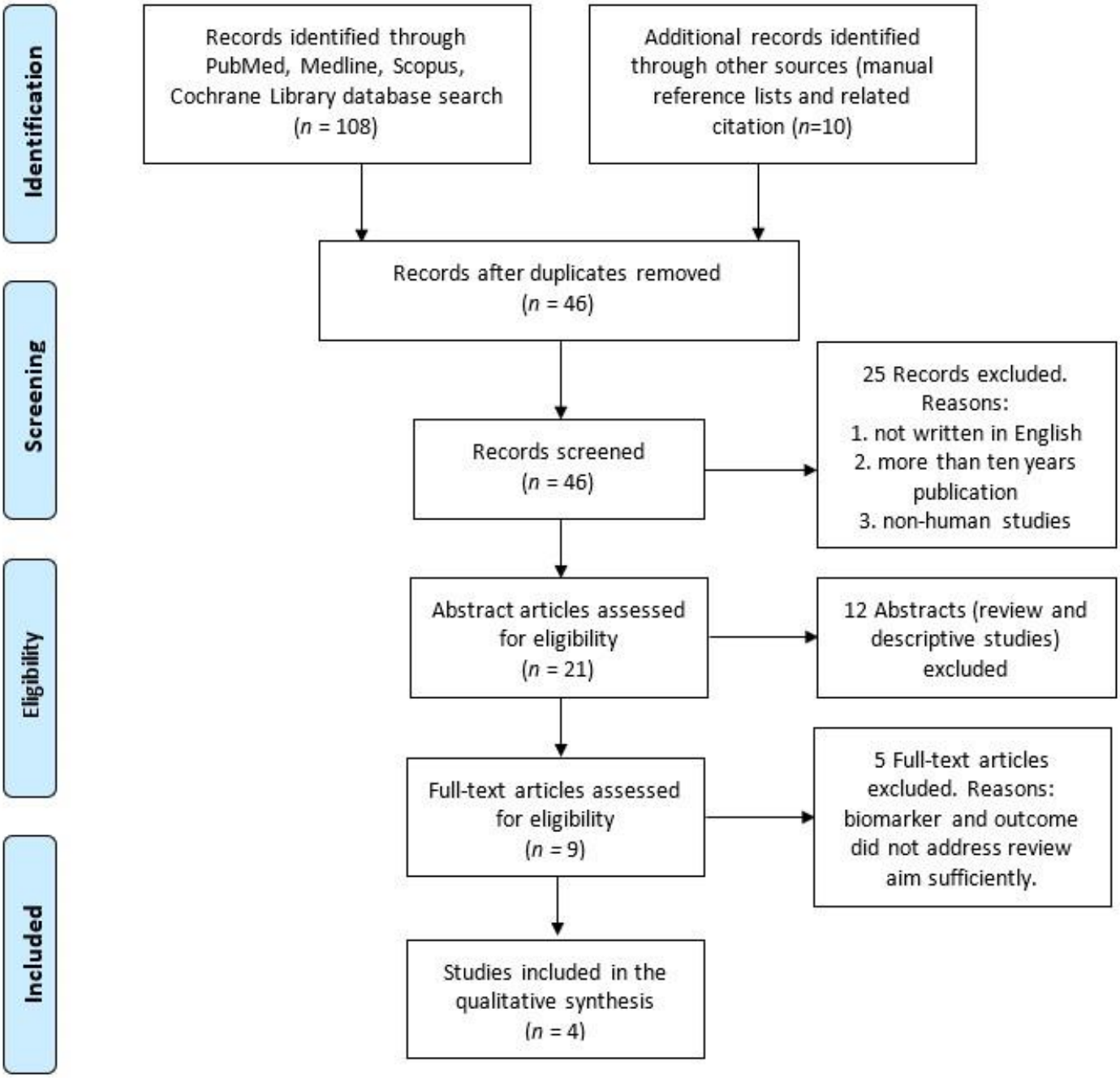
Of the 46 records after the removal of duplicates, the examination of titles and abstracts of the latter according to eligibility criteria resulted in the removal of 37 additional articles, leaving nine articles. A complete reading of the entire contents of the last number of papers further excluded five additional articles because biomarkers and outcomes did not address the review's aim sufficiently, thus, leaving four studies for consideration.

Data extraction and quality assessment

Data were extracted by two authors (EPBM and IPD) independently, then reviewed and validated by the third author (MB). All four studies were classified according to author/year, country/setting, the methodology of the study, sample size, statistical analysis, and outcome. The authors also conducted an assessment of the quality of each research article. We assessed the quality of the methods and research of studies by using Newcastle Ottawa Quality Assessment Scale (NOS) using predefined criteria, namely:

selection (representativeness of population), comparability (adjustment for confounders), and ascertainment of the outcome. The NOS allocates a maximum of four points for selection, two points for comparability, and three points for the outcome. Nine points of NOS reflects the highest study quality.

Figure 1 Flowchart of study selection



RESULTS

Four studies with a total of 1430 patients met all inclusion and exclusion criteria. Three were of case-control and one was a prospective cohort study. The sample size varied between studies. The setting of research was well described in all publications. In Table 1, the research setting was either classified as a hospital or academic medical center (utilizing laboratory). The outcome of the studies was the incidence of VTE (Table 1).

In spite of the fact that all of the articles were published in peer-reviewed journals in this review, we still carried out the examination of the methodological quality using NOS. The four qualifying reports good quality in all of the studies, as shown in table 2.

There are several biomarkers of NETS formation that are different from each study, namely: citrullinated histone H3,[20] circulating nucleosome and activated neutrophils,[21] plasma DNA,[22] and neutrophil adhesion with inflammatory cytokines.[23] All biomarkers were measured at baseline only, with no longitudinal measurements reported. One study used a specific population of cancer patients,[20] while 3 others did not mention special populations.[21–23] One study of cancer patient demonstrated that NETs increase VTE risk,[20] two other studies demonstrated NETs correlate with DVT,[21-22] and one study demonstrated there is increasing NETs in residual vein obstruction (RVO) and increased D-dimer.[23] Table 3 showed that all four studies found a significant association of NETs and VTE occurrence (p=0.003; p=0.018; p<0.01; p<0.001). Two studies used DVT as an outcome; one study used VTE in general as an outcome, while the rest used residual vein obstruction and increased D-dimer as an outcome. Increased biomarkers of NET formation were correlated with a threefold risk of DVT and biomarker of NETs formation is increased in DVT patients and correlates with DVT biomarkers.[21–23]

Table 1 Description of the studies

Author (year)	Country/Setting	Method	Subject (n)	% VTE
Mauracher et al. (2018)[20]	Austria/ Medical University	Cohort	946	9.4
Zapponi et al. (2014)[23]	Brazil/ Medical University	Case Control	58	50
Diaz et al. (2013)[22]	Switzerland/ Medical University	Case Control	94	50
van Montfoort et al. (2013)[21]	Netherlands/ Academic Medical Center	Case Control	332	45

Table 2 Quality assessment of studies using NOS

Author (year)	Selection	Comparability	Outcome/Exposure	Summary
Mauracher et al. (2018)[20]	4	2	3	9
Zapponi et al. (2014)[23]	4	2	3	9
Diaz et al. (2013)[22]	4	2	2	8
van Montfoort et al. (2013)[21]	4	2	3	9

Table 3 Study Results

Author (year)	NETs biomarkers	OR; 95% Confidence Interval (CI); *P*-value	Outcome
Mauracher et al. (2018)[20]	citrullinated histone H3	1.13; 95%CI 1.04–1.22; p=0.003	Biomarker of NETs formation is associated with VTE development in cancer patients.
Zapponi et al. (2014)[23]	neutrophil adhesion with inflammatory cytokines	24.68 vs 19.29 *vs* 18.23**; p=0.018	Increased biomarkers of NETs formation profile among patients with RVO and increased D-dimer.
Diaz et al. (2013)[22]	plasma DNA	57.7±6.3 vs 23.9±2.1 *vs* 17.9±3.5*; p<0.01	Biomarker of NETs formation is increased in DVT patients and correlates with DVT biomarkers.
van Montfoort et al. (2013)[21]	circulating nucleosome and activated neutrophils	3.0; 95%CI 1.5–3.9; p<0.001	Increased biomarkers of NETs formation were correlated with a threefold risk of DVT.

data are expressed as
*mean ± SD
** median % adhesion

Discussion

Significant correlations between NETs and the incidence of VTE were demonstrated in all included studies. Citrullinated histone H3 (H3Cit) has been proposed as a target biomarker, which reflects the formation of NET.[24,25] Mauracher et al. mentioned cancer patients with elevated H3Cit levels experiencing higher cumulative VTE events than patients with H3Cit levels under this limit with a 2-year risk of 14.5% and 8.5%, respectively. Competing-risk regression analysis showed that for each increase in H3Cit levels of 100 ng / mL, there is a relative increase of 13% in the risk of VTE (subdistribution hazard ratio [SHR] 1.13; 95% CI 1.04-1.22). This relationship remained after adjustment for tumor site with very high VTE risk and high VTE risk, dissolved P-selectin levels, and D-dimer levels.[20]

Histones are the most abundant protein in NETs. Histones are positively charged proteins that have the function to wrap DNA in the nucleus. This helps regulate the activation of platelet and thrombin in a dose-dependent manner.[26] Platelets reveal several characteristics associated with activation such as aggregation, phosphatidylserine presentation, and P-selectin surface expression after treatment with histones.[27] Importantly, the compilation of histones complexes with DNA (such as those encountered in NETs), their ability to support platelet and generation of thrombin is strengthened.[27] It has also been proposed that histone contributes to thrombin activation by isolating protein C and thrombomodulin and preventing the activation of thrombomodulin-dependent protein C.[28] Varied experiments in animal models showed that intravenous histones promote DVT risk in the context of inferior vena cava in mice.[7]

The population used in the study by Mauracher et al. is cancer patients.[20] Cancer populations have an increased risk of VTE. A review by Horsted et al. stated that each year, VTE develops in more than 1% of cancer populations, but it differs significantly by time since diagnosis, type of cancer, stage of the disease, and treatment modality. Horsted et al. also stated that brain and pancreatic cancer had the highest risk of VTE.[29] Tumor cells release cell-free DNA, growth factors, and procoagulant factors, which increase VTE. These factors include tissue factor (TF)-positive microvesicles, granulocyte-colony stimulation factors (G-CSF), thrombopoietin (TPO), and IL-6. The release of G-CSF enhances the release of NETs from neutrophils and the expression of tissue factors by monocytes. Other drugs, including chemotherapy used to treat cancer, can also injure the endothelium and increase the level of plasma cell-free DNA, which may contribute to the mechanism of cancer-related thrombosis.[30]

Van Montfoort et al. explore the relationship between elastase complex - α1-antitrypsin (EA) and nucleosomes, and the presence of DVT. Activation of neutrophils consequently leads to the NETs formation and nucleosome exposure to NETs contributes to in vivo coagulation activation and DVT development. Activated neutrophils were measured by EA. Patients with nucleosome levels more than 80th percentile from controls had an increase in DVT risk compared to patients with levels less than equal 80th percentile (OR 3.0; 95% CI 1.5-3.9). Besides, patients with EA complex levels more than 80th percentile from controls had an increase in DVT risk compared to patients with levels less than equal 80th percentile (OR 2.4; 95% CI 1.5-3.9). Increased levels of EA complex

and circulating nucleosomes are associated with a threefold DVT risk, and the relationship remains the same after adjusting for potential confounders (smoking, malignancy, recent hospitalization, and recent immobilization). This risk increased with a higher level of EA and nucleosomes, showing a relationship between circulating nucleosomes, activated neutrophils, and DVT in a dose-dependent way.[21]

Diaz et al. showed that circulating DNA (a surrogate marker for NETs) was increased in patients with DVT significantly, compared with patients without DVT (57.7 ± 6.3 vs. 17.9 ± 3.5 ng/mL; $p<0.01$) and controls (57.7 ± 6.3 vs. 23.9 ± 2.1 ng/mL; $p<0.01$). Strong positive correlations were found with D-dimers, C-reactive protein, vWF, myeloperoxidase/MPO, and Wells scores, and also strong negative correlations with disintegrin and metalloproteinase with type 1 thrombospondin motifs, member 13 (ADAMTS13) and ADAMTS13/vWF ratio. A strong relationship between plasma DNA and the presence of DVT was showed in logistic regression analysis (receiver operating characteristics/ROC curve 0.814).[22]

Plasma contains circulating DNA (cDNA) and is increased in cardiovascular, inflammatory, and thromboembolic disorders. In vivo and in vitro data showed the vital role of cDNA in thrombosis and inflammation. DNase infusion reduces tissue inflammation and prevents thrombus formation in murine models by decreasing cDNA.[12,31] Circulating DNA may be produced from dead cells and release of NETs from neutrophils, which is described as DNA fibers that contain enzymes released from neutrophil granules and histones. A study by Jiménez-Alcázar et al. showed that cDNA levels increased in patients with VTE compared with healthy controls. However, the relationship of cDNA to VTE level and outcome is poorly understood. They measured cDNA and nucleosomes as well as plasma circulating NETs from acute VTE patients. Their study suggested that cDNA levels but not NETs showed the level of inflammation and may function as a biomarker for stratification of patients with VTE who are at risk of death.[32]

Fibronectin (FN) is a high molecular weight plasma glycoprotein produced by hepatocytes from the extracellular matrix that binds to integrins (membrane-spanning receptor proteins). Fibronectin plays a vital role in many cellular processes, including tissue repair, embryogenesis, cell migration/adhesion, and blood clotting. Because fibronectin is on the surface of endothelial cells and is secreted into the cell matrix, the adhesive properties of FN are modulated and enhanced by cell retaining to collagen or proteoglycan substrates.[33] Similar neutrophil adhesive properties of fibronectin (FN) between VTE and control patients were demonstrated in a study by Zapponi et al. Subgroup evaluation of patients who showed high D-dimer levels and residual venous occlusion (RVO) (n = 15; 51%), a significant increase in neutrophil adhesive properties when compared with the controls and the remaining patients could be observed, respectively (24.68%, 19.29%, 18.23%, p = 0.0179).[23]

For average-risk studies, the prevalence of VTE varied between 9.4%[20] to 50%[21,22]. Some studies use case-control designs that usually use case and control with almost the same comparison; therefore, three studies showed a VTE prevalence of around 50%. One cohort of cancer patient showed that NETs increase VTE risk (9.4%), two case-controls showed NETs correlate with DVT (45% and 50%),[21,22] and one case study showed there is increasing NETs in

RVO and increased D-dimer with 50% prevalence.[23]

Several limitations of our study include non-random trial assignments, various populations, and small sample sizes. Four included studies also using various biomarkers in NETs measurement and having various study outcomes.

Conclusions

Neutrophils' role in thrombogenesis is the recent advances in understanding VTE. Neutrophils are unique and essential; NETs formation has been shown to have a causative role in increasing VTE risk. More research is necessary to determine the NETs' role in VTE as a diagnostic biomarker or target of therapy.

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