

REVIEW

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Clinical significance of neutrophil extracellular traps biomarkers in thrombosis

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Abstract

Neutrophil extracellular traps (NETs) may be associated with the development of thrombosis. Experimental studies have confirmed the presence of NETs in thrombi specimens and potential role of NETs in the mechanisms of thrombosis. Clinical studies also have demonstrated significant changes in the levels of serum or plasma NETs biomarkers, such as citrullinated histones, myeloperoxidase, neutrophil elastase, nucleosomes, DNA, and their complexes in patients with thrombosis. This paper aims to comprehensively review the currently available evidence regarding the change in the levels of NETs biomarkers in patients with thrombosis, summarize the role of NETs and its biomarkers in the development and prognostic assessment of venous thromboembolism, coronary artery diseases, ischemic stroke, cancer-associated thromboembolism, and coronavirus disease 2019-associated thromboembolism, explore the potential therapeutic implications of NETs, and further discuss the shortcomings of existing NETs biomarkers in serum and plasma and their detection methods.

Keywords: Neutrophil, Neutrophil extracellular traps, Thrombosis, Citrullinated histones, Myeloperoxidase

Introduction

Thrombosis, which refers to the formation of blood clots in arterial and venous vessels, is a consequence of inherited or acquired imbalance of procoagulant, anticoagulant, and fibrinolytic factors [1], and results in high morbidity and mortality [2, 3]. Knowledge regarding underlying mechanisms of thrombosis is necessary to improve its management strategy. Traditionally, it is thought that thrombus should be formed by the interaction of platelets, fibrin, and red blood cells. Neutrophils are the first-line defense against invading pathogens [4]. Recently, it has been recognized that the release of

neutrophil extracellular traps (NETs) may contribute to the development of thrombosis [5–8]. NETs release is caused by stimulated neutrophils which form web-like structures mainly composed of extracellular DNA, histones, and granular proteins, such as neutrophil elastase (NE), myeloperoxidase (MPO), and calprotectin, etc [9, 10]. The current review paper primarily aims to summarize the role of NETs and its biomarkers in the development and prognostic assessment of venous thromboembolism (VTE), coronary artery diseases (CAD), ischemic stroke (IS), cancer-associated thromboembolism, and coronavirus disease 2019 (COVID-19)-associated thromboembolism, explore the potential therapeutic implications of NETs, and further discuss the shortcomings of existing NETs biomarkers in serum and plasma and their detection methods.

Mechanisms of NETs formation

NETs formation, a unique form of cell death process [11], release decondensed chromatin and granular

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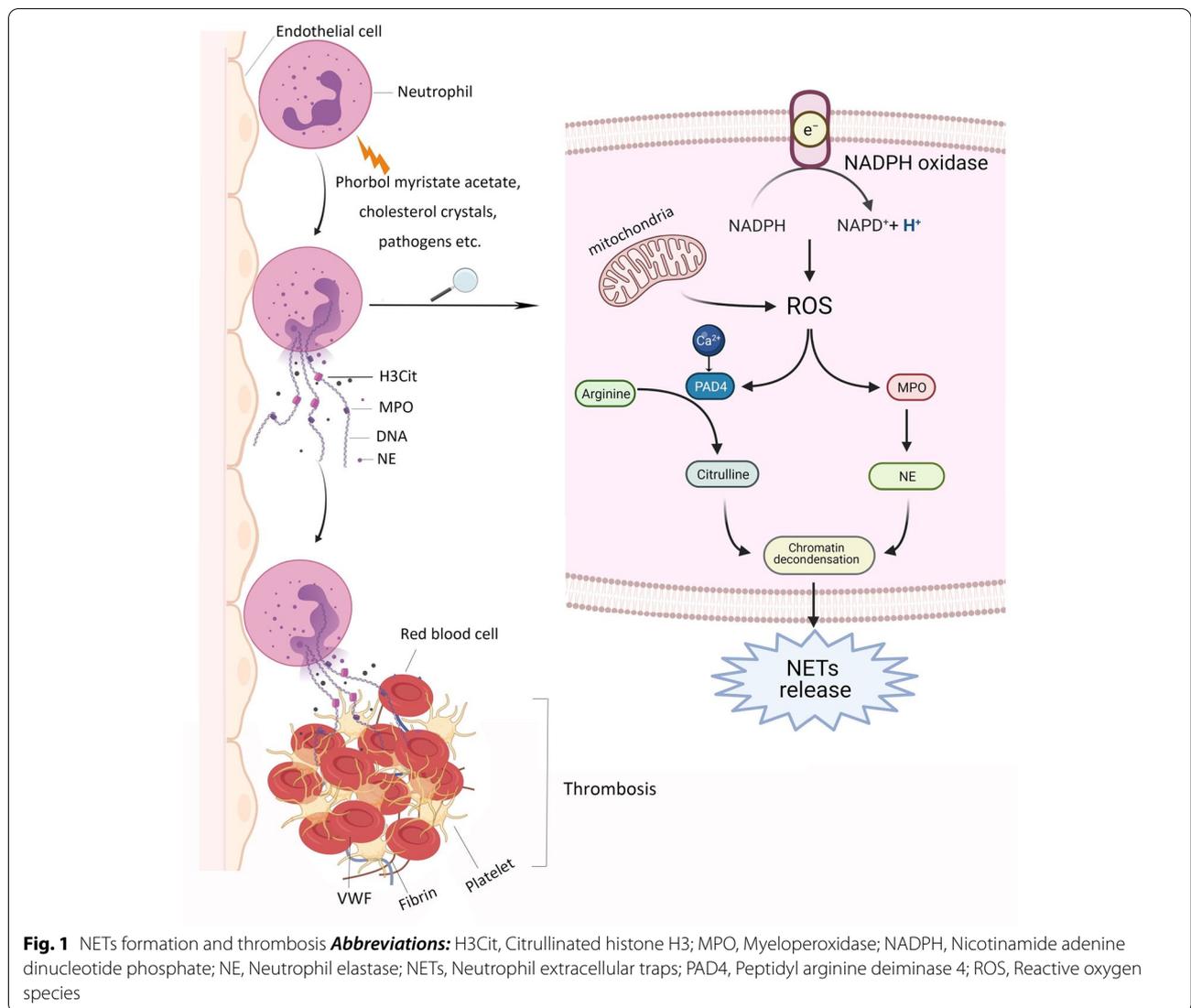
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proteins with nuclear materials [12]. Until now, there are two potential mechanisms of NETs formation [13]. The first mechanism is lytic-NETs formation, which can be induced by phorbol myristate acetate or cholesterol crystal. Peptidyl arginine deiminase 4 (PAD4) may be activated by reactive oxygen species (ROS) [13–15], which can be generated by nicotinamide adenine dinucleotide phosphate (NADPH) or mitochondria [16, 17], or calcium ionophore [18], thereby leading to the citrullination of arginine residues of histones [18]. Notably, gasdermin D is required for ROS generation [19]. Meanwhile, MPO and NE can be translocated by ROS into the nucleus [20]. Subsequently, neutrophils exhibit rapid disassembly of the actin cytoskeleton, followed by shedding of plasma membrane microvesicles, disassembly and remodeling of the microtubule and vimentin

cytoskeletons, endoplasmic reticulum vesiculation, chromatin decondensation and nuclear rounding, and progressive permeabilization of plasma membrane and nuclear envelope [21]. Then, protein kinase C α -mediated lamin B phosphorylation drives nuclear envelope rupture to release chromatin [22]. Finally, NETs are released after plasma membrane rupture [21] (Fig. 1). The second mechanism is non-lytic NETs formation, which can be induced by certain bacteria, such as *E. coli*, *S. aureus*, or *Candida albicans*, through the activation of neutrophils mediated by Toll-like receptors (TLRs) or complement receptors [23], independent of NADPH oxidase activation. By this way, neutrophils are still alive and preserve their functions to move and phagocytose to some extent [23]. Besides, autophagy may provide another insight into the mechanisms of NETs formation [24, 25]. Collectively,

some biomarkers involved in the NETs formation should include independent extracellular DNA, proteins derived from neutrophils (i.e., MPO and NE), proteins required for NETs formation (i.e., PAD4 and citrullinated histones), and their complexes.

NETs promote thrombosis

NETs may contribute to the development of thrombosis by forming a “scaffold”, which induces platelets adhesion, activation, and aggregation, recruits red blood cells, and maintains the stability of thrombus together with fibronectin, fibrinogen, and von Willebrand factor (VWF) [26]. The interaction of neutrophils with platelets depends on the adhesion molecules, such as P-selectin, P-selectin glycoprotein ligand 1, glycoprotein Ib, and macrophage-1 antigen [27]. Additionally, platelet-derived high mobility group box 1 (HMGB1) mediates both NETs formation and thrombosis [28, 29]. HMGB1 can interact with TLR4 [30], enabling neutrophils to release NETs. Furthermore, HMGB1 can promote early recruitment of platelets [31], thereby enhancing the pro-thrombotic effect and promoting the development of thrombosis.

The components of NETs themselves can also affect the formation of thrombosis. Histones are responsible for tissue factor activity [32], platelet activation via mediating TLR2 and TLR4 [33], platelet aggregation via inducing calcium influx and fibrinogen recruitment [34], reduction of thrombomodulin-dependent protein C activation [35], and release of activated thrombin [36, 37]. Furthermore, histone 4 promotes prothrombin autoactivation to thrombin [38]. DNA, which is deemed as another component of NETs, is reported to shorten clotting time, promote FXII activation and FXIa generation, and amplify tissue factor-initiated thrombin generation [12]. Both histones and DNA can increase the median fiber diameter of plasma clots [39]. PAD4 can accelerate the development of thrombosis via protecting VWF-platelets string from the cleavage of endogenous a disintegrin and metalloproteinase with thrombospondin type-1 motif-13 (ADAMTS13) [40]. Other components of NETs, including NE, cathepsin G, and nucleosomes, are responsible for promoting coagulation and intravascular thrombus growth through enhancing intrinsic and extrinsic coagulation pathways [41].

Collectively, NETs can affect the development of thrombosis via multiple ways. Additional evidence regarding how NETs promote thrombosis is also emerging.

NETs biomarkers and VTE

VTE primarily comprises of deep vein thrombosis (DVT) and pulmonary embolism (PE). Experimental and clinical studies have confirmed the presence of

NETs biomarkers in VTE specimens. Extracellular DNA was in close proximity to neutrophils, together with positive staining of MPO, NE, and histones by immunostaining assay after induction of venous thrombosis [42]. Additionally, citrullinated histone H3 (H3Cit) was observed in the red [43] or fresh red fibrin-rich parts of thrombi [44]. In baboons with iliac vein thrombosis, dotted and diffuse staining of DNA and positive staining of DNA-histone could be observed in thrombi [26]. Human venous thrombi from surgical samples or autopsies revealed the colocalization of DNA, DNA-histone complexes, and MPO [45], that of DNA, MPO, CD11b, and H3Cit [46], and that of DNA, MPO, H3Cit, pan-Cit, and PAD4 in organizing thrombi [46].

NETs biomarkers have been quantitatively evaluated in VTE patients in several clinical studies [44, 47–58] (Table 1). The levels of plasma DNA [53], H3Cit-DNA [58], and NE [58] were elevated in VTE patients. The level of plasma MPO had a good diagnostic performance of VTE [59], while the diagnostic accuracy of H3Cit-DNA and NE was not superior to that of D-dimer [58]. On the other hand, the expression of NETs biomarkers may depend on the locations of VTE. The levels of plasma DNA and nucleosomes were significantly different between elderly patients with PE and DVT [50]. Besides, the levels of plasma DNA and calprotectin were higher in patients with splanchnic vein thrombosis (SVT) than those with DVT, whereas the level of MPO was much higher in patients with DVT of the lower limbs than those with SVT [52]. Clinical evidence regarding NETs biomarkers in patients with VTE at various locations are separately reviewed in the following paragraphs.

DVT

In a case-control study, the levels of plasma NE- α 1-antitrypsin complexes and nucleosomes \geq 80th percentile (odds ratio [OR] = 3.0 and OR = 2.4) significantly increased the risk of symptomatic DVT regardless of adjustment for potential confounders [49]. By contrast, another study did not show any significant difference in the levels of serum NE and nucleosomes between patients with DVT and healthy controls [51]. Thus, more evidence is necessary to clarify the association of NE and nucleosomes with DVT. Notably, among the currently published studies, the levels of serum MPO, MPO-DNA, and DNA were significantly higher in patients with DVT than those without DVT or healthy controls [48, 51, 52]. Furthermore, the levels of plasma H3Cit and DNA can be used for the diagnosis of DVT in patients with traumatic fractures [55].

Table 1 Studies evaluating NETs biomarkers in VTE

First author/year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
Arnalich et al (2013) [47]	Case-control and cohort	Patients with acute massive or sub-massive PE, confirmed with computed tomographic pulmonary angiography	Massive PE (n = 37) vs. Sub-massive PE (n = 37) vs. HC (n = 37)	Plasma, 4 °C, 1800 × g, 10 min	Mitochondrial DNA Nuclear DNA	qPCR qPCR	2970 vs. 870 vs. 185 GE/mL 3325 vs. 1245 vs. 520 GE/mL
Diaz et al (2013) [48]	Case-control	Patients performed duplex ultrasound to confirm the presence of DVT	DVT (n = 47) vs. Negative DVT (n = 28) vs. HC (n = 19)	Plasma, 4 °C, 2000 × g, 10 min	MPO DNA	ELISA SytoxGreen fluorimetry	31.7 vs. 15.5 vs. 5.7 AU 57.7 vs. 17.9 vs. 23.9 ng/mL
van Montfoort et al (2013) [49]	Case-control	Adult patients with and without acute symptomatic DVT of the leg	DVT (n = 150) vs. No DVT (n = 195)	Plasma, RT, 1500 × g, 15 min	NE-α1-antitrypsin Nucleosomes	ELISA ELISA	53 vs. 45 ng/mL 17 vs. 9 U/mL
Jiménez-Alcázar et al (2018) [50]	Case-control and cohort	Patients aged > 65 years with acute, symptomatic VTE	Distal DVT (n = 51) vs. Proximal DVT (n = 133) vs. PE (n = 427)	Plasma	DNA-histone-MPO Nucleosomes DNA	ELISA ELISA SytoxGreen fluorimetry	NA NA NA
Lee et al (2018) [51]	Case-control	Patients with sepsis and thrombosis	DVT (n = 25) vs. HC (n = 23)	Serum, 4 °C, 1500 × g, 15 min	MPO MPO-DNA Nucleosomes	ELISA ELISA ELISA	250.5 vs. 120.4 ng/mL 0.07 vs. 0.05 OD 0.3 vs. 0.1 U/L
Martos et al (2020) [52]	Case-control	Patients with VTE	DVT (n = 192) vs. SVT (n = 61) vs. HC (n = 249)	Plasma, 4 °C, 1811 × g, 30 min	NE DNA MPO	ELISA SytoxGreen fluorimetry ELISA	370.8 vs. 162.4 ng/mL 22.3 vs. 8.1 ng/mL 1728.5 vs. 1882.5 vs. 1250.0 ng/mL
Medeiros et al (2020) [53]	Case-control	Patients with VTE and anticoagulation therapy	VTE off warfarin (n = 263) vs. VTE on warfarin (n = 245) vs. HC (n = 50)	Plasma, 20 °C, 1700 × g, 15 + 5 min	Calprotectin DNA	ELISA QIAamp	NA 5.53 vs. 3.11 vs. 2.77 µg/mL
Ząbczyk et al (2020) [54]	Case-control and cohort	Patients with acute PE	Acute PE (n = 126) vs. HC (n = 25)	Plasma, 2500 × g, 10 min	H3Cit	ELISA	2.77 vs. 0.59 ng/mL
Liu et al (2021) [55]	Case-control	Patients with traumatic fracture	Trauma non-DVT (n = 37) vs. Trauma DVT (n = 39) vs. DVT (n = 34) vs. HC (n = 24)	Plasma, 2500 × g, 15 min	H3Cit Nucleosomes DNA	ELISA ELISA PicoGreen fluorimetry	0.38 vs. 0.87 vs. 1.88 vs. 1.79 ng/mL 1.20 vs. 1.03 vs. 1.29 vs.—ratio 185.56 vs. 165.70 vs. 216.15 vs. 135.08 ng/mL

Table 1 (continued)

First author/year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
Sharma et al (2021) [44]	Case-control	Patients with stable CTEPH	CTEPH (n = 141) vs. Controls (n = 60)	Plasma, 2000 × g, 10 min	MPO H3Cit	ELISA ELISA	NA NA
Turon et al (2021) [56]	Cohort	Patients with cirrhosis	PVT (n = 23) vs. No PVT (n = 287)	Plasma	DNA MPO-DNA	SytoxGreen fluorimetry ELISA	NA 0.21 vs. 0.29 AU
Xing et al (2022) [57]	Case-control	Patients with cirrhosis	PVT (n = 28) vs. No PVT (n = 44)	Plasma, 1000 × g, 1.5 min	MPO NE H3Cit	PicoGreen fluorimetry ELISA ELISA ELISA	0.89 vs. 0.89 µg/mL NA NA NA
Smith et al (2022) [58]	Case-control	Patients with VTE	VEBIO ER Cohort: VTE (n = 51) vs. No VTE (n = 96) vs. HC (n = 30) DFW-VTE Cohort: VTE (n = 61) vs. No VTE (n = 86) vs. HC (n = 30)	Plasma, 3000 × g, 1.5 min	H3Cit-DNA	ELISA	VEBIO ER Cohort: 110 vs. 73 vs. 38 ng/mL DFW-VTE Cohort: 102 vs. 54 vs. 38 ng/mL
					NE	ELISA	VEBIO ER Cohort: 31 vs. 24 vs. 21 ng/mL DFW-VTE Cohort: 49 vs. 38 vs. 21 ng/mL
					DNA	PicoGreen fluorimetry	VEBIO ER Cohort: 423 vs. 405 vs. 421 ng/mL DFW-VTE Cohort: 396 vs. 392 vs. 421 ng/mL

Abbreviations: AU Absorbance unit, CTEPH Chronic thromboembolic pulmonary hypertension, DVT Deep vein thrombosis, HC Healthy control, H3Cit Citrullinated histone H3, ELISA Enzyme-linked immunosorbent assay, Min Minute, MPO Myeloperoxidase, NA Not available, NE Neutrophil elastase, NETs Neutrophil extracellular traps, OD Optical density, PE Pulmonary embolism, PVT Portal vein thrombosis, qPCR Quantitative polymerase chain reaction, RT Room temperature, SVT Splanchnic vein thrombosis, VTE Venous thromboembolism

PE

A recent study found that the levels of plasma neutrophils, MPO, and DNA, rather than H3Cit, were significantly elevated in patients with chronic thromboembolic pulmonary hypertension as compared with healthy controls [44]. By contrast, another study demonstrated that the level of plasma H3Cit was almost fivefold higher in patients with acute PE than healthy controls [54]. Such a difference in the expression of plasma H3Cit between the two studies might be attributed to the stage of disease (chronic versus acute). On the other hand, NETs biomarkers can also reflect the severity of PE. The levels of plasma DNA deriving from mitochondria and nucleus were higher in patients with massive PE than those with sub-massive PE [47]. Notably, it should be acknowledged that this change of NETs biomarkers might also be derived from damaged tissues during severe PE. Additionally, higher level of plasma DNA was independently associated with increased PE-related mortality [50] and all-cause mortality [47, 50], but not the recurrence of VTE during a 3-year follow-up period [50]. Similarly, the level of plasma H3Cit could also predict acute PE-related death [54].

PVT

A European prospective cohort study did not find any significant relationship between the levels of plasma MPO-DNA and DNA at baseline and the development of portal vein thrombosis (PVT) in patients with liver cirrhosis during a mean follow-up period of 48 months [56]. However, it should be noted that a majority of patients included in this cohort study had Child–Pugh class A, suggesting that they had well-preserved hepatic function [60]. By comparison, a Chinese cross-sectional study, in which a majority of cirrhotic patients included had Child–Pugh class B + C (69.4%), demonstrated that the levels of plasma H3Cit, NE, and MPO were significantly higher in patients with PVT than those without PVT, and positively correlated with thrombin-antithrombin (TAT) complex and FX, which are well-known markers for hypercoagulability [57]. Such a controversy should be further clarified in cirrhotic patients according to the severity of liver dysfunction.

NETs biomarkers and CAD

CAD encompasses stable angina, unstable angina, myocardial infarction (MI), and sudden cardiac death due to the occurrence of atherosclerosis or thrombosis in coronary arteries [61]. NETs formation has been detected by positive staining of Ly6G, DNA, MPO, and H3Cit in mice's atherosclerotic lesions [62–64]. Immunostaining assay found the colocalization of CD177, NE, and DNA in patients' carotid plaques [65]. By immunostaining of

patients' carotid and coronary plaques, another study also demonstrated that CD66b, NE, H4Cit, and DNA were in contact with the luminal surface of erosion-prone plaques and localized within rupture-prone plaques [66]. Additionally, the colocalization of histones, NE, and MPO was commonly observed in fresh and lytic coronary thrombi from MI patients, rather than organized coronary thrombi [67]. The other colocalizations of MPO, H3Cit, and DNA [68] and DNA, DNA-histone complexes, and MPO [45] were also detected in coronary thrombi.

Some clinical studies have been performed to evaluate the importance of NETs biomarkers in CAD patients [69–80] (Table 2). It seems that NETs biomarkers could predict the disease severity, hypercoagulability, and worse clinical outcomes in CAD patients. The levels of plasma MPO-DNA, nucleosomes, and DNA were significantly elevated in patients with more severe CAD, and could predict the number of diseased coronary artery segments and the incidence of major adverse cardiac events (MACE). Among them, only higher level of plasma nucleosomes was an independent risk factor for severe coronary stenosis, and only higher level of plasma DNA was independently associated with prothrombotic state [71]. Another large-scale study involving 1001 CAD patients found that higher level of serum DNA was significantly associated with hypercoagulability and predicted worse clinical outcomes [73]. Both studies suggested that DNA could predict hypercoagulability, and other NETs biomarkers, such as nucleosomes and MPO-DNA, might be useful to predict CAD progression.

MI

MI is primarily associated with plaque rupture and erosion [81]. Until now, the role of NETs biomarkers in patients with MI has been more comprehensively explored as compared to those with other types of CAD. The level of plasma DNA was higher in patients with acute MI (AMI) than healthy controls [69] and stable angina [72], and positively correlated with Gensini and GRACE scores [72] and peak levels of creatine kinase (CK) and troponin-T [70]. Particularly, in ST elevation MI (STEMI) patients admitted for percutaneous coronary intervention (PCI), the levels of plasma H3Cit [77], MPO-DNA [76, 78], and DNA [76–78] were significantly higher in infarct-related coronary arteries than peripheral arteries [76, 77] or in anterior MI than other locations of infarction [78]. The levels of serum MPO-DNA and DNA became the highest in STEMI patients before PCI, and decreased after PCI [74]. Both H3Cit and DNA levels positively correlated with infarct size [74, 77], and high level of DNA was usually associated with increased risk of developing lower left ventricular ejection fraction [74],

Table 2 Studies evaluating NETs biomarkers in CAD

First author/ year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
Antonatos et al (2006) [69]	Case-control and cohort	Patients with acute MI and underwent thrombolysis with reteplase within 6 h from onset of pain	Acute MI (n = 13) vs. HC (n = 30)	Plasma, 800 x g and 16,000 x g	DNA	qPCR	6873 vs. 4112 GE/mL
Shimony et al (2010) [70]	Case-control	Patients with acute STEMI	STEMI (n = 16) vs. HC (n = 47)	Serum	DNA	Sybr Gold fluorimetry	747 vs. 471 ng/mL
Borissoff et al (2013) [71]	Case-control and cohort	Patients with chest discomfort symptoms, suspected for CAD	Extremely calcified (n = 37) vs. Severe CAD (n = 45) vs. Moderate CAD (n = 74) vs. Mild CAD (n = 75) vs. No CAD (n = 51)	Plasma, 2000 x g, 15 min, 11,000 x g, 10 min	MPO-DNA Nucleosomes DNA	ELISA ELISA SytoxGreen fluorimetry	NA NA 79.37 (Extremely calcified) vs. 69.59 (Severe CAD) vs. 50.09 (No CAD) ng/mL
Cui et al (2013) [72]	Case-control	Patients with ACS and SA controls	ACS (n = 137) vs. SA (n = 13) vs. HC (n = 45)	Plasma, 25 °C, 1600 x g, 10 min, 16,000 x g, 1 min	DNA	Alu sequence-based bDNA assay	2285.0 vs. 202.3 vs. 118.3 ng/mL
Ramirez et al (2016) [79]	Case-control	Patients with STEMI underwent PCI within 1-6 h from the onset of chest pain and chronic SA controls	STEMI vs. Chronic SA vs. HC	Plasma, 4 °C, 320 x g, 15 min, 100,000 x g, 5 min	H4Cit MPO-DNA	ELISA ELISA	NA NA
Langseth et al (2018) [73]	Cohort	Patients with angiographically verified CAD, on aspirin monotherapy for at least 1 w	Clinical endpoint (n = 402) vs. No clinical endpoints (n = 394)	Serum, 2500 x g, 10 min	MPO-DNA DNA	ELISA PicoGreen fluorimetry	NA 402 vs. 394 ng/mL
Helseth et al (2019) [74]	Cohort	Patients with first-time STEMI within 6 h of symptom onset admitted for PCI	Before PCI (n = 259) vs. After PCI vs. (n = 258) vs. After PCI 1 d (n = 251/254) vs. After PCI 4 m (n = 258)	Serum, 2500 x g, 10 min	MPO-DNA DNA	ELISA PicoGreen fluorimetry	NA NA
Lim et al (2019) [75]	Case-control	Patients with newly diagnosed ACS or AIS	ACS (n = 37) vs. AIS (n = 58) vs. HC (n = 25)	Plasma, 1600 x g, 15 min	DNA-histone DNA	ELISA PicoGreen fluorimetry	19.73 vs. 13.71 vs. 14.32 mU 743.28 vs. 524.22 vs. 216.48 ng/mL
Liu et al (2019) [76]	Cohort	Patient was enrolled within 12 h of the onset of clinical signs and had STEMI with TIMI flow 0 before emergent PCI	Infarct-related artery (n = 36) vs. Peripheral arteries (n = 36)	Plasma	MPO-DNA DNA	ELISA SytoxGreen fluorimetry	0.44 vs. 0.28 0.41 vs. 0.31 µg/mL
Hofbauer et al (2019) [77]	Cohort and case-control	Patients with STEMI undergoing primary PCI for a coronary TIMI flow of 0	Culprit site (n = 48) vs. Femoral site (n = 48) vs. HC (n = 21)	Plasma, 1000 x g, 10 min	H3Cit DNA	ELISA PicoGreen fluorimetry	332 vs. 235 vs. 192 ng/mL 529 vs. 404 vs. 291 ng/mL

Table 2 (continued)

First author/ year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
Langseth et al (2020) [78]	Cohort	Patients diagnosed with STEMI admitted for PCI	Anterior MI (n = 413) vs. Other locations of infarction (n = 543)	Serum, 2500 × g, 10 min	H3Cit MPO-DNA	ELISA ELISA	9.71 vs. 8.69 ng/mL 0.188 vs. 0.171 OD
Hally et al (2021) [80]	Case-control	Patients diagnosed with MACE post-AMI within 1-year follow-up period	MACE (n = 100) vs. No MACE (n = 200)	Serum, 1500 × g, 12 min	DNA MPO-DNA NE-DNA H3Cit	PicoGreen fluorimetry ELISA ELISA ELISA	424 vs. 409 ng/mL 5.09 vs. 4.67 (% of NETs standard) 2.05 vs. 1.97 (% of pooled serum standard) 7.07 vs. 5.44 (% of NETs standard)

Abbreviations: ACS Acute coronary syndrome, AIS Acute ischemic stroke, AMI Acute myocardial infarction, CAD Coronary artery disease, D Day, ELISA Enzyme-linked immunosorbent assay, H Hour, H3Cit Citrullinated histone H3, HC Healthy control, M Month, MACE Major adverse cardiovascular events, MI Myocardial infarction, Min Minute, MPO Myeloperoxidase, NA Not available, NE Neutrophil elastase, NETs Neutrophil extracellular traps, OD Optical density, PCI Percutaneous coronary intervention, qPCR Quantitative polymerase chain reaction, SA Stable angina, STEMI ST-segment elevation myocardial infarction, TIMI Thrombolysis in myocardial infarction, W Week

adverse clinical events [76], and all-cause mortality [78]. Importantly, DNA level had a predictive value for in-hospital mortality in STEMI patients, which was equivalent to that of troponin I [75], troponin T, and CK-MB [76]. Higher level of serum DNA is also associated with hypercoagulability indicated by elevated D-dimer and prothrombin fragment 1 + 2 levels in STEMI patients [78]. A composite score of NETs biomarkers and platelet count showed the most favorable predictive value for MACE in non-ST and STEMI patients [80].

NETs biomarkers and IS

IS can be caused by cardiac embolism, atherosclerosis of cerebral circulation, and occlusion of small vessels resulting in high mortality and disability worldwide [82]. In a rat model, a significant increase in the level of serum DNA was observed at 24 h after the onset of IS. DNA level was positively associated with the total infarct volume, brain edema, and neurologic severity score (correlation coefficient = 0.78, 0.91, and 0.73, respectively) [83]. Abundant neutrophils and NETs were also found in thrombi from patients with acute IS (AIS) by the colocalization of CD66b, H3Cit, and DNA, that of H3Cit and NE [84], or that of H4Cit, MPO, and DNA [85]. Meanwhile, neutrophils and H3Cit were especially higher in older thrombi than fresh thrombi by calculating the area of H3Cit positive staining [84].

NETs biomarkers have been explored in AIS patients (Table 3) [75, 86, 87]. In a prospective cohort study, the level of plasma DNA was elevated by threefold in AIS patients compared with non-AIS patients, and exhibited a positive correlation with infarct size [86]. Besides, the levels of plasma nucleosomes and H3Cit were also elevated in AIS patients with a history of atrial fibrillation, NIHSS score > 14 at onset, NIHSS score \geq 6 at discharge, and mRankin scale score > 2 at discharge [87]. The highest quartile level of plasma H3Cit was independently associated with atrial fibrillation (OR = 6.7) and all-cause mortality (OR = 7.1) during one-year follow-up period [87]. Furthermore, the levels of plasma H3Cit, MPO, and DNA were significantly increased in IS patients with elevated hypersensitive troponin T levels as compared to those with normal hypersensitive troponin T levels [88].

NETs biomarkers and cancer-associated thromboembolism

Thromboembolism is one of the most common comorbidities associated with cancer and also a leading cause of death for cancer patients [89, 90]. NETs formation has been detected in animal models of cancer and patients with cancer-associated thrombosis. Increased levels of plasma H3Cit, NE, and DNA were found in mice bearing pancreatic tumors [91]. Additionally, in murine models of chronic myelogenous leukemia and breast and

lung cancers, NETs formation was implied by the colocalization of DNA, fibrin, and VWF in thrombi as well as web-like patterns [92]. In patients with gastric cancer, the levels of NETs biomarkers released by neutrophils cultured in vitro were positively associated with TAT complex and D-dimer levels, indicating that NETs might contribute to hypercoagulability [93]. Besides, in patients with cancer, NETs formation was indicated by the colocalization of H3Cit and DNA in cerebral, coronary, and pulmonary microthrombi [88].

Recently, clinical studies have focused on the association between NETs biomarkers and cancer-associated thromboembolism [88, 94–98] (Table 4).

VTE

The level of plasma nucleosomes was an independent risk factor for DVT, irrespective of malignancy [49]. However, in a large-scale study of 946 patients with malignancy, higher levels of plasma nucleosomes and DNA could only predict a higher risk of VTE, including PE, DVT, and SVT, during the first 6-month follow-up period, but only higher level of plasma H3Cit was an independent predictor of VTE during the overall follow-up period and comparable to D-dimer, soluble P-selectin, FVIII, and prothrombin fragment 1 + 2 for predicting VTE. More importantly, H3Cit significantly increased the risk of VTE in patients with pancreatic and lung cancer, but not those with cancers in other sites [94]. The levels of plasma DNA-histone and DNA, rather than NE, were significantly higher in hepatocellular carcinoma patients with PVT than those without PVT [97]. In patients with colorectal cancer, the levels of plasma MPO-DNA and DNA also positively correlated with the levels of plasma TAT complex and D-dimer, suggesting that NETs may contribute to coagulation activation and increased risk of VTE [99].

Arterial thrombosis

The levels of plasma nucleosomes and DNA were significantly elevated in cancer-related stroke patients compared with healthy-, cancer-, and stroke-controls. High plasma DNA level was independently associated with the risk of cancer-related stroke [95]. Furthermore, the levels of plasma H3Cit, MPO, and DNA were significantly elevated in IS patients with cancer as compared to those without [88]. Conversely, a prospective observational cohort study revealed that the levels of plasma H3Cit, DNA, and nucleosomes at baseline could not predict a composite outcome of MI, IS, and peripheral arterial occlusion in patients with malignancy, although H3Cit and DNA significantly increased the risk of death [96]. The level of plasma MPO-DNA was higher

Table 3 Studies evaluating NETs biomarkers in IS

First author/ year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
O'Connell et al (2017) [86]	Case-control	Patients experiencing AIS and those identified as stroke mimics	AIS (n = 43) vs. Negative AIS (n = 20)	Plasma, 2000 × g, 10 min and 10,000 × g, 10 min	DNA	qPCR	NA
Vallés et al (2017) [87]	Case-control and cohort	Patients with AIS during the acute phase of brain ischemia and suffering stroke < 24 h before admission	AIS (n = 243) vs. HC (n = 27)	Plasma, 22 °C, 2500 × g, 10 min	H3Cit Nucleosomes DNA	ELISA ELISA SytoxGreen fluorimetry	0.080 vs. 0.039 AU 0.329 vs. 0.209 AU 432.11 vs. 324.2 ng/mL
Lim et al (2020) [75]	Case-control and cohort	Patients with newly diagnosed ACS or AIS	ACS (n = 37) vs. AIS (n = 58) vs. HC (n = 25)	Plasma, 1600 × g, 15 min	DNA-histone DNA	ELISA PicoGreen fluorimetry	19.73 vs. 13.71 vs. 14.32 mU 743.28 vs. 524.22 vs. 216.48 ng/mL

Abbreviations: ACS Acute coronary syndrome, AIS Acute ischemic stroke, AU Absorbance unit, H3Cit Citrullinated histone H3, ELISA Enzyme-linked immunosorbent assay, HC Healthy control, IS Ischemic stroke, Min Minute, NA Not available, NETs Neutrophil extracellular traps, qPCR Quantitative polymerase chain reaction

in myeloproliferative neoplasms (MPNs) patients with a history of arterial and venous thrombosis than those without [98].

NETs biomarkers and COVID-19-associated thromboembolism

Thromboembolism is common in COVID-19 patients [100] and independently associated with hospitalized mortality [101]. Immunostaining of lung, kidney, and heart tissues of COVID-19 patients revealed positive staining of H3Cit, MPO-DNA, NE, and DNA [102, 103]. Additionally, H3Cit, MPO, and DNA were colocalized with platelet and fibrin in blood vessels, indicating the involvement of NETs formation in the development of immunothrombosis [104].

The levels of plasma MPO-DNA and H3Cit were significantly higher in COVID-19 patients than healthy controls [105], and the level of plasma MPO-DNA positively correlated with the severity of COVID-19 [104]. Furthermore, the levels of plasma H3Cit-DNA, DNA, and NE correlated with those of widely recognized plasma markers for coagulation and fibrinolysis (i.e., D-dimer, TAT complex, and plasmin-antiplasmin) and endothelial activation and damage (i.e., VWF and ADAMTS13) [106]. The levels of plasma H3Cit and MPO-DNA were significantly higher in COVID-19 patients with VTE than those without. The areas under the curve of H3Cit and MPO-DNA for predicting VTE were 0.791 and 0.769, respectively [105]. The levels of serum H3Cit, MPO-DNA, DNA, and calprotectin were still higher in COVID-19 patients with both arterial thrombosis and VTE than those without thrombotic events, despite prophylactic

anticoagulation was prescribed at the time of diagnosis of thrombotic events [107]. But such an association was not confirmed by a prospective cohort study, which demonstrated that the baseline level of plasma MPO-DNA could not predict the development of thrombosis [108] (Table 5).

Potential therapeutic implications

NETs may be a potential therapeutic target for the management of thrombosis. First, DNase I can dissolve NETs structure, thereby compromising the formation of arterial thrombosis [109, 110], and reducing the weight of venous thrombus [42, 91] in mice. Ex vivo experiments measured the change of thrombus weight after thrombolysis of human PE, CAD, and IS thrombi and showed that either DNase I or tissue plasminogen activator (tPA) alone could induce thrombolysis [85], and a combination of DNase I and tPA further accelerated thrombolysis [45, 84, 85, 111]. This phenomenon may be attributed to the capacity of tPA to remove fibrin and that of DNase I to remove the "scaffold" of NETs connecting red blood cells and platelets [26]. Second, heparin, a frequently used anticoagulant, can remove histones in chromatin, then dismantle NETs [26]. Third, Cl-amidine, a PAD inhibitor, shows its ability to prevent thrombosis by inhibiting the NETs formation. Treatment with Cl-amidine can reduce the area of atherosclerotic lesion, prolong the time to carotid artery thrombosis in atherosclerosis mice [62], maintain the stability of cerebral perfusion, reduce the size of the ischemic lesion, and prevent from the development of thrombosis in IS mice [112]. GSK484, another potent and selective inhibitor of PAD4, strongly inhibits

Table 4 Studies evaluating NETs biomarkers in cancer-associated thromboembolism

First author/ year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
Thålin et al (2016) [88]	Case-control	Patients with IS	Cancers (n = 8) vs. No cancers (n = 23)	Plasma, 2000 × g, 20 min	H3Cit MPO DNA	ELISA ELISA PicoGreen fluorimetry	0.22 vs. 0.07 OD 74.1 vs. 37.8 ng/mL 504.0 vs. 407.9 ng/mL
Mauracher et al (2018) [94]	Cohort	Adult patients with newly diagnosed malignancy or progression of disease after remission	VTE (n = 89) vs. No VTE (n = 857)	Plasma, 3000 × g, 10 min	H3Cit Nucleosomes DNA	ELISA ELISA PicoGreen fluorimetry	52.4 vs. 24.1 ng/mL 1.3 vs. 1.2 MoM 384.5 vs. 355.8 ng/mL
Bang et al (2019) [95]	Case-control	Patients with active cancer	Cancer-stroke (n = 38) vs. Stroke-control (n = 40) vs. Cancer-control (n = 27) vs. HC (n = 33)	Plasma, 2000 × g, 15 min	Nucleosomes DNA	ELISA PicoGreen fluorimetry	0.379 vs. 0.189 vs. 0.251 vs. 0.194 OD 40.35 vs. 34.38 vs. 34.52 vs. 30.48 mg/mL
Grilz et al (2019) [96]	Cohort	Adult patients with newly diagnosed malignancy or a progression of disease after complete or partial remission	ATE (n = 22) vs. No ATE (n = 935)	Plasma, 3000 × g, 10 min	H3Cit Nucleosomes DNA	ELISA ELISA PicoGreen fluorimetry	NA NA NA
Guy et al (2019) [98]	Case-control	Patients with MPN	Thrombosis (n = 16) vs. No thrombosis (n = 15)	Plasma, 2400 × g, 15 min	DNA MPO-DNA	PicoGreen fluorimetry ELISA	NA NA
Seo et al (2019) [97]	Case-control	Patients with HCC	PVT (n = 77) vs. No PVT (n = 100)	Plasma, 1550 × g, 15 min	DNA-histone NE DNA	ELISA ELISA PicoGreen fluorimetry	159 vs. 83 AU NA 142.1 vs. 127.0 ng/mL

Abbreviations: ATE Arterial thromboembolism, AU Absorbance unit, ELISA Enzyme-linked immunosorbent assay, H3Cit Citrullinated histone H3, HC Healthy control, HCC Hepatocellular carcinoma, IS Ischemic stroke, Min Minute, MPN Myeloproliferative neoplasms, MPO Myeloperoxidase, NA Not available, NE Neutrophil elastase, NETs Neutrophil extracellular traps, OD Optical density, PVT Portal vein thrombosis, VTE Venous thromboembolism

the NETs formation and thrombus deposition in mouse lungs [113]. Forth, ruxolitinib, a JAK1/JAK2 inhibitor, is a second-line drug for the treatment of MPN [114]. It can also abrogate the NETs formation and decrease the rate of stenosis-induced venous thrombosis in JAK2V617F-driven MPN mice [115]. Notably, all the above-mentioned evidence comes from animal and ex vivo experiments, and clinical studies of NETs as a therapeutic target for thrombosis have not been carried out yet.

Limitations of current NETs biomarkers

Circulating NETs biomarkers include serum or plasma PAD4, H3Cit, MPO, NE, nucleosomes, or DNA, but their specificity of reflecting NETs formation remains uncertain. First, among the published studies, NETs biomarkers have been measured in human serum and plasma samples. However, it should be noted that neither

serum nor plasma is the exact position of NETs formation. Second, PAD4 is not only involved in citrullination of histones during NETs formation, but also participates in other physiological processes, such as activation of vascular smooth muscle cells [116] and regulation of hematopoietic stem cell proliferation [117]. On the other hand, Cl-amidine, which has been widely used for the inhibition of PAD4 in NETs studies, is not a PAD4-specific inhibitor, but a pan-PAD inhibitor [118]. Third, citrullinated histones have been also observed during apoptosis [119]. Furthermore, in the absence of NETs-dependent stimulation, Western blot assay also shows positive expression of citrullinated histones in liver tissues [120]. Fourth, NE may be unnecessary for NETs formation, because NE deficiency or inhibition does not prevent NETs formation [121]. Fifth, MPO, which plays an important role in antimicrobial responses, is also

Table 5 Studies evaluating NETs biomarkers in COVID-19 associated thromboembolism

First author/ year	Study design	Included patients	Groups (No. patients)	Samples processing	NETs biomarkers	Analytical methods for NETs biomarkers	Detailed values
Ouwendijk et al (2021) [108]	Case-control and cohort	Critically ill patients with COVID-19	Thrombosis (n = 44) vs. No thrombosis (n = 33) vs. HC (n = 7)	Plasma	MPO-DNA	ELISA	NA
Petito et al (2021) [105]	Case-control and cohort	Hospitalized patients with COVID-19	VTE (n = 8) vs. No VTE (n = 27) vs. HC (n = 31)	Plasma, 4000 × g, 10 min	H3Cit MPO-DNA	ELISA ELISA	NA NA
Zuo et al (2021) [107]	Case-control	Hospitalized patients with COVID-19 and thrombosis	Thrombosis (n = 11) vs. No thrombosis (n = 33)	Serum	Calprotectin MPO-DNA H3Cit DNA	ELISA ELISA ELISA PicoGreen fluorimetry	NA NA NA NA

Abbreviations: COVID-19 Coronavirus disease 2019, ELISA Enzyme-linked immunosorbent assay, H3Cit Citrullinated histone H3, Min Minute, MPO Myeloperoxidase, NA Not available, NETs Neutrophil extracellular traps, VTE Venous thromboembolism

expressed in monocytes and macrophages [122]. Sixth, nucleosomes may also originate from lymphocytes, red blood cells, and tumor cells, etc. [123]. Last, DNA can be either cell-free or bound with histones or other proteins in plasma and serum. Extracellular DNA is often considered a NETs biomarker, but can also be released during other cell death processes (i.e., apoptosis, necrosis, and pyroptosis) and active secretion (i.e., phagocytosis and egestion of DNA) [124]. Therefore, considering low specificity of a single NETs biomarker, it may be more reliable to combine two or more biomarkers for reflecting NETs formation.

Quantitative analyses of NETs biomarkers are clinically more useful and valuable. H3Cit, MPO-DNA, NE, and nucleosomes are often measured by enzyme-linked immunosorbent assay (ELISA), and DNA by quantitative polymerase chain reaction or fluorimetry assays. However, the type of samples, preanalytical sample preparation, and analytical methods used for measuring NETs biomarkers are heterogeneous among the published studies. First, plasma was employed for measuring NETs biomarkers in some studies, but serum in others. However, DNA levels are comparable in both plasma and serum of the same individuals [51]. Second, the methods on sample preparation, including the time from blood collection to sample processing, processing temperature, and centrifugal force, time, and frequency, often vary by study, which might influence experimental results. Prescriptive methods will helpfully improve the quality of samples and minimize preanalytical errors associated with sample preparation. Third, antibodies, assays, detection instruments, and manufacturers for detecting the same NETs biomarker are often diverse, thereby leading

to the heterogeneity in experimental results among studies. Notably, the specificity of ELISA for the detection of some NETs biomarkers, such as the measurement of MPO-DNA complexes in human plasma, is questionable [125]. Therefore, robust, accurate, reproducible, well-standardized, and highly specific assays for measuring NETs biomarkers are required before drawing solid conclusions.

Conclusion

Taken together, the effect of NETs formation on thrombosis is supported by a growing number of experimental and clinical studies, in which NETs biomarkers have been qualitatively and quantitatively measured. Particularly, H3Cit, MPO, MPO-DNA, NE, nucleosomes, and DNA, which are deemed as NETs biomarkers, have been evaluated in VTE, CAD, IS, cancer-associated thromboembolism, and COVID-19 associated thromboembolism (Fig. 2). Collectively, circulating NETs biomarkers seem to be associated with the presence and severity of thrombosis and correlate with hypercoagulability, but it remains unclear whether they can exactly reflect the NETs formation related to thrombosis, especially in patients with cancers and COVID-19. Instead of case-control or cross-sectional studies comparing between patients with thrombotic event and healthy population, cohort studies, where the development of a thrombotic event has been observed in the same population during follow up, should be more conducive in drawing more accurate and clinically relevant conclusions regarding diagnostic performance and predictive ability of NETs biomarkers. Routine detection of NETs biomarkers in patients with thrombosis cannot be considered until

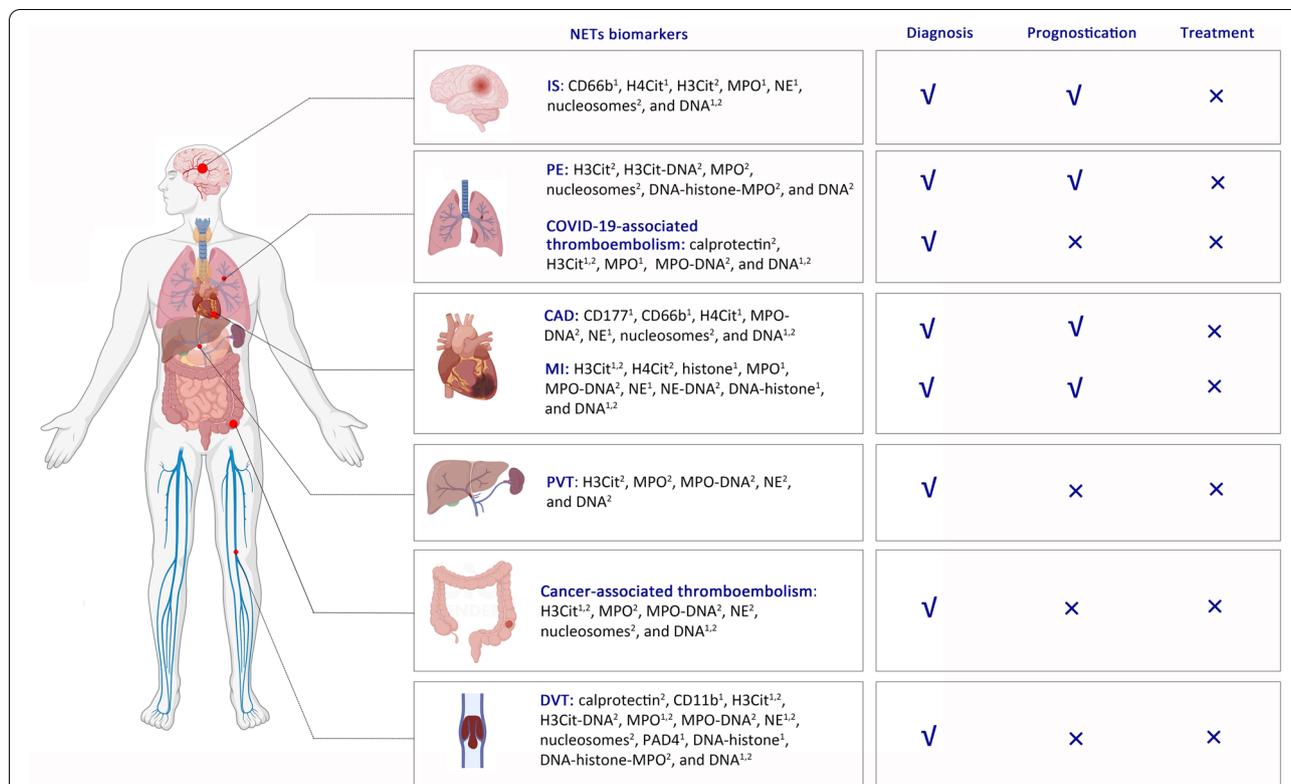


Fig. 2 A schematic diagram of NETs biomarkers detected in human VTE, CAD, MI, IS, cancer-associated thromboembolism, and COVID-2019-associated thromboembolism **Notes:** ¹ NETs biomarkers that have been explored in human thrombi specimens. ² NETs biomarkers that have been explored in human plasma/serum. ✓ NETs biomarkers that have been explored for diagnosis, prognostication, and/or treatment. ✗ NETs biomarkers that have not been explored for diagnosis, prognostication, or treatment. **Abbreviations:** CAD, Coronary artery diseases; COVID, Coronavirus disease 2019; DVT, Deep vein thrombosis; H3Cit, Citrullinated histone H3; H4Cit, Citrullinated histone H4; IS, Ischemic stroke; MI, Myocardial infarction; MPO, Myeloperoxidase; NE, Neutrophil elastase; NETs, Neutrophil extracellular traps; PAD4, Peptidyl arginine deiminase 4; PE, Pulmonary embolism; PVT, Portal vein thrombosis

more robust evidence has been produced. Notably, it should be acknowledged that existing NETs biomarkers in serum and plasma and their detection methods are unsatisfactory. Besides, concomitant infection or inflammation, use of anticoagulants, antiplatelet drugs, and anti-cancer therapies, and effect of invasive or surgical procedures may influence the reliability of the current findings. In future, well-designed studies should also be necessary to clarify whether the change of NETs biomarkers is a cause or consequence of thrombosis by collecting blood samples before and after thrombosis.

Abbreviations

ADAMTS13: A disintegrin and metalloproteinase with thrombospondin type-1 motif-13; AMI: Acute myocardial infarction; AIS: Acute ischemic stroke; CAD: Coronary artery diseases; CK: Creatine kinase; COVID-19: Coronavirus disease 2019; DVT: Deep vein thrombosis; ELISA: Enzyme-linked immunosorbent assay; HMGB1: High mobility group box 1; H3Cit: Citrullinated histone H3; H4Cit: Citrullinated histone H4; IS: Ischemic stroke; MACE: Major adverse cardiac events; MI: Myocardial infarction; MPN: Myeloproliferative neoplasms; MPO: Myeloperoxidase; NADPH: Nicotinamide adenine dinucleotide

phosphate; NE: Neutrophil elastase; NETs: Neutrophil extracellular traps; OR: Odds ratio; PAD4: Peptidyl arginine deiminase 4; PCI: Percutaneous coronary intervention; PE: Pulmonary embolism; PVT: Portal vein thrombosis; ROS: Reactive oxygen species; STEMI: ST-elevation myocardial infarction; SVT: Splanchnic vein thrombosis; TAT: Thrombin-antithrombin; TLRs: Toll-like receptors; tPA: Tissue plasminogen activator; VTE: Venous thromboembolism.

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Authors' contributions

Conceptualization: Xingshun Qi; Data curation: Xiangbo Xu and Xingshun Qi; Writing—original draft: Xiangbo Xu; Writing—review and editing: Xiangbo Xu, Shixue Xu, Yuting Wu, Yue Yin, Walter Ageno, Valerio De Stefano, Qingchun Zhao, and Xingshun Qi; Supervision: Qingchun Zhao and Xingshun Qi. All authors have made an intellectual contribution to the manuscript and approved the submission.

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