Therapeutic Targeting of Neutrophil Extracellular Traps in Atherogenic Inflammation

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Abstract

Neutrophils and neutrophil extracellular traps (NETs) have a robust relationship with atherothrombotic disease risk, which led to the idea that interfering with the release of NETs therapeutically would ameliorate atherosclerosis. In human studies, acute coronary events and the pro-thrombotic state cause markedly elevated levels of circulating deoxyribonucleic acid (DNA) and chromatin, suggesting that DNase I might produce cardiovascular benefit. DNase I reproduced the phenotype of peptidylarginine deiminase 4 (PAD4) deficiency and showed a significant benefit for atherothrombotic disease in experimental mouse models. However, the mechanisms of benefit remain unclear. Insights into the mechanisms underlying NET release and atherogenic inflammation have come from transgenic mouse studies. In particular, the importance of neutrophil NET formation in promoting atherothrombotic disease has been shown and linked to profound pro-inflammatory and pro-thrombotic effects, complement activation and endothelial dysfunction. Recent studies have shown that myeloid deficiency of PAD4 leads to diminished NET formation, which in turn protects against atherosclerosis burden, propagation of its thrombotic complications and notably macrophage inflammation in plaques. In addition, oxidative stress and neutrophil cholesterol accumulation have emerged as important factors driving NET release, likely involving mitochondrial reactive oxidants and neutrophil inflammasome activation. Further elucidation of the mechanisms linking hyperlipidaemia to the release of NETs may lead to the development of new therapeutics specifically targeting atherogenic inflammation, with likely benefit for cardiovascular diseases.

Keywords
- neutrophil
- NETosis
- atherogenic inflammation
- atherothrombotic disease

Introduction

Despite current therapies, cardiovascular diseases (CVDs) have remained the leading cause of mortality globally for many years. In addition to the major impact on personal health, CVDs constitute a serious social and economic burden worldwide. Coronary artery and cerebrovascular disease—causative for myocardial infarction and stroke, respectively—are the most common and severe complications of CVDs. Atherosclerosis is recognized as the primary...
pathophysiology of CVD originating from a lipid-driven chronic inflammation of the vessel wall.\textsuperscript{1–2} Hyperlipidaemia can damage endothelial cells promoting lipid deposition and plaque formation, and represents the initial spark in atherosclerosis; however, chronic inflammation fuels progression of the disease. Many recent studies have linked hyperlipidaemia to atherogenic inflammation,\textsuperscript{3–6} and neutrophils are likely activated during hyperlipidaemia to promote atherogenic inflammation.\textsuperscript{4,7,8} Indeed, hyperlipidaemia drives neutrophilia, and circulating neutrophil counts are directly related to atherosclerosis burden.\textsuperscript{4,7} Infusion of neutrophil-depleting antibody reduces atherosclerosis in animal models.\textsuperscript{7} In addition, hypercholesterolaemia can trigger the synthesis of granulocyte colony-stimulating factor (G-CSF), a master regulator of granulopoiesis.\textsuperscript{7,9–12} G-CSF stimulates the proliferation of myeloid precursor cells and suppresses the clearance of aged neutrophils.\textsuperscript{13} Also, hypercholesterolaemia increases serum levels of CXCL1 promoting mobilization of neutrophils.\textsuperscript{14} Indeed, in experimental mouse models neutrophils accumulate in atherosclerotic lesions.\textsuperscript{7,15–19} Within human atheroma, neutrophil infiltrates are detected less frequently.\textsuperscript{20–24} Nevertheless, they have been detected at the sites of plaque erosion or rupture,\textsuperscript{20,24–26} and in clinical cohort studies, neutrophil blood counts show a robust relationship with increased risk of acute coronary events.\textsuperscript{27–30} Despite the recognition that infiltration of leukocytes acts as a driving force of atherothrombotic disease, the contribution of neutrophils to CVDs has, however, been under-estimated.

Neutrophils are the most abundant population of leukocytes in human circulation and form an essential part of the inflammatory response to combat invading pathogens through their functional properties such as phagocytosis, degranulation and the generation of reactive oxygen species (ROS).\textsuperscript{31,32} Given their limited lifespan, neutrophils have in the context of chronic inflammation long been over-shadowed by other leukocyte populations, such as monocytes and macrophages. However, the last 10 years witnessed a revival of neutrophils as multi-functional innate immune cells that can greatly influence the course of chronic inflammation via their crosstalk with other immunocompetent cells.\textsuperscript{33–36} Among several new neutrophil interactions discovered, the finding that neutrophils can release threads of chromatin covered with proteins of nuclear, cytoplasmic or granular origin—named neutrophil extracellular traps (NETs)—has placed neutrophils back in the spotlight of cutting-edge immunological research.\textsuperscript{37} The release of NETs by neutrophils is called NETosis, and neutrophil NETosis is an emerging mechanism underlying atherogenic inflammation. Recent studies have highlighted the importance of neutrophil activation and NETosis in acute coronary events\textsuperscript{38,39} while other studies have suggested a role of NETosis in atherogenesis\textsuperscript{8,40–42} and plaque erosion.\textsuperscript{43,44} Yet, few studies have examined directly the effect of NETs on the formation, development and complication of atherosclerosis. This highlights the need to elucidate potential inflammatory mechanisms underlying neutrophil NETosis in atherogenesis and to explore the potential clinical and therapeutic implications of NETosis for CVDs.

### Formation of Neutrophil Extracellular Traps

Neutrophils contribute to an acute inflammatory cascade by several different mechanisms, including phagocytosis, chemokinesis and degranulation.\textsuperscript{31,32} In response to damage, neutrophils not only secrete inflammatory mediators, but can also release their cytoplasmic content and extrude their deoxyribonucleic acid (DNA) in a process named NETosis.\textsuperscript{45} Besides other types of cell death such as necrosis and apoptosis, NETosis is an alternative form of programmed cell death wherein neutrophils release NETs.\textsuperscript{46} Depending on the inciting event, the host membrane receptors, signalling cascades and effector proteins involved, NETosis unfolds in a ‘vital’ or ‘suicidal’ manner. ‘Suicidal’ NETosis is preceded by hours of oxidant generation by the multi-component nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and ends when NETs are expelled from the neutrophil; the outcome is neutrophil cell death.\textsuperscript{36,47} In contrast, without compromising membrane integrity, ‘vital’ NET formation involves the secreted expulsion of chromatin via vesicles. The surface membrane reseals and results in a viable anuclear neutrophil.\textsuperscript{48–50}

Irrespective of the mechanism of NETosis, NETs are networks of extracellular fibrous material composed of neutrophil DNA and granule-derived peptides and proteolytic enzymes. Many effector mediators cover this extracellular neutrophil DNA such as histones, multiple proteases such as neutrophil elastase (NE), proteinase 3, cathepsin G and gelatinase, and the pro-oxidant enzyme myeloperoxidase (MPO). Neutrophils use these fibrous structures to trap extracellular pathogens and prevent bacterial dissemination. NETs are released during inflammation and occur in vivo following infections.\textsuperscript{51} Moreover, NETs can trigger coagulation, cause endothelial dysfunction and amplify local inflammation, and NETosis not only plays a role in the elimination of pathogens, but also contributes to sterile inflammation, cancer, autoimmunity and thrombosis.\textsuperscript{36,44}

**Cholesterol, oxidized low-density lipoprotein (oxLDL) and platelets as drivers of neutrophil NET formation:** The idea that NETs might mediate atherothrombotic disease by stimulating an overall process of inflammation and thrombosis has moved studies to expand beyond the original characterization of NETosis as a mechanism of defence against bacteria. Over the subsequent decade, the factors controlling NET formation and the molecular underpinnings of mechanisms linking NETosis to atherogenic inflammation have in part been revealed (\textbf{Fig. 1}). In vitro, neutrophils release NETs in response to cholesterol crystals and oxLDLs in a manner that depends on NADPH oxidase activity\textsuperscript{40,52},\textbf{(Fig. 1A)}, and inhibition of mitochondrial oxidative stress reduces the formation of NETs by cultured neutrophils when exposed to 7-ketocholesterol, an oxysterol found in human atheroma.\textsuperscript{53} Also, interactions with activated platelets commit neutrophils to undergo NETosis\textsuperscript{54,55} (\textbf{Fig. 1A}). This can be propagated through high-mobility...
group box 1 (HMGBl) exposed on the surface of activated platelets and by interactions of neutrophil P-selectin glycoprotein ligand-1 with platelet P-selectin.\textsuperscript{56,57} In addition, CCL5 and CXCL4 are chemokines stored in platelet α-granules and CCL5/CXCL4 heterodimers are potent neutrophil attractants. Upon adhesive contact with platelets, the CCL5/CXCL4 complex also triggers neutrophils to release NETs\textsuperscript{58} and blocking the heterophilic interaction between CCL5 and CXCL4 was recently shown to prevent NETosis in a mouse model of myocardial infarction.\textsuperscript{59} Vice versa, expelled NETs enhance the adhesion of additional platelets via von Willebrand factor\textsuperscript{60,61} and promote their activation, thereby coordinating a pro-thrombotic cycle of coagulation and inflammation.\textsuperscript{56,60,62,63} (\textit{Fig. 1B}). Important insights have been gained by the elucidation that neutrophil NETosis participates in path physiological thrombosis, including deep vein thrombosis\textsuperscript{61,64} and atherothrombosis.\textsuperscript{39,65} NETs have been suggested to participate in thrombus growth and stabilization by providing a scaffold for fibrin formation and platelet aggregation.\textsuperscript{60,66} Indeed, elevated levels of circulating DNA have been detected in patients with acute coronary events\textsuperscript{65} and also in situ in the thrombus mass of patients with acute myocardial infarction.\textsuperscript{67} Moreover, mediators associated with NETs can stimulate inflammatory cells ranging from plasma- cytoid dendritic cells\textsuperscript{68} to macrophages,\textsuperscript{40} and recent insight suggests a role of NETs in macrophage activation, potentiating plaque formation in murine atherosclerosis.\textsuperscript{40} However, the importance of NETs in macrophage priming in early atherosclerosis was not found in another study. Instead, it appeared that neutrophil-derived proteases directly contributed to interleukin (IL)-1β maturation.\textsuperscript{69} In sum, understanding of the role of NET formation in atherogenic inflammation remains scant, and only recently studies emerged that established causation
linking NETosis to inflammation, plaque disruption and thrombosis with different strategies (i.e. pharmacologically blocking NET formation, use of genetic approaches to interfere with NETosis, etc.).

**NETs in Atherosclerosis**

Atherosclerosis is a chronic inflammatory pathology of the medium- and large-sized arteries that underlies CVDs.\(^1\) Activation of the vascular endothelium is the initial spark in atherosclerosis; however, chronic inflammation fuels progression of the disease. Circulating leukocytes anchor to and infiltrate the inflamed vessel wall. Adherent monocytes continually migrate and accumulate inside the atherosclerotic lesion, driving progression and enlargement of the plaque with the formation of a collagenous fibrous cap (►Fig. 2A). Activated macrophages within the lesion damage the fibrous cap making the atherosclerotic plaque vulnerable to rupture.\(^70\) Plaque rupture—with subsequent exposure of tissue factor and collagen resulting in thrombus formation (►Fig. 2B and C)—causes a myocardial infarction or stroke within seconds. Although plaque instability is responsible for two-thirds of sudden coronary death,\(^71,72\) plaque rupture does not always lead to major acute clinical events.\(^73\) Indeed, large plaque burden and severe lumen narrowing are essential criteria for the development of acute coronary events, rendering the residual lumen unable to host the thrombus.\(^74\)

Only recently neutrophils have been forwarded as important regulators in atherosclerosis and particularly in atherothrombosis.\(^34,75\) Histological studies on murine atherosclerotic plaques in experimental models of atherosclerosis revived the discussion on the role of neutrophils in human atherosclerotic disease. While experimental plaques in severely hypercholesterolaemic mice contain neutrophils,\(^7,17–19\) investigations on human atherosclerotic plaques failed to demonstrate the presence of large numbers of neutrophils in intact plaques.\(^20–22\) Neutrophils are found only scarcely scattered as solitary cells throughout the intima, suggesting that neutrophils do not play a prominent role during early stages of human atherosclerosis.\(^20,23,24\) Yet, the paucity of validated selective markers for human neutrophils has rendered their identification in pathological studies difficult, and the extent and timing of potential neutrophil involvement in human atherosclerosis remains unsettled.\(^76,77\) In addition, neutrophils may undergo phenotypic changes, and hence, lose expression of specific markers in response to inflammation. For example, a recent study reported similar staining patterns for NE and MPO—generally considered as markers to identify neutrophils in situ\(^17,19,20,67\)—in complicated atherosclerotic plaques.\(^24\) However, CD177—a neutrophil-specific antigen involved in cell migration\(^78\)—reacted with only a sub-population of the neutrophils.\(^24\)

In fact, the clinical relevance of neutrophil NETosis remains poorly understood and hard to prove experimentally. The potential non-specificity of NETosis markers still precludes understanding of the causes of NET formation in men and their contribution to human disease. Although extracellular DNA is found in several acute and chronic, sterile and infective disease compartments, yet it is still unclear whether free DNA truly derives from NET formation. Also, it remains experimentally challenging to dissect cellular origins of free extracellular DNA. Finally, even if putative NET-associated markers, (e.g. citrullinated histones or proteases) support the presence of NETs, their impact on disease pathologies is still hard to assess.

**Fig. 2** The involvement of neutrophil extracellular traps (NETs) during the different stages of atherosclerosis and its complications. In the early stages of plaque formation (A), neutrophils accumulate throughout the atheroma. Activated neutrophils further stimulate the recruitment and activation of monocytes through the release of granular proteins such as cathelicidin-related antimicrobial peptide (CRAMP). The atheroma builds up and a core of lipids, living and dead cells and a fibrous cap with collagen expands. Deposition of oxidized low-density lipoprotein (LDL) and cholesterol causes neutrophil inflammasome activation, increases oxidative stress and triggers NETosis. Once the necrotic core progresses, extracellular deoxyribonucleic acid (DNA) accumulates. Sensing of double stranded DNA (dsDNA) by lesional macrophages through the cytosolic absence in melanoma 2 (Aim2) inflammasome results in the release of interleukin (IL)-1β, further amplifying local inflammation. During plaque erosion (B), activation of endothelial cells (for example, through engagement of Toll-like receptor [TLR] 2) propagates the recruitment of neutrophils. Neutrophils localized near the inflamed intimal surface degranulate and generate reactive oxidants, leading to endothelial cell death and detachment. Superficial plaque erosion exposes pro-thrombotic factors, and activated platelets trigger neutrophils to form NETs and the release of tissue factor. Through complement, NETs induce continued endothelial erosion. Further complement activation drives neutrophil recruitment to the site of atherothrombosis (C). Here, tissue factor-covered NETs entrap platelets and provide a nidus for platelet aggregation and thrombus formation.
Neutrophils lay down the tracks in atherogenic inflammation: Several experimental mouse models of atherogenesis have demonstrated the presence of neutrophils in arterial plaques, and that neutrophil-derived factors are able to modulate murine plaque size and composition. Neutrophils may contribute to plaque formation through promoting inflammatory monocyte recruitment, and may also participate in lesion evolution and complication. Within atherosclerotic plaques, NET components such as cathespin G and cathelicidins exhibit monocyte-chemotactic activity. The neutrophil granular cathelicidin-related antimicrobial peptide (CRAMP) affects the recruitment and activation of other immune cells, including monocytes and dendritic cells. In atherosclerotic vessels, CRAMP is deposited on the intima, suggesting a novel role for cholesterol in atherogenic inflammation. NETosis in atherosclerotic lesions exerts direct pro-inflammatory effects on macrophages to release ROS and other pro-inflammatory cytokines. In turn, reactive oxidants modify LDL to generate oxLDL that drives the differentiation of foam cells.

Oxidative burden, DNA sensing and NETosis in atherogenic inflammation: Oxidative stress is present in aging and human atherosclerotic vascular diseases. Recent findings underscore a relationship between mitochondrial oxidative stress and neutrophil NETosis in animal models for atherosclerosis. Irradiation of recipient animals to ablate endogenous haematopoietic tissues, followed by reconstitution of aged atheroprone Ldr−/− mice with mitochondrial catalase transgenic bone marrow cells suppresses oxidative stress and protects against atherosclerosis development. The higher oxidative burden in these old mice correlates with enhanced NETosis. In vitro, exposure of neutrophils to 7-ketocholesterol led to the formation of NETs (Fig. 1A), and suppression of mitochondrial oxidative stress reduced NETosis in response to 7-ketocholesterol. Along these lines, further studies have revealed that cholesterol crystal-triggered NETosis in atherosclerotic lesions exerts direct pro-inflammatory effects on macrophages leading to cytokine release such as IL-1β and IL-6, thus further amplifying local inflammatory cascades in the artery and exacerbating and propagating arterial intimal injury and thrombosis. Finally, recent studies have shown that defective cholesterol efflux pathways lead to neutrophil cholesterol accumulation, inflammasome activation and prominent NET formation in atherosclerotic lesions, suggesting a novel role for cholesterol accumulation in atherogenic inflammation. Links between inflammasome activation and NETosis, however, need to be more clearly delineated. In this regard, recent studies in Aim2−/− mice have revealed a major role of the double stranded DNA (dsDNA), absent in melanoma 2 (Aim2) inflammasome in lesional macrophages. At later stages of atherosclerosis, ApoE−/− mice showed prominent lesional deposition of extracellular dsDNA, and this was echoed by parallel Aim2 expression in macrophages at advanced stages of the disease (Fig. 1C). Aim2 deficiency on the ApoE−/− background diminished the production of IL-1β and reduced plaque destabilization suggesting a novel role for Aim2 in inflammation associated with atherosclerosis. At present, the possibility that dsDNA is primarily released by accumulating dead cells within the expanding necrotic core cannot be ruled out, and whether NETs promote Aim2 activation in atherosclerosis remains unclear.

Peptidylarginine deiminase 4 (PAD4), a nodal intervention point to target NET formation: NETs can be detected in atherosclerosis, and given their pro-inflammatory and pro-thrombotic properties, the presence of NETosis could potentiate atherosclerotic plaque formation via enhanced inflammation and increased monocyte recruitment. Yet, few studies have attempted to establish a direct link between NETosis and atherogenic inflammation. The enzyme PAD4 participates in NET formation by citrullination of histones, releasing the electrostatic bonds that constrain nuclear DNA to nucleosomes. Loss of these positive charges due to PAD4 activity frees the chromatin to unfold and form the threads of chromatin furnished by NETs. Indeed, NET formation in mice depends on PAD4 activity. Cl−-amidine, a pan-PAD inhibitor administered systemically, prevented NETosis, retarded neutrophil and monocyte recruitment to arteries and reduced experimental atherosclerosis and the pro-thrombotic phenotype of ApoE−/− mice. However, Cl−-amidine could potentially have off-target effects, and findings obtained with Cl−-amidine—targeting all PAD isotypes—should be translated with caution. PAD-mediated citrullination can drive T cell polarization and cytokine production. Also, PAD4 activity can affect dendritic and smooth muscle cell activation. Thus, the lack of specificity of Cl−-amidine limits unambiguous probing of the role of PAD4 and NETs in atherothrombosis. Recently, one study reported a novel selective PAD4 inhibitor to block NETosis by human and murine neutrophils in vitro. However, more work is needed to validate the enzymatic role of PAD4 in the formation of NETs. It will therefore be critically important to test the causal contributions of neutrophil NETosis to atherogenic inflammation at different stages of lesion development using new tools for the detection and manipulation of NET formation in key model systems for human atherosclerosis. Finally, given the many functions of PAD4 other than NET formation, any phenotype in mice lacking PAD4 cannot undoubtedly be taken as an unambiguous demonstration of the involvement of NETs.

To evaluate more rigorously the participation of PAD4 and NETosis in atherothrombotic disease, more recent studies used mice with genetic deficiency of PAD4 in blood cells but not in intrinsic vascular wall cells and other tissues. Backcrossing of atheroprone ApoE−/− mice to mice that lack PAD4 specifically in myeloid cells protects against atheroma burden that is intimately linked to diminished NETosis and reduced atherogenic inflammation in the artery. In the same model, NETs provoked macrophages to release pro-inflammatory cytokines such as IL-1β and CXCL1 facilitating further local inflammatory responses. These findings are in line with previous work that suggested a link between NET formation and macrophage inflammation in atherosclerotic plaques. By contrast, other results indicate that myeloablative irradiation and reconstitution with Pad4-deficient bone marrow cells, and hence,
NETosis does not alter atherogenesis in hypercholesterolaemic Ldlr<sup>−/−</sup> mice, but are involved causally in endothelial erosion.<sup>43</sup> Previously, NETs were shown to directly induce endothelial dysfunction and to kill endothelial cells in vitro,<sup>58,59</sup> and this was associated with endothelial damage in systemic lupus erythematosus.<sup>100,101</sup> An effect mediated in particular by matrix-degrading factors contained in NETs such as matrix metalloproteinases.<sup>58,59</sup> Now, genetic deficiency of Pad4 in blood cells has been shown to reduce intimal damage in mice with arterial lesions, without affecting plaque size and atherogenic inflammation.<sup>43</sup> On balance, while the role of NETs in early atherosclerosis and plaque erosion has been studied intensely, their role in plaque progression is unclear and future studies will be needed to determine the involvement of NETosis in the development of unstable lesions.

In mice, experimental atherosclerotic plaques are usually devoid of thrombosis.<sup>102</sup> On the other hand, neutrophil contribution in human atherosclerotic disease appears to be a prominent feature of complicated thrombosed plaques.<sup>20,24</sup> In contrast to intact atherosclerotic plaques, complicated plaques contain large numbers of neutrophils, which frequently also express markers for NET formation such as citrullinated histone H3 and PAD4.<sup>24</sup>

**NETs in Atherothrombosis**

For decades, research has focused primarily on the so-called ‘vulnerable plaque’, a morphology associated with plaque rupture and thrombosis, which may be triggered by neutrophil NETosis.<sup>44,56</sup> Clinical cohort studies reported that neutrophil blood counts associated with an increased risk of acute coronary events, heart failure and death.<sup>27–30</sup> However, the involvement of neutrophils, and particularly NETosis, in plaque destabilization and rupture remains scant, and only supported by associative data. Moreover, contradictory findings have been observed. For example, some have reported neutrophils to accumulate in rupture-prone human atherosclerotic lesions.<sup>25</sup> In contrast to those studies, others have shown neutrophils and markers for NETosis to colocalize with apoptotic endothelial cells in lesions complicated by superficial erosion, but not in plaques considered rupture-prone.<sup>26</sup> Thus, experimental studies are clearly warranted to unambiguously establish the contribution of neutrophil NETosis to plaque destabilization, rupture and its complications.

Decades of therapies to lower exposure to traditional risk factors may have altered human atheromatosa, increasing the proportion of acute coronary events by superficial plaque erosion. Indeed, recent data indicate that up to one-third of acute coronary events currently result from erosion rather than plaque rupture.<sup>103</sup> A growing body of evidence underscores that neutrophils and NETs pertain to the propagation of thrombotic complications of atheroma prompted by superficial plaque erosion.<sup>24,26,43,104</sup> NETs appear to be associated with eroded or erosion-prone plaques in endarterectomy specimens of carotid arteries<sup>26</sup> and recently in coronary specimens from patients with acute myocardial infarction.<sup>24</sup> In the same study, ligation of Toll-like receptor 2 was found to activate endothelial cells and potentiate neutrophil recruitment (<sup>►</sup> Figs. 1D and 2B). Participation of neutrophils led to endothelial cell death and detachment, implicating a role for neutrophil NETosis in superficial plaque erosion. Also, cells bearing CD66b, MPO and NE were found to localize near luminal endothelial cells within human plaques harvested from carotid arteries supporting the presence of neutrophils at the intimal surface of complicated plaques that required endarterectomy.<sup>104</sup> Finally, genetic loss of Pad4 function in haematopoietic cells and NETosis protects against endothelial desquamation and thrombus formation in a mouse model of atherosclerosis.<sup>43</sup> Here, NETs trigger endothelial cell death and detachment in a manner that depends on complement deposition (<sup>►</sup> Figs. 1D and 2B).

In addition, further complement activation also triggers neutrophil recruitment to the site of atherothrombosis in acute myocardial infarction;<sup>105</sup> and pathological studies showed high numbers of neutrophils in coronary specimens from patients with acute myocardial infarction,<sup>20,106–108</sup> or with complicated thrombosed plaques.<sup>20</sup> Also, thrombectomy specimens retrieved from patients with acute myocardial infarction contain neutrophils in the thrombus mass.<sup>109</sup> Similarly, neutrophils were found more frequently associated with the presence of occlusive thrombi.<sup>21</sup> The presence of NETs has been reported in thrombectomy specimens of patients with acute myocardial infarction<sup>39,67</sup> or with stent thrombosis.<sup>110</sup> Elevated levels of circulating DNA, chromatin and MPO–DNA complexes are independently associated with severe coronary events and the pro-thrombotic state.<sup>65</sup> However, the possibility that DNA and nucleosomes are released as a result of other cell death programs, for example, endothelial cell apoptosis and cardiomyocyte necrosis, cannot be ruled out, and it remains unclear to what extent neutrophil NETosis contributes to thrombus formation. NETs exposed to blood gather the potent pro-coagulant tissue factor, the initiator of the extrinsic coagulation pathway (<sup>►</sup> Fig. 1B). Local accumulation of tissue factor-covered NETs occurs at sites of coronary thrombosis,<sup>111</sup> and neutrophils release NETs bearing tissue factor within thrombi of infarcted regions.<sup>39</sup> In addition, cleavage and inactivation of the endogenous anticoagulant protein, tissue factor pathway inhibitor by NETs-contained neutrophil proteases (such as NE and cathepsin G) drive and amplify intravascular clot formation<sup>54</sup> (<sup>►</sup> Fig. 1B). Studies with mice that lack factor XII (FXII), the starting point of the intrinsic coagulation pathway, suggest that NETs also contribute to the propagation of intravascular blood coagulation by promoting FXII activation.<sup>61</sup> Plaque rupture during acute myocardial infarction triggers platelet aggregation and deposition of fibrin at the initial site of the vulnerable atheroma. In turn, activated platelets present HMGB1 protein to neutrophils provoking the formation of NETs and the release of tissue factor.<sup>39,56</sup> Together, these events may contribute to plaque rupture and subsequent thrombus formation (<sup>►</sup> Fig. 2B and C). Indeed, platelet-derived HMGB1 protein facilitates NETosis and coagulation.<sup>62</sup> Results from other studies suggest that the formation of NETs may promote the growth of a thrombus mass after the onset of a rupture of the plaque,<sup>60,64</sup> by providing a
scaffold for erythrocytes binding and platelet aggregation. Thrombus growth and expansion leads to the reduction of blood flow and thus the onset of ischaemic heart failure. In line herewith, NETosis has emerged as an important contributor in a mouse model of myocardial ischaemia-reperfusion injury.\textsuperscript{59,112} Neutrophils are able to produce a large array of cytokines, chemotactic factors and proteolytic enzymes and therefore play a role in inflammation, fibrogenesis and angiogenesis.\textsuperscript{75,79} Thus, given the array of matrix-degrading enzymes that neutrophils contain, one can anticipate a destabilizing impact of neutrophils during thrombus evolution. Release of these enzymes could lead to thrombus disintegration and embolization. Indeed, lytic thrombi with features of tissue necrosis have been reported to contain highest concentrations of NETs\textsuperscript{67} together with matrix metalloproteinase.\textsuperscript{113}

**Clinical Perspective and Future Challenges**

Despite current therapies that have successfully lowered LDL, there remains a large burden of residual risk, and atherothrombotic disease is still the leading cause of morbidity and mortality globally. Attempts to lower exposure to additional risk factors such as hypertension and smoking have only met with modest success. Although diet and lifestyle certainly contribute to atherothrombosis, these alone cannot account for the entire burden of atherosclerosis.\textsuperscript{114–116} Indeed, genetic and environmental factors (such as co-morbidity, infection and products of the endogenous microbiome) are now emerging as risk factors to atherosclerosis. In fact, normal aging—a process that drives a state of chronic systemic low-grade inflammation—is receiving more attention as perhaps the greatest risk factor for a wide variety of chronic disease, including atherothrombotic disease. Without challenging traditional risk factors, inflammation and immunity provide pathways that connect traditional with these emerging risk factors that give rise to the disease and its complications.

There is a great deal of excitement about new targets linked to atherogenic inflammation that have emerged from animal and human studies. For example, multiple studies have shown that IL-1β plays important roles in atherosclerosis.\textsuperscript{117,118} And the recent CANTOS trial strongly underscores the concept of anti-inflammatory therapy to treat CVs.\textsuperscript{6} Yet, broadly immunosuppressive therapies carry the risk of excess deaths from infections limiting the clinical impact of these strategies. This heightens the need to understand more clearly the inflammatory mechanisms that are specific to atherogenesis. Perhaps the most obvious candidates for future studies are inflammatory mechanisms that are linked to hyperlipidaemia and oxidative stress. Neutrophil NETosis is an emerging mechanism underlying atherogenic inflammation, and likely interacts with hyperlipidaemia and oxidative stress to promote atherogenic inflammation.\textsuperscript{8,53}

Neutrophils and NETosis have been implied to contribute to atherogenesis as well as thrombotic plaque complications, and thus represent novel targets for the treatment and/or prevention of atherothrombotic disease. Given the profound pro-inflammatory and pro-thrombotic effects that have been identified in earlier studies, systemic treatment with DNase I merits consideration as a therapeutic approach. Indeed, formulations of DNase I (Pulmozyme)—approved for the treatment of cystic fibrosis\textsuperscript{119}—exert beneficial effects in mice with experimental atherogenic inflammation and thrombosis. Moreover, NETs appear to jeopardize normal endothelial functions. In this regard, the complement pathway and NETosis are intimately linked. NETs can activate the alternative complement pathway\textsuperscript{120} and promote endothelial damage affecting glomeruli in anti-neutrophil cytoplasmic antibody-associated vasculitis.\textsuperscript{121} NETs could constitute a critical scaffold promoting the local activation of the complement pathway in the vicinity of vascular endothelial cells, exacerbating endothelial cell death, detachment and thrombosis (\textsuperscript{Fig. 1D}). Thus, strategies that limit complement activation also merit consideration as an adjunct to treatment of atherosclerosis and its thrombotic complications. Recent studies have linked oxidative stress, neutrophil cholesterol accumulation and NETosis to atherogenic inflammation. However, this area remains poorly understood, therapy is challenging and there is a tremendous need for further research.

Pulmozyme is used in the clinic to treat patients with cystic fibrosis, where it has a beneficial effect, suggesting that—in this setting—NETs do more harm than good. Initially, NETosis was demonstrated to be involved in anti-bacterial responses. Yet, the initial finding that NETs are protective for host immunity has been challenged by recent studies, and the clinical relevance of NETosis in infective diseases, particularly chronic infections, is hard to judge. Hence, the net clinical impact of therapeutically preventing NET formation in NETosis-associated diseases remains the most important, unanswered question in the field to be resolved.

Perhaps the biggest challenge facing the field is translating findings from mouse to human into novel and effective therapies. The principles of evolution, as well as the scientific literature, suggest that there are many similarities between both mammal species, but also significant differences.\textsuperscript{122} When it comes to modelling immunity and inflammation in atherothrombotic disease, this is by no means surprising or new. Although there are surely important differences of opinion, mouse models do provide enough similarities in their immune responses, and clinical and histological manifestations to be of value as a relevant model organism in understanding mechanisms of atherosclerosis. Clearly, however, the physiology and pathophysiology of mice is also sufficiently different to mandate an awareness of potential resulting pitfalls. Nevertheless, despite these differences between mice and men, immunologic research in mice led—among many other relevant insights—to the discovery of the major histocompatibility complex and, ultimately, successful organ transplants. The challenges ahead in understanding the genetic and/or environmental factors accounting for heterogeneity in man (e.g. age, sex and co-morbid factors) and faithfully modelling them in pre-clinical studies to best fit the human condition loom large. With technology and innovation, the research community will hopefully overcome them.
Conclusion

In summary, recent work has contributed to a growing body of evidence that NETosis participates in atherogenic inflammation and the propagation of atherothrombosis. NETs can perpetuate activation of endothelial cells, macrophages and platelets, trigger coagulation and complement activation and cause endothelial dysfunction. Therefore, interfering with the formation of NETs may result in numerous beneficial clinical effects for patients with CVD. In animal experimental models, DNase I and Cl-amidine restrict neutrophil NETosis, and thus protect against atheroma burden and thrombotic plaque complications. By contrast, other studies indicate that neutrophils also have beneficial effects in complications associated with atherosclerosis.\(^1\)\(^2\)\(^3\)\(^4\) NETs have been found at each stage of atherothrombotic disease. Nevertheless, whether NETosis plays different roles at different stages remains unknown. In addition, it will be a challenge to explore whether NETs are involved in crosstalk with intrinsic vascular wall cells or other cell types such as smooth muscle cells. The identification of endogenous triggers of NETosis remains an interesting prospect. Thus, future studies will be needed to establish a better understanding of the role of NETosis in atherosclerotic plaques and will be of paramount importance for the identification of the best candidates for therapeutic targeting.

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Conflict of Interest

None declared.

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Van Avont et al.


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Van Avondt et al.