

# Neutrophil Extracellular Traps in Atherosclerosis and Atherothrombosis

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**Abstract:** Neutrophil extracellular traps expelled from suicidal neutrophils comprise a complex structure of nuclear chromatin and proteins of nuclear, granular, and cytosolic origin. These net-like structures have also been detected in atherosclerotic lesions and arterial thrombi in humans and mice. Functionally, neutrophil extracellular traps have been shown to induce activation of endothelial cells, antigen-presenting cells, and platelets, resulting in a proinflammatory immune response. Overall, this suggests that they are not only present in plaques and thrombi but also they may play a causative role in triggering atherosclerotic plaque formation and arterial thrombosis. This review will focus on current findings of the involvement of neutrophil extracellular traps in atherogenesis and atherothrombosis. (*Circ Res.* 2017;120:736-743. DOI: 10.1161/CIRCRESAHA.116.309692.)

**Key Words:** atherosclerosis ■ coronary artery disease ■ extracellular trap ■ neutrophils ■ thrombosis

Phagocytes and especially neutrophils belong to the first line of defense against invading pathogens and use a broad spectrum of weaponry to eliminate microorganisms and further activate adaptive immunity.<sup>1,2</sup> Besides the discharge of granule proteins (eg, antimicrobial peptides), proteases, and reactive oxygen metabolites, neutrophils have also been recognized to respond to infectious challenges via the formation of neutrophil extracellular traps (NETs). NETs are defined as large web-like structures composed of decondensed chromatin and neutrophil-derived nuclear, cytoplasmic, and granular proteins, which are capable of ensnaring and killing pathogens. In this context, NET structures may function as a platform providing high local concentrations of effector molecules eliminating the intruder.<sup>3-5</sup> The process of NET formation is termed NETosis and was introduced to discriminate this route of dying from other forms of cell death, eg, necrosis and apoptosis.<sup>3</sup> Notably, other cell types such as macrophages<sup>6</sup> or mast cells<sup>7</sup> can also release decondensed chromatin, which is referred to as ETosis. Hence, NETs (and ETs) are believed to represent an important defense tool of innate immunity fighting pathogens<sup>8</sup> but have also been implicated in the pathophysiology of cancer,<sup>9</sup> autoimmunity,<sup>10</sup> and chronic inflammation.<sup>11</sup> The latter also encompasses a variety of cardiovascular diseases and syndromes including coronary artery disease and stroke as the most frequent entities. The underlying pathophysiology termed atherosclerosis refers to a lipid-driven inflammatory disease of arteries, which develops at predilection sites with disturbed flow, where endothelial activation facilitates intimal retention of lipoproteins. Modified lipoproteins, eg, oxidized low-density lipoprotein, augment

endothelial damage and trigger recruitment of leukocytes, which eventually fail to clear lipoproteins, undergo cell death, and maintain inflammation. Ultimately, growing lesions will lead to vessel occlusion and subsequent ischemia or (arterial) thrombosis.<sup>12,13</sup> Neutrophils and their specific contribution to the pathophysiology of atherosclerosis have long been denied; however, during the last years, we<sup>14-18</sup> and others<sup>19-26</sup> have provided substantial evidence underlining presence and actions of neutrophils in early and established human and murine atherosclerotic lesions. In a nutshell, neutrophils aggravate endothelial dysfunction, attract leukocytes, in particular monocytes, to atherosclerotic lesions and promote foam cell formation. Advanced plaques are exposed to neutrophil-derived proteases and reactive oxygen species leading to plaque destabilization.<sup>17,27,28</sup> NET formation may occur at all stages of disease progression and particularly high local concentrations of effector molecules are suspected to be proinflammatory<sup>29</sup> and therefore also atherogenic.

This review will focus on current findings of the involvement of NETs in atherosclerosis, arterial thrombosis (atherothrombosis), and myocardial infarction.

## Mechanisms and Initiators of NET Formation

Among the 2 types of NETosis (lytic and vital) defined to date, lytic or suicidal NETosis is best known as a slow (hours) active form of cell death distinct from, for example, necrosis or apoptosis.<sup>8</sup> Most of the mechanistic insight on lytic NETosis stems from studies investigating NETosis by treating isolated neutrophils with phorbol 12-myristate 13-acetate in vitro, which activates the neutrophil respiratory burst leading to the assembly

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**Nonstandard Abbreviations and Acronyms**

<b>IL</b>	interleukin
<b>NADPH</b>	nicotinamide adenine dinucleotide phosphate (reduced form)
<b>NETs</b>	neutrophil extracellular traps
<b>NE</b>	neutrophil elastase
<b>PAD4</b>	peptidyl arginine deiminase 4
<b>PR3</b>	proteinase 3
<b>r-tPA</b>	recombinant tissue-type plasminogen activator

of multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and subsequent reactive oxygen species formation.<sup>30</sup> Reactive oxygen species can liberate nuclear, granular, and cytoplasmic contents, including neutrophil elastase (NE) and myeloperoxidase, which degrade linker histones and enhance chromatin decondensation.<sup>31</sup> Furthermore, peptidyl arginine deiminase 4 (PAD4) mediates histone deimination by citrullinating histones, which then mix with granule proteins and are expelled from the neutrophil.<sup>32</sup> However, phorbol 12-myristate 13-acetate is an artificial stimulus, and although in vivo NET formation in mice seems to indeed depend on NE and PAD4 activity, it may not rely on reactive oxygen species generated by NADPH oxidase.<sup>33</sup>

More recently the term vital NETosis has been introduced, describing a fast (minutes) process in vivo (humans and mice) where cell viability and functions are retained. Discharge of nuclear material is enabled by bleb formation and vesicular exportation. Vital NETosis can be specifically induced by microbial-specific molecular patterns (eg, lipopolysaccharides), which bind to host pattern recognition receptors and does not necessarily involve NADPH oxidase. Remaining anuclear neutrophils show integrity of the plasma membrane and still contain granules.<sup>34–37</sup> Hence, the question remains, whether vital NETosis might even be the more physiologically relevant of the 2 processes because conclusions on lytic NETosis are mainly based on phorbol 12-myristate 13-acetate-stimulated in vitro assays. On the contrary, recent study suggests that NETosis in general may have been confused or misleadingly recognized as leukotoxic hypercitrullination and defective mitophagy leaving us with the notion that real NETosis is dependent on NADPH oxidase but not associated with hypercitrullination.<sup>38</sup>

Another phenomenon solely observed in vitro seems to be the formation of (N)ETs composed of mitochondrial DNA,<sup>39,40</sup> whereas in vivo mitochondrial DNA released by neutrophils, for example, after major surgery, rather triggers NET formation.<sup>37,41,42</sup> Besides the well-documented NET-inducers (phorbol 12-myristate 13-acetate, lipopolysaccharide, and mitochondrial DNA), many other stimuli have been reported to initiate NET formation in vitro and in vivo (human and mouse), including pathogens such as Gram-positive and Gram-negative bacteria,<sup>3,8,34,43</sup> fungi,<sup>44</sup> parasites,<sup>45</sup> and viruses.<sup>46</sup> Moreover, NET formation may also be propagated by intrinsic mediators, such as hydrogen peroxide,<sup>8</sup> cytokines,<sup>47,48</sup> chemokines,<sup>3,49</sup> cholesterol,<sup>50</sup> monosodium urate crystals,<sup>51</sup> autoantibodies,<sup>52</sup> and antibody-antigen complexes.<sup>53</sup> Initiators of NET formation that have already been described in the context of atherosclerosis/atherothrombosis comprise antibody-antigen complexes,<sup>54</sup> cholesterol crystals,<sup>50</sup> or activated platelets.<sup>55</sup>

**NET Content**

Decondensed nuclear chromatin consisting of nucleic acids (DNA and RNA) and proteins (predominantly positively charged histones) constitute the backbone of a NET. Histones (accounting for  $\approx 70\%$  of the NET protein content) facilitate the adhesion of (negatively charged) microbial and viral pathogens and both; histones<sup>56</sup> and nucleic acids<sup>57</sup> can per se exert bactericidal activity. Other NET-associated antimicrobial peptides comprise granule, cytoplasmic and cytoskeletal proteins, and metabolic enzymes of neutrophil origin.<sup>31,58,59</sup> In addition, it is conceivable that NETs contain effector molecules or constituents released by neighboring cells, as evidenced by a study showing trapping and degradation of proinflammatory mediators by aggregated NET structures in vitro and in vivo (mouse).<sup>51</sup> Thus, not only the stimuli inducing NET formation are manifold but also the NET proteome may differ depending on the localization (tissue versus circulation) and disease (acute versus chronic).<sup>29,43,60,61</sup> Nevertheless  $\approx 20$  (mostly neutrophil-derived) proteins have been proposed as a core NET proteome including histones, myeloperoxidase, NE, proteinase 3, cathepsin G, and  $\alpha$ -defensins.<sup>61–63</sup> Yet, disease-specific NETome patterns await to be properly defined.

**NETs in Innate Immune Defense**

Initially, NETs were discovered and described as an important tool to fight invading pathogens, in particular bacteria.<sup>3</sup> On the contrary, NET-associated microbes have also been found to remain alive and pathogenic.<sup>64,65</sup> Nevertheless, restoration of NET formation in a patient with chronic granulomatous disease (strong decrease in NADPH oxidase function) and *Aspergillus* infection significantly improved the immune response against the fungi.<sup>66</sup> Accordingly, PAD4-deficient mice, which are incapable of generating NETs,<sup>32</sup> are more susceptible to bacterial infection, as shown in a mouse model of infectious necrotizing fasciitis.<sup>67</sup> However, another study using PAD4 knockout mice did not reveal differences in morbidity or mortality compared with wild-type mice in a model of peritonitis (cecal ligation puncture).<sup>68</sup> Similarly, patients having Papillon-Lefèvre Syndrome are unable to generate NETs—except in saliva—because of the lack of all neutrophil serine proteases but do not exhibit severe immunodeficiencies. Instead, they have pronounced juvenile periodontitis caused by fungi and bacteria although saliva (containing NETs) is supposed to prevent periodontal disease.<sup>69–71</sup> In conclusion, the overall importance of NET-mediated killing of microbes may differ depending on the type of pathogen and the specific location versus a more systemic affliction; it is hence conceivable that NETs deficiency may be compensated by other antimicrobial mechanisms during inflammatory immune responses.

**NETs in Atherosclerosis**

Atherosclerosis, widely recognized as a lipid-driven inflammatory disease of the arterial vessel wall, results in intimal lesion growth. Progressing lesions may eventually rupture, thereby inducing intraluminal thrombosis leading to acute cardiac events and ischemic stroke.<sup>72</sup> Of note, extracellular DNA (eg, NET derived) exerts cytotoxic and prothrombotic effects, possibly providing a causative link between inflammation and

coagulation. In line with this, NETs were identified in luminal location in murine and human atherosclerotic lesions<sup>50,73,74</sup> and implicated in arterial thrombosis (see subsequent section).

In *apolipoprotein E*-deficient knock-out (*Apoe*<sup>-/-</sup>) mice, we could elucidate a mechanism of NET-driven atherogenesis, involving the autoimmune activation of plasmacytoid dendritic cells. Mechanistically, complexes of self-DNA (presumably NET-borne DNA but also self-DNA from dying cells) and neutrophil-derived granule proteins (eg, cathelicidin) stimulate plasmacytoid dendritic cells in the vessel wall, resulting in a strong type I interferon response, which drives atherogenesis.<sup>54</sup> Depletion of plasmacytoid dendritic cells instead resulted in reduced plaque burden and type I interferon response.<sup>54,75</sup> Moreover, NETs have been suggested to directly induce endothelial dysfunction (as a starting point for atherosclerosis) by activation and damage of endothelial cells.<sup>76–78</sup> Findings by Knight et al<sup>79</sup> further underscore the importance of NETs in atherosclerotic lesion development by showing that inhibition of PAD4 (described to be important in NET formation<sup>32</sup>) by chloramidine treatment prevents NET formation, thereby decreasing atherosclerotic lesion size and delaying carotid artery thrombosis in a mouse model of atherosclerosis. Comparable effects could not be reproduced in mice treated with a neutrophil-depleting antibody or in mice lacking a functional type I interferon receptor. In summary, these data suggest an important role of NETs in the instigation of a type I interferon response driving atherogenesis.<sup>79</sup> On the contrary, the importance of a NET-driven type I interferon response in atherogenesis is called into doubt by Warnatsch et al,<sup>50</sup> instead showing that cholesterol crystals, as a sterile danger signal, induce lesional NET formation in *Apoe*<sup>-/-</sup> mice after a high-fat diet for only 8 weeks. These NETs are thought to subsequently prime macrophages for production of interleukin (IL)-1 $\beta$ , resulting in activation of a Th17 response, which further amplifies immune cell recruitment into atherosclerotic lesions. To render *Apoe*<sup>-/-</sup> mice devoid of NETs, the authors used different approaches; however, in their hands, only inhibition of NADPH oxidase with diphenylene iodonium or blocking of the neutrophil-specific proteases NE and proteinase 3 (PR3)<sup>31</sup> abrogated NET formation, whereas chloramidine treatment was ineffective. As a consequence, they used *Apoe*<sup>-/-</sup> mice lacking NE and PR3 (*Apoe*<sup>-/-</sup>*Elane*<sup>-/-</sup>*Prtn3*<sup>-/-</sup>) to study atherosclerosis in the absence of NETs, revealing reduced lesions size in *Apoe*<sup>-/-</sup>*Elane*<sup>-/-</sup>*Prtn3*<sup>-/-</sup> animals after 8 but not after 4 weeks of high-fat diet feeding. Likewise, they found diminished plaque growth in *Apoe*<sup>-/-</sup> mice treated with deoxyribonuclease to degrade NETs. Excluding the possibility that NE/PR3 deficiency causes intrinsic defects in neutrophil chemotaxis or extravasation, which would add to reduced lesions sizes in *Apoe*<sup>-/-</sup>*Elane*<sup>-/-</sup>*Prtn3*<sup>-/-</sup> animals, the authors conclude that NET-mediated priming of macrophages induces a strong IL-1 $\beta$ /Th17 response driving atherogenesis.<sup>50</sup> In our hands,<sup>80</sup> however, *Apoe*<sup>-/-</sup>*Elane*<sup>-/-</sup>*Prtn3*<sup>-/-</sup> animals showed reduced atherosclerotic lesion size only at early but not at advanced stages of lesion development and lack of NE alone did not affect plaque growth. In addition, we could not observe any effect on lesion size after NET degradation through repetitive deoxyribonuclease injection. Hence, our findings do not support the

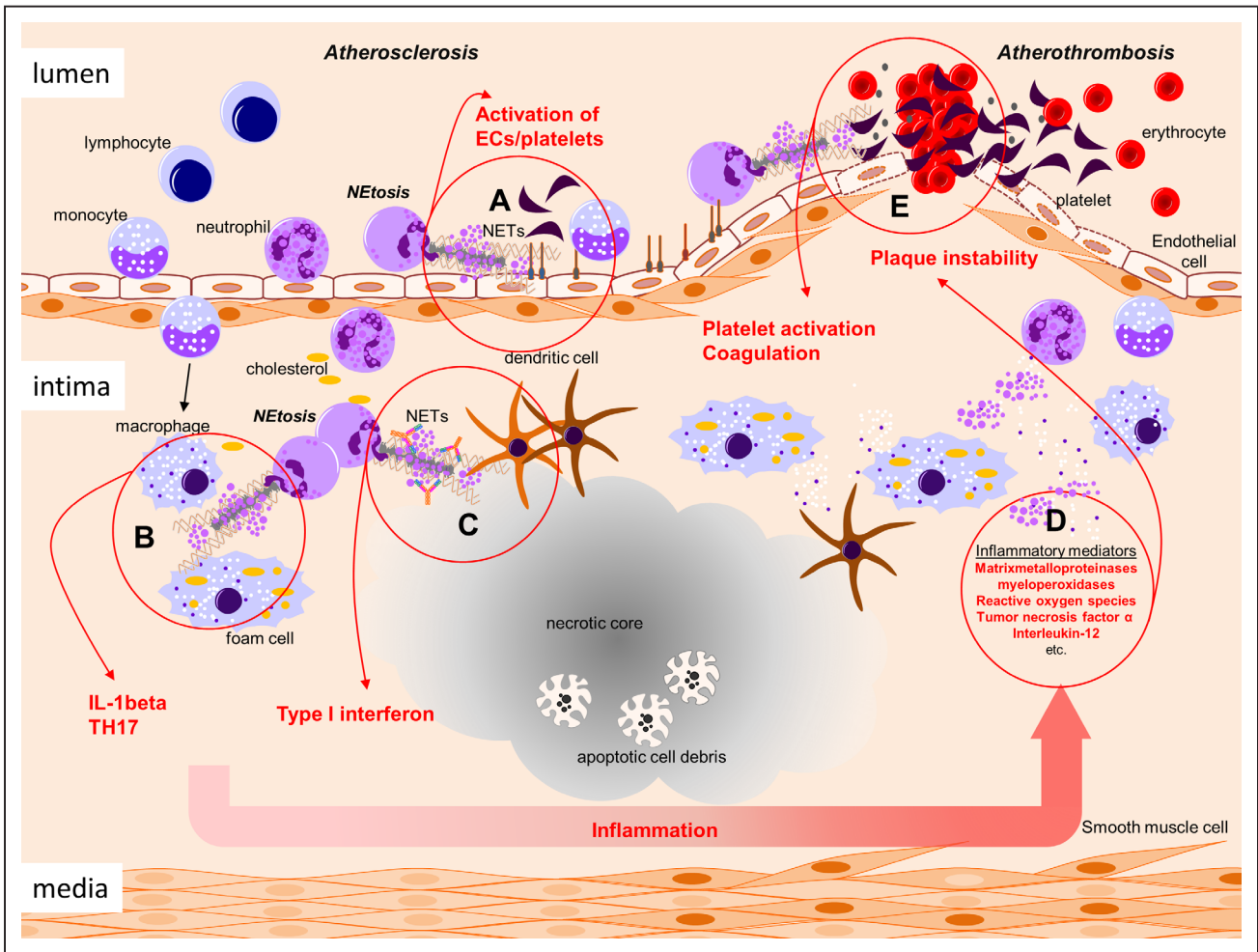
conclusion that the absence of elastase-provoked NET release or deoxyribonuclease treatment alters atherosclerotic lesion development. In summary, these results point toward PR3 rather than toward NE triggering early atherosclerosis, giving rise to the notion that PR3-mediated cytokine maturation (in particular cleavage of pro-IL-1 $\beta$ <sup>81</sup>) seems to be more important for lesion formation than NET-mediated macrophage priming.<sup>80</sup>

At a diagnostic or prognostic level, NETs (or their components) have been suggested to serve as biomarkers predicting the severity of atherosclerosis and the risk of future cardiovascular events.<sup>82</sup> In this study, 282 human individuals with suspected coronary artery disease were examined about the prevalence of double-stranded DNA, nucleosomes, citrullinated histone H4, and myeloperoxidase–DNA complexes in coronary atherosclerosis. Furthermore, plasma markers of coagulation activation and inflammation were determined. The results demonstrated that plasma levels of double-stranded DNA, nucleosomes, and myeloperoxidase–DNA complexes were positively associated with thrombin generation and significantly elevated in patients with severe coronary atherosclerosis or extremely calcified coronary arteries. In addition, high plasma nucleosome levels were found to be an independent risk factor of severe coronary stenosis, and the load of myeloperoxidase–DNA complexes correlated with the number of atherosclerotic coronary vessels and the occurrence of major adverse cardiac events.<sup>82</sup> A synopsis of potential NET-mediated mechanisms driving atherosclerosis has been visualized in a Figure. However, NET localization in atherosclerosis (mouse and human) was mostly luminal or—in a lesional context—somewhat associative by identifying single NET relevant structures/proteins rather than a real NET-releasing neutrophil. Clearly, further in-depth studies will be required to detail the role of lesional NETs and their interactions with potential immune cells (plasmacytoid dendritic cells and macrophages) triggering proinflammatory immune responses such as type I interferon and Th17 responses, which can fuel atherosclerosis.

### NETs in Atherothrombosis and Myocardial Infarction

Studies specifically investigating the role of NETs in the context of atherothrombosis are scarce, whereas more detailed knowledge is available about their involvement in venous thrombosis.<sup>83–85</sup> However, circulating leukocytes, in particular monocytes, play a crucial role in atherothrombosis,<sup>86,87</sup> and systemic neutrophil counts are robust predictors of acute coronary events<sup>88</sup> and impact outcomes<sup>89</sup> in humans. Furthermore, neutrophils are present in coronary thrombi<sup>90,91</sup> and have been detected in surgical thrombectomies and abdominal aortic aneurysms.<sup>92</sup> Following on these findings, recent research specifically addressed whether and how NETs (and NETosis) may affect arterial thrombus formation and its complications.

Riegger et al<sup>91</sup> analyzed 253 samples from patients with stent thrombosis after percutaneous coronary intervention. Around 23% of the specimens investigated contained NETs, but no differences in the number of NETs could be detected within these thrombi with respect to the timing of stent



**Figure. Emerging roles of neutrophil extracellular traps (NETs) in atherosclerosis and atherothrombosis.** (A) Luminally netting neutrophils activate leukocytes, platelets, and endothelial cells creating a proinflammatory milieu presumably resulting in endothelial dysfunction, the initial trigger of lesion development. (B/C) Lesional NETs may initiate an interleukin-1 $\beta$ /TH17 (T helper 17) and type I interferon response, which leads to further activation of lesional leukocytes, releasing more proinflammatory mediators. (D/E) Furthermore, it may be assumed that NET-driven proinflammatory responses will cause an inflammatory environment that favors plaque destabilization and rupture. During atherothrombosis, NETs may trigger activation of the coagulation cascade and increase thrombus stability thus orchestrating arterial occlusion.

thrombosis (early versus late), the stent type, or in comparison to samples from patients with spontaneous myocardial infarction. Nevertheless, analysis of thrombi from 111 patients with ST-segment elevation as a sign of acute coronary syndrome and subjected to the primary percutaneous coronary intervention revealed highly activated neutrophils at culprit lesional sites compared with femoral neutrophils. Moreover, the NET burden in these thrombi correlated positively with infarct size and negatively with ST-segment resolution, whereas deoxyribonuclease activity in these lesions showed a negative correlation with infarct size but positive with ST-segment resolution. Notably, *ex vivo* addition of deoxyribonuclease to these thrombi accelerated their lysis. In conclusion, the authors postulate that the balance of NETing neutrophils at rupture-prone lesion sites and endogenous deoxyribonuclease activity is predictors of ST-segment resolution and myocardial infarct size.<sup>93</sup>

Along these lines, Maugeri et al<sup>94</sup> performed histology on 26 thrombectomies from patients after acute myocardial infarction, revealing that activated platelets present

high-mobility group box 1 protein to neutrophils thereby inducing NET formation. The authors speculate that these NETs may have contributed to plaque rupture with subsequent thrombus formation. In accordance with these findings, platelet-derived high-mobility group box 1 protein has also been shown to facilitate NET formation and coagulation in a mouse model of venous thrombosis.<sup>95</sup> Another study examined 45 coronary thrombectomy specimens (15 fresh, 15 lytic, and 15 organized thrombi) obtained from patients after acute myocardial infarction and detected NETs in fresh and lytic, but not in organized thrombi.<sup>96</sup> Notably, these NETs were found to be coated with IL-17A and IL-17F, which has been described to promote thrombosis by enhancing platelet aggregation.<sup>96</sup>

As the main initiator of coagulation critically involved in arterial thrombosis,<sup>97</sup> tissue factor has also been investigated in patients with acute ST-segment-elevation myocardial infarction. Samples of thrombotic material and surrounding blood from the infarct-related coronary artery and the noninfarcted area of 18 patients were collected during the primary

percutaneous revascularization. Analysis of these specimens revealed local accumulation of tissue factor–bearing NETs at sites of coronary thrombosis and blood neutrophils releasing NETs and exposing tissue factor in the infarct-related area, but not in the noninfarcted area of these patients. In addition, neutrophil islets and NETs decorated with tissue factor were detected in thrombi obtained from the infarcted region.<sup>98</sup> As a conclusion, the authors state that the interactions of activated platelets with neutrophils at sites of plaque rupture during acute myocardial infarction result in local NET formation and delivery of active tissue factor altogether fostering thrombus formation.<sup>98</sup> Interestingly, another study investigating carotid culprit plaque samples from 157 patients showed the presence of periodontal bacteria especially in hemorrhagic atherosclerotic carotid plaques, correlating with an increased prevalence of activated neutrophils, as evidenced for example by measuring myeloperoxidase–DNA complexes, which would be indicative of NET release.<sup>99</sup> Taken together, these data suggest a potential role of periodontal microorganisms, inducing NET formation and subsequent plaque rupture.<sup>99</sup>

In mice, neutrophil-derived externalized nucleosomes, which may turn into NETs, have been studied in an arterial vessel injury model induced by ferric chloride application. Infusion of the anti–H2A–H2B–DNA antibody (neutralizing histones as major components of these nucleosomes) into ferric chloride–treated wild-type mice leads to prolonged time to occlusion and lower thrombus stability in carotid arteries. Notably, no effect of antibody infusion is observed in the NE/cathepsin G–deficient mice after induction of vessel injury. Mechanistically, externalized nucleosomes can enable the coassembly of NE and its substrate tissue factor pathway inhibitor on the surface of activated neutrophils triggering thrombosis. Hence, in sterile inflammation, neutrophil-derived serine proteases and nucleosomes may contribute to large-vessel thrombosis, leading to myocardial infarction and stroke.<sup>55</sup>

The role of NETs has also been examined in an alternative model of myocardial ischemia-reperfusion and myocardial no-reflow. Male Wistar rats were treated with deoxyribonuclease, recombinant tissue-type plasminogen activator (r-tPA), a combination of deoxyribonuclease and r-tPA or left untreated for 45 minutes after induction of myocardial ischemia. Comparing control rats with those treated with a combination of deoxyribonuclease and r-tPA revealed reduced NET density and no-flow area in the ischemic region, as well as reduced infarct size in deoxyribonuclease/r-tPA–treated animals. In addition, deoxyribonuclease/r-tPA treatment significantly ameliorated ischemia-reperfusion injury–induced left ventricular remodeling, as compared to controls. The authors conclude that NETs have a detrimental effect in ischemia-reperfusion–challenged myocardium and suggest deoxyribonuclease treatment regimens in patients with myocardial ischemia reperfusion injury and coronary no-reflow.<sup>100</sup> Similarly and again using a mouse model of myocardial ischemia reperfusion, Savchenko et al<sup>101</sup> showed a significant cardioprotective effect of deoxyribonuclease I treatment on myocardial ischemia reperfusion injury. In the same study, PAD4-deficient mice, which are not capable of producing NETs, were significantly protected from

myocardial ischemia reperfusion injury and deoxyribonuclease I treatment had no added beneficial effect in these animals. Deoxyribonuclease I treatment also protects mice from cerebral ischemia reperfusion injury,<sup>102</sup> suggesting that chromatin/NET degradation by deoxyribonuclease and consequently removal of extracellular histones significantly alleviates tissue damage after ischemia-reperfusion injury. Hence, removal of extracellular chromatin generated at the sites of infarction not only implicates NETs in the myocardial damage but may also offer promising therapeutic options.

### Summary, Outlook, and Conclusion

As evident from the studies highlighted and discussed above, NETs clearly do matter and significantly affect the initiation and progression of atherosclerotic lesions. Moreover, NETs can induce and contribute to arterial thrombus formation, stability, and growth (Figure). Although deoxyribonuclease treatment, likely enhancing thrombus lysis, seems to harbor a relevant therapeutic potential in acute cardiac events,<sup>103</sup> its utility and applicability in preventing NET formation or in digesting already established NETs to reduce atherosclerotic lesion growth is at least debatable and will remain controversial. Undoubtedly, more in-depth studies are needed to meticulously dissect the exact mechanisms of in vivo NET formation<sup>36</sup> and to clarify the importance of histone citrullination for NETosis.<sup>38</sup> A better understanding of in vivo NETosis, both with regards to the structural constituents and to their context-specific functional decoration, will be a prerequisite to further elucidate the role of NETs in the development of atherosclerotic plaques and will be of paramount importance to identify, validate, and enable the best molecular candidates for therapeutic targeting.

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### Disclosures

None.

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