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COMPENDIUM ON INCREASED RISK OF CARDIOVASCULAR COMPLICATIONS IN CHRONIC KIDNEY DISEASE

NETs-Induced Thrombosis Impacts on Cardiovascular and Chronic Kidney Disease

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ABSTRACT: Arterial and venous thrombosis constitute a major source of morbidity and mortality worldwide. Association between thrombotic complications and cardiovascular and other chronic inflammatory diseases are well described. Inflammation and subsequent initiation of thrombotic events, termed immunothrombosis, also receive growing attention but are still incompletely understood. Nevertheless, the clinical relevance of aberrant immunothrombosis, referred to as thromboinflammation, is evident by an increased risk of thrombosis and cardiovascular events in patients with inflammatory or infectious diseases. Proinflammatory mediators released from platelets, complement activation, and the formation of NETs (neutrophil extracellular traps) initiate and foster immunothrombosis. In this review, we highlight and discuss prominent and emerging interrelationships and functions between NETs and other mediators in immunothrombosis in cardiovascular disease. Also, with patients with chronic kidney disease suffering from increased cardiovascular and thrombotic risk, we summarize current knowledge on neutrophil phenotype, function, and NET formation in chronic kidney disease. In addition, we elaborate on therapeutic targeting of NETs-induced immunothrombosis. A better understanding of the functional relevance of antithrombotic mediators which do not increase bleeding risk may provide opportunities for successful therapeutic interventions to reduce thrombotic risk beyond current treatment options.

Key Words: cardiovascular diseases ■ inflammation ■ kidney ■ neutrophils ■ thrombosis

Intravascular NETs (neutrophil extracellular traps) have been shown to play an important role in initiating and accelerating thrombosis in arteries[1] and veins.[2] They are widely accepted as an essential player in immunothrombosis; this is due to their complex interplay with both pathogens and components of the coagulation system and due to the release of their intracellular components to the immune system.[3,4] Immunothrombosis is thought to be a host defense mechanism to trap invading pathogens; however, its aberrant activation, termed thromboinflammation, increases the risk of thrombotic events in sterile inflammatory conditions like cardiovascular disease (CVD). For example, NETs in plaques of atherosclerotic CVD lead to an increased proinflammatory macrophage phenotype[5] and trigger the generation of inflammatory mediators, which enhance platelet activation and thrombus formation.[6] Over the past decade, studies have also analyzed neutrophil activation, NETs formation, and a potential association of NETs components to CVD risk in patients with chronic kidney disease (CKD, since these patients present with an increased cardiovascular risk.[7] They display chronic low-grade inflammation[8] and enhanced serum titers of NETs inducers like interleukin (IL)8[9–11] and show increased expression of neutrophil activation markers on circulating blood neutrophils.[12–15]

Central to thromboinflammation is the loss of the antithrombotic and anti-inflammatory functions of endothelial cells, leading to dysregulation of coagulation, complement, platelet activation, and leukocyte recruitment. Binding between platelets and recruited neutrophils advances changes in neutrophil function promoting arrest at sites of thrombus formation but also initiation of thrombosis through NETs formation. NETs form a scaffold for the activation of platelets and the coagulation system, boosting their prothrombotic properties.[16] Furthermore, NETs activate the contact pathway of coagulation via electrostatic interactions between the NET histones and platelet phospholipids,[17,18] and

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Nonstandard Abbreviations and Acronyms

ADAMTS13	A disintegrin and metalloprotease with a thrombospondin type 1 motif 13
CVD	cardiovascular disease
GFR	glomerular filtration rate
MI	myocardial infarction
NE	neutrophil elastase
PAD4	peptidyl arginine deaminase 4
PMA	phorbol myristate acetate
TLR	Toll-like receptor
VTE	venous thromboembolism
vWF	von Willebrand factor

NETs-derived NE (neutrophil elastase) digests the coagulation inhibitors antithrombin and TF (tissue factor) pathway inhibitor.[19] In addition, the extracellular DNA backbone of NETs provides a platform for platelet adhesion and thereby promotes their aggregation.[20,21] In turn, the disruption of NETs, that is, by administration of DNase (deoxyribonuclease) diminishes the activation of the coagulation system thereby limiting thrombotic events.[22] Hence, a better understanding of the mechanisms initiating and driving thromboinflammation to allow for the identification of potential new therapeutic targets is an unmet clinical need. This is particularly true because thrombotic complications can only be prevented in part with the use of conventional antithrombotic therapies and carry the risk of bleeding complications.[23,24]

Therefore, this review focuses on the prominent and emerging interrelationships and functions between NETs and other mediators of immunothrombosis in CVD, extends this to currently available observations in CKD, and elaborates on therapeutic targeting of NETs-induced immunothrombosis to shed light on current advances and developments.

THROMBOTIC COMPLICATIONS IN CVD

CVDs of atherosclerotic origin like coronary artery disease, cerebrovascular disease, and peripheral artery disease can be complicated by myocardial infarction (MI), ischemic stroke, and limb ischemia, respectively. These manifestations result from arterial occlusion for example due to atherothrombosis. Two major steps involved in the onset of atherothrombotic events are atherosclerotic plaque disruption and thrombus formation. Plaque disruption leads to exposure of subendothelial matrix and release of TF at the site of vascular damage.[25] Extensive release of TF to the blood initiates the extrinsic pathway of coagulation to generate thrombin and promote fibrin clot formation. Additionally, upon plaque rupture, platelets and myeloid cells are also recruited to the exposed subendothelial matrix via GP (glycoprotein)Ibα-vWF (von Willebrand factor) and GPVI (glycoprotein VI)-collagen interactions and adhesion molecules like P-selectin.[1,26] These processes are involved in thrombotic complications leading to MI and ischemic stroke, both among the leading causes of mortality worldwide.[27]

Another manifestation of thrombotic complications is venous thromboembolism (VTE), which is triggered by endothelial damage, altered blood flow, and hypercoagulability named the Virchow triad.[28] VTE includes deep vein thrombosis that mostly arise from veins of the lower extremities. This thrombus can travel to the lungs and cause pulmonary embolism. Patients with symptomatic proximal deep vein thrombosis have silent pulmonary embolism in 30% to 60% of cases.[29]

NETS AND ABERRANT IMMUNOTHROMBOSIS TRIGGER ATHEROTHROMBOTIC AND VENOTHROMBOTIC COMPLICATIONS IN CHRONIC INFLAMMATION

NETs formation is an innate immune response of the host defense mechanism during pathogenic challenge when it occurs in controlled manner. However, persistent NETs formation can damage the endothelium and other tissues using multiple proteases.[30–32] NETs are proinflammatory and are involved in the pathogenesis of sterile and nonsterile inflammatory conditions, including atherosclerosis,[33] arterial thrombosis,[34,35] and venous thrombosis.[36–38]

For example, in an inflammatory environment, prothrombotic NETs lead to the various thrombotic complications including ischemic arterial disease,[39] ischemic stroke,[40] and VTE.[41] Recent studies suggest the prevalence of NETs in mouse deep vein thrombosis (DVT),[42] human cerebral arteriovenous malformations,[43] and MI.[44] In the following subsections, we will elaborate on explaining NETs, immunothrombosis, and the role of NETs in immunothrombosis.

Neutrophil Extracellular Traps

NETs are extracellular web-like structures consisting of DNA scaffold, granular proteins including myeloperoxidase, NE, defensins, calprotectin, cathelicidins, cathepsin G, lactoferrin, matrix metalloproteinase-9, peptidoglycan recognition proteins, pentraxin, and LL-37 (37-amino-acid peptide)[45,46] as well as histones H1, H2A, H2B, H3, and H4.[47]

NETs formation involves both NADPH oxidase–dependent and NADPH-independent pathways.

NADPH oxidase–dependent NETs formation is stimulated by phorbol myristate acetate (PMA), hydrogen peroxide, lipopolysaccharide, IL (interleukin)-8, bacteria, fungus, immune complexes, and cholesterol crystals.[46,48–51] This leads to the activation of PKC (protein kinase C), and the Raf-Mek-Erk (rapidly accelerated fibrosarcoma/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase) pathway resulting in the production of reactive oxygen species (ROS) through NADPH oxidase.[49,52] Generated ROS promote the activation of MPO[53] and release of NE in cytoplasmic granules.[54] Released NE enters the nucleus and thereby promotes DNA unwinding by cleaving histones.[55,56] This frees disintegrated DNA and exposes histones to the cytoplasm, initiating lysis of the cell membrane to generate NETs.[51] Release of NE from the granules during NETs formation is also regulated by pore-forming protein gasdermin D, which can initiate NE release by localizing to the plasma, nuclear, and granular membrane to induce permeabilization.[57]

NADPH oxidase–independent NETs formation can be initiated by the calcium ionophore A23187 (ionomycin) or the potassium ionophore nigericin.[51] HMGB1 (high-mobility group box 1) also induces NET formation in vitro as well as in vivo through a TLR (Toll-like receptor) 4–dependent mechanism and is independent of NADPH oxidase ROS production.[58] One important feature of NADPH oxidase–independent NETs formation is calcium fluxes which activate the PAD4 (peptidyl arginine deaminase 4) enzyme which converts arginine (positively charged amino acid) to citrulline (neutral amino acid) on histones. This conversion interferes with the association of histones with the negatively charged DNA scaffold and thus promotes unwinding of DNA, allowing for nuclear expansion and chromatin condensation.[59–62] A recent review has provided more detailed information on these mechanisms leading to NETs release and also discussed the connection of NETs release with other types of cell death mechanisms[50]

NETs-Induced Immunothrombosis

NETs have been demonstrated to play a critical role in mediating thrombosis in blood vessels, which has been attributed to the procoagulant nature of histone proteins.[63] NETs are implicated in thrombotic events in VTE, acute respiratory distress syndrome, atherosclerosis, and sepsis and their high levels were related to high mortality.[64,65] This has been further highlighted by the current COVID-19 pandemic, where NETs form in abundance and contribute to severe disease and immunothrombosis in the lungs and other organs.[63,66,67]

During pathogenic challenge, the host defense system can entrap pathogens and stop their dissemination by activating the coagulation system. This involvement of immune cells and coagulation proteins leads to the formation of immunothrombosis. Physiological immunothrombosis can become dysregulated resulting in excessive formation of immunothrombi which mainly affect the microvasculature.[23] NETs are central contributors to immunothrombosis; in fact, they are prevalent in human thrombi and are usually colocalized with platelets, vWF, and coagulation factors, including TF, factor XII, and fibrin/fibrinogen. Different approaches to NETs-induced thrombosis are depicted in Figure 1 and described in more detail below.

NETs-driven immunothrombosis is triggered by the interaction of platelets and neutrophils. During this interaction, PSGL-1 (P-selectin glycoprotein ligand-1) receptor on neutrophils recognizes and binds to P-selectin expressed by activated platelets and initiates NETs formation in mice.[68] Additionally, under flow and static conditions, activated platelets lead to NETs release.[69–73] This platelet-mediated NETs release could also be P-selectin independent. Activated platelets release platelet factor 4, thromboxane A_2, and vWF, which are soluble mediators of NETs formation.[72] NETs interact with vWF, released by endothelial cells and platelets, which promotes platelet adhesion, platelet aggregation, fibrin formation, and consecutive immunothrombosis.[20,74] This positive feedback loop in activation of platelets by NETs was first shown by perfusing whole blood over NETs, which resulted in platelet adhesion, activation, and aggregation.[20] Histone proteins in the DNA fragments of NETs can also activate and aggregate platelets in a P-selectin- and TLR2/4–dependent manner[20,75] or by associating with crosslinking molecules, such as fibrinogen, that in turn interact with platelets through αIIbβ3 integrin.[20,76] This interaction of histones with TLR2 and TLR4 induces platelet-mediated thrombin generation, thus further supporting activation of the coagulation cascade.[75] Finally, NETs also activate platelets via the cathelicidin LL-37, which binds to the GPVI receptor on the platelet surface.[77] The GPVI receptor promotes the proinflammatory role of platelets and mediates platelet adhesion to subendothelial collagen and thrombosis at the site of vascular injury.[78] NETs also impact the endothelium and thereby on the thrombotic risk through induction of endothelial cell activation and induction of tissue factor,[79] as well as inducing endothelial cell damage or vascular leakage.[80,81] Hence NET interference could also reduce thrombotic risk through less endothelial cell activation and reduction of vascular leakage.

vWF has also been found to colocalize with NETs within thrombi.[20] Indeed, under flow conditions, histones and DNA from NETs bind the A1 domain of vWF; this leads to the anchoring of NETs to the vasculature via vWF allowing NETs-mediated vascular damage.[74] In a systemic infection mouse model, vWF lines the liver microvasculature, which

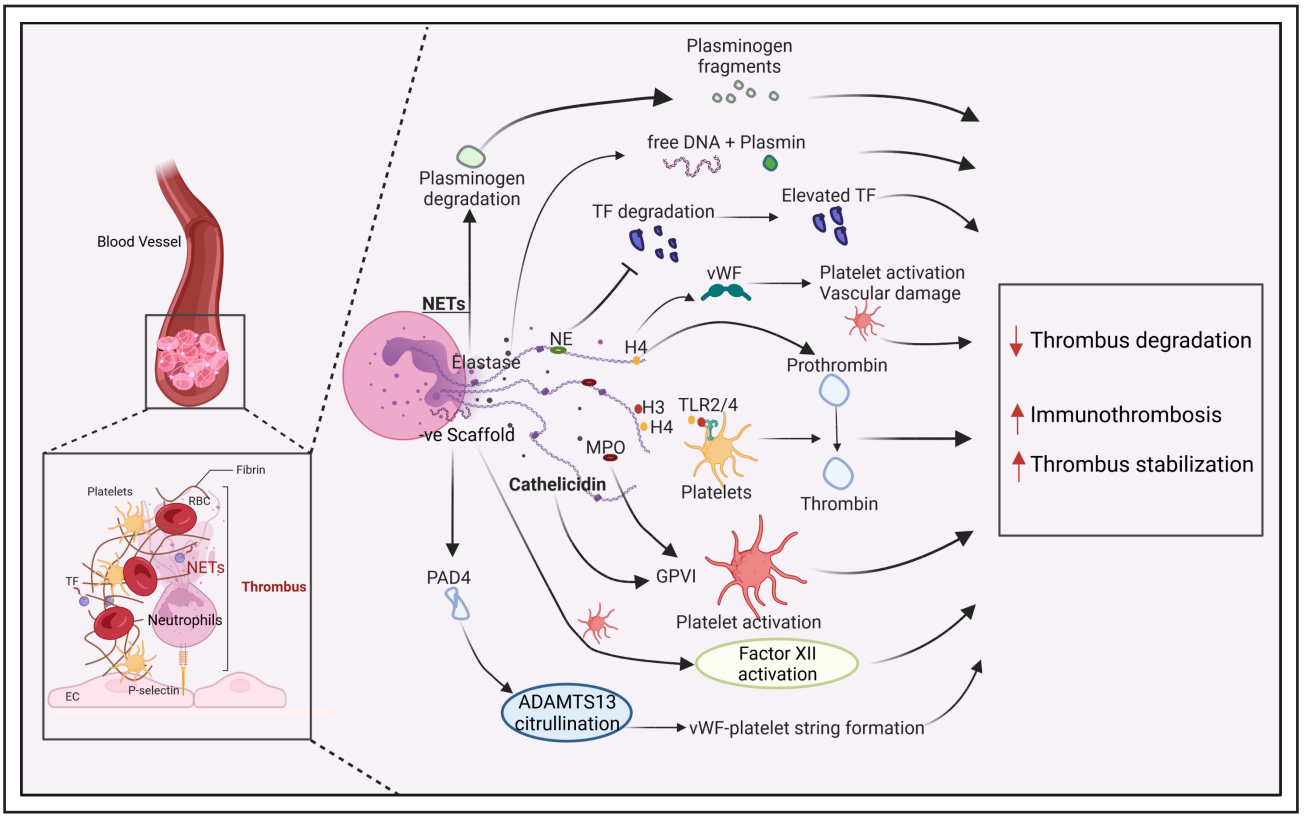

Figure 1. NETs (neutrophil extracellular traps) components induce immunothrombosis.
NETs components like negatively charged DNA scaffold, cathelicidin, MPO (myeloperoxidases), histones (H3 and H4), and NE (neutrophil elastase) act via different pathways including vWF (von Willebrand factor)-platelet string formation, platelet activation, aggregation, thrombin generation and TF (tissue factor) release to form immunothrombi. Elastases and cell-free DNA also promote thrombosis by reducing fibrinolysis. ADAMTS13 indicates A disintegrin and metalloprotease with a thrombospondin type 1 motif 13; EC, endothelial cell; GPVI, glycoprotein VI; and TLR, toll-like receptor. Made with biorender.com.

upon treatment with vWF blocking antibody reduces the histone binding (by 50%) and reduces liver damage by around 80%.[82] Histones themselves can promote the release of Weibel-Palade bodies containing vWF, thereby stimulating thrombus formation and immunothrombosis.[83,84] Physiologically, ultralarge vWF is cleaved by the protease ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13), which is endogenously active in the circulation. In pathological conditions like sepsis high vWF levels and low ADAMTS13 activity are associated with inflammation and disseminated intravascular coagulation.[85] ADAMTS13 administration before systemic bacterial infection resulted in reduced vWF-dependent NETs adherence to the hepatic vascular wall.[82] PAD4, which is released with NETs, can citrullinate ADAMTS13 and can reduce its activity levels, thus promoting ultralarge vWF-platelet string formation and microvascular thrombosis after vessel injury.[86] vWF, in turn, by binding to platelet GPIbα and neutrophil αMβ2, also promotes platelet interaction with neutrophils and platelet-induced NETs formation.[72]

NETs also interact with various coagulation factors and lead to clot formation.[20,22] For example, NETs release NE which interferes with the degradation of TF by the TF pathway inhibitor, thus increasing TF and supporting the extrinsic pathway of coagulation.[19] This NETs-related increase in TF is also observed in the liver microvasculature of mice with sepsis or vessel injury.[19,22,87] NETs also interfere with the intrinsic pathway of coagulation. For instance, in the presence of activated platelets, the negatively charged DNA scaffold of NETs can trigger the autoactivation of factor XII.[21,88] This charge-driven interaction induces the generation of thrombin. This effect was reversed in factor XII–deficient plasma or with the blocking of factor XII.[21,89,90] By promoting factor XII–driven thrombin generation, NETs lead to clot formation. Factor XII in turn can promote additional NETs formation via uPAR (urokinase plasminogen activator receptor)–induced phosphorylated Akt2 signaling.[88] NETs can enhance thrombin formation via several other mechanisms, for instance, intact NETs or histones H3 and H4 can interact with platelet TLR2 and TLR4 and lead to cleavage of prothrombin to generate thrombin.[89] Histones reduce protein C activation by binding to thrombomodulin[91]; this leads to a reduced inhibition of factor Va and factor VIIIa formation and results in increased thrombin generation. Histone H4 is also depicted to bind and autoactivate prothrombin.[92] The involvement of NETs in the generation of thrombin has been further demonstrated by reduced formation of

thrombin-antithrombin complexes and reduced thrombin activity when NETs formation was abolished using DNases[90,93] and in conditions of PAD4-deficiency.[22,94] Host DNases like Dnase1 and DNase1-like 3 provide protection to the host against NETs-induced vascular occlusion.[95] Reduced activity of these DNases, for example, in patients with systemic lupus erythematosus leads to reduced NET degradation and, therefore, contributes towards a higher susceptibility in these patients to form thrombi.[96–98] Different studies support the idea that NETs also promote coagulation by preventing fibrin degradation (fibrinolysis). Elastase bound to NETs-DNA backbone degrades plasminogen needed for fibrinolysis and results in a high amount of NE-derived plasminogen fragments in the plasma of septic shock patients.[99] Additionally, clot lysis assays showed that cell-free DNA can bind plasmin and fibrin, thereby impairing plasmin-mediated fibrinolysis.[100] To confirm these observations, treatment with DNase has been able to restore thrombolysis in different settings.[100,101] Furthermore, histones have shown the ability to inhibit tPA (tissue-type plasminogen activator)-driven fibrinolysis in vitro assays,[101,102] thus again promoting thrombus formation. Considering the detrimental implications of NETs, inhibiting NETs formation is crucial in preventing immunothrombosis. NET interference, however, should be restricted to thrombus-specific NETs to reduce the detrimental side effects of NET degrading agents.

NETs and Immunothrombosis in CVD

NETs and NETs-induced immunothrombosis are implicated in the progression of cardiovascular and other diseases. Uncontrolled NETs formation leads to arterial, venous, and cancer-associated thrombosis, which has been widely discussed.[103] NETs can trigger chronic inflammation by releasing intracellular components to the immune system, thus aggravating inflammation, and by producing autoantibodies such as in rheumatoid arthritis.[3,4] NETs in atherosclerotic plaques lead to an increased proinflammatory macrophage phenotype, since macrophages isolated from NETs-positive areas in the atherosclerotic plaque show an enrichment of the proinflammatory M1-like macrophage phenotype and have an augmentation in inflammasome-related genes.[5] Furthermore, CRP (C-reactive protein), an inflammatory marker and predictor of cardiovascular events as well as an inducer of NETs formation, is increased in patients with chronic heart failure.[104] NETs can lead to the generation of monomeric CRP (from circulating pentameric CRP), which enhances platelet activation, thrombus formation, and inflammatory responses.[6] NETs can also lead to systemic hypercoagulability in SARS-CoV-2 infection and reduced NETs clearance was associated with increased mortality during SARS-Cov-2 infection as shown by plasma proteomic profiling in these patients.[105] These thrombotic complications in patients with COVID-19 were recently summarized in excellent reviews.[66,105–107]

NETs also play an important role in patients with acute coronary syndrome, as NETs prevalence is higher in coronary thrombi as compared to in vitro clots and deep venous thrombi.[44] In ST-segment–elevation MI, NETs-related components like double-stranded DNA, and myeloperoxidase/DNA complexes are significantly higher in the coronary blood and are associated with in-hospital major adverse cardiovascular events.[108] Human atherosclerotic plaques obtained through endarterectomy showed the prevalence of NETs in atherosclerotic plaques.[109] Furthermore, NETs trigger the production of proinflammatory cytokines that result in a significant increase in immune cell recruitment to the atherosclerotic plaque.[110] PAD4-dependent NETs formation leads to destabilization and erosion of plaques.[111] Additionally, myeloid-specific PAD4 deficiency in mice significantly reduces atherosclerotic burden by diminishing NETs formation.[112]

Neutrophil activation and inflammation are also involved in the propagation of venous thromboembolism. A mouse model of venous thrombosis induced by reduced flow in the inferior vena cava shows that platelets deposit reduced HMGB1 on the luminal side of venous endothelial cells. This reduced HMGB1 is rapidly oxidized to the disulfide and sulfonyl forms. Disulfide HMGB1 is crucial in DVT as it promotes the release of NETs, triggers monocyte recruitment as well as enhances the expression of TF by monocytes.[113] Furthermore, disturbed flow leads to the upregulation of inflammatory NF-κB (nuclear factor κB) pathways and could lead to chronic inflammation with constant macrophage recruitment.[114] The activation of the coagulation system in venous thrombosis involves release of TF from various cells, mainly released by monocytes and locally activated by protein disulfide-isomerase.[21,115] Together, this immune response triggers the activation of the extrinsic and intrinsic coagulation pathways, resulting in clot formation through a dense fibrin network.[21] NETs enhance thrombus formation and the stability of venous thrombi, as they are present in the organizing stage of thrombus formation and surrounded by vWF-positive platelet islands in human DVT samples.[41] In conclusion, NETs and immunothrombosis lead to atherothrombotic and venothrombotic complications in various vascular beds.

CKD INCREASES THE RISK FOR CVD AND THROMBOTIC COMPLICATIONS

CKD entails kidney damage or a reduced kidney filtration function expressed by the glomerular filtration rate (GFR in mL/[min·1.73 m^2]) over at least 3 months.[116] The classification of this progressive disease is based on the remaining kidney filtration function: CKD stage 1 with normal kidney filtration function (GFR ≥90 mL/min); CKD stage 2 with mild kidney impairment (GFR, 60–89); CKD stage 3 with mild-moderate or moderate-severe kidney impairment (GFR, 30–59 mL/min); CKD stage 4 with severe kidney impairment (GFR, 15–29 mL/min) and CKD stage 5 with

kidney failure (GFR <15 mL/min). In this end-stage of renal disease, patients depend on dialysis or kidney transplantation. Patients with CKD have a highly increased risk of cardiovascular and thrombotic complications,[7,117] with 40% to 45% of patients in advanced CKD stage (CKD4–5) dying from CVD.[118] As underlying causes, patients with CKD display a higher prevalence and progression of atherosclerotic lesions with increasing CKD stage.[119] The prospective, observational Reduction of Atherothrombosis for Continued Health (REACH) registry enrolled outpatients with established coronary artery disease, peripheral artery disease, or cerebrovascular disease (transient ischemic attack or stroke) and identified that most patients presented with coronary artery disease in non-CKD as well as along all CKD stages, followed by cerebrovascular disease and peripheral artery disease. However, the number of atherothrombotic locations increased with decreasing kidney function, and at 1 year of follow-up, patients in CKD stage 4 and beyond had a significantly increased risk of cardiovascular death, nonfatal MI, other ischemic arterial events as well as revascularizations for peripheral artery disease and lower limb amputations after adjustment for differences in cardiovascular risk factors at baseline.[117] Furthermore, patients with mild CKD (CKD stage 2) as well as moderate to severe CKD (CKD stages 3–4) displayed an increased risk of VTE including deep vein thrombosis and pulmonary embolism, even after adjusting for VTE- and CKD-associated risk factors (relative risk for CKD stage 2, 1.29; for CKD stages 3–4, 1.71).[120]

Underlying pathophysiological mechanisms that may contribute to increased atherothrombotic risk in CKD include a dysregulation of coagulation and platelet function,[121] endothelial cell dysfunction, and increased inflammation. Being mostly derived from endothelial cells, vWF is considered an endothelial injury marker. It initiates platelet adhesion to sites of vascular injury. Also, vWF serves as carrier protein for clotting factor VIII, a procoagulant factor of the intrinsic coagulation pathway. Thereby vWF extends the half-life of factor VIII by protecting it from proteolytic degradation and may direct it to the site of injury to support hemostasis.[122] Both, vWF[123–129] and factor VIII[129] were shown to be increased in plasma of patients with CKD and high plasma vWF levels are correlated with increased mortality in CKD.[124,130,131] Also, the anticoagulant endothelial injury marker thrombomodulin, which is proteolytically cleaved from the endothelial surface, was shown to be increased in plasma of CKD stages 4 to 5 patients.[128,129]

NEUTROPHILS AND NETS AS CONTRIBUTORS TO THROMBOTIC RISK IN CKD?

In the context of acute kidney injury, DNA released by necrotic kidney tubular epithelial cells during ischemia-reperfusion injury of the kidney triggers platelet activation with subsequent NETs formation by neutrophils, kidney inflammation, and further kidney damage.[132] NETs induced upon ischemic acute kidney injury can also trigger remote organ injury, with NET accumulation in lungs and neutrophil infiltration in lung, liver, brain, and heart detected.[133] Furthermore, macrophage extracellular trap formation has been identified in rhabdomyolysis-induced acute kidney injury, triggered by platelets that had been activated by heme released by damaged muscle cells.[134] Over the past decade, studies have analyzed neutrophil activation and NETs formation, as well as a potential association of NETs markers to cardiovascular risk, also in patients with CKD, with a focus on patients requiring dialysis. Overall, this suggested increased basal neutrophil activation and NETs formation in patients with CKD. However, a closer analysis of the underlying mechanisms of NETs formation in CKD, mechanistic studies of NETs formation in animal models of CKD, and an investigation of a potential contribution to CKD-associated cardiovascular risk are still lacking.

Neutrophil Activation and Apoptosis in CKD

Multiple groups reported a signature of increased neutrophil activation in blood of patients with CKD with increased expression of CD11b[12,13] and its interaction partner CD18,[14,15] 2 integrins with important role in neutrophil adhesion to activated endothelium. Furthermore, circulating neutrophils from patients with CKD on hemodialysis showed increased expression of the activation markers CD35,[135] CD66b and CD15.[15] In contrast, surface expression of the CXCR1 (C-X-C Motif Chemokine Receptor 1) on neutrophils of dialysis patients was reduced,[136,137] with CXCR1 known to be downregulated on the neutrophil surface upon inflammatory stimulation.[138] Furthermore, neutrophils in predialysis patients in CKD stages 4 to 5 showed an increased rate of apoptosis.[14,139]

Extracellular DNA and NETs Formation in CKD

Extracellular DNA, as consequence of either cellular apoptosis or NETs formation, was mostly found to be unaltered in both predialysis and dialysis patients with CKD compared to healthy controls or comparing early vs. advanced CKD stages, although it temporarily increased in plasma after dialysis sessions[140–145] (Table 1). Jeong et al[146] did detect increased cell-free DNA in plasma of CKD5 patients on hemodialysis, although especially in patients who also presented with diabetes or cardiovascular complications.

When focusing on H3cit (citrullinated histone H3) and PAD4 as markers of NETs formation, patients in CKD stage 5 revealed increased levels of H3cit and PAD4 in blood compared to healthy controls, without significant

Table 1. Levels of Extracellular DNA and NET Formation Markers in Plasma or Neutrophils of Patients With CKD

	Cohort			NET formation markers		
Author	CKD population	Dialysis	Analysis of	Marker	Predialysis	Postdialysis
Atamaniuk et al[140]	CKD 5D	HD	Plasma	cfDNA	No difference (1)	↑ (2)
Moreira et al[141]	CKD 5D	HD	Plasma	cfDNA	No difference (1)	↑ (2)
Korabecna et al[142]	CKD (no dialysis), CKD 5D	PD, HD	Plasma	cfDNA	No difference (1); ↑ in HD (3)	↑ in most patients (2)
Opatrna et al[143]	CKD 5D	HD	Plasma	cfDNA	No difference comparing long vs. short dialysis interval (4)	↑ after long-interval HD session
Atamaniuk et al[144]	CKD 5D	HD	Plasma	cfDNA	n.a.	↑ (1)
McGuire et al[145]	CKD 2-5 and 5D	PD, HD	Plasma	cfDNA	No difference between CKD stages	No difference (2)
Jeong et al[146]	CKD 5D	HD	Plasma	cfDNA	↑ (1), especially in patients with CKD with diabetes and CV complications	n.a.
Jeong et al[147]	CKD 5D	HD	Plasma	cfDNA	No difference (1)	n.a.
				Histone-DNA	↑ (1)	
				NE conc.	No difference (1)	
Kim et al[135]	CKD 5D	HD	Resting neutrophils	H3Cit	↑ *(1)*	…
Kim et al[148]	CKD 5D	HD	Serum	Histone-DNA	↑ (1)	n.a.
			Resting and PMA-stimulated neutrophils	NE activity	↑ *(1)*	
Vega-Roman et al[149]	CKD 5 and 5D	HD, PD	Serum	H3cit	↑ (1)	n.a.
				PAD4	↑ (1)	
Lee et al[150]	CKD 5D	HD	Plasma	Histone-DNA	↑ (1), ↑ (8)	n.a.
				MPO-DNA	↑ (1), ↑ (8)	
Talal et al[15]	CKD 5D	HD	Resting neutrophils	NE conc.	↓ (1)	n.a.
				PAD4	↓ (1)	
				MPO	↓ (1)	
				MMP-9	↓ (1)	

(1) CKD versus healthy control, (2) postdialysis versus predialysis, (3) CKD HD versus (CKD and CKD PD), (4) long interval versus short interval between dialysis, (5) CKD4 versus CKD2, (6) HD versus healthy control or CKD4, (7) CKD5 versus CKD1, (8) HD patients reaching composite endpoint (all-cause mortality or loss of vascular access) versus those who do not. cfDNA indicates cell-free DNA (extracellular DNA); CKD, chronic kidney disease; CKD5D, CKD 5 on dialysis; conc., concentration; CV, cardiovascular; H3cit, citrullinated histone H3; HD, hemodialysis; MMP, matrix metallopeptidase; MPO, myeloperoxidase; n.a., not analyzed; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD4, peptidyl arginase deiminase 4; and PD, peritoneal dialysis

differences between predialysis patients or patients on hemodialysis or peritoneal dialysis.[149] Also, increased levels of histone-DNA complexes[147,148,150] as well as myeloperoxidase-DNA complexes[150] have been detected in hemodialysis patients, with even higher levels in those reaching a composite endpoint of all-cause mortality or loss of vascular access, compared to those who did not[150] (Table 1).

There are contrasting findings on the stimuli-induced NETs formation capacity of neutrophils in CKD stage 5 (Table 2). Some authors observed increased NETs production by neutrophils isolated from dialysis patients upon phorbol-12-myristate-13-acetate (PMA) stimulation,[148,149] along with increased PAD4 levels in serum[149] or increased NE activity in both resting and PMA-stimulated neutrophils.[148] In contrast, others reported a decreased NETs formation capacity of isolated neutrophils from dialysis patients upon stimulation with PMA, lipopolysaccharide, or the Ca^{2+} ionophore A23187 compared with healthy controls[15] (Table 2). The latter was observed along with a reduced PAD4 expression in neutrophils as well as a reduced capacity of agonist-induced ROS production, a decreased basal cellular content of the neutrophil granule proteins myeloperoxidase, NE, and MMP-9 (matrix metallopeptidase-9) but an increased surface expression of activation and degranulation markers (CD66b, CD15, CD18) by isolated neutrophils. Based on these latter findings, it was suggested that neutrophils from hemodialysis patients have a constant potent immune activation, which leads to substantial neutrophil degranulation, a subsequent limited

Table 2. Analysis of In Vitro NET Formation Capacity in Patients With CKD

Author	Cohort	In vitro NET formation capacity	
	CKD population	Stimuli	CKD vs control
Kim et al[135]	CKD 5D (HD)	Basal	↑
		PMA	↓ fold-upregulation
Kim et al[148]	CKD 5D (HD)	Basal	↑
		PMA	↑
Vega-Roman et al[149]	CKD5 and 5D (HD/PD)	PMA	↑
Talal et al[15]	CKD 5D (HD)	Ca^{2+} ionophore A23187	↓
		PMA	↓
		LPS	↓

NET formation by isolated neutrophils was analyzed by cell-free DNA quantification using Sytox Green or microscopic analysis of NETs formation after staining for myeloperoxidase or citrullinated histone H3. CKD indicates chronic kidney disease; CKD5D, CKD 5 on dialysis; HD, hemodialysis; LPS, lipopolysaccharide; NETs, neutrophil extracellular traps; and PMA, phorbol-12-myristate-13-acetate

preservation of degranulation capacity and a malfunctioning ROS cascade, and thereby a reduction in both NADPH oxidase-dependent and NADPH oxidase-independent NET formation.[15] Another study also concluded on an increased basal activation of neutrophils in hemodialysis patients upon observation of increased NETs formation, ROS production, surface activation marker expression, and H3cit levels by unstimulated neutrophils when isolated from hemodialysis patients compared to controls, along with a reduced fold-upregulation of NET formation upon neutrophil stimulation with PMA[135] (Table 2). Overall, reasons for the divergent findings on NETs formation capacity of neutrophils isolated from dialysis patients (being either increased or decreased upon PMA stimulation) remain currently unclear. Factors that may influence the neutrophil phenotype may include the neutrophil isolation method; heterogeneity in the examined patient cohorts (eg, CKD cause, comorbidities, medication); as well as dialysis-related factors (procedure itself, time of blood withdrawal with respect to dialysis, time of dialysis therapy). Hence, more well-powered clinical studies and comparable isolation and NET formation assays are needed to understand if and how NET formation capacity is generally impacted in CKD.

Finally, altered host DNAse activity in patients may impact the ability of NET degradation. However, to our knowledge, there are currently no reports on DNAse activity in patients with CKD or respective animal models. In the context of acute kidney injury, reduced urinary DNAse activity has been reported in animal models, whereas children with acute kidney injury did not show altered DNAse activity in urine of plasma compared with healthy controls[151]

Potential Mechanisms of Neutrophil Priming and NETs Formation in CKD

Uremic serum (but not healthy control serum)[150] as well as the uremic toxins indoxyl sulfate and ADMA could induce NETs formation in isolated neutrophils from healthy donors,[147] suggesting a contribution to increased neutrophil priming and basal NETs formation in patients with CKD. However, the uremic toxin uric acid was identified as a negative regulator of neutrophil function: it did not impact NETs formation but instead impaired β2 integrin activity through effects on intracellular pH and cytoskeletal dynamics, thereby reducing neutrophil adhesion capacity.[152]

Furthermore, patients with CKD present with chronic low-grade inflammation,[8] and IL8[9–11] and HMGB1[11,153] as known drivers of NETs formation have been reported to be increased in blood of patients with CKD. Also, patients with advanced CKD frequently display hyperphosphatemia and have an increased risk of medial calcification, characterized by a deposition of calcium and phosphate in the medial layer of the vasculature[47,154] Calcium phosphate-based particles—along with other crystalline nanoparticles and microparticles—can induce NETs formation by neutrophils[155,156] by increasing ROS production[156] and RIPK1/MLKL (receptor-interacting protein kinase 1/mixed-ineage kinase like)–dependent necroptosis.[155] The contribution of altered platelet reactivity to NET formation in CKD has not yet been investigated, and highly variable results have been obtained in terms of platelet activity in patients with CKD. This varies from reduced platelet aggregation and activity over no changes to increased platelet responses in CKD—as discussed in detail elsewhere[121]—with heterogeneity in CKD pathophysiology, severity, or potential comorbidities as potential explanations. On the other hand, frequent comorbidities of CKD, such as CVD, diabetes, and hypertension could contribute to increased basal NETs formation in patients with CKD. Along this line, hemodialysis patients with major adverse cardiovascular events showed a higher level of histone-DNA complexes in blood compared with patients without major adverse cardiovascular events.[147] Furthermore, diabetes was shown to prime neutrophils for NETs production, with neutrophils from diabetic patients as well as healthy donor-derived neutrophils treated with high glucose demonstrating increased basal as well as agonist-induced NETs formation.[157] On mechanistic level, neutrophils from patients with type 2 diabetes reveal higher levels of PAD4 expression,[157] an increased ROS production[158] and higher basal calcium levels[159] (supporting ROS production and PAD4 chromatin citrullination activity[160]). Also, extracellular DNA in plasma correlated significantly with systolic blood pressure in hemodialysis patients[146] and patients with essential hypertension displayed increased levels of NETs in plasma.[161] To the latter patient group,

their plasma, as well as angiotensin II, stimulated NETs formation by neutrophils from healthy donors in vitro, with a role for ROS, PAD4 activity, and autophagy in NETs formation identified.[161]

NETs Formation and Cardiovascular Risk in Patients With CKD

In patients with CKD with coronary artery disease, a strong relation was identified between plasma levels of double-stranded DNA, NETs formation markers (myeloperoxidase-DNA complexes, citH4 [citrullinated histone H4]), and thrombin-antithrombin complex and vWF as markers of a prothrombotic state. Also, increased baseline levels of double-stranded DNA, nucleosomes, and myeloperoxidase-DNA complexes, but not of citH4, correlated with the occurrence of major adverse cardiovascular events over a median follow-up of 1.5 years.[162] In the same line, plasma levels of DNA-histone complexes were an independent predictor of major adverse cardiovascular events (MI, ischemic stroke, cardiovascular death) in hemodialysis patients over a follow-up of 24 months, after adjustment for age, sex, dialysis duration and efficiency, diabetes, ischemic CVD and stroke history, and the inflammatory marker high-sensitivity CRP (hs-CRP).[147] Of note, although this may raise the hypothesis of a role for NETs formation in increased cardiovascular risk in CKD, rigorous mechanistic in vivo studies still remain to be performed in future.

POTENTIAL FUTURE THERAPEUTIC OPTIONS TARGETING NETS-INDUCED IMMUNOTHROMBOSIS

Increasing evidence shows that current therapies targeting thrombosis also modulate inflammatory processes[163] and, vice versa, anti-inflammatory approaches can prevent thrombotic events.[164] Colchicine, for example, was described to inhibit extravascular NETs formation,[165] and IL-1β blocking by anakinra, canakinumab, and rilonacept[166] may potentially prevent NETs formation in general.[167] Selective inhibition of coagulation factors[168] Xia or XIIa represents a promising target in the context of NETs-mediated immunothrombosis. On one hand, inhibition of the intrinsic contact pathway of coagulation provides safe thromboprotection with no or only mildly increased bleeding risk.[169,170] Importantly, FXI/FXII leads to the activation of the proinflammatory bradykinin-producing kallikrein-kinin system[171] and endothelial inflammation by PAR (protease-activated receptor) signaling.[172] Findings showing an intimate connection between inflammation and thrombosis and the role of NETs therein have been accumulating. Hence, targeting the interface between these processes to prevent thrombosis seems a promising new therapeutic field (Figure 2).

Our understanding of NETs formation and function is incomplete, but substances targeting NETs formation or destroying NETs structures are available. A well-known agent that degrades the DNA backbone of NETs is Dnase I, which was shown to suppress thrombus growth.[20,21,173] Dornase alfa, the recombinant human DNase I, is FDA-approved for cystic fibrosis treatment and investigated for COVID-19–associated acute respiratory distress syndrome.[174] Bioinformatic investigations of the cytokine profile of SARS-CoV-2 infected lung epithelial cells have even suggested that the large number of NETs are the most important factor for vascular injury, thrombosis, and organ damage in severe thrombotic pulmonary complications of patients with COVID-19.[175,176] Although Dnase I preferentially digests naked cell-free DNA, Dnase 1L3 cleaves nuclear DNA with high activity and chromatin in an internucleosomal manner without proteolytic help.[177] Moreover, combining Dnase I with tPA treatment seems to be even more effective in the treatment of thrombosis in stroke and myocardial ischemia-reperfusion injury models.[178–180] Nevertheless, DNase I treatment also triggers the release of NETs components into the bloodstream, which may elicit procoagulant activity and intravascular thrombosis as free extracellular DNA fragments enhance the intrinsic coagulation pathway.[2,181] Others provide evidence that proinflammatory macrophages degrade NETs by enhanced micropinocytosis, engulfment, and subsequent uptake. Conversely, inhibition of micropinocytosis by imipramine treatment in mice corresponded to increased NET amounts in murine thrombi.[182] Another NETs inhibiting agent could be RE31, a 31-nt DNA aptamer, which inhibits thrombin formation, accelerates fibrinolysis in vitro, and suppresses thrombosis in vivo.[183]

As an alternative to the destruction of NETs, NETs formation itself could be targeted. For example, the administration of Cl-amidine, a pan-PAD inhibitor, effectively diminished NETs release and progression of sepsis, atherosclerosis, and thrombosis in mice.[184,185] Arterial occlusion induced by use of ferric chloride ($FeCl_3$) in mice revealed lower NET abundance and a reduction of arterial thrombi when exposed to Cl-amidine.[34] Thus, Cl-amidine shows potential to minimize arterial and atherosclerotic thrombi in experimental mouse models. Two other more specific PAD4 inhibitors, GSK 199 and GSK484, have shown beneficial effects in reducing disease burden of collagen-induced arthritis[186] and in heparin-induced thrombocytopenia in animal models[173,187] by interfering with NETs formation. Down this road, most promising seems to be the PAD4 inhibitor BMS-P5 (Bristol-Myers Squibb ((cis)-5-Amino-2-methylpiperidin-1-yl)(2-(1-(cyclopropylmethyl)-1H-pyrrolo[2,3-b]pyridine-2-yl)-7-methoxy-1-methyl-1H-benzo[d]imidazole-5-yl) methanone, hydrochloride)), which is found to be just as effective in reducing NETs levels and citrullinated histones as less novel PAD4 inhibitors such as Cl-amidine

and GSK484.[188] Results of PAD4 inhibitors are paralleled by data from PAD4 deficient mice showing reduced intravascular coagulation and organ damage compared with control littermates in sepsis and deep vein thrombosis.[22,189] More recently, gasdermin D was shown to be critical in NETs formation.[57] Yet, inhibitors are still in preclinical phase, but disulfiram, approved for the treatment of alcohol abuse, was reported to prevent NETs formation[190,191] and thereby protect rodents from acute lung injury and thrombosis.[192] Similar necrostatin-1 a small molecule inhibitor of the RIP1 (receptor-interacting protein 1) kinase, known to block necroptosis, was shown to inhibit NET formation through interference with MLKL phosphorylation and perforation in a murine model of asthma.[193]

Inhibiting the (toxic) protein components associated with NETs, such as granular proteins and histones, may provide an additional spectrum of therapeutic opportunities. Inhibitors of NE and anti-histone antibodies have been shown to reduce organ damage and lethality in mice with sepsis.[30] The NE inhibitor sivelestat was approved to treat acute respiratory distress syndrome in Japan and South Korea but did not increase survival in a meta-analysis of clinical trials.[194] However, newer, and more specific NE inhibitors are under investigation in phase I clinical trials and may not only improve treatment of patients with cystic fibrosis but may also allow for interference with NETs-associated immunothrombosis.[195] Another neutrophil-derived protein is the cathelicidin LL-37 (mouse Cramp); it is also able to stimulate NETs formation, was implicated in thrombotic and complications of patients with COVID-19,[196] and found in thrombi from patients with acute MI.[77] Its depletion in mice reduced platelet recruitment, diminished platelet-neutrophil interactions, and abolished thrombosis in mice.[19]

Further, neutrophils that are attracted to inflamed vasculature of atherosclerotic lesions are stimulated to undergo NETs formation, which frees nuclear proteins. These histones, and in particular histone H4, weaken plaque stability by inducing smooth muscle cell death, which increases the thrombotic risk. This process could be inhibited by a histone-inhibitory peptide administered in vivo.[197] Neutrophil recruitment to sites of inflammation is mainly mediated by CCL5 (C-C Motif Chemokine

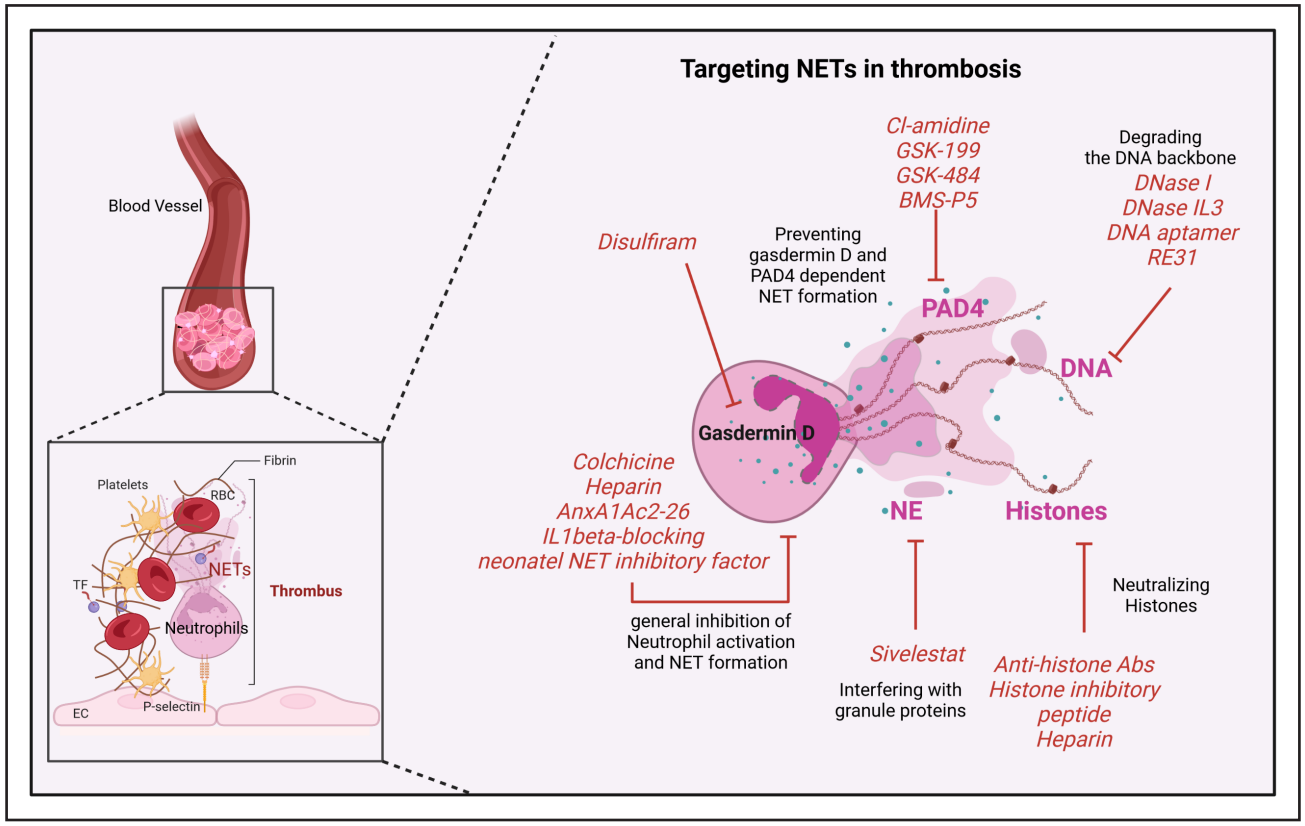

Figure 2. NETs (neutrophil extracellular traps) targeting in thrombosis.
Colchicine and other more general NETs inhibitors interfere with neutrophil activation, migration, and infiltration into sites of inflammation. IL (interleukin)-1β blockers will prevent an inflammatory loop between NETs and IL-1β. PAD4 (peptidyl arginase deiminase 4) and gasdermin D inhibitors will avert NETs formation more specifically while DNase (deoxyribonuclease) has been used safely to digest NETs. Interfering with toxic effects of histones and neutrophile elastase decorating the NET backbone also reduce thrombus formation and tissue damage. Abs indicates antibodies; AnxA1Ac2-26, annexin A1 mimetic peptide; BMS-P5, Bristol-Myers Squibb ((cis)-5-Amino-2-methylpiperidin-1-yl)(2-(1-(cyclopropylmethyl)-1H-pyrrolo[2,3-b]pyridine-2-yl)-7-methoxy-1-methyl-1H-benzo[d]imidazole-5-yl)methanone, hydrochloride); EC, endothelial cell; GSK, glycogen synthase kinase; RBC, red blood cell; RE31, 31-nt DNA aptamer; and TF, tissue factor. Made with biorender.com.

Ligand 5); thus, attenuating inflammation could consist in the CCL5-CXCL4 heterodimer blockage by MKEY, as it was demonstrated via restriction of leukocyte recruitment and NETs formation in a mouse model of MI.[198] In addition, heparin was reported to not only inhibit thrombosis by activation of antithrombin but also to partly mediate NETs disruption.[20,21] In addition, heparin neutralizes detrimental effects of histone-mediated thrombosis independent of its other anticoagulant properties.[199] vWF serves as a mediator to bring NETs and fibrin into proximity, facilitating NETs to modify fibrin structure and perform its procoagulant effect. Moreover, Grässle et al. reported that vWF directly binds and immobilizes extracellular DNA (eg, from NETs) thereby mediating leukocyte adhesion to endothelial cells, supporting leukocyte extravasation and inflammation. Heparin may interfere with DNA-vWF binding, thereby blocking leukocyte adhesion.[74] Similar, use of recombinant ADAMTS13, which targets ultralarge vWF, was shown to reduce ischemic brain injury in experimental stroke.[200] Infusion of recombinant human ADAMTS13 markedly dissolved the tPA-resistant thrombi in a dose-dependent manner, resulting in reduced cerebral infarct sizes in a thrombosis mouse model.[201] Hence, targeting vWF-NETs interaction could represent a potential target for antithrombotic therapies.

Further, still preliminary strategies to target NETs in thrombosis could be the treatment with the AnxA1 (annexin A1) mimetic peptide AnxA1Ac2-26 which targets the human FPR (formyl peptide receptor) FPR2/ALX. FPR2 is a G-protein–coupled receptor expressed in different cells and tissues, propagating resolution of inflammation. Treatment with the AnxA1 mimetic reduces H3Cit1-rich NETs production by transforming the neutrophil phenotype from pro-NETotic into pro-apoptotic, thereby driving thromboinflammation resolution.[202]

Neutrophil activation including NETs formation was also described to be regulated by transcription factor KLF2 (Krüppel-like factor 2). Neutrophil activation through genetic loss of KLF2 or administration of antiphospholipid antibodies resulted in clustering of PSGL-1 by cortical actin remodeling, thereby increasing neutrophil adhesion potential at sites of thrombosis. Targeting clustered PSGL-1 using nanoparticles attenuated neutrophil-mediated thrombosis in KLF2 knockout mouse models, illustrating the importance and feasibility of targeting activated neutrophils to prevent pathological thrombosis.[203] In addition, the neonatal nNIF (NETs-inhibitory factor) identified in umbilical cord blood was shown to inhibit key terminal events in NETs formation, including PAD4 activity, neutrophil nuclear histone citrullination, and nuclear decondensation in vitro and in vivo.[204]

Taken together, the involvement of NETs in arterial and venous thrombosis offers new therapeutic opportunities. Strategies could be aimed at either reducing thrombosis or enhancing thrombolysis. Digestion of the NET structures (eg, DNAse) or inhibition of NET formation (eg, cl-amidine) represent promising thrombolytic options that, most importantly, do not increase the risk of bleeding.[205,206] Hence, these measures could be added to already available therapeutic options which target fibrin breakdown or interfere with the coagulation cascade. In addition in patients with recurrent thrombosis, a problem in both arterial and venous thrombosis, interfering with NET formation could also be a preventive therapeutic measure. Preclinical and clinical studies investigating these new therapeutic options are now needed to fully understand the efficacy and safety of targeting NETs in immunothrombosis.

CONCLUSIONS

Altered immune thrombosis, particularly in the context of chronic inflammation, contributes significantly to cardiovascular events in CVD patients and represents a major cause of death. In particular, the identification of specific targets that inhibit immunothrombosis without concomitant immunosuppressive side effects would be crucial. Possibly, thrombus-specific application or even a targeted delivery of drugs (coated nanoparticles) could be a future approach.

Whereas mechanistic insights into NETs formation and contribution to cardiovascular risk have accumulated over the past years, relatively few studies have addressed NETs formation in patients with CKD. A better understanding of NETs formation in CKD and underlying CKD-specific mechanisms is required. Furthermore, a contribution of CKD-associated NET formation to increased cardiovascular risk in CKD remains currently only a reasonable hypothesis; it remains to be investigated whether (and if, how) NETs interference through the potential therapeutic targets mentioned above would impact thrombotic risk in patients with CKD. Of note, patients with CKD suffer not only from increased thrombotic risk but also from a higher bleeding risk, with increased bleeding reported to impact the application of antiplatelet therapy in these patients, as discussed in more detail elsewhere.[121] Thus, also the effect of NETs interference on bleeding risk in patients with CKD requires attention. Finally, in the context of vascular leakage and endothelial cell dysfunction, CKD negatively impacts endothelial health, for example, through the accumulation of uremic toxins[207] and other mechanisms (as discussed in more detail in the review by Baaten et al. within this Compendium). Also, NETs induced by neutrophil treatment with uremic serum from hemodialysis patients reduced endothelial viability and increased levels of soluble ICAM-1 (intercellular adhesion molecule-1), soluble E-selectin, and vWF in endothelial cell supernatant,[150] suggesting that NET inhibition may also offer endothelial protective effects in CKD.

In summary, various studies have focused on NET components like DNA scaffold, histones, NE, myeloperoxidase, and their interaction with coagulant proteins

to enhance thrombus formation. Promising results with anti-inflammatory drugs could even further be enhanced by combination with antithrombotic drugs. To make it more specific, refinement in this direction could include future trials or studies combining anticoagulants with NETs/ NETs component inhibitors. Maybe also phenotypic switching of the neutrophils and inhibition of platelet activation, or platelet aggregation could resolute thromboinflammation. However, many mechanistic studies on this topic have so far originated in mice and have yet to be validated in the human context, which would be important to allow treatment development without the risk of bleeding.

ARTICLE INFORMATION

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Disclosures

None.

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