

Neutrophil extracellular traps (NETs) and thrombosis in COVID-19

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ABSTRACT

Here, we report on five patients whose hospitalizations for COVID-19 were complicated by venous thromboembolism (**VTE**). All demonstrated high levels of D-dimer as well as high neutrophil-to-lymphocyte ratios. For four patients we were able to test sera for neutrophil extracellular trap (**NET**) remnants and found significantly elevated levels of cell-free DNA, myeloperoxidase-DNA complexes, and citrullinated histone H3. Neutrophil-derived S100A8/A9 (calprotectin) was also elevated. Given strong links between hyperactive neutrophils, NET release, and thrombosis in many inflammatory diseases, the potential relationship between NETs and VTE should be urgently investigated in COVID-19.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (**SARS-CoV-2**) causes the disease known as coronavirus disease 2019 (**COVID-19**). It most commonly presents with influenza-like illness and viral pneumonia, but in its most severe manifestation progresses to acute respiratory distress syndrome (**ARDS**) and multi-organ failure¹. To date the viral pandemic has resulted in more than three million infections worldwide (<https://coronavirus.jhu.edu/map.html>).

In COVID-19, elevated levels of blood neutrophils predict severe respiratory disease and unfavorable outcomes^{2, 3}. Neutrophil-derived neutrophil extracellular traps (**NETs**) play a pathogenic role in many thrombo-inflammatory states including sepsis^{4, 5}, thrombosis⁶⁻⁸, and respiratory failure^{9, 10}. NETs are extracellular webs of chromatin and microbicidal proteins that are an evolutionarily conserved aspect of innate immune host-defense¹¹; however, NETs also have potential to initiate and propagate inflammation and thrombosis^{12, 13}. NETs deliver a variety of oxidant enzymes to the extracellular space, including myeloperoxidase, NADPH oxidase, and nitric oxide synthase¹⁴, while also serving as a source of extracellular histones that carry significant cytotoxic potential^{15, 16}. NETs are drivers of cardiovascular disease by propagating inflammation in vessel walls¹⁷. Furthermore, when formed intravascularly, NETs can occlude arteries¹⁸, veins¹⁹, and microscopic vessels²⁰. Early studies of COVID-19 suggest a high risk of morbid arterial events²¹, and the risk of venous thromboembolism (**VTE**) is increasingly revealing itself as more data become available²².

Descriptive and mechanistic studies to date that examine COVID-19 pathophysiology have focused on monocytes and lymphocytes more so than neutrophils and their effector products—including NETs²³. Here, we describe five cases of VTE in patients hospitalized with COVID-19 and provide evidence for neutrophil hyperactivity.

RESULTS

We identified five patients admitted to a large academic medical center with COVID-19 who also developed VTE (either deep vein thrombosis or pulmonary embolism) despite immediate initiation of prophylactic-dose heparin. Three of the patients were diagnosed with VTE within 48 hours of admission. All three had markedly elevated D-dimers and neutrophil-to-lymphocyte ratios (**Table 1**). For two of the early VTE patients, we were able to access sera for measurement of NET remnants. Three different NET-associated markers (cell-free DNA, myeloperoxidase-DNA complexes, and citrullinated-histone H3) were elevated in both patients

(**Table 1**). We also detected elevated levels of the classic marker of neutrophil activation, S100A8/A9 (also known as calprotectin). Neither patient had positive testing for traditional antiphospholipid antibodies²⁴.

We identified two additional patients who developed VTE several weeks into their hospitalizations. Patient 4 was a 66-year-old man whose course was complicated by respiratory failure requiring mechanical ventilation beginning on day 6. He received prophylactic-dose heparin throughout his hospitalization, but was nevertheless diagnosed on day 20 with extensive right-lower-extremity deep vein thrombosis. We were able to test serum from day 8 of his hospitalization for NET remnants (**Table 1**). Interestingly, markers of neutrophil activation including NET remnants were already significantly elevated at day 8 (**Table 1**); this is in contrast to D-dimer and neutrophil count, which were only mildly elevated at the time. Both D-dimer and neutrophil count were elevated by the time the patient was diagnosed with deep vein thrombosis on day 20 (18.1 mg/L and 10.9 K/ul, respectively).

Patient 5 was a 49-year-old man who was intubated at the time of admission. On hospital day 9, extracorporeal membrane oxygenation (ECMO) was initiated. ECMO was required for 14 days, and then discontinued. He received unfractionated heparin with goal PTT 40-45 for thrombosis prophylaxis. 29 days into the hospitalization, he had a bleeding complication and unfractionated heparin was discontinued. A chest CT scan was obtained on the same day to assess for additional etiologies of his ongoing respiratory failure, and he was found to have acute bilateral segmental and subsegmental pulmonary embolism. We were able to measure NET remnants 12 days before his diagnosis of pulmonary embolism, and found them to be significantly elevated (**Table 1**).

DISCUSSION

Hyperactivity of the coagulation system is a common finding of severe COVID-19²⁵. Indeed, many patients have a profile to suggest a prothrombotic diathesis including high levels of fibrin degradation products (D-dimer), elevated fibrinogen levels, and low antithrombin levels^{25, 26}. Here, we report five cases of COVID-19-associated VTE. We measured three markers commonly used to detect NET remnants in blood (cell-free DNA, myeloperoxidase-DNA complexes, and citrullinated-histone H3)²³, as well as a fourth marker, S100A8/A9 (calprotectin), which is commonly used to track neutrophil activation. All tests were elevated in patients diagnosed with VTE, including as early as 12 days prior to detection of VTE in two cases.

Given the known link between NETs and venous thrombosis in many inflammatory diseases, these data suggest that the role of NETs in COVID-19-associated thrombophilia warrants systematic investigation. While a recent report suggested that traditional antiphospholipid antibodies may be drivers of thrombosis in some COVID-19 patients²⁴, such antibodies were not detected here.

Examples of NETs as drivers of thrombosis are myriad, as intravascular NET release is responsible for initiation and accretion of thrombotic events in arteries, veins, and microvessels, where thrombotic disease can drive end-organ damage in lungs, heart, kidneys, and other organs^{27, 28}. Mechanistically, DNA in NETs may directly activate the extrinsic pathway of coagulation²⁹, while NETs also present tissue factor to initiate the intrinsic pathway³⁰. Serine proteases in NETs such as neutrophil elastase dismantle brakes on coagulation such as tissue factor pathway inhibitor³¹. Bidirectional interplay between NETs and platelets might also be critical for COVID-19-associated thrombosis as has been characterized in a variety of disease models^{28, 29}.

Approaches to combatting NETs³²⁻³⁴ include the dismantling of NETs with deoxyribonucleases and strategies that prevent initiation of NET release such as neutrophil elastase inhibitors and peptidylarginine deiminase 4 inhibitors. As we await definitive antiviral and immunologic solutions to the current pandemic, we posit that anti-neutrophil therapies may be part of a personalized strategy for some individuals affected by COVID-19. Furthermore, those patients with hyperactive neutrophils may be at particularly high risk for VTE and might therefore benefit from more aggressive anticoagulation while hospitalized.

METHODS

Human samples. Blood was collected into serum separator tubes by a trained hospital phlebotomist. After completion of biochemical testing ordered by the clinician, the remaining serum was stored at 4°C for up to 48 hours before it was deemed “discarded” and released to the research laboratory. Serum samples were immediately divided into small aliquots and stored at -80°C until the time of testing. This study complied with all relevant ethical regulations, and was approved by the University of Michigan Institutional Review Board (HUM00179409), which waived the requirement for informed consent given the discarded nature of the samples. Healthy volunteers were recruited through a posted flyer; exclusion criteria for these controls included history of a systemic autoimmune disease, active infection, and pregnancy. For

preparation of control serum, blood was collected in a similar manner as the COVID-19 patient samples. These serum samples were divided into small aliquots and stored at -80°C until the time of testing.

Quantification of cell-free DNA. Cell-free DNA was quantified in sera using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, P11496) according to the manufacturer's instructions.

Quantification of citrullinated-histone H3. Citrullinated-histone H3 was quantified in sera using the Citrullinated Histone H3 (Clone 11D3) ELISA Kit (Cayman, 501620) according to the manufacturer's instructions.

Quantification of myeloperoxidase-DNA complexes. Myeloperoxidase-DNA complexes were quantified similarly to what has been previously described³⁵. This protocol used several reagents from the Cell Death Detection ELISA kit (Roche). First, a high-binding EIA/RIA 96-well plate (Costar) was coated overnight at 4°C with anti-human myeloperoxidase antibody (Bio-Rad 0400-0002), diluted to a concentration of 1 µg/ml in coating buffer (Cell Death kit). The plate was washed two times with wash buffer (0.05% Tween 20 in PBS), and then blocked with 4% bovine serum albumin in PBS (supplemented with 0.05% Tween 20) for 2 hours at room temperature. The plate was again washed five times, before incubating for 90 minutes at room temperature with 10% serum or plasma in the aforementioned blocking buffer (without Tween 20). The plate was washed five times, and then incubated for 90 minutes at room temperature with 10x anti-DNA antibody (HRP-conjugated; from the Cell Death kit) diluted 1:100 in blocking buffer. After five more washes, the plate was developed with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Invitrogen) followed by a 2N sulfuric acid stop solution. Absorbance was measured at a wavelength of 450 nm using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek). Data were normalized to *in vitro*-prepared NET standards included on every plate, which were quantified based on their DNA content.

Quantification of antiphospholipid antibodies. Antiphospholipid antibodies were quantified in sera using the Quanta Lite® ACA IgG, ACA IgM, ACA IgA, β2GPI IgG, β2GPI IgM, β2GPI IgA Kits (Inova Diagnostics) according to the manufacturer's instructions. Manufacturer-recommended cut off was used to determine positive values.

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AUTHORSHIP

YZ, MZ, SY, KG, JM, and HS conducted experiments and analyzed data. YZ, MZ, YK, and JSK conceived the study and analyzed data. All authors participated in writing the manuscript and gave approval before submission.

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Table 1: Characteristics of five patients diagnosed with VTE					
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	44	38	67	66	49
Type of VTE	bilateral LE DVT	bilateral LE DVT	PE	right LE DVT	PE
Hospital day	1	2	2	20	29
Quantification of neutrophil extracellular traps (NETs)					
Timing (relative to VTE)	same day	same day	same day	12 days prior	12 days prior
Cell-free DNA (<1.1 µg/ml)*	2.75	3.01	n/a	1.36	2.19
MPO-DNA (<0.91 µg/ml)*	9.42	0.95	n/a	3.68	1.88
Cit-histone H3 (<27.7 ng/ml)*	36.3	41.2	n/a	28.0	89.5
S100A8/A9 (<1427 ng/ml)*	22259.18	9503	n/a	3352	32694
Clinical status	mechanical vent.	mechanical vent.	high-flow O ₂	ECMO	mechanical vent.
D-dimer (<0.59 mg/L)	>35	>35	>35	0.43	30.9
CRP (≤0.6 mg/dl)	31.6	30.8	13.9	14.6	51.6
ANC (≤7.2 K/µl)	17.4	8.1	6.4	4.6	n/a
ALC (≥1.2 K/µl)	0.7	0.8	0.5	0.7	n/a
N-to-L ratio	24.9	14.7	12.8	6.6	n/a
Antiphospholipid antibodies					
aCL IgG/IgM/IgA	negative	negative	n/a	negative	negative
aβ ₂ GPI IgG/IgM/IgA	negative	negative	n/a	negative	negative
*defined as mean + 2 standard deviations for 30 healthy volunteers n/a=data not available VTE=venous thromboembolism; LE=lower extremity; DVT=deep vein thrombosis; PE=pulmonary embolism; ECMO=extracorporeal membrane oxygenation; CRP=C-reactive protein; ANC=absolute neutrophil count; ALC=absolute lymphocyte count; MPO=myeloperoxidase; Cit-histone H3=citrullinated-histone H3; aCL=anticardiolipin; aβ ₂ GPI=anti-beta-2 glycoprotein I					