Neutrophil extracellular traps (NETs) in cardiovascular diseases: From molecular mechanisms to therapeutic interventions

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ABSTRACT

Neutrophil extracellular traps (NETs), which are net-like structures composed of DNA, histones, and antimicrobial proteins, in particular myeloperoxidase (MPO) and elastase, have been demonstrated in bacterial, viral, protozoal, and fungal infections as a potent innate immunity mechanism of pathogen elimination associated with enhanced inflammation. Growing evidence indicates the contribution of NETs formation (NETosis), driven by protein-arginine deiminase type 4, to thrombosis, ischemia, and atherosclerosis. NETs are considered new players involved in the development and progression of cardiovascular diseases (CVDs), including coronary artery disease (CAD) and its acute manifestations in particular acute myocardial infarction (MI), peripheral artery disease (PAD) along with ischemic stroke, heart failure, aortic stenosis, and atrial fibrillation (AF). Formation of NETs and elevated levels of their circulating markers, e.g. citrullinated histone 3 and MPO-DNA complexes, have been observed in chronic and acute manifestations of CVD. NETs accumulation was associated with plaque rupture, infarct size, and impaired myocardial function. NETs have been identified within human stenotic aortic valves, like in atherosclerotic plaques and arterial thrombi. Moreover, circulating NETs markers in association with prothrombotic markers, including fibrin clot properties, predicted adverse clinical events in AF. Several NETs inhibitors, including recombinant human DNase, an enzyme degrading NETs, reactive oxygen species scavengers, together with antithrombotic and antiplatelet drugs, have been shown to reduce uncontrolled NETosis. This review summarizes the current evidence on the role of NETosis in CVDs, its significance as a risk factor for clinical outcomes, and finally, the potential of NETs as a target for future therapeutic interventions.

Key words: atherosclerosis, atrial fibrillation, myocardial infarction, NETs, stroke

INTRODUCTION

Cardiovascular diseases (CVDs) represent a range of common conditions being the leading cause of death worldwide, accounting for about one-third of all death in 2019, as reported by the World Health Organization [1]. CVDs encompass coronary artery disease (CAD) and its acute manifestations, in particular acute coronary syndrome, myocardial infarction (MI), and peripheral artery disease (PAD), increasing the risk of ischemic stroke, as well as heart failure (HF) and atrial fibrillation (AF). The prevalence of CVD rises with age, starting at around 1% of individuals aged 20–39 and increasing to 42.9% among males and 31.3% among females aged 80 years and older, excluding cases related to hypertension [2]. In 2019, the primary cause of CVD-related deaths was CAD, which accounts for 41.3% of fatalities, followed by stroke at 17.2%, high blood pressure at 11.7%, HF at 9.9%, and arterial diseases at 2.8% of all deaths [2]. It is worth mentioning that CVD risk factors, such as obesity and diabetes, were more frequently associated with CVD-related deaths, as became evident during the COVID-19 pandemic [3].

Chronic inflammation within the arterial wall is a key pathophysiological mechanism underlying the development and progression of CVDs [2]. Extremely intricate and

intertwined processes involved in atherosclerosis, which are still incompletely elucidated encompass enhanced oxidative stress, lipid accumulation, immune responses, fibrosis, calcification and many others [4].

While monocytes have been extensively studied in the context of CVD, neutrophils, the most abundant white blood cells, play a crucial role in innate defense against infections. Their functions include phagocytosis, the process of engulfing and ingesting pathogens like bacteria, fungi, and cellular debris [5].

Neutrophils migrate to sites of infection or inflammation in response to chemical signals, such as chemokines and cytokines, through a process called chemotaxis [5]. In response to specific stimuli neutrophils can produce large amounts of reactive oxygen species (ROS) upon activation of NADPH oxidase 2 (NOX2) [6]. ROS are toxic to pathogens and destroy bacteria and fungi inside the neutrophil. As inflammation subsides, neutrophils undergo apoptosis (programmed cell death), and then macrophages remove the apoptotic neutrophils [6].

In 2004 Brinkmann et al. [7] described a new activity of neutrophils called neutrophil extracellular traps (NETs) formation, suggesting that this mechanism of the innate immunity is of key importance in pathogen elimination. NETs are web-like structures composed of DNA, histones, and antimicrobial proteins [7]. Initially it was thought that NETs exclusively capture and immobilize microbes, such as bacteria, fungi, and some viruses, and thus preventing their spread [7]. However, in the following years growing evidence supported the concept that NETosis is implicated in sepsis, autoimmune diseases, venous thromboembolism, cancer, and also CVDs [8]. It is important to emphasize that neutrophil activation and NETosis are two different processes involving neutrophils. Neutrophil activation is associated with their degranulation, oxidative burst, and phagocytosis. Prolonging neutrophil activation associated with enhanced ROS production leads to NETs generation [9].

In this review we summarized available data on the role of NETosis in a broad spectrum of CVDs involving CAD, acute arterial thromboembolism, and AF in search for novel biomarkers and potential treatment options targeting NETosis inhibition.

Molecular mechanisms of NETosis

The molecular basis of NETosis involves a series of complex events. Neutrophil activation triggers intracellular signaling pathways that lead to the activation of an enzyme called nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH generates ROS, including singlet oxygen ($1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid, and others, which are about to destroy microbes [9]. It is important to note that ROS, apart from its bactericidal function, play a crucial role in the process of "suicidal" NETosis, which persists for 3–4 hours and is fatal for neutrophils [9]. An enzyme, called myeloperoxidase (MPO), activated by H_2O_2, leads to the release of neutrophil elastase (NE) from neutrophil azurophilic granules. NE migrates to the cell nuclei to induce chromatin decondensation and degrade neutrophil actin filaments, thereby inhibiting neutrophil chemotaxis [10]. After nucleus disintegration, neutrophils release NETs into the extracellular environment and both MPO and NE on chromatin fibers stabilize NETs and ensure their antibacterial properties [11]. Complexes of cell free DNA and MPO or NE are considered as specific biomarkers of NETs, while citrullinated histones H3 (citH3), although associated with NETosis, covers only protein arginase deiminase 4 (PAD4)-dependent citrullination of H3 histones [9]. Moreover, Kenny et al. [9] reported that NETosis can occur without histone H3 citrullination and pharmacological inhibition of PADs did not inhibit NETosis.

PAD4, catalyzes the conversion of arginine residues in histones to citrulline, which weakens their interaction with DNA, promoting chromatin decondensation [11]. Citrullinated histones have been demonstrated to exert antimicrobial properties [11]. Of note, several proteins, including fibrinogen, can be citrullinated by PAD4. Citrullinated fibrinogen, together with citrullinated vimentin were observed within human atherosclerotic plaques [12]. Moreover, *in vitro* fibrinogen citrullination led to formation of more compact clots with reduced porosity and susceptibility to fibrinolysis [13].

An alternative form of NETosis is the "vital" or NOX-independent pathway. In contrast to the "suicidal", the "vital" NETosis occurs in about 30 minutes [14]. "Vital" NETosis is ROS-independent and can be induced by activated platelets, microorganisms, and complement proteins. This leads to the influx of Ca^{2+} *via* the small conductance potassium channel member three [14], which activates PAD4, and results in histone citrullination and chromatin decondensation [15]. Neutrophils remain viable after this form of NETosis [8].

NETs are eliminated by deoxyribonucleases (DNases), Ca^{2+}/Mg^{2+}-dependent enzymes degrading circulating DNA, and phagocyted by macrophages [16]. There are three main types of DNases: DNase-I, DNase-II, and DNase-1L3. DNase-I preferentially digests cell-free DNA (cfDNA), DNase-II degrades DNA from apoptotic bodies, while DNase-1L3 degrades chromatin and chromatin-bound DNA [17, 18]. It was shown that genetic defects in the DNase-I or DNase-1L3 genes are associated with severe forms of autoimmune diseases e.g. rheumatoid arthritis and scleroderma [19]. Insufficient clearance of NETs may contribute to the development of autoimmune diseases primarily because of the exposure to intracellular antigens present on NETs [20]. Moreover, in mouse models, DNase deficiency was associated with massive NET-related thrombosis in lungs, liver or kidneys [21].

Factors triggering NETosis

NETosis is triggered by a variety of factors, primarily associated with the recognition of pathogens such as pathogen-associated molecular patterns (PAMPs), like bacterial

lipopolysaccharides, lipoproteins, viruses, and fungal cell wall components. NETosis can also be activated by receptors, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), complement receptors (CRs), receptors for immunoglobulin c-terminal fragment (FcR) and Cys-X-Cys motif chemokine receptors. Recently nucleotide-binding oligomerization domain-like receptors have been shown to activate NETosis [22]. Moreover, cytokines released upon inflammation, such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-8 (IL-8) that can activate neutrophils trigger NETosis [23]. The complement system, a component of the immune system, was also shown to induce NETosis. It has been demonstrated that complement component 3 (C3) knock-out mice and receptor for C3a fragment (C3aR) knock-out mice display reduced NETs formation [24]. Other complement components such as C3b or C5a were shown to stimulate NETosis by binding to their receptors on neutrophils, while C1q can prevent NETs from DNases. Moreover, physical factors such as shear stress and mechanical stretching can contribute to NETosis. Factors like hypoxia (low oxygen levels) and nutrient deprivation can also induce NETosis, especially during tissue damage or inflammation.

Platelets in NETosis

Platelets are the interface between blood coagulation and inflammation in atherosclerosis [25]. Neutrophils and platelets interact together *via* the glycoprotein Ib. Moreover, neutrophils recognize P-selectin expressed by activated platelets, which was shown to facilitate NETosis [25] but P-selectin blockage does not inhibit NETs formation [26]. Another platelet-derived protein, high mobility group box 1 (HMGB1), was independently associated with NETs formation. A study by Maugeri et al. [26] performed on 26 patients with acute MI showed that besides activated platelets, NETing neutrophils were a main cellular component of thrombi. Additionally, studies have demonstrated that platelets are recruited to NETs and bind to them in a histone-dependent manner via complement component C3b deposited on NETs and CR1 receptor expressed by platelets [27, 28]. Histones H3 and H4 have been shown to activate platelets, which in turn stimulated NETosis in a positive-feedback loop [27, 28]. Moreover, those histones induced secretion of platelet-derived polyphosphate (poly P) and activated blood coagulation by factor XII (FXII) and render fibrin clots more compact [28, 29]. Factors triggering NETosis and receptors involved are summarized in Figure 1.

A link between NETosis and coagulation

NETs formed after neutrophil activation provide a scaffold for thrombus formation [30]. Interestingly, only DNA and histones alone can induce thrombin generation, while these components in a complex of nucleosomes did not have the same effect [28]. Moreover, the selective exposure of tissue factor (TF), which initiates blood coagulation *in vivo*, on NETs determines the ability to initiate coagulation. However, it is important to note that not all NETs exhibit TF. It has been shown that neutrophils treated with cytokines increased the expression of TF mRNA and its release on NETs [31]. Therefore, the exposure of TF on NETs appears to be contingent on the specific stimulus used to induce NETosis, which may explain an importance of the experimental approach used to research. On the other hand, another mechanisms of direct coagulation activation by NETs is that they provide a negatively charged surface to activate FXII, the initial factor of the intrinsic coagulation pathway [32]. FXII or FXI inhibition attenuated thrombin generation on NETs [33]. Finally, it was shown that fibrinogen binds to DNA-rich NETs [34]. It is plausible that fibrin formation takes place on NETs following coagulation activation regardless of the pathway involved. Fibrin may serve to stiffen the structure of NETs, creating a mesh that entraps pathogens, thus preventing their dissemination. Using scanning electron microscopy imaging it has been demonstrated that fibrin clots formed *in vitro* in the presence of NET components displayed denser structure, which is more resistant to lysis [35].

It is worth mentioning that positively-charged nucleosomes located at the site of injury can attract negatively-charged tissue factor pathway inhibitor (TFPI) [2]. NET-associated proteins, namely NE and cathepsin G, play a role in promoting fibrin formation on NETs, since they have been demonstrated to inhibit TFPI, a major inhibitor of the extrinsic coagulation pathway [2]. Further research is needed to clarify the role of TFPI in the cross-talk between NETosis and blood coagulation.

Enhanced NETosis exerts potent proinflammatory effects. An overabundant discharge or malfunction of NETs may initiate and enhance inflammatory reactions, leading to potential harm to tissues and various disease conditions. Components of NETs, such as histones, of circulating DNA, can transform into self-antigens, resulting in inflammation, tissue toxicity and thrombosis [29]. Therefore, maintaining a balance between NETosis activation and inhibition seems to be of major importance in a variety of disease states, including atherosclerotic vascular disease and its thrombotic manifestations.

NETosis in CVDs

Growing evidence has established a link between enhanced NETs formation or insufficient NETs degradation and the underlying mechanisms of various CVDs driven by inflammatory responses (Figure 2).

Coronary artery disease (CAD)

The involvement of NETosis in atherosclerosis may result from infections and stimulation of neutrophils by bacterial components [36] or from a non-infectious activation of neutrophils within atherosclerotic plaques by cholesterol crystals and the interplay between neutrophils and macrophages, amplifying the immune response [37].

In 2012, Megens and co-workers [38] were the first to report the presence of NETs within atherosclerotic plaques,

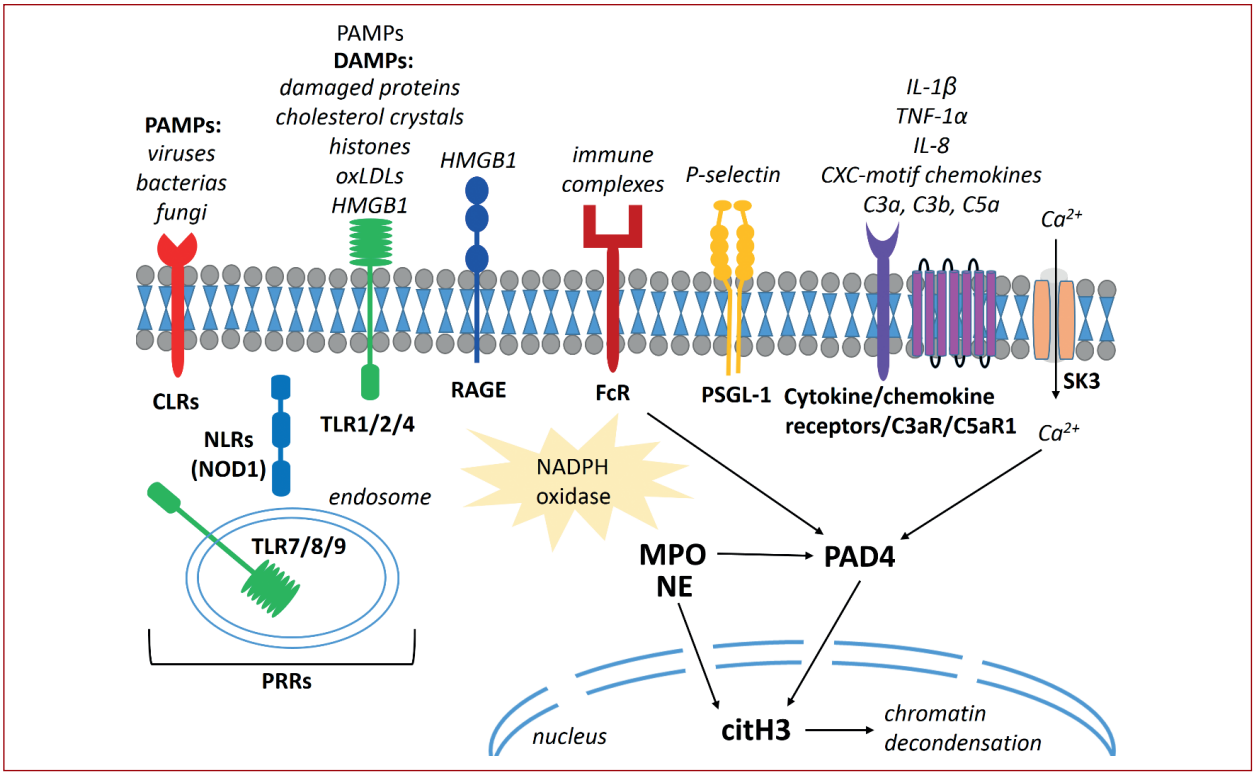

Figure 1. Receptors and corresponding stimuli activating the release of neutrophil extracellular traps (NETs). Pathogen-associated molecular patters (PAMPs) and damage-associated molecular patterns (DAMPs) activate receptors such as C-type lectin (CLRs), cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors as well as toll-like receptors (TLRs), called together as pathogen-recognition receptors (PRRs). Moreover, transmembrane receptors such as receptor for advanced glycation end products (RAGE) binding platelet-derived high mobility group box 1 (HMGB1) protein, Fc fragments receptors (FcR), P-selectin glycoprotein ligand-1 (PSGL-1) binding P-selectin released by activated platelets, and cytokine (for interleukin [IL]-1β, tumor necrosis factor-1α [TNF-1α], and IL-8) or chemokine receptors, including receptors for complement components C3a and C5a (C3aR and C5aR1) are able to activate enzymes involved in NETs generation, such as myeloperoxidase (MPO), neutrophil elastase (NE), and protein arginine deiminase 4 (PAD4) in nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase)-dependent mechanism. PAD4 can be also activated directly by an influx of calcium ions (Ca^{2+}) through the small conductance potassium channel member three (SK3). MPO, NE, and PAD4 are responsible for chromatin decondensation and histone citrullination, leading to NETs formation

precisely in the luminal region. Borissoff et al. [39] in a study comprising 282 individuals with CAD showed associations between elevated levels of circulating DNA, nucleosomes, citrullinated histone H4, and MPO-DNA complexes with thrombin-antithrombin complexes. These were linked to the severity of CAD and the occurrence of major adverse cardiac events. This study strongly suggested that NETs contribute to the progression of atherosclerosis.

Fibrin forms the primary matrix of thrombi intertwined with DNA, derived from NETs, which has been shown in intracoronary thrombi from acute MI patients, particularly within fresh and lytic but not in organized thrombi [40]. Two studies provided additional evidence that NETs are involved in acute MI and the no-reflow phenomenon after reperfusion [41, 42]. Savchenko et al. [42] showed in a mouse model that cardiac ischemia induced NETs formation and DNase treatment reduced NETs accumulation. Similarly, Ge et al. [41] reported that addition of DNase I to thrombolytic therapy reduced NETosis, limited the no-reflow area, and showed beneficial effects for left ventricular function in rats. Mangold et al. [43] investigated 111 patients with ST-segment elevation MI (STEMI) who were undergoing primary percutaneous coronary intervention. They found that markers of NETosis and neutrophil activation, including nucleosomes, double-stranded DNA, NE, and MPO, are elevated in the culprit lesion site. NETs were identified within coronary thrombi retrieved during thrombectomy and the extent of NETosis was positively associated with the infarct size and negatively with ST-segment resolution [43]. Stakos et al. [44] in 18 patients with STEMI showed that thrombi from the culprit artery were rich in NETs expressing TF. The largest study performed on 253 thrombi from patients with stent thrombosis revealed the presence of NETs in 23% of samples, highlighting the role of NETs in coronary thrombosis [45]. It is unclear as to whether NETosis markers can predict major adverse coronary events in CAD.

Peripheral arterial disease (PAD)

PAD, often secondary to atherosclerosis, coexists commonly with CAD and shows association with a prothrombotic state [46]. Little is known about the role of NETosis in PAD. In 79 patients with PAD citH3 and cell-free DNA levels tended to be higher compared to healthy controls [47]. Circulating citH3 were associated with P-selectin

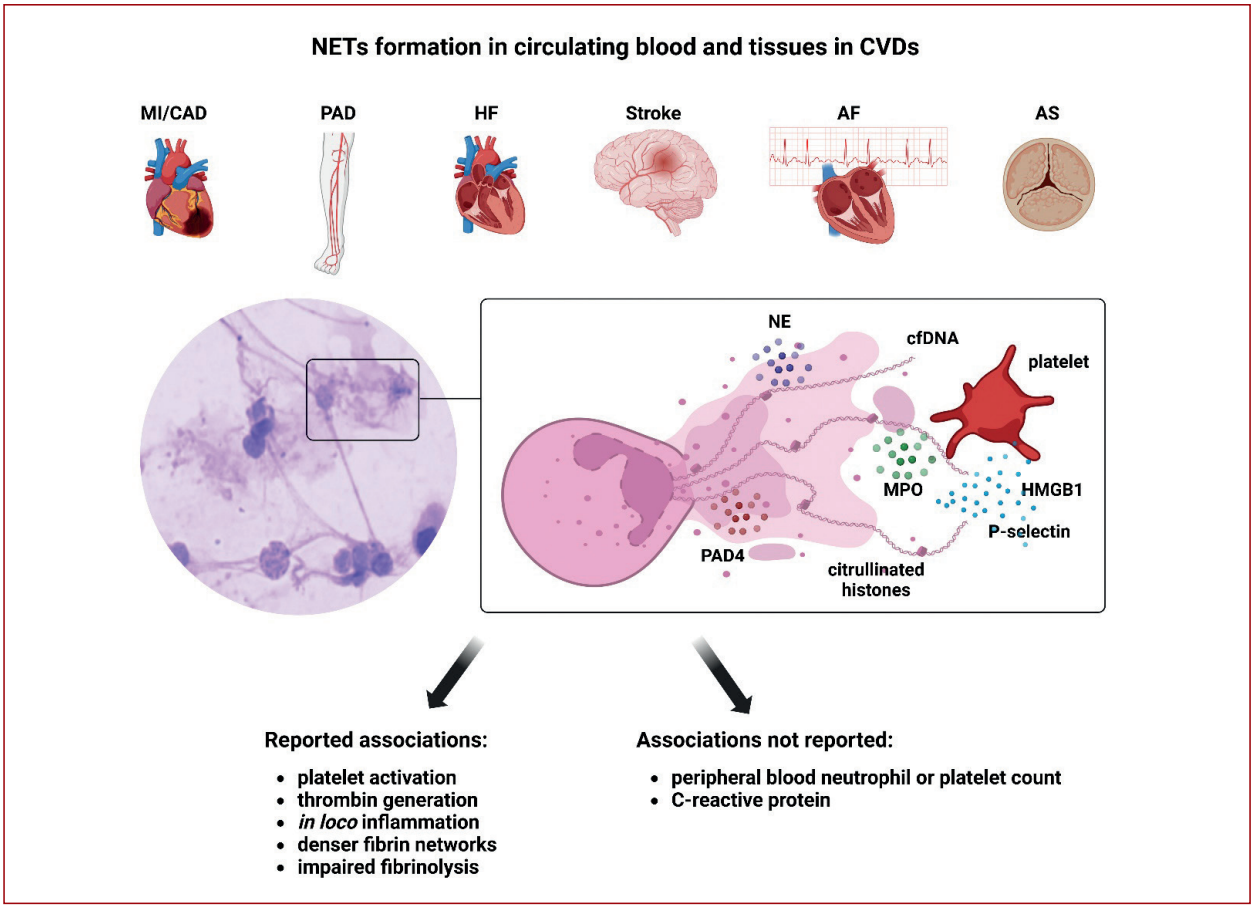

Figure 2. The involvement of NETosis in cardiovascular diseases (CVDs). NETs formation in circulating blood or tissues contributes to platelet activation, thrombin generation, and *in loco* inflammation in myocardial infarction (MI), coronary artery disease (CAD), and stroke. In peripheral arterial disease (PAD) no differences in circulating NETs markers were observed compared to controls, however, levels of cell-free DNA (cfDNA) and citrullinated histone H3 (citH3) correlated with P-selectin following platelet activation. Myeloperoxidase (MPO) was identified as an independent predictor of heart failure (HF) development. In ischemic stroke MPO-DNA complexes were associated with platelet-derived high mobility group box 1 (HMGB1) levels and neurological function. Prothrombotic fibrin clot phenotype, involving formation of more compact fibrin network and reduced susceptibility to fibrinolysis, in association with enhanced NETosis predisposed to cerebrovascular events in atrial fibrillation (AF). In aortic stenosis (AS) elevated plasma citH3 levels, together with increased valvular expression of citH3, MPO, and neutrophil elastase (NE) were observed. The image presents human neutrophils stimulated for 180 min with phorbol 12-myristate 13-acetate (PMA, final concentration 600 nM) and hematoxylin stained after stimulation. Magnification, 40×. Created with BioRender.com (agreement No. YZ262U4X94)

expression in response to exogenous thrombin-receptor activating peptide or arachidonic acid, while cell-free DNA was associated with P-selectin expression and activated glycoprotein IIb/IIIa after *in vitro* platelet activation using arachidonic acid [47]. Following infrainguinal angioplasty with stent implantation, increased levels of citH3 and cell-free DNA on admission predicted ischemic outcomes during 2-year follow-up [47]. Toth et al. [48] assessed 19 PAD and 18 CAD patients and reported that in PAD and CAD patients with dyslipidemia (n = 15) and high prevalence of atherothrombosis, DNA content was positively associated with the amounts of von Willebrand factor within thrombi.

No difference in circulating MPO-DNA levels was shown between symptomatic PAD patients compared with healthy controls. However, increased neutrophil degranulation was found to be related with PAD along with its prognostic role for major adverse cardiac events [49].

Heart failure

Mechanisms leading to HF involve inflammation, endothelial dysfunction, abnormal cardiac metabolism, cardiomyocyte hypertrophy or cardiac fibrosis [50, 51]. To the best of our knowledge there are few reports linking HF with NETosis.

Increased circulating MPO levels were identified as an independent risk factor for the onset and persistence of chronic HF [52, 53]. La Rocca et al. [54] suggested that this phenomenon is associated with MPO-associated chlorination or nitration of protein tyrosine, resulting in protein malfunction and endothelial damage. In an animal experiment involving lipodystrophic mice, which are prone to development of HF with preserved EF, showed that NETs-associated interstitial fibrosis contributes to ventricular stiffness. Moreover, NETs can be deposited within cardiac tissue as large amorphous structures [55]. A role of NETs in cardiac fibrosis has been demonstrated

in PAD4-knockout mice [56]. The authors showed that in this model protection from heart/lung fibrosis and improvement in left ventricular ejection fraction was found compared to wild-type mice, with a similar effect after DNase 1 supplementation [56]. Langseth et al. [57] reported in 61 STEMI patients with symptomatic acute HF that circulating levels of cell free DNA, MPO-DNA complexes, and IL-8 correlated with myocardial function but not myocardial recovery.

Ischemic stroke

Neutrophils are the initial immune cells that enter into the brain tissue shortly following an acute ischemic stroke (AIS), and contribute to brain injury within the ischemic region [58]. In 2012 De Meyer et al. [59] showed increased levels of circulating NETs components, such as nucleosomes, cell-free DNA, and histones in mice with experimental ischemic stroke. Moreover, histones contributed to cerebral ischemia/reperfusion injury and targeting of histones and DNA improved stroke outcome [59]. Laridan et al. [60] have shown for the first time that NETs are present in cerebral thrombi during AIS. Interestingly, NETs amount was largely higher in strokes of cardioembolic compared to non-cardioembolic origin. Additionally, thrombi older than 1 day exhibited higher neutrophil count compared to fresh thrombi [60].

In the study by Vallés et al. [61] performed on 243 AIS patients who were compared to 27 healthy controls, the highest quartile of citH3 (>0.284 AU) was independently associated with all-cause mortality at one-year follow-up (odds ratio [OR], 7.06; 95% confidence interval [CI], 1.63–30.5). In contrast, no associations with cfDNA or nucleosomes were found [61].

Elevated citH3 along with higher von Willebrand factor levels assessed on admission predicted AF-related cerebrovascular ischemic events, including ischemic stroke or transient ischemic attack during long-term anticoagulation [62]. This observation suggests a potential predictive value of NETosis markers in AF. Kollikowski et al. [63] showed in AIS patients that MPO concentrations assessed in cerebral arterial blood samples correlated positively with the number of neutrophils infiltrating the ischemic brain area, local platelet count, and neutrophil-activating peptide 2 (NAP-2), a primary platelet-derived neutrophil chemoattractant. Moreover, brain MPO levels were associated with functional clinical outcome assessed using modified Rankin scale. A recent study by Denorme et al. [64] performed in AIS patients and on a mouse model supported a pathological role of NETs in AIS. NETs were detected in the brain tissue samples of patients, irrespectively of stroke severity, and platelet-derived HMGB1 was correlated with NETs formation in this group of patients [64]. This study also showed on a model of HMGB1-knockout mice that platelets are a critical source of HMGB1 and that HMGB1-knockout mice had reduced plasma NETs and improved stroke outcomes [64]. In conclusion, NETs contribute to AIS and further studies are needed to elucidate whether therapeutic strategies aimed at NETosis inhibition may be beneficial to reduce stroke severity or improve stroke outcomes.

Atrial fibrillation

Little is known about the role of NETs in AF. Both experimental and clinical data have shown that MPO, an enzyme released upon neutrophil activation, plays a role in the pathogenesis of AF [62, 65]. In experiments involving right atrial electrophysiological stimulation, mice deficient in MPO did not develop AF [65]. However, when MPO was administered, this protective effect was reversed, leading to a similar degree of atrial fibrosis as observed in wild type mice treated with MPO for 7 days [65]. This observation suggests that MPO is a vital factor in myocardial remodeling, ultimately increasing susceptibility to AF. Also NE was shown to be elevated in AF patients compared to healthy subjects and the highest NE concentrations (>55.3 ng/ml) predicted ACEs (HR, 1.84; 95% CI, 1.01–3.76) in this group of patients [66]. Increased levels of NETs markers, including elevated citH3 concentrations characterized AF patients at high thromboembolic risk during long-term follow-up (Figure 3) [62]. Moreover, citH3 levels were associated with formation of denser fibrin clots, explaining about 5% of variation in clot porosity [62]. More compact fibrin clots were composed of thinner fibers and were more resistant to fibrinolysis [62]. These observations suggest that enhanced NETosis may contribute to a prothrombotic state observed in AF patients. Of note, in AF patients positive correlations between citH3 levels, 3-nitrotyrosine, as a marker of protein oxidation, and NAP-2 were found, emphasizing the role of oxidative stress and an interplay between neutrophils and platelets in AF [67]. Whether NETosis may be involved in the pathogenesis of AF remains to be established.

Aortic stenosis

Aortic stenosis (AS) is the most common acquired valvular heart disease in the adult population [68]. AS is closely linked to atherosclerosis, with similar risk factors and underlying pathomechanisms, such as chronic inflammation driven by oxidatively modified low-density lipoproteins (oxLDLs) followed by an influx of monocytes transforming into macrophages and the resulting calcification [68]. Moreover, prothrombotic state and hypofibrinolysis have been shown to contribute to AS progression [69]. The activity of MPO can also contribute to LDL modifications, which can effectively function as damage-associated molecular patterns (DAMPs) known to initiate atherosclerosis [70]. Awasthi et al. [71] reported that oxLDLs, especially lysophosphatidylcholine, facilitate NETs formation in humans, which favored endothelial inflammation in a vicious cycle. In 2019 Kopytek et al. [72] showed that AS patients compared to healthy controls had above 80% higher plasma citH3 levels and the presence of citH3/MPO- and citH3/NE-positive NETs was demonstrated within stenotic aortic valves

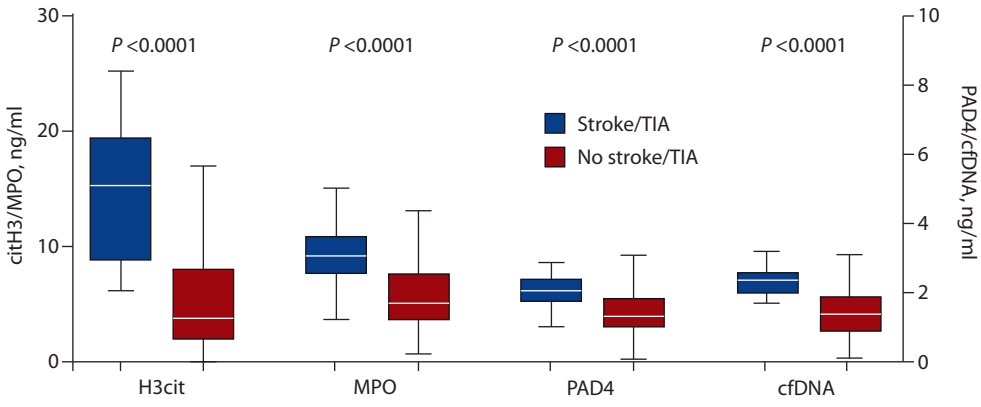

Figure 3. Elevated levels of circulating NETosis-related markers in patients with AF who experienced thrombotic cerebrovascular events during long-term follow-up. Circulating NETs markers were assessed in 243 AF patients (aged, 69 [64–75], 44% women) off anticoagulation. As many as 20 patients (8.2%) experienced thrombotic cerebrovascular events (ischemic stroke or transient ischemic attacks) during a median follow-up of 53 months despite anticoagulation (based on [62])

Abbreviations: TIA, transient ischemic attack; other — see Figures 1 and 2

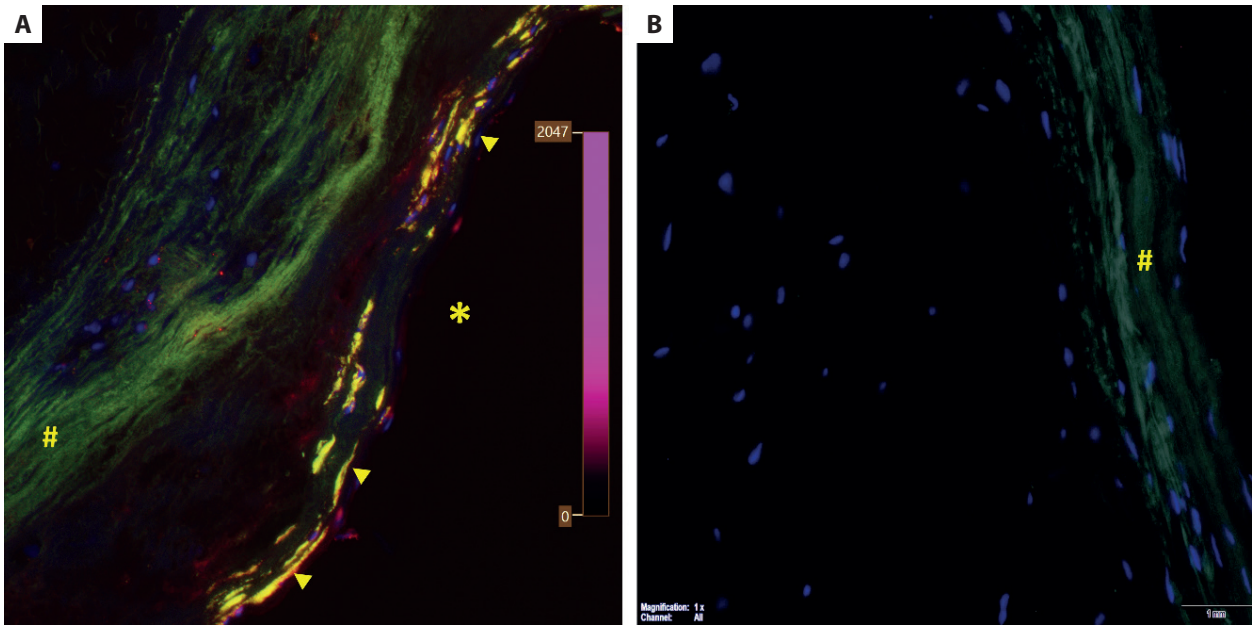

Figure 4. Specific biomarkers of neutrophil extracellular traps (NETs) within stenotic aortic valves. Aortic valve samples, dissected during surgical aortic valve replacement, were embedded in Tissue Tec-OCT compound (Sakura, Torrance, CA, USA) and cryosectioned onto SuperFrost slides (5 µm) (Menzel-Glaser, Braunschweig, Germany) by a Leica CM 1520 cryostat (Wetzlar, Germany). Single-label fluorescence was performed using monoclonal antibodies against citrullinated histones H3 (citH3; 1:250; Abcam, Cambridge, UK, cat. No. ab219407) or myeloperoxidase (MPO; 1:1000; GeneTex, Irvine, CA, USA, cat. No. GTX75318). Primary antibodies, incubated overnight at 4°C, were followed by the corresponding secondary antibodies conjugated with AlexaFluor 488 or AlexaFluor 594 (1:1500, Abcam, Cambridge, UK, cat. No. ab150073 and ab150116) at room temperature for 1 hour. Double-label immunofluorescence was performed using the same antibodies. A negative control (without primary antibody) was included. The semi-quantitative analyses were performed using Olympus BX 43 microscope equipped with software Cell Sense Standard (version 11.0.06). citH3 stained in green, MPO stained in red, Δ merged citH3 and MPO stained in yellow (**A**). A negative control stained without citH3 primary antibody (**B**). Cell nuclei are stained in blue (DAPI, Sigma Aldrich, Co, St. Louis, MO, US). Images were performed for the purposes of the study described previously [72]. #Autofluorescence of collagen fibers; *Aortic site of the leaflet. Original magnification 40×

(Figure 4). Importantly, the valvular expression of NETs markers correlated with AS severity. The authors concluded that the small amount of valvular NETs may contribute to AS progression [72]. Further studies are needed to establish whether valvular NETosis results from neutrophil interaction with oxLDL or with platelets/macrophages.

Therapeutic interventions to suppress NETosis

Controlling NETosis could present a promising approach for the treatment of NETs-associated cardiovascular manifestations especially in cases resistant to available strategies. The use of agents that can inhibit NETosis or degrade NETs to reduce inflammation and prevent thrombosis has

been assessed in cardiovascular patients or animal models of CVD. However until now there have been no reliable studies able to confirm that any of the therapies tested can substantially suppress NETosis in such patients leading to clinical benefits, though a few candidates deserve further investigation.

Recombinant human DNase-I (rhDNase-I)

Elevated double stranded-DNA levels within the 2nd to 4th quartiles were independently linked to a two-fold increase in the risk of experiencing the composite outcome of unstable angina, non-hemorrhagic stroke, acute MI, or all-cause mortality. This relationship was observed independently of the specific treatment received and markers associated with hypercoagulability [73]. DNase-I, an enzyme that selectively cleaves extracellular DNA, serves multiple roles, such as reducing neutrophil infiltration, regulating biofilm formation, assisting in pathogen invasion, degrading DNA matrices, and modulating immune functions via effective break down of DNA-nucleoprotein complexes and immune complexes [74]. Studies have demonstrated that rhDNase-I provides therapeutic benefits in conditions like lupus nephritis or systemic lupus erythematosus. Despite numerous studies evaluating the effect of rhDNase on NETs, which showed reduced NETosis and inflammatory response [75–77], to our knowledge, there have been no studies investigating DNases in CVD patients. This approach may be controversial due to studies showing that neutrophil components can limit inflammatory response, restrict the area of myocardial injury or initiate repair in a process of conversion macrophages to a reparative phenotype [78]. Moreover, a mouse model showed that neutrophil depletion impairs myocardial function and HF development [78]. However, it was demonstrated in HF patients that statins [79] and metformin [80] can effectively decrease NETosis by exerting anti-inflammatory effects and additionally by their ability to reduce plasma MPO levels. Metformin also reduced the levels of NE, proteinase-3, histones, and cfDNA, while a similar effect was not observed for glucose control with insulin [80].

Acetylsalicylic acid (Aspirin)

This antiplatelet agent can also affect NETs formation. Platelets take part in activating NETosis, therefore the inhibition of this action, using antiplatelet therapy, has the potential to inhibit NET formation [81]. Lapponi et al. [82] demonstrated that aspirin prevented NETs formation by inhibiting a transcriptional pathway controlled by nuclear factor kappa B (NFκB). Interestingly, dexamethasone had no effect on NETosis in this model [83]. On the other hand, dexamethasone, an inhibitor of TLR-2 and 4, reduced NETosis in COVID patients [83].

Antithrombotic drugs, such as heparin have been shown to suppress histone-induced diseases, e.g., sepsis [84]. Unfractionated heparin, low-molecular-weight heparin (parnaparin) and non-anticoagulant heparin protected from organ damage and death, neutralizing a toxic effect of circulating histones [85, 86]. Heparin, especially the non-anticoagulant type, represents a novel and promising approach to treating patients with high levels of circulating histones without increasing the bleeding risk [84].

Activated protein C (APC), a serine protease displaying anticoagulant, cytoprotective, and anti-inflammatory activities, has been demonstrated to cleave extracellular histones. Moreover, pretreatment of neutrophils with APC prevented activated platelets from adhering to neutrophils and from NETosis [87], suggesting that APC could serve as NETosis inhibitor. Recombinant human thrombomodulin (rhTM) can also inhibit NETosis. Helms et al. [88] showed in a rat model of septic shock that rhTM reduced NETosis and protected inner organs from dysfunction.

Chlor-amidine (Cl-amidine), a pharmacological inhibitor of PAD4, administered daily for 11 weeks, reduced thrombosis and the size of atherosclerotic lesions in a mouse model of atherosclerosis [89]. In a murine model of systemic lupus erythematosus, Cl-amidine protected against NET-induced kidney injury, endothelial dysfunction, and vascular damage [90]. Another therapeutic option to suppress PAD4 activity is hydroxychloroquine [91], used to treat malaria. Hydroxychloroquine has been shown to inhibit NETs formation in a mouse model of hepatic ischemia/reperfusion injury [91]. PAD4 inhibitors as a therapeutic option are an area of ongoing research with unclear safety and efficacy in humans.

Anti-HMGB1 antibodies also diminished NET formation in the bronchoalveolar lavage fluid of lipopolysaccharide-treated mice, measured as decreased levels of TNF-α, cell-free DNA and citH3 [92]. This observation allows to consider anti-HMGB1 antibodies as a potential therapy against excessive NETosis, though their value in CVDs is unknown.

C1 esterase inhibitor (C1INH), an endogenous inhibitor of C1 component of the complement system and a regulator of the contact activation pathway, has been reported to bind histones *in vitro* as well as C1INH-histone complexes were detected in the bronchoalveolar lavage fluid from acute respiratory distress syndrome patients [93]. The positive charge of histones could be used to deliver negatively charged inhibitors, which might not only bind to and neutralize histones but also be coupled with other potential therapeutic agents to augment their effectiveness at the site of inflammation [94].

N-acetylcysteine used primarily in patients with chronic obstructive pulmonary disease, was shown to exert antioxidant properties, consequently diminishing ROS-associated NETosis, however, its effect was not observed in the presence of H_2O_2 [95]. N-acetylcysteine reduced thrombus formation *in vivo* and decreased NETs formation in human neutrophils obtained from patients with hematologic malignances and healthy controls [96]. Also ROS scavengers, like octyl gallate [97] or methotrexate (NCT00470522) have been shown to reduce NETosis.

Antibiotics, such as chloramphenicol, azithromycin, and gentamicin, were shown to reduce NETs formation,

probably by decreased cytokine release and respiratory burst in a concentration-dependent manner [98].

Statins, potent cholesterol-lowering agents with pleiotropic effects used in primary and secondary CVD prevention [99], have shown anti-inflammatory effects in part by reducing NETosis, along with several antithrombotic and anti-inflammatory actions [79, 100].

SUMMARY

NETosis is a crucial part of the innate immune response and now is considered as the important mediator in atherothrombosis and atherosclerosis. The most compelling evidence supports the role of enhanced NETs formation in STEMI and ischemic stroke in particular in the context of resistance to thrombolysis. The role of NETosis in the progression of atherosclerotic plaques and AS appears to be less pronounced. Of importance is a potential predictive value of circulating NETs markers in AF despite anticoagulation. Further work is needed to develop more refined and well standardized diagnostic tools to detect excessive NETosis in cardiovascular patients. Modulation of NETosis attracts attention though the development of such targeted therapies may take some time, but it holds promise for improving the treatment and management of thromboembolic manifestations of CVDs in the future. It is tempting to speculate that combining NETosis-targeted treatment with existing therapies for CVD, such as aspirin, statins or antithrombotic drugs, could maximize the benefits. Large clinical trials to evaluate the safety and efficacy of NETosis-targeted treatments, alone or in combination, also in patients with CVDs are warranted in the next years.

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