

# The Role of Neutrophil Extracellular Traps in Atherosclerosis: From the Molecular to the Clinical Level

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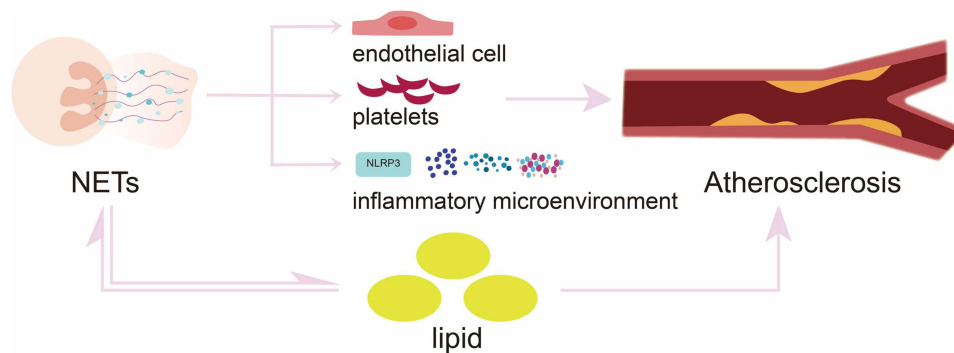
**Abstract:** Atherosclerosis is a chronic inflammatory condition that is typified by the deposition of lipids and the subsequent inflammation of medium and large arteries. Neutrophil extracellular traps (NETs) are fibrous meshworks of DNA, histones, and granzymes expelled by activated neutrophils in response to a variety of pathogenic conditions. In addition to their role in pathogen eradication, NETs have been demonstrated to play a pivotal role in the development of atherosclerosis. This article presents a review of the bidirectional interactions in which atherosclerosis-related risk factors stimulate the formation of NETs, which in turn support disease progression. This article emphasizes the involvement of NETs in the various stages of atherogenesis and development, influencing multiple factors such as the vascular endothelium, platelets, the inflammatory milieu, and lipid metabolism. The findings of this study offer new insights and avenues for further investigation into the processes underlying the formation and regulation of the vascular inflammatory microenvironment in atherosclerosis. Finally, potential targeted therapeutic strategies for NETs are discussed to facilitate their progression to clinical practice (Graphical Abstract).

**Keywords:** atherosclerosis, neutrophil traps, inflammation, lipid metabolism, therapeutic strategies

## Introduction

Atherosclerosis represents the primary pathophysiology of acute cardiovascular disease, including conditions such as myocardial infarction and stroke. It is characterized by the accumulation of lipids and subsequent inflammation in medium and large arteries.<sup>1</sup> Atherosclerosis is a complex multifactorial disease in which risk factors for atherosclerosis commonly cause an inflammatory response. Inflammation is involved in all stages of atherosclerosis.<sup>2</sup> The deposition of low-density lipoprotein (LDL) induces endothelial dysfunction and the expression of adhesion molecules, resulting in the selective recruitment of circulating monocytes into the intima of the vessel wall, the subsequent activation of the inflammatory response, and their transformation into macrophages. Macrophages have been shown to internalise lipids, forming cholesterol-rich foam cells.<sup>3</sup> The release of inflammatory cytokines and pro-inflammatory mediators by both activated macrophage foam cells serves to further exacerbate the inflammatory response, leading to the formation of plaques. Subsequently, the accumulation of foam cells in the intima of the arterial wall promotes the development of fatty streaks. Furthermore, a number of chemokines and growth factors are implicated in the modulation of the inflammatory response, as well as in the induction of the migration and proliferation of smooth muscle cells (SMCs). The aforementioned cells contribute to the formation of a fibrous cap in atherosclerotic plaques.<sup>4,5</sup> The involvement of a range of inflammatory components plays a pivotal role in fibrous cap growth and instability. A ruptured fibrous cap may result in thrombus formation, leading to myocardial infarction or ischaemic stroke.<sup>2</sup>

## Graphical Abstract



Neutrophils, the most prevalent type of leukocyte in circulation, have been demonstrated to serve as a prognostic indicator for cardiovascular incidents and have been observed to exhibit a substantial correlation with atherosclerosis.<sup>6</sup> These cells exert anti-inflammatory effects through various mechanisms such as phagocytosis, degranulation, and the formation of fibrous mesh structures known as neutrophil extracellular traps (NETs)<sup>7</sup> composed of DNA, histones, and granzymes.<sup>8</sup> NETs act as barriers inhibiting the proliferation and spread of pathogens including bacteria,<sup>9</sup> fungi,<sup>10</sup> and parasites.<sup>11,12</sup> However, their release in the vascular system can also lead to disease by directly damaging the vascular endothelium<sup>13</sup> and inducing thrombosis.<sup>14</sup> NETs have potent pro-inflammatory, cytotoxic, and pro-thrombotic effects.<sup>15,16</sup> Some studies have reported the presence of neutrophils and NETs in atherosclerotic plaques,<sup>17</sup> and the NET component can lead to plaque fragility and vulnerability to dislodgement.<sup>18</sup> NETs may play an important role in the relationship between inflammation and atherosclerosis and have been the subject of considerable attention in recent years.

In this article, we present a synthesis of our current understanding of the mechanisms of NET formation, with a particular focus on elucidating the impact of NETs on the development of atherosclerosis through a multifactorial approach. We then discuss the role of atherosclerotic risk factors in NET formation. We use NETs, a novel regulatory mechanism of neutrophils, to explore in depth the formation and regulation of the vascular inflammatory milieu during atherosclerosis and hope to provide new ideas and methods for the prevention and treatment of atherosclerosis. Finally, we examine the potential of NETs as a therapeutic target in clinical practice.

## NETs Structure Formation and Release

### Structure and Function of NETs

Brinkmann et al were the first to identify NETs as a fibrous meshwork outside activated neutrophils, which consists mainly of a DNA backbone, histones, and antimicrobial proteins, including neutrophil elastase (NE) and myeloperoxidase (MPO).<sup>8,19</sup> The DNA backbone of NETs can result in rapid cell death by isolating surface-bound cations and disrupting the integrity of the bacterial envelope.<sup>20</sup> Histones possess antimicrobial properties and also exhibit cytotoxicity.<sup>21</sup> NE degrades virulence factors to kill bacteria, and MPO helps to create an oxidative environment and synergistically with NE promotes chromatin de-condensation and degradation of pathogen proteins.<sup>22</sup> Additionally, it is involved in histone modification, which enhances antimicrobial activity.<sup>23</sup> Other proteases (eg, cathepsin G, CG) associated with NETs also help to activate pro-inflammatory cytokines, which increase inflammation and recruitment of immune cells.<sup>24</sup> Therefore, NETs are involved in the immune response through direct or indirect effects on pathogens.

## Formation of NETs

### Lytic NETosis

NETs are released by neutrophils through a process known as NETosis. The formation of NETs involves a complex series of molecular mechanisms, primarily through the neutrophil death pathway, which is also termed “lytic NETosis”.<sup>25</sup>

A variety of pathogens, pro-inflammatory factors, and chemical inducers have been observed to promote the production of reactive oxygen species (ROS) by activating protein kinase C isoforms and mitogen-activated protein kinase (MAPK) cascade signaling pathways, which ultimately lead to the activation of Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NADPH oxidase).<sup>26</sup> Subsequently, NE and MPO are released from azurophilic granules and migrate towards the nucleus, where they exert a synergistic effect on nuclear chromatin decondensation and rupture of the nuclear and cytoplasmic membranes.<sup>27</sup> Additionally, NE dismantles the actin cytoskeleton, immobilizing neutrophils to facilitate precise deployment of NETs. MPO is indispensable for NET formation and NE release. Although the enzymatic activity of MPO may not be indispensable for chromatin dissociation, its enzymatic activity and products are of paramount importance in the transfer of NE to the nucleus.<sup>28</sup> Additionally, calcium-dependent peptidylarginine deiminase 4 (PAD4)-promoted citrullination of histones contributes to chromatin decondensation.<sup>29</sup> Eventually, the neutrophil undergoes lysogenic death and releases NETs into the extracellular space. It has been demonstrated that neutrophils stimulated by non-inflammatory vesicles, such as lipopolysaccharide or Gram-negative bacteria, activate caspase 11, which in turn activates gasdermin D (GSDMD) and forms pores in the plasma and nuclear membranes, thereby allowing caspase 11 to enter the chromatin and perform a function analogous to that of NE, which ultimately results in the rupture of the neutrophils and the release of NETs.<sup>30</sup> Additionally, it has been demonstrated that the GSDMD pathway is not essential for NET formation.<sup>31</sup>

### Vital Netosis

NETs may additionally be generated in a markedly abbreviated timeframe using modifications in nuclear morphology, the disintegration of the inner and outer nuclear membranes, and the ejection of DNA-laden vesicles from the intracellular milieu to the extracellular space.<sup>32</sup> This process, termed vital Netosis, is oxidant-independent and is not accompanied by cell lysis and death.<sup>32</sup>

### Other Mechanisms

Upon stimulation of neutrophils by granulocyte-macrophage colony-stimulating factor and subsequent toll-like receptor 4 (TLR4) or complement factor 5a receptor, mitochondria are able to release mitochondrial DNA in a ROS-dependent manner, resulting in the formation of NETs and live neutrophils.<sup>33</sup>

### Biomarkers of NETs

Double-stranded DNA (dsDNA), citrullinated histones, MPO-DNA complexes, NE, and other NET components are one way to evaluate the involvement of NETs in cardiovascular disease. A prospective, observational, cross-sectional cohort study of patients with coronary artery disease revealed that four biomarkers—dsDNA, MPO-DNA, nucleosomes, and citrullinated histone H4—were associated with the severity of atherosclerosis and major adverse cardiac events and these biomarkers may prove useful in predicting cardiovascular risk in patients presenting with chest discomfort.<sup>34</sup> The presence of coronary artery dsDNA is an independent predictor of the occurrence of major adverse cardiovascular events in hospital settings.<sup>35</sup> One study found that dsDNA levels in stable coronary artery disease were significantly associated with poor clinical outcomes at 2 years.<sup>36</sup> Furthermore, an increase in dsDNA expression levels in patients diagnosed with an ST-segment elevation myocardial infarction is similarly indicative of an adverse prognosis.<sup>37</sup> The presence of citrullinated histones can serve as a specific indicator of PAD4-mediated NET formation.<sup>38</sup> Measurement of MPO-DNA complexes using enzyme-linked immunosorbent assay is considered the most specific, objective, and quantitative marker for NET formation, with other forms of NET residues including DNA and NE complexes.<sup>38</sup> It has been demonstrated that the MPO-DNA complex can be employed as a predictive indicator of both the prevalence of atherosclerotic coronary arteries and the occurrence of major adverse cardiovascular events.<sup>34</sup> In a prospective multi-center cohort study, the expression of MPO-DNA, a marker of NETs, was reported to be positively associated with plaque fragility in patients with carotid stenosis (odds ratio = 2.08, 95% confidence interval 1.04–4.17).<sup>39</sup> Elevated levels of MPO have been independently shown to predict the risk of developing endothelial and coronary arterial disease, among other conditions.<sup>40</sup> Given the limited specificity of a single NET biomarker, combining two or more biomarkers

that react to NET formation may offer a more reliable approach. Moreover, quantitative analysis of NET biomarkers is likely to hold greater value in clinical practice.<sup>41</sup>

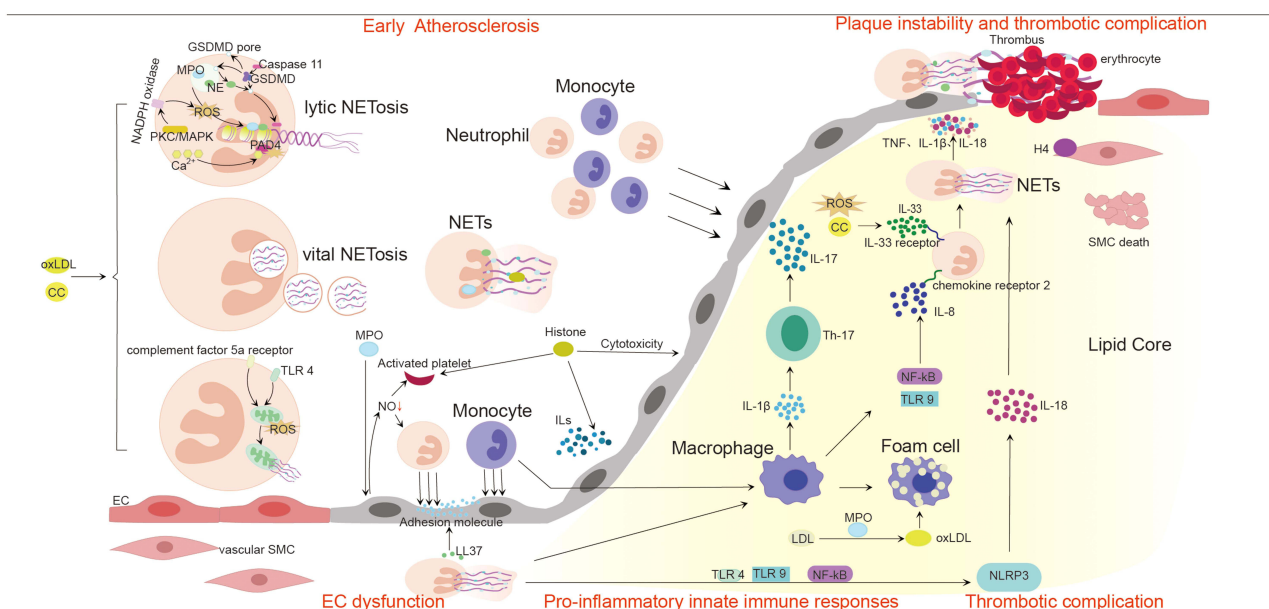
## NETs in Atherosclerosis

Atherosclerosis is a chronic inflammatory disease. The presence of NETs in the lumen of atherosclerotic lesions in mice and humans was first reported by Megens et al.<sup>17</sup> NETs and their components are associated with atherosclerosis severity and adverse cardiovascular events.<sup>34</sup>(Figure 1)

## Effects of NETs on Vascular Endothelial Cells

The vascular endothelium is comprised of a heterogeneous monolayer of endothelial cells (ECs), which serves as the initial barrier to molecules, cells, and pathogens in the circulation.<sup>42</sup> Healthy ECs play a crucial role in maintaining vascular homeostasis. They impede the adhesion of platelets and leukocytes to the vascular surface, maintain equilibrium between profibrinolytic and prothrombotic activities, and demonstrate anti-inflammatory and anti-atherosclerotic properties.<sup>43</sup> Nitric oxide (NO), synthesized by ECs via endothelial nitric oxide synthase (eNOS), retards endothelial-leukocyte interactions, platelet activation, and SMCs proliferation.<sup>44</sup> EC activation resulting from disturbances such as alterations in blood composition or blood flow is a crucial factor in the development of atherosclerosis.<sup>45</sup> NETs play a significant role in vascular EC inflammation during the progression of atherosclerosis.<sup>46</sup>

It has been demonstrated that histones present in NETs are capable of inducing cytotoxicity in ECs.<sup>47</sup> Furthermore, histone binding to phospholipids present in EC membranes alters the permeability of the membrane and increases the flux of calcium, which can ultimately result in cell death and tissue damage.<sup>48</sup> The binding of histones to TLR 2 and 4 activates the myeloid differentiation primary response 88 signaling pathway, which culminates in the release of pro-inflammatory cytokines.<sup>49</sup> MPO-derived oxidant hypochlorous acid reduces the bioactivity of NO through biochemical modification of L-Arginine substrates.<sup>50</sup> Additionally, MPO enhances the adhesion of ECs to leukocytes via the activation of the  $\mu$ -calpain pathway, reduces the phosphorylation of eNOS, and results in a decline in NO levels and



**Figure 1** The formation of neutrophil extracellular traps (NETs) and their interaction with atherosclerotic risk factors. The formation of NETs is stimulated by risk factors such as lipid metabolism imbalance, which contribute to the vascular inflammatory environment and participate in the formation and destabilisation of atherosclerotic plaques through multiple effects on vascular endothelial cells and platelets. The black arrow indicates the trend of the process.

**Abbreviations:** SMC, Smooth muscle cell; NETs, Neutrophil extracellular traps; NE, Neutrophil elastase; MPO, Myeloperoxidase; ROS, Reactive oxygen species; PKC, Protein Kinase C; MAPK, Mitogen-activated protein kinase; PAD4, Peptidylarginine deiminase 4; GSDMD, Gasdermin D; TLR, Toll-like receptor; EC, Endothelial cell; NO, Nitric oxide; NLRP3, NOD-like receptor protein 3; IL, Interleukin; TNF, Tumor necrosis factor; LDL, low-density lipoprotein; CC, Cholesterol crystal; Th-17, T-helper 17; NF- $\kappa$ B, Nuclear Factor kappa-light-chain-enhancer of activated B cells.

endothelial dysfunction.<sup>51</sup> The interaction of polymorphonuclear neutrophils (PMNs) with ECs is prevented by an electrostatic repulsion between the glycocalyx of PMNs, which has a negative charge, and the vessel wall. The binding of MPO to glycosaminoglycans reduces the negative surface charge, resulting in PMN being electrostatically attracted to ECs and triggering the release of NETs.<sup>52</sup> The cathelicidin LL37, which is present in greater quantities on the chromatin fibers of NETs, can recruit leukocytes and monocytes in the ECs by activating the expression of adhesion molecules and chemokines. This enables it to function as an immunomodulator, enhancing innate immunity to atherosclerosis.<sup>53</sup> The various NET components exert direct cytotoxicity on ECs, recruit monocytes, and mediate pro-inflammatory responses and NET formation. Additionally, NETs can indirectly promote EC inflammation and atherosclerosis formation through pro-inflammatory factors such as NOD-like receptor protein 3 (NLRP3) inflammatory vesicles and interleukin (IL)-8.<sup>54</sup>

Endothelial-to-mesenchymal transition (EndMT) represents a process whereby ECs undergo a series of molecular changes, resulting in the formation of a mesenchymal-like cell phenotype.<sup>55</sup> It has been demonstrated that EndMT, induced by the inflammatory component of atherosclerosis, intensifies the inflammatory environment of atherosclerotic lesions, thereby facilitating the progressive expansion of atherosclerotic plaques.<sup>56</sup> The excessive release of NETs beyond their phagocytosis by ECs compromises the integrity of the endothelial monolayer, stimulates the development of vascular leakage through proteolytic hydrolysis of VE-cadherin, and activates  $\beta$ -catenin signaling, which in turn induces EndMT.<sup>57</sup>

## Effects of NETs on Platelets

In addition to their role in hemostasis and thrombosis, platelets act as immune cells to promote an inflammatory environment and play a role in the formation of atherosclerosis.<sup>58</sup> In response to alterations in the vascular microenvironment, platelets exhibit a rapid adhesion to damaged vessels, thereby maintaining vascular integrity. Additionally, they facilitate the migration of immune cells across the intima, which in turn accelerates the process of atherosclerosis.<sup>59</sup> Interactions between NETs and platelets have been demonstrated to be instrumental in the formation and progression of atherosclerotic plaques, which, in turn, increases the risk of adverse cardiovascular events. A comprehensive understanding of these interactions is imperative for the development of targeted therapeutic strategies aimed at mitigating the prothrombotic and inflammatory effects of NETs in the context of cardiovascular disease.

Following endothelial activation in the context of vascular injury or inflammatory conditions, platelets adhere to subendothelial molecules, thereby initiating platelet activation.<sup>60</sup> Activation of platelets has been demonstrated to facilitate the development of atherosclerosis through the production of platelet-derived extracellular vesicles.<sup>61</sup> The P-selectin glycoprotein ligand-1 receptor on neutrophils recognizes and binds to activated platelet-expressed P-selectin, thereby promoting the formation of NETs in mice.<sup>62</sup> Upon activation, platelets release soluble mediators, including von Willebrand Factor (vWF).<sup>60</sup> NETs interact with ECs and vWF to facilitate platelet adhesion and aggregation, fibrin formation, and serial immune thrombosis.<sup>15</sup> Histones in NETs induce platelet activation and accelerate thrombin production by interacting with TLR2 and TLR4, which in turn promote thrombin cascade reaction activation.<sup>63</sup> Additionally, histones facilitate thrombin generation by attenuating the activation of thrombomodulin-dependent protein C.<sup>64</sup> In addition to hemostasis, thrombin can also induce a variety of vascular proinflammatory reactions leading to the amplification of atherosclerosis.<sup>65</sup> NETs also engage platelets to enhance coagulation through the interaction of the cathelicidin LL37 with the glycoprotein VI receptor on the platelet surface.<sup>66</sup> Furthermore, DNA directly activates platelets and stimulates coagulation.<sup>67</sup> NETs-associated CG is also a physiological regulator of platelet activation and thrombosis in vivo, NETs-associated CG also accelerates platelet recruitment and vascular occlusion at the site of injury.<sup>68</sup> Furthermore, NE and CG can promote platelet activation and enhance coagulation activity by affecting platelet surface molecules and degrading coagulation inhibitors.<sup>69</sup>

It has been demonstrated that NETs serve to promote thrombus growth by virtue of their capacity to bind to platelets and erythrocytes.<sup>15</sup> NETs can promote thrombin production and accelerate thrombus formation by activating platelets and coagulation factors XI and XII.<sup>70</sup> In the case of atherosclerosis, the formation of blood clots leads to further blockages and an increased risk of myocardial infarction and stroke, among other complications.



## NETs-Induced Changes in the Vascular Inflammatory Microenvironment

Inflammation has an important role in the development of atherosclerosis. A variety of cells and cytokines, such as ECs, macrophages, neutrophils, vascular SMCs, ILs, matrix metalloproteinases (MMPs), adhesion molecules, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), are involved in the relevant inflammatory processes.<sup>71</sup> The TLR signaling pathway and the NLRP3 inflammatory vesicle pathway have been associated with atherosclerotic lesions.<sup>72</sup>

Macrophages secrete chemokines, cytokines, and MMPs, thereby playing a crucial role in the sustained local inflammatory response and plaque rupture.<sup>73</sup> Macrophages stimulated by highly oxidized LDL (oxLDL) adopt an inflammatory M4 phenotype, facilitating neutrophil recruitment and NET formation.<sup>74</sup> Additionally, macrophages can be pretreated with DNA enzymes and phagocytosed for the purpose of enhancing NET degradation, which is further enhanced by pro-inflammatory polarization.<sup>75</sup> Furthermore, MMP9, which is contained in NETs, damages ECs in atherosclerosis by activating endothelial MMP2, which leads to its dysfunction.<sup>76</sup> In co-culture with cholesterol crystals (CCs), NETs can enhance macrophage to release cytokines and activate T-helper 17 (Th-17) cells to produce IL-17, thereby promoting the recruitment of immune cells in atherosclerotic plaque and amplifying systemic inflammation.<sup>46</sup> Furthermore, it has been demonstrated that NETs inhibit autophagosome generation and autophagosome-lysosome fusion in macrophages through modulation of epidermal growth factor receptor activity, thereby accelerating inflammatory vesicle activity and promoting atherogenesis.<sup>77</sup>

It has been demonstrated that the activation of the NLRP3 inflammasome results in the enhanced recruitment of neutrophils, which subsequently leads to the formation of NETs within atherosclerotic plaques, and this process is associated with a heightened risk of accelerated atherosclerosis development.<sup>78</sup> NETs have been shown to activate NLRP3 inflammasomes through the TLR4/TLR9/ Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling pathway.<sup>79</sup> The maturation of the pro-inflammatory cytokine IL-18 is driven by inflammatory vesicles, which in turn initiate the formation of NETs.<sup>80</sup> The release of NETs by phorbol-12-myristate-13-acetate has been observed to directly regulate the TLR9/ NF- $\kappa$ B signaling cascade in macrophages, subsequently triggering the secretion of IL-8.<sup>81</sup> IL-8 binds to neutrophils via a protein-coupled chemokine receptor 2 and is dependent on Src family of tyrosine kinases and MAPK signaling pathways to form NETs and promote the progression of atherosclerosis in vivo.<sup>82</sup>

One of the components of NETs is a serine protease, which contains NE, proteinase 3 (PR3), and CG.<sup>54</sup> PR3 functions as a positively charged protein that, independent of its catalytic enzymes, activates TNF- $\alpha$ , IL-18, and IL-1 $\beta$ , thereby initiating a pro-inflammatory response.<sup>83</sup> CG stimulates the activation of platelets and other cells, which subsequently results in the onset of inflammation in the ECs. Moreover, it synergistically exacerbates inflammation and atherosclerosis in conjunction with NE.<sup>54</sup>

## NETs-Mediated Crosstalk with Lipid Metabolism

Imbalances in lipid metabolism are a critical factor in the development of atherosclerotic plaques.<sup>84</sup> The accumulation of cholesterol and lipids in the arterial wall is primarily derived from atherogenic-modified LDL, which results in the aggregation of lipoprotein particles, endothelial damage, leukocyte recruitment, foam cell formation, and inflammation.<sup>2</sup> Cholesterol, fatty acids, and modified lipids can directly activate inflammatory pathways, while inflammatory signals also influence lipid metabolism.<sup>85</sup>

The production of ROS and NET formation in human PMN is dependent on oxLDL and NADPH oxidase.<sup>86</sup> NET formation may be followed by amplification of oxidized and protein hydrolysis-modified LDL production via MPO, which further increases ox- and NET formation.<sup>87</sup> Compared to natural LDL, oxLDL has a reduced ability to enhance the proteolytic activity of platelet reactive protein type 1 motif (ADAMTS13) and competes with natural LDL, which in turn increases platelet adhesion to oversized vWF.<sup>88</sup> NET-associated PAD4-mediated citrullination also inhibits the proteolytic activity of ADAMTS13 and reduces vWF-platelet string clearance, which accelerates NET-mediated thrombosis.<sup>89</sup> The presence of NETs has been associated with an increased likelihood of LDL oxidation, aggregation, and foam cell formation.<sup>90</sup> oxLDL also regulates the expression and activity of MMPs in human coronary ECs through activation of lectin-like oxidized LDL lipoprotein receptor-1, which in turn promotes inflammatory responses.<sup>91</sup> Furthermore, NETs have been shown to directly interact with lipids, resulting in their deposition and clearance. NETs have been observed to capture and immobilise lipids, thereby preventing their normal circulation and promoting their deposition in the arterial

wall.<sup>92</sup> This process is further exacerbated by the pro-inflammatory cytokines released by activated macrophages in response to NETs, which have been shown to enhance the expression of scavenger receptors and promote the uptake of oxidised lipids by macrophages, leading to foam cell formation.<sup>93</sup>

The formation of CCs occurs at an early stage of atherosclerosis, where they induce inflammation by promoting the activation of inflammatory vesicles, which in turn leads to the secretion of IL-1 $\beta$ .<sup>94</sup> CCs function as an endogenous danger signal, inducing the expression of IL-33, which in turn triggers the formation of NETs by binding to IL-33 receptors on neutrophils and upregulating CCs function as an endogenous danger signal, inducing the expression of IL-33. This cytokine, in turn, triggers the formation of NETs by binding to IL-33 receptors on neutrophils and upregulating the expression of CD16 (Cluster of Differentiation 16).<sup>93</sup> Additionally, it has been demonstrated that CCs are necessary for the translocation of ROS, NE, and PR3 to the nucleus, thereby inducing NET release.<sup>46</sup>

## Interaction of NETs with Other Atherosclerotic Factors

During the development of atherosclerosis, SMCs play a role in the growth of necrotic cores and the thinning of fibrous caps, which can contribute to plaque instability. In addition, activated diseased SMCs attract neutrophils and trigger the ejection of nucleoprotein-containing NETs, which in turn contain histone H4 that interacts with the SMC plasma membrane and induces cell lysis and death, leading to plaque instability.<sup>18</sup> Furthermore, NETs promote the proliferation of vascular SMCs in rats via protein kinase B / Cyclin-dependent kinase inhibitor 1b / thymidine kinase 1 and are associated with the development of hypertension.<sup>95</sup>

One study observed that the ability of neutrophils isolated from patients with coronary artery disease to form ROS-dependent NETs is positively correlated with blood pressure levels and is mediated by the Angiotensin II (Ang II) receptor.<sup>96</sup> Hypertension has been demonstrated to result in the release of NETs within the arteries, with NETs-induced phenotypic alterations in vascular SMCs identified as a causative factor in the development of hypertension.<sup>95</sup> Ang II has been demonstrated to enhance the proliferation of vascular SMCs and is involved in the pathogenesis of hypertension.<sup>97</sup> Neutrophils activated by the environment of essential hypertension expose active tissue factor (TF) via NETs, and the NETs/TF/thrombin axis can further amplify the prothrombotic state of essential hypertension.<sup>98</sup> Ang II induces the release of in vitro, NETs are released via the ROS/PAD4 and autophagy-dependent pathways. In patients who commenced treatment with an Ang II receptor blocker, circulating NETs, and thrombin generation levels were significantly reduced in patients with essential hypertension.<sup>98</sup>

The formation of NETs is dependent on glucose,<sup>99</sup> and in the diabetic setting, neutrophils can increase PAD4 protein expression and basal calcium levels to participate in the formation of NETs at multiple levels.<sup>100</sup> Diabetes mellitus is a risk factor for atherosclerosis, and studies involving diabetic patients have demonstrated an increased risk and accelerated progression of atherosclerosis.<sup>101</sup>

During the process of arterial aging, there is an increase in both Ang II signaling and MMP2 activity, which subsequently leads to an increase in the expression of fibronectin and collagen in the aging aortic wall.<sup>102</sup> Chronic low-grade inflammation that develops with age is a principal factor in the development of age-related diseases such as atherosclerosis. This is typified by aberrant activation of the innate immune system, augmented expression of pro-inflammatory cytokines (eg, IL-6, IL-1 $\beta$ ), and activation of immune complexes (NLRP3 inflammatory vesicles).<sup>103</sup> Pro-inflammatory cytokines and NLRP3 inflammatory vesicles are involved in the formation of NETs. The promotion of inflammation by aging is accompanied by an increase in the prevalence of NET release in aged mice.<sup>104</sup>

## Targeting NETs in Atherosclerosis

In clinical practice, lipid-lowering drugs, especially statins, are the cornerstone of atherosclerosis prevention. A multicentre cohort study demonstrated that levels of NETs were positively associated with plaque vulnerability index in a statin-naïve subgroup (odds ratio = 2.08, 95% confidence interval 1.04–4.17), whereas no significant association was found in a statin-treated population (odds ratio = 1.10, 95% confidence interval 0.68–1.79).<sup>39</sup> Statins have been demonstrated to exert anti-inflammatory, antioxidant, and antithrombotic effects by reducing NETs formation through the inhibition of PAD4 and MPO.<sup>92</sup>

Given the important role of NETs in the development of atherosclerosis, approaches targeting NETs are emerging therapeutic avenues. Disulfiram, the first Food and Drug Administration (FDA)-approved compound to block NET formation, is a potent inhibitor of GSDMD pore formation, prevents the release of IL-1 $\beta$ ,<sup>105</sup> and inhibits NET release.<sup>106</sup> PAD4 mediates histone citrullination and contributes to chromatin decondensation, and deletion of PAD4 in mice reduces NET formation and significantly reduces atherosclerotic burden.<sup>107,108</sup> It has been shown that PAD4 can represent the presence of NETs, and that elevation decreases plaque stability in patients with carotid stenosis.<sup>109</sup> In a myocardial infarction model, pharmacological treatment of mice with the PAD inhibitor Cl-amidine resulted in the elimination of NET formation, a reduction in arterial thrombosis, and a limitation of injury.<sup>110</sup> The binding of novel selective PAD4 inhibitors, GSK484 and GSK199, to calcium-deficient PAD4 enzymes, provides confirmation of the pivotal role of PAD4 enzymes in histone citrullination and NET formation in humans and mice.<sup>111</sup> GSK484 administration reduced the accumulation of NETs at the site of endothelial injury and preserved endothelial continuity.<sup>112</sup> However, no drugs targeting PAD4 are currently approved for use in humans. The serum nucleic acid endonuclease DNase 1 degrades NETs and reduces intimal damage in mouse models of atherosclerosis.<sup>113</sup> DNase and the anticoagulant heparin have been shown to degrade NET scaffolds, separate NET-platelet aggregates, and prevent thrombus formation in an experimental setting.<sup>15</sup> FDA-approved inhaled DNase 1 formulations can digest formed NETs but have little use outside the airway.<sup>114</sup> Colchicine is an anti-inflammatory agent that binds to microtubule proteins to affect the expression of inflammatory vesicles and ILs and disrupts NET formation, in addition, colchicine may interfere with neutrophil-platelet interactions to affect atherosclerotic thrombosis.<sup>115</sup> In addition, the novel histone peptidic inhibitor (Cyclical Histone H2A Interference Peptide) also reduces the progression of atherosclerosis by binding to NET-resident histone H2A, thereby blocking monocyte adhesion.<sup>116</sup> A plethora of research has been conducted on the potential of pharmaceuticals to impede the process of NET formation. These studies have explored various pathways associated with NETs. However, certain inhibitors, such as disulfiram, have been observed to demonstrate a lack of specificity for NETs. Consequently, there is a necessity for further research to be conducted in order to identify drugs that can selectively inhibit NET formation. This would facilitate the development of targeted therapeutic interventions for the management of cardiovascular manifestations associated with NETs.<sup>117</sup>

## Conclusions

The study of NETs has received considerable attention. Atherosclerosis-related risk factors, such as dyslipidemia and hypertension, stimulate the formation of NETs. At the same time, many studies have demonstrated that NETs play an important role in the initiation and development of atherosclerosis. NETs may promote the inflammatory milieu and participate in the formation and instability of atherosclerotic plaque by affecting vascular ECs, platelets, and other multiple effects. NETs offer novel insights and avenues for advancing our comprehension of the genesis and modulation of the vascular inflammatory microenvironment in atherosclerosis and may represent promising novel therapeutic targets in this field. Presently there are only two FDA-approved compounds that target NETs, and as our understanding of the formation of NETs and the molecular mechanisms of NETs in disease continues to improve, the development of more specific NET-targeting drugs will be possible. However, further efforts are needed to bring them into clinical practice, such as standardization of the NET detection protocol. Under certain circumstances, NETs may have protective or adverse effects. Therefore, clinical evaluation and further molecular biology research are still needed. Notwithstanding these challenges, it is anticipated that the study of NETs will offer novel insights into the diagnostic and therapeutic approaches for atherosclerotic diseases and other cardiovascular disorders.

## Abbreviations

SMCs, Smooth muscle cells; NETs, Neutrophil extracellular traps; NE, Neutrophil elastase; MPO, Myeloperoxidase; CG, Cathepsin G; ROS: Reactive oxygen species; MAPK, Mitogen-activated protein kinase; NADPH oxidase, Nicotinamide Adenine Dinucleotide Phosphate Oxidase; PAD4, Peptidylarginine deiminase 4; GSDMD, Gasdermin D; TLR: Toll-like receptor; dsDNA, Double-stranded DNA; ECs, Endothelial cells; NO, Nitric oxide; eNOS, Endothelial nitric oxide synthase; PMN, Polymorphonuclear neutrophils; NLRP3, NOD-like receptor protein 3; IL, Interleukin; EndMT, Endothelial-to-mesenchymal transition; vWF, von Willebrand Factor; MMPs, Matrix metalloproteinases; TNF, Tumor



necrosis factor; oxLDL, Oxidized low-density lipoprotein; PR3, Proteinase 3; CCs, Cholesterol crystals; Th-17, T-helper 17; NF- $\kappa$ B, Nuclear Factor kappa-light-chain-enhancer of activated B cells; CD16, Cluster of Differentiation 16; Ang II, Angiotensin II; TF, Tissue factor; FDA, Food and Drug Administration.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no conflicts of interest.

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