Neutrophil Extracellular Traps in Atherosclerosis and Thrombosis

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Abstract

Despite effective therapeutic and preventive strategies, atherosclerosis and its complications still represent a substantial health burden. Leukocytes and inflammatory mechanisms are increasingly recognized as drivers of atherosclerosis. Neutrophil granulocytes within the circulation were recently shown to undergo neutrophil extracellular trap (NET) formation, linking innate immunity with acute complications of atherosclerosis. In this chapter, we summarize mechanisms of NET formation, evidence for their involvement in atherosclerosis and thrombosis, and potential therapeutic regimens specifically targeting NET components.

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Keywords

Atherosclerosis · Cl-amidine · Deoxyribonuclease · Heparin · Myocardial infarction · Neutrophil extracellular traps · Neutrophils · Thrombosis

Abbreviations

AAA	Abdominal aortic aneurysm
citH3	Citrullinated histone H3
DNase	Deoxyribonuclease
dsDNA	Double-stranded DNA
DVT	Deep vein thrombosis
ET	Extracellular traps
HIT	Heparin-induced thrombocytopenia
IL	Interleukin
MI	Myocardial infarction
MPO	Myeloperoxidase
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
PAD	Peripheral artery disease
PAD-4	Peptidylarginine deiminase 4
PE	Pulmonary embolism
PMA	Phorbol myristate acetate
ROS	Reactive oxygen species
tPA	Tissue plasminogen activator
VTE	Venous thromboembolism

1 Introduction

Atherosclerosis accounts for a substantial global disease burden. In recent decades, significant progress in understanding atherosclerosis was made. The identification of modifiable risk factors (Catapano et al. 2016), e.g. arterial hypertension or hypercholesterolemia, and the advent of percutaneous primary coronary intervention for treating acute complications of atherosclerosis like acute myocardial infarction (MI) (Beran et al. 2002) have led to a further reduction in incidence, cardiovascular morbidity, and markedly improved prognosis (Herrington et al. 2016). Still, due to the growing global population, the absolute number of deaths attributed to atherosclerotic disease is rising (Barquera et al. 2015) and increasing the need for novel therapies.

Besides traditional risk factors, inflammation and leukocytes are increasingly recognized as contributors to vascular disease and its complications. Pathomechanisms are manifold, with T cells (Bullenkamp et al. 2016), B cells

(Sage et al. 2019), dendritic cells (Gil-Pulido and Zernecke 2017), monocytes, and macrophages (Moroni et al. 2019) being implicated. Recently, neutrophils were suggested to significantly promote atherosclerosis (Doring et al. 2015), particularly its acute vascular syndromes (Mangold et al. 2015). Upon activation, neutrophils are able to undergo drastic morphological changes, leading to cellular disintegration and release of intracellular content into the extracellular space, in a process called neutrophil extracellular trap (NET) formation (Brinkmann et al. 2004). Besides atherosclerosis (Doring et al. 2017), NETs have emerged as important drivers of disease (Papayannopoulos 2018), including auto-immunity, sepsis, and cancer.

In this chapter, we aim to give a broad overview of the role of vascular NETs in atherosclerotic disease and its specific manifestations. Finally, we discuss potential therapeutic regimens targeting NETs and their components.

2 Neutrophils and Neutrophil Extracellular Traps

Under physiological conditions, neutrophil granulocytes comprise approximately 60% of total leukocytes in humans (Bainton et al. 1971). Neutrophils have a rather short lifespan and are released from the bone marrow into the circulation (Ley et al. 2018). Neutrophils are crucial fighters of the innate immune system, being the first cell population recruited to sites of inflammation and injury (Distelmaier et al. 2014). Neutrophils are critical for host defense (Wang and Arase 2014).

Their abundance and cellular properties enable them to effectively fight pathogens by phagocytosis, degranulation, or cytokine secretion (Witko-Sarsat et al. 2000). Neutrophils are equipped with numerous types of granules (Cowland and Borregaard 2016) containing catalytic enzymes, such as myeloperoxidase (MPO) and serine proteases. Upon phagocytosis of pathogens, neutrophils can fuse their granules with phagolysosomes, resulting in intracellular degradation of pathogens. Alternatively, neutrophils can release their granular content into the extracellular space (Cowland and Borregaard 2016).

Another effector mechanism of neutrophils was identified only 15 years ago, although first evidence had already emerged in 1996: It was reported that phorbol myristate acetate (PMA) could rapidly induce cell death of neutrophils. Thereby, neutrophils underwent substantial morphological changes different from apoptosis or necrosis, including signs of nuclear decondensation (Takei et al. 1996). Extending this finding, the group of Arturo Zychlinsky demonstrated that not only PMA, but also lipopolysaccharide (LPS) or interleukin (IL)-8 led to the appearance of extracellular structures described as fragile fibers of decondensed DNA covered in histones and granule proteins. Gram-positive as well as gram-negative bacteria could be ensnared by these NETs, and presence of neutrophil elastase (NE) or MPO promoted degradation of important bacterial virulence factors (Brinkmann et al. 2004).

Using live-cell imaging, NET formation was found to be an active process, different from apoptosis and necrosis. After stimulation, isolated neutrophils first flattened, forming intracellular vacuoles, followed by loss of the nuclear lobular

shape and further expanding in the cytoplasm. In this stage, neutrophils were still viable, containing the vital dye calcein blue but were not positive for Annexin V. This was reversed upon ultimate rupture of the plasma membrane. Furthermore, and in contrast to apoptosis, chromatin was decondensed. Intracellular membranes were fragmented, enabling mixing of nuclear, granular, and cytoplasmic components, which was not a feature of necrosis (Fuchs et al. 2007).

An important pre-requisite for NET formation is the presence of reactive oxygen species (ROS) (Fuchs et al. 2007; Hakkim et al. 2011). Inhibiting ROS production diminished NET formation, and neutrophils isolated from chronic granulomatous disease patients carrying mutations in the phagocyte NADPH oxidase did not produce NETs in response to PMA. However, when hydrogen peroxide was added to the system, the ability to form NETs was restored. These experiments indicated that NETosis is dependent on assembly and activation of the NADPH oxidase (Fuchs et al. 2007) and can be triggered by protein kinase C signaling via the raf-MEK-ERK pathway (Hakkim et al. 2011).

ROS serve as substrate for MPO, but presence of both is an essential stimulus for activation of NE (Metzler et al. 2014). Upon nuclear translocation, NE degrades core histones, facilitating decondensation of chromatin in synergy with histone citrullination by the calcium-dependent enzyme peptidyl arginine deiminase 4 (PAD-4) (Wang et al. 2004). Subsequent disintegration of intracellular membranes enables adsorption of granular proteases and antimicrobials onto chromatin. Rupture of the outer cell membrane finally leads to expulsion of cellular meshwork resulting in formation of NETs (Remijsen et al. 2011). Many of these released NET-associated proteins were shown to be degraded by neutrophil proteases *in vitro*, probably reducing their capacity to act as autoantigens *in vivo* (de Bont et al. 2020).

Since their discovery, pathways of NET formation are under thorough debate. Numerous triggers were reported; however, key events essential for NETosis could hardly be connected by signaling molecules to describe defined intracellular cascades. Even the absolute necessity of PAD-4 activity for NET formation is in question. Knockout or inhibition of PAD-4 was, on the one hand, reported to disrupt mouse and human NET formation (Lewis et al. 2015; Li et al. 2010; Martinod et al. 2013) while, on the other hand, other groups still observed NETs in response to the same stimuli independent of PAD-4 activity (Claushuis et al. 2018; Kenny et al. 2017). These conflicting data highlight the problems regarding different methods and also different interpretations of results. Nevertheless, citrullinated histone H3 (citH3) is still considered the most specific marker for NET formation. Differentiation between “vital” and “suicidal” NETosis even questions terminology itself by indicating that NET formation does not have to result in immediate cell death (Desai et al. 2016; Madhusoodanan 2017). Despite uncertainty regarding NET formation, it is increasingly recognized that presence of NETs fundamentally influences disease, including atherosclerosis and thrombosis.

Recently, the exclusivity of extracellular trap (ET) formation to neutrophils came into debate. Among granulocytes, mast cells (Campillo-Navarro et al. 2017) and eosinophils (Mukherjee et al. 2018) were reported capable of forming ETs. Another group even found monocytes to release ETs containing myeloperoxidase and citH3

(Granger et al. 2017). Furthermore, evidence implicated macrophages to equally expel their intracellular content in a process of ET formation (Doster et al. 2018). However, the significance of these non-neutrophil-associated ETs so far remains incompletely understood.

3 NETs in Venous Thrombosis

Deep vein thrombosis (DVT) and its major complications are prevalent in Europe and associated with high morbidity and mortality. Virchow's triad (Kumar et al. 2010) serves as an excellent framework for understanding risk factors of thrombosis, which are hypercoagulability, vascular dysfunction, and stasis. Recently, however, it was proposed to extend this triad to a tetrad, taking into account the paramount influence of the immune system and its dysregulation on thrombosis (Kapoor et al. 2018). In a rat model of inferior vena cava ligation, pro-inflammatory neutrophils were observed in emerging thrombi and vein walls (Wakefield et al. 1995). In humans, the pro-inflammatory markers interleukin (IL)-6 and C-reactive protein in plasma were increased in DVT and gradually declined after disease onset (Roumen-Klappe et al. 2002), while C-reactive protein predicted post-thrombotic syndrome (Roumen-Klappe et al. 2009).

The potential role of NETs in venous thrombosis was indicated by a flow chamber experiment, where NETs provided a fibrous scaffold for fibrin, von Willebrand factor (vWF) and platelets. NETs were then observed in thrombi of baboons subjected to experimental DVT (Fuchs et al. 2010). In a mouse model of DVT, large amounts of DNA were observed in thrombi, forming NET-like structures (von Bruhl et al. 2012). These observations were complemented by another group showing that fresh parts of thrombi were rich in the NET-specific marker citH3, which co-localized with vWF (Brill et al. 2012).

The first observation of NETs in human venous thrombosis was presented in a case report of a patient suffering from microscopic polyangiitis and DVT: both in kidney and thrombus samples, NETs were abundantly present (Nakazawa et al. 2012). Characterizing thrombi based on histological methods, DNA webs and citH3 were concentrated in organizing sections of thrombi, but not in already organized parts (Savchenko et al. 2014). Extending these findings, plasma DNA levels were found increased, diagnosing DVT with a sensitivity of 81%. Also, thrombus DNA positively correlated with D-dimer, vWF activity, the clinical Wells score, and neutrophil-derived MPO (Diaz et al. 2013). Similar results were obtained with concentrations of nucleosomes, which were elevated in DVT patients and positively correlated with neutrophil activation (van Montfoort et al. 2013).

Venous thromboembolism, mainly presenting as pulmonary embolism (PE), is a major complication of DVT (Di Nisio et al. 2016). Levels of nuclear DNA were shown to be elevated in PE (Arnalich et al. 2013) and independently predictive of mortality (Jimenez-Alcazar et al. 2018). In chronic thromboembolic pulmonary hypertension, a long-term sequela of PE (Lang 2004) characterized by the apposition of non-resolving, organized clots (Galie et al. 2016), neutrophils were shown to be

hyperresponsive (Rose et al. 2003) and present in superficial areas of thrombi (Quarck et al. 2015), while soluble NET surrogates were increased compared to healthy controls (Aldabbous et al. 2016).

4 NETs in Atherosclerosis and Arterial Thrombosis

4.1 Atherosclerosis

First evidence for the importance of neutrophils in human atherosclerosis arose indirectly, when increased MPO levels predicted risk of coronary artery disease independently of traditional risk factors (Zhang et al. 2001). High numbers of circulating neutrophils as important source of MPO were linked to both formation and severity of atherosclerotic lesions (Huang et al. 2001) and chronic stable angina pectoris (Avanzas et al. 2004). Direct assessment of human atherosclerotic lesions revealed presence of MPO (Daugherty et al. 1994) and neutrophils (Tavora et al. 2009) producing pro-inflammatory IL-8 (Marino et al. 2015). The extent of neutrophil infiltration was associated with a pro-inflammatory state and rupture-prone lesions (Ionita et al. 2010). Furthermore, levels of the NET surrogate markers dsDNA and chromatin were independently associated with the severity of coronary atherosclerosis and occurrence of adverse cardiovascular events (Borissoff et al. 2013).

Experimental models to identify mechanistic pathways of atherosclerosis typically rely on mice deficient for Apo E or low-density lipoprotein receptor, which are fed with a high-fat diet to develop atherosclerotic lesions. This led to the identification of a plethora of contributors underlying lesion formation and progression and confirmed a significant role for neutrophils in plaque development.

MPO-positive neutrophils were predominantly found in lesional caps of plaques (van Leeuwen et al. 2008) and plaque regions with already high inflammatory activity outnumbering present macrophages (Rotzius et al. 2010). Depletion of neutrophils was shown to attenuate lesion formation (Zernecke et al. 2008); however, protective effects were only apparent if depletion was performed within the first weeks, confining the effect of neutrophil activity to early stages of plaque development (Drechsler et al. 2010).

In *Apo E* knockout mice, neutrophils adhered to the luminal site of carotid atherosclerotic lesions and released DNA, indicative of NET formation, an interpretation which was supported by visualization of NETs in human endarterectomy samples (Megens et al. 2012). Presence of NETs was further verified in atherosclerotic lesions of mice in conjunction with an increase of the pro-inflammatory markers IL-1 α , IL-1 β , and IL-6 (Warnatsch et al. 2015). NETs, via histones, induced lytic cell death of smooth muscle cells in atherosclerotic lesions, leading to decreased plaque stability (Silvestre-Roig et al. 2019).

Recently, atherosclerotic lesions were classified into rupture-prone or erosion-prone phenotypes (Quillard et al. 2017). A growing body of evidence suggests that pathomechanisms are profoundly different in these two entities. Rupture-prone

lesions typically contain many macrophages, harbor large lipid pools but have low interstitial collagen and few smooth muscle cells covered by thin fibrous caps. Disruption of fibrous caps makes up about two thirds of coronary events (Prati et al. 2013; Virmani et al. 2000). Conversely, eroded plaques typically present with a thick or even intact fibrous cap with a discontinuous endothelial layer (Libby 2017). Neutrophil infiltration appears to be critical for erosion as shown by an optical coherence tomography study to distinguish between plaque rupture and erosion in acute coronary syndrome. Of 25 included patients, seven exhibited erosion, and levels of systemic MPO were strikingly increased compared to patients with plaque rupture (Ferrante et al. 2010). Characterization of human endarterectomy samples demonstrated that presence of NETs was positively correlated with endothelial cell apoptosis, a hallmark feature of eroded plaques. Yet, the extent of NET burden was not different in both lesion types, emphasizing the power of neutrophil effector function (Quillard et al. 2015).

4.2 Arterial Thrombosis

The role of NETs in arterial thrombosis was mostly studied in two major conditions, acute myocardial infarction and ischemic stroke.

4.2.1 Acute Myocardial Infarction

Apart from atherosclerosis itself, neutrophils are critically involved in myocardial infarction, an acute manifestation of stable disease. Naruko et al. discovered that neutrophils were abundantly present in both ruptured and eroded plaques of patients who have died from MI (Naruko et al. 2002). The emergence of catheter-based thrombus aspiration in MI (Beran et al. 2002) made a detailed examination of thrombi in a high number of patients possible, and enabled a new view on atherosclerosis outside of autopsy specimens. This provided crucial insights into pathomechanisms underlying coronary thrombosis. Analyses revealed that leukocytes are a major component of fresh thrombi (Rittersma et al. 2005). The majority of thrombus leukocytes were neutrophils which co-localized with large quantities of endothelin-1 (Adlbrecht et al. 2007), a potent vasoconstrictor and pro-inflammatory mediator associated with left ventricular dysfunction after MI (Taylor et al. 2004). Neutrophil accumulation at the culprit site was associated with a local increase in pro-thrombotic complement factors and infarct size (Distelmaier et al. 2009). This was corroborated by the observation that thrombus neutrophil count was associated with impaired coronary microcirculation and reduced left ventricular function at six-month follow-up (Arakawa et al. 2009).

Ultimately, the presence of NETs was demonstrated in culprit site thrombi (de Boer et al. 2013). NETs were decorated with pro-inflammatory interleukin-17, which drives neutrophil accumulation (Liao et al. 2012) and is suggested to be important in the pathogenesis of MI (Mora-Ruiz et al. 2019). NETs in culprit site thrombi were confirmed by another group, which demonstrated NET formation to be induced by high mobility group box 1, an important danger-associated molecular

pattern (Maugeri et al. 2014). In comparison with venous thrombi, NET burden was significantly higher in coronary thrombi and positively correlated with infarct size (Mangold et al. 2015). Recently, levels of dsDNA measured one day after MI were also associated with microvascular obstruction, myocardial salvage index, and left ventricular ejection fraction at four months (Helseth et al. 2019). Furthermore, neutrophils isolated from the culprit lesion site were more prone to undergo NETosis ex vivo in comparison with neutrophils harvested from a non-infarct related coronary artery (Stakos et al. 2015). The same group also found NETs to be decorated with tissue factor, an important mediator of coagulation. NETs were also shown to contribute to myocardial fibrosis by leading to increased activation and differentiation of fibrocytes at the culprit site (Hofbauer et al. 2019).

Circadian rhythms and neutrophil aging were recently proposed to modulate neutrophil migratory properties into tissues with substantial influence on vascular health and thrombo-inflammatory reactions in ischemia reperfusion (Adrover et al. 2019; Steffens et al. 2017).

4.2.2 Acute Ischemic Stroke

NETs were also shown to be associated with acute ischemic stroke. Concentrations of plasma DNA identified patients at risk of death at follow-up (Rainer et al. 2003), while nucleosomes were correlated with neurological dysfunction and infarction volume (Geiger et al. 2006). Another group demonstrated that DNA was increased after stroke (Tsai et al. 2011). Correspondingly, immunohistological analysis of thrombectomy samples revealed large numbers of neutrophils positive for citH3 (Laridan et al. 2017). NET burden in ischemic stroke thrombi retrieved via endovascular therapy was associated with the complexity of intervention, measured as duration of procedure and number of required wire passes (Ducroux et al. 2018). With respect to prognosis and outcome, soluble NET markers were associated with a higher NIHSS score, an indicator of stroke severity. Increased all-cause mortality was reported in patients with citH3 levels ranging in the upper quartile (Valles et al. 2017). Mechanistically, a mouse model of cerebral artery ischemia/reperfusion revealed significant exacerbation of brain injury after infusion of exogenous histones, highlighting the cytotoxic properties of NET components and their devastating influence on vascular tissues (De Meyer et al. 2012).

4.2.3 Other Conditions Associated with Arterial Thrombosis

In other diseases associated with arterial thrombosis, most evidence is available in *abdominal aortic aneurysm* (AAA). AAA, characterized by vessel dilation and formation of multilayered intraluminal thrombi (Delbosc et al. 2011), is increasingly being recognized as an inflammatory condition (Piechota-Polanczyk et al. 2015). Neutrophils appear to be crucial, as their depletion using a specific, cytotoxic antibody resulted in drastically reduced AAA formation (Eliason et al. 2005). NETs were shown to be abundantly present in the luminal part of human AAA thrombi and adventitia, and to be induced by periodontal pathogens (Delbosc et al. 2011) associated with AAA progression (Nakano et al. 2011). Finally, NETs co-localized with IL-1 β in AAA thrombi (Meher et al. 2018), a pro-inflammatory

mediator that drives AAA (Johnston et al. 2013) and NET (Keshari et al. 2012) formation.

In other pathologies associated with arterial thrombosis, evidence for the influence of NETs is scarce. *Peripheral artery disease* thrombi contain NETs to a similar extent as coronary and stroke thrombi (Farkas et al. 2019). Comparing plasma samples of DVT and PAD patients, neutrophil elastase alpha1 anti-trypsin complex, a specific marker for NETs, was increased in PAD (Kremers et al. 2019). Increased NET markers were also observed in *thrombotic microangiopathies* like thrombotic thrombocytopenic purpura or hemolytic uremic syndrome, with plasma levels being reflective of disease activity (Fuchs et al. 2012).

5 Neutrophil Extracellular Traps as a Therapeutic Target

Given their importance in the pathogenesis of atherosclerotic vascular disease, there is an interest in finding therapeutic compounds to inhibit NETs and block their detrimental effects. Several pathways have been suggested, targeting various NET components.

5.1 PAD-4 Inhibitors: Cl-Amidine

Since activation of PAD-4 is regarded critical for efficient uncoiling of chromatin in NETosis (Wang et al. 2009), inhibition of this key enzyme was suggested as a potential therapeutic regimen. Initially, synthetic PAD-4 inhibitors such as Cl-amidine were envisioned for treatment of rheumatoid arthritis (Kearney et al. 2005; Luo et al. 2006), a disease exacerbated by PAD-mediated excessive formation of citrullinated proteins that promote auto-immunity (Turunen et al. 2016). As Cl-amidine was characterized to irreversibly block PAD4, it critically interferes with NET formation (Wang et al. 2009). Cl-amidine was shown to attenuate disease severity in mouse models of sepsis (Biron et al. 2017), collagen-induced arthritis (Willis et al. 2011) and systemic lupus erythematosus, where it decreased NET formation and reduced deposition of inflammatory immunoglobulin and complement factors in the kidney (Knight et al. 2013). Daily subcutaneous treatment of ApoE knockout mice with Cl-amidine could alleviate atherosclerotic lesions under high-fat diet, while accumulation of neutrophils and macrophages into lesions was reduced (Knight et al. 2014). Thrombus formation induced by photochemical injury of the carotid artery could be significantly delayed by pre-treatment with Cl-amidine (Knight et al. 2013). It was demonstrated that Cl-amidine treatment reduces infarct size in a mouse model of coronary artery ligation and was associated with improved cardiac function (Novotny et al. 2018). In a model of ischemic stroke, administration of Cl-amidine prevented thrombotic occlusions (Pena-Martinez et al. 2019). These observations emphasize a certain dependency of NETosis on enzymatic PAD4 activity despite conflicting results and render pharmaceutical PAD-4 inhibition, a potentially promising target for treatment of human atherosclerotic and thrombotic disease.

5.2 Deoxyribonuclease

Deoxyribonuclease (DNase) degrades NETs by hydrolysis of the DNA backbone (Fuchs et al. 2010). Two isoforms of different cellular origins target DNA strands *in vivo*. DNase 1, secreted by the non-hematopoietic compartment, preferentially degrades protein-free DNA, while leukocyte-derived DNase 1 like 3 (DNase 1L3) cleaves DNA:protein complexes (Napirei et al. 2009). Adequate DNase activity was suggested to be crucial for a homeostatic balance between NET formation and degradation. Indeed, neutrophilic mice deficient in both plasmatic DNases developed severe disseminated thrombosis. However, reconstitution with or presence of just one functional DNase type was sufficient to protect mice from vascular occlusion (Jimenez-Alcazar et al. 2017). Targeting chromatin and NETs by DNase 1 was shown to be beneficial in experimental DVT (Brill et al. 2012; von Bruhl et al. 2012) and ischemic injury, including intestinal ischemia (Boettcher et al. 2017a), testicular torsion (Boettcher et al. 2017b), and ischemic stroke (Pena-Martinez et al. 2019).

In atherosclerotic mice, injection of DNase reduced lesion size and attenuated lesional NET burden as well as pro-inflammatory cytokines (Warnatsch et al. 2015). Furthermore, DNase 1 was used in rodent models of cardiac ischemia. Although one study reports reduction of neutrophil infiltration by DNase 1 alone in a model of ischemia/reperfusion, the authors could only show improved cardiac function by co-administration of tissue plasminogen activator (tPA) (Ge et al. 2015). In contrast, DNase 1 treatment improved cardiac function in mice after coronary artery ligation, without any reduction in neutrophil infiltration to the ischemic myocardium (Vogel et al. 2015). These apparently contradictory results may be due to variations in methodology of ligation, duration of ischemia, and timing of DNase application. *In vitro*, DNase 1 was shown to accelerate tPA-mediated thrombolysis of human coronary (Mangold et al. 2015) and cerebral (Laridan et al. 2017) thrombi in comparison with tPA alone. Importantly, in MI patients, low DNase activity was associated with increased infarct size (Mangold et al. 2015).

Considering the mounting evidence on the benefits of DNase application in different well-established disease models, these data raise the possibility of DNase 1 treatment of patients in neutrophil-driven disease settings in which NET formation plays a pathogenic role.

5.3 Heparin

Due to their anticoagulant properties, unfractionated heparin and low molecular weight heparins are long recognized therapeutic cornerstones for deep vein thrombosis (Mazzolai et al. 2018) and MI (Neumann et al. 2019). Given the inflammatory component of thrombotic diseases, it is intriguing that heparins have a variety of anti-inflammatory effects (Mulloy et al. 2016; Rao et al. 2010) which seem to be unrelated to their anticoagulant activity (Rao et al. 2010). In a model of acute inflammation, diminished accumulation of neutrophils was at least in part ascribed to the ability of heparin oligosaccharides to block L- and P-selectin (Nelson et al.

1993). Likewise, CD11b-dependent adhesion could be attenuated by interaction with heparin (Salas et al. 2000; Wang et al. 2002). Furthermore, heparins can not only limit NET formation itself as shown by treatment in vitro and in vivo (Manfredi et al. 2017), but can also target existing NETs in various ways: heparin was shown to inhibit enzymatic activity of neutrophil elastase and Cathepsin G (Fryer et al. 1997), two major enzymes present in NETs (Folco et al. 2018). Binding and displacement of histones by heparin promotes NETs disassembly, limiting their pro-thrombotic properties in vitro (Fuchs et al. 2010) and in vivo (von Bruhl et al. 2012). At the same time, excessive release of histones during cell death and NETosis was suggested to counteract the anti-thrombotic function of heparin, potentially explaining non-responsiveness to heparin (Longstaff et al. 2016).

These observations stimulated the development of heparinoids that lack anticoagulant properties while keeping their anti-inflammatory effects (Rao et al. 2010). Indeed, sevuparin, a low-anticoagulant heparin analog, inhibited NE and histone H4, proteins associated with NETs (Rasmuson et al. 2019). In another study, non-anticoagulant heparin prevented histone-mediated cytotoxicity and improved survival in a murine sepsis model (Wildhagen et al. 2014). Thus, it might be a crucial therapeutic add-on to reduce NET burden without increasing risk of bleeding complications.

6 Summary

A summary of the content of this chapter is provided in the central Fig. 1. Taken together, NETs are important for the initiation and progression of thrombosis in the setting of atherosclerosis, including VTE, MI, and stroke. For these reasons, NETs

Fig. 1 The central figure summarizes mechanisms of NET formation, its pathways and consequences in atherosclerosis and thrombosis. The figure was constructed using Biorender and Microsoft PowerPoint

have emerged as potential targets for treatment. Despite promising in vitro and experimental in vivo data, adequately powered clinical trials are required to assess clinical benefits and safety of anti-NETotic regimens.

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