

# Platelet–Neutrophil Crosstalk in Atherothrombosis

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## Abstract

Atherothrombosis is a frequent cause of cardiovascular mortality. It is mostly triggered by plaque rupture and exposure of the thrombogenic subendothelial matrix, which initiates platelet aggregation and clot formation. Current antithrombotic strategies, however, target both thrombosis and physiological hemostasis and thereby increase bleeding risk. Thus, there is an unmet clinical need for optimized therapies. Neutrophil activation and consecutive interactions of neutrophils and platelets contribute mechanistically to thromboinflammation and arterial thrombosis, and thus present a potential therapeutic target. Platelet–neutrophil interactions are mediated through adhesion molecules such as P-selectin and P-selectin glycoprotein ligand 1 as well as glycoprotein Ib and macrophage-1 antigen, which mediate physical cell interactions and intracellular signaling. Release of soluble mediators as well as direct signaling between platelets and neutrophils lead to their reciprocal activation and neutrophil release of extracellular traps, scaffolds of condensed chromatin that play a prothrombotic role in atherothrombosis. In this article, we review the role of neutrophils and neutrophil-derived prothrombotic molecules in platelet activation and atherothrombosis, and highlight potential therapeutic targets.

## Keywords

- arterial thrombosis
- atherothrombosis
- thrombo-inflammation
- platelet–leukocyte interaction

## Introduction

Cardiovascular diseases are the most common cause of death in developed countries. About 80% of cardiovascular deaths are caused by myocardial infarction and stroke following rupture of an atherosclerotic plaque and subsequent arterial occlusion. While an increased understanding of the mechanisms underlying these conditions has resulted in the development of novel antithrombotic strategies that reduced cardiovascular death, for example, thienopyridines, mortality has remained on a significant level. The relevance of these conditions is fueled by the increasing prevalence of risk factors for atherosclerosis in developing countries, such as diabetes and adipositas. Therefore, there is an unmet clinical and socioeconomical need for new treatment strategies. This article highlights recent find-

ings on novel therapeutic targets in atherothrombosis with focus on neutrophil-derived activators of platelets and thrombus formation.

## Established Pathophysiological Aspects and Current Therapeutic Strategies

In atherosclerosis, sustained inflammation drives formation of atheromatous plaques. The latter are typically composed of lipids, leukocytes, smooth muscle cells, and covered by a fibrous cap.<sup>1–4</sup> Eventually plaque rupture triggers arterial thrombosis, which manifests clinically as myocardial infarction, ischemic stroke, or limb ischemia in patients with peripheral artery disease. Mechanistically, highly thrombogenic plaque content

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becomes exposed to the blood stream and components such as collagen and von Willebrand factor (vWF) then mediate platelet adhesion and activation (**►Fig. 1A**).<sup>5–7</sup> Activated platelets release adenosine diphosphate, serotonin, and thromboxane A2 (TxA2), which amplify activation and induce platelet aggregate formation. Additionally, P-selectin is highly upregulated on the surface membrane of platelets to mediate interactions with leukocytes and endothelial cells.<sup>8</sup> Simultaneously, plaque-released tissue factor activates the coagulation cascade and triggers thrombin generation and subsequent conversion of fibrinogen into insoluble fibrin.<sup>9,10</sup> Thrombin stabilizes the forming clot by amplifying blood coagulation and by activating platelets which further propagates coagulation.<sup>11</sup>

In addition to urgent mechanical revascularization of the obstructed vessel to restore blood flow, current pharmacological strategies for the treatment of acute atherothrombotic events include platelet inhibition and anticoagulation (**►Fig. 1A**). Current guidelines recommend intravenous administration of unfractionated heparin in the acute setting and platelet inhibition with aspirin, P2Y12 inhibitors, and, as bailout therapy, glycoprotein (GP) IIb/IIIa inhibitors.<sup>12,13</sup> Yet, all antiplatelet drugs as well as anticoagulants are associated with an increased risk of bleeding, which underlines the necessity for further research into antithrombotic strategies.<sup>14</sup>

## **Neutrophil Activation and Platelet–Neutrophil Interactions in Atherothrombosis**

Neutrophils play a role in different pathophysiological processes of atherosclerosis.<sup>15–19</sup> They support the development of early atherosclerotic lesions,<sup>15</sup> but have also been associated with features of plaque rupture.<sup>16</sup> Upon plaque rupture, together with platelets, neutrophils rapidly accumulate at sites of injury, and can induce and amplify platelet activation and blood coagulation<sup>20–25</sup> (**►Fig. 1A**). Notably, neutrophils represent the most abundant leukocyte subset also in arterial thrombi of human patients with myocardial infarction.<sup>26</sup> Neutrophils are stimulated by inflammatory cytokines such as interleukin-1 $\beta$  or tumor necrosis factor- $\alpha$ , which are elevated in plasma in atherosclerosis.<sup>1,27</sup> Activated neutrophils express adhesion molecules on their surface, which promote binding to platelets and the endothelium.<sup>24</sup> Vice versa, also activated platelets express adhesion molecules on their surface membrane, such as P-selectin, which mediate mechanical interactions with neutrophils.<sup>28</sup> Indeed, Sreeramkumar et al showed that neutrophils scan for activated platelets and foster their recruitment to inflamed or injured vessel wall thereby initiating and promoting inflammation.<sup>29</sup> It is therefore not surprising that circulating platelet–neutrophil aggregates represent an independent predictor of atherothrombotic events.<sup>24,30–32</sup> However, it is unclear whether these aggregates are a result of systemic inflammation during development of atherosclerotic lesions.

On the molecular level, platelet–neutrophil interactions lead to reciprocal activation of either cell type (**►Fig. 1B**). Several studies have described how platelets affect leukocyte functions and recruitment.<sup>33,34</sup> Platelets have a repertoire of

cytokines and chemokines such as CCL5 (RANTES) and CXCL7, that are stored in their granules (mainly  $\alpha$ -granules) and are able not only to recruit but also to activate neutrophils.<sup>35,36</sup> Additionally, platelet-derived microparticles and extracellular vesicles can transport and provide neutrophil activating molecules.<sup>37</sup> Moreover, fragments of dying platelets foster neutrophil aggregation after ischemia reperfusion injury.<sup>38</sup> Antiplatelet therapy in acute atherothrombosis effectively inhibits platelet aggregation and inhibiting platelet activation reduces the release of neutrophil-activating substances from  $\alpha$ -granules.<sup>8</sup> In a mouse model of acute lung injury, where platelet–neutrophil interactions play a key mechanistic role, platelet depletion as well as aspirin therapy reduced neutrophil infiltration and provided protective effects.<sup>39–41</sup> This aspect was recently confirmed in human models of acute respiratory distress syndrome.<sup>42</sup> Thus, platelet–neutrophils interactions play a role in the wider context of thromboinflammation, and their inhibition could represent an interesting concept in these conditions.

Less is known about the effects of neutrophils on platelets as a result of aforementioned interactions. Activated neutrophils can combat microbes by releasing antimicrobial molecules (by exocytosis), phagocytosis of microbes (which are destroyed intracellularly by reactive oxygen species [ROS] and antimicrobial proteins), and by formation of neutrophils extracellular traps (NETs).<sup>43</sup> These mechanisms could differentially influence different aspects of atherothrombosis including platelet function. Specific targeting of leukocyte-derived platelet activating agents could represent an alternative approach in atherothrombosis and may have reduced hemorrhagic side effects.

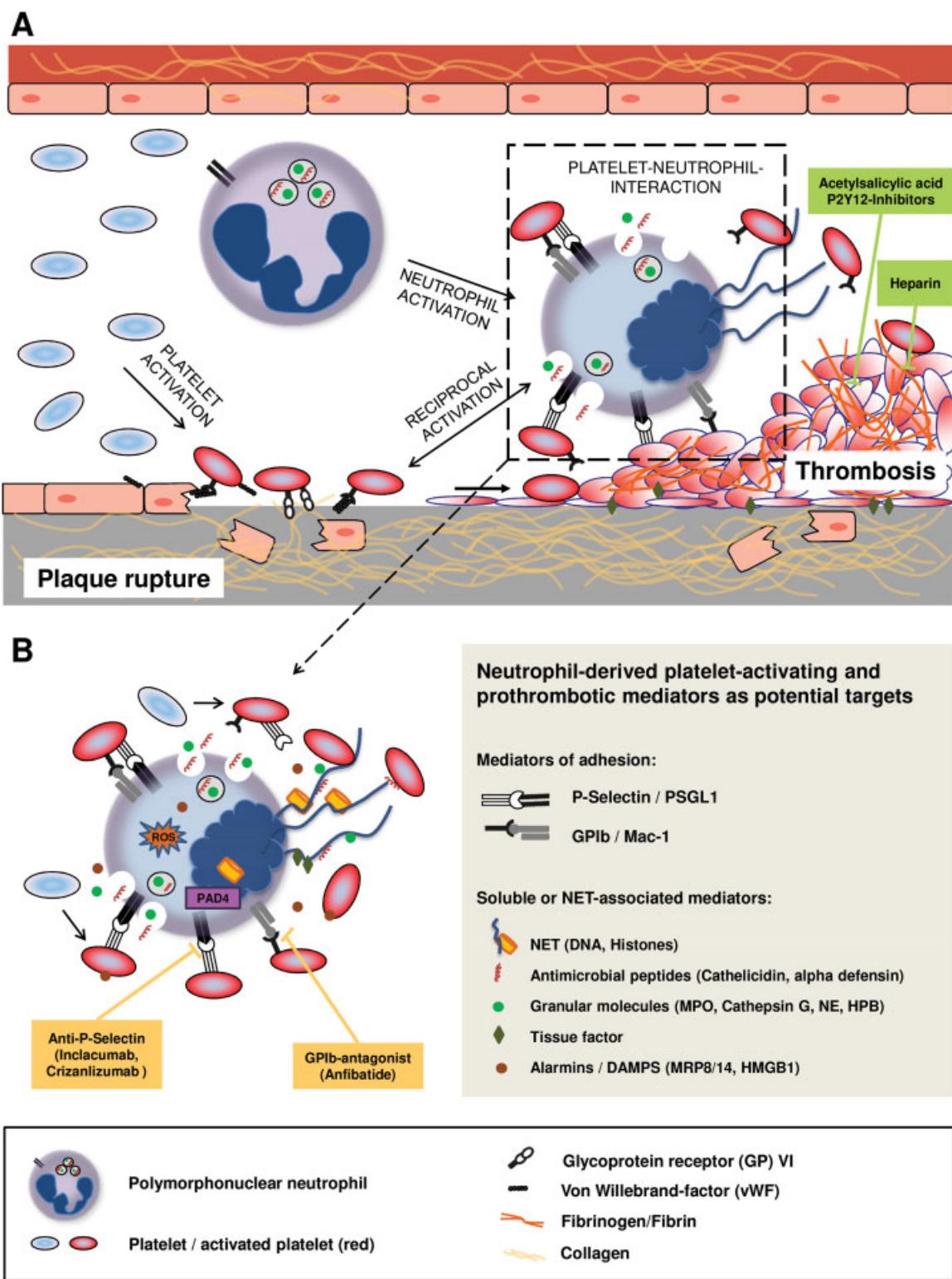
## **Platelet Activation through Physical Interaction with Neutrophils**

To prevent or reduce platelet–neutrophil interactions and their consequences, targeting the adhesion molecules that mediate the physical interactions between the two cells would be the most straightforward approach. A therapeutic challenge is the fact that most adhesion molecules do not have a single respective ligand but share several counter-receptors including those expressed on other cell types.

### **P-Selectin and P-Selectin Glycoprotein Ligand 1**

Binding of platelet P-selectin and neutrophil P-selectin glycoprotein ligand 1 (PSGL-1) might constitute the most important physical interaction between platelets and neutrophils.<sup>44</sup> P-selectin is stored in platelet  $\alpha$ -granules with little surface expression under resting conditions and is highly upregulated on the surface membrane when platelets become activated.<sup>8</sup>

While PSGL-1-induced activation of neutrophils via downstream signaling of tyrosine kinases and consecutive integrin activation is well described,<sup>45,46</sup> less is known about platelet activation after binding of P-selectin. Recombinant PSGL-1 induced integrin activation on platelets in a P-selectin-dependent manner and increased platelet aggregation-driven large artery thrombosis *in vivo*.<sup>47</sup> Further, disruption



**Fig. 1** (A) Upon rupture of an atherosclerotic plaque, thrombogenic proteins of the subendothelial matrix are exposed to the blood stream and initiate thrombus formation. Together with platelets, neutrophils rapidly accumulate at sites of injury, become activated, and can induce and amplify platelet activation and blood coagulation. Current pharmacological approaches include anticoagulation and platelet inhibition (green boxes). (B) Upon activation, platelets and neutrophils interact through adhesion molecules and soluble mediators and thereby reciprocally amplify activatory signals. The illustration depicts adhesion molecules and neutrophil-derived mediators that play a role in platelet activation and atherothrombosis and constitute potential therapeutic targets (gray box). Targets that have already been tested in first clinical trials are highlighted (orange boxes). DAMP, damage-associated molecular pattern; GP1b, glycoprotein 1b; HMGB1, high-mobility group box 1; HPB, heparin-binding protein; MPO, myeloperoxidase; MRP8/14, myeloid-related protein 8 and 14 (calprotectin, S100A8/A9); NE, neutrophil elastase; NET, neutrophil extracellular traps; PSGL-1, P-selectin glycoprotein ligand 1; vWF, von Willebrand-factor.

of P-selectin-PSGL-1-binding reduced fibrin incorporation upon laser injury-induced thrombosis in arterioles.<sup>48,49</sup> First clinical trials targeting platelet–leukocyte interactions using monoclonal antibodies against P-selectin have been initiated and proven safe.<sup>50</sup> Crizanlizumab lowered the rate of pain crisis in patients with sickle cell disease<sup>51</sup> and the monoclonal P-selectin antibody inclacumab reduced myocardial damage after percutaneous coronary intervention in patients with non-ST-segment elevation myocardial infarction.<sup>52,53</sup> In mice, deficiency in PSGL-1 improved outcome in thromboinflammation models such as acute lung injury and ischemic stroke.<sup>29</sup> Targeting P-selectin seems promising also in atherothrombosis as treatment of mice with anti-P-selectin antibodies has been shown to directly reduce thrombus size in a mouse model of ferric chloride-induced arterial thrombosis.<sup>54</sup>

### GPIb and Macrophage-1 Antigen

Platelet GPIb receptor is constitutively active for ligand binding and represents an important mediator of platelet–neutrophil interactions. Interestingly, platelet-derived protein disulfide isomerase (PDI) regulates the binding function of GPIbα by reducing its disulfide bonds. Consequently, inhibition or genetic absence of PDI abrogated platelet–neutrophil interactions and inhibited vascular occlusion under thromboinflammatory conditions.<sup>55</sup> Among various potential partners, GPIb binds to the β-2 integrin receptor macrophage-1 antigen (Mac-1) on neutrophils to form platelet–neutrophil aggregates.<sup>7,24,29</sup> Mac-1 deficient mice were protected from thrombosis after glomerular injury<sup>56</sup> and show delayed thrombosis after carotid artery and cremaster microvascular injury.<sup>57</sup> These effects could also be observed by targeting Mac1–GPIb interaction with antibodies and the small-molecule inhibitor glucosamine. Importantly, no effects on hemostasis parameters were observed, making Mac-1 an interesting therapeutic target.<sup>57</sup> Besides binding to GPIb, neutrophil Mac-1 has also been described to bind platelet GPIIb/IIIa-integrin via bridging of soluble fibrinogen<sup>7,58</sup> and might contribute to platelet activation through outside-in signaling.<sup>59</sup> Further, microparticles derived from activated neutrophils express an active form of Mac-1 that can induce P-selectin surface expression and integrin activation in platelets via GPIb-mediated Akt phosphorylation.<sup>60</sup>

Direct targeting of GPIb could represent another perspective for treatment of atherothrombosis. Such approach would not only affect platelet–neutrophil crosstalk but simultaneously inhibit GPIb interaction with vWF, which is known to contribute to atherothrombotic events.<sup>5,6</sup> Several clinical trials with therapeutics targeting the GPIb–vWF axis have been made.<sup>61</sup> The humanized anti-vWF bivalent nanobody caplacizumab (ALX-0081) has already undergone first clinical tests with promising results.<sup>62,63</sup> Similarly, the GPIb antagonist anfibatide reduced thrombus formation in mice without prolonging bleeding time<sup>64</sup> and safety and efficacy trials in humans are ongoing (ClinicalTrials.gov Identifier: NCT01585259, NCT02495012).

Platelet–neutrophil interaction is promoted and stabilized by several other ligand–receptor interactions, such as

GPVI and extracellular matrix metalloproteinase inducer (EMMPRIN) or intercellular adhesion molecule 2 (ICAM-2) and lymphocyte function-associated antigen 1 (LFA-1).<sup>7,24,65</sup> While EMMPRIN–GPVI has been investigated in the context of platelet–monocyte interactions,<sup>65</sup> little is known about its role in platelet–neutrophil crosstalk. Binding of platelet ICAM-2 with neutrophil LFA-1 can mediate platelet–neutrophil adhesion under flow,<sup>66</sup> however, the physiological relevance for platelet and neutrophil function is not yet understood.

### Platelet Activation through Soluble Neutrophil-Derived Mediators

Neutrophil granules store a broad repertoire of molecules, which upon activation are released or translocated to the surface.<sup>43</sup> In the context of atherothrombosis, these molecules will come in contact with platelets and coagulation factors and might differentially affect thrombus formation.

Neutrophil-derived peptides such human α-defensins (HNP 1–3) or heparin-binding protein have been shown to affect fibrinogen binding to platelets resulting in platelet aggregation and activation of the coagulation system.<sup>67–70</sup> In contrast, myeloperoxidase (MPO) has been described to induce weak activation of platelets without inducing aggregation, which primes platelets by potentiating agonist-induced platelet aggregation.<sup>71,72</sup> The neutrophil granular peptide cathepsin G has been shown to activate platelets in vitro<sup>73</sup> and arterial thrombosis in vivo,<sup>67</sup> which was explained by proteolysis of the coagulation suppressor tissue factor pathway inhibitor (TFPI)<sup>20</sup> and activation of factor XII (FXII).<sup>74</sup> Neutrophil elastase apart from cleaving TFPI also enhances coagulation by cleavage of another anticoagulant, antithrombin,<sup>75</sup> and degradation of proteoglycans in the arterial media, thereby exposing collagen.

Another abundant neutrophil-derived protein is S100A8/A9 (MRP8/14), which can also be secreted by platelets.<sup>76</sup> It increases neutrophil recruitment<sup>77</sup> as well as platelet activation and thrombosis in photochemical injury of the carotid artery in mice.<sup>76</sup> MRP8/14 is elevated in early steps of myocardial infarction and could again be a target to treat the acute atherothrombotic event as well as consecutive inflammation.<sup>78</sup>

Recently, we detected neutrophil-derived cathelicidins, a family of antimicrobial peptides, in thrombi from coronary arteries in acute myocardial infarction and demonstrated a functional role in platelet activation and arterial thrombosis which is in part mediated by reciprocal activation of neutrophils and platelets and by consecutive formation of NETs. Interestingly, these effects seem less dependent on classical cathelicidin receptors but rather are mediated by functional GPVI.<sup>25</sup> We are currently investigating whether other peptides with similar properties could play a role in atherothrombosis.

Upon interaction with platelets, neutrophils have been shown to release extracellular vesicles containing arachidonic acid which can be internalized into platelets and serve as a substrate for cyclooxygenase 1, an enzyme that synthesizes

TxA2.<sup>79</sup> TxA2 is strong paracrine amplifier of platelet aggregation and endothelial activation.<sup>80</sup>

### NETs in Arterial Thrombosis

NET release (or NETosis) was first described by Brinkmann et al as an active form of cell death that releases a scaffold of condensed chromatin and antimicrobial proteins.<sup>81</sup> However, NET formation is clearly distinct to apoptosis and necrosis.<sup>82</sup> Neutrophils undergo histone citrullination by an enzyme called protein arginine deiminase 4 (PAD4).<sup>83</sup> In detail, PAD4 converts arginyl residues in histones (particularly H3 and H4) to citrulline which releases the ionic bonds that usually constrain nuclear deoxyribonucleic acid (DNA) to nucleosomes. This leads to dissociation of linking histones and heterochromatin from the nucleosome structure,<sup>84,85</sup> and eventually the nuclear chromatin network is released into the extracellular space and the surrounding tissue.<sup>81,82</sup> ROS and neutrophil granular enzymes significantly aid this process.<sup>86</sup> Several neutrophil activating agents triggering NET formation have been identified; however, differences in the kinetics may occur.<sup>87</sup> More rapid formation of NETs has been described when neutrophils are exposed to Gram-positive bacteria suggesting involvement of other so far not well-known mechanisms.<sup>88</sup>

While NETs are protective in infectious conditions, in recent years they have been shown to be involved in the pathology of various inflammatory diseases including atherothrombosis.<sup>19,87,89</sup> NETs have been detected in the carotid artery in apolipoprotein E-deficient mice on high-fat diet<sup>90</sup> and are clinically associated with coronary atherosclerosis.<sup>91,92</sup> Further, NETs have been found within arterial thrombi of mice and humans in acute myocardial infarction.<sup>18,25,26,93</sup> The majority of neutrophils in arterial thrombi stained positive for citrullinated histone 3,<sup>25</sup> a marker for neutrophil priming toward NET formation (NETosis),<sup>83</sup> indicating a highly activated state of neutrophils in arterial thrombosis. It has been shown that inhibition of the enzyme PAD4 (which is required for NET formation) with Cl-amidine, which abrogated NET formation, reduced atherosclerosis burden and arterial thrombosis in mice, and further limited injury in a model of myocardial infarction.<sup>23,26</sup> Genetic deficiency of PAD4 decreased deep vein thrombosis<sup>22,94</sup> and reduced acute thrombotic complications of intimal atherosclerotic lesions in mice.<sup>95</sup> It has been intensively studied how or which components of NETs are responsible for its prothrombotic effects.

### Prothrombotic and Platelet Activating Properties of NETs

NETs are composed of a core DNA element, to which histones and neutrophil granular proteins (e.g., lactoferrin, cathepsins, cathelicidins, MPO, and neutrophil elastase) are attached.<sup>43</sup> NETs can serve as mechanical scaffolds for the entrapment and aggregation of platelets and erythrocytes in thrombosis, but additionally bind plasma proteins like fibrinogen, fibronectin, and vWF and thereby can stabilize

clots.<sup>22</sup> Theoretically, however, NET components can separately participate in atherothrombosis and contribute to the activation of platelets, endothelial cells, or the coagulation system, and not least, different types of leukocytes. Negatively charged nucleic acids can activate the contact-dependent coagulation pathway in vitro, which surprisingly was not observed by entire NETs.<sup>96</sup> However, whether the DNA backbone itself contributes to atherothrombosis in vivo is not fully understood. Even though DNase treatment did not attenuate arterial thrombus formation in healthy mice,<sup>97</sup> arterial thrombosis was impaired in mouse models of lupus and atherosclerosis, where animals showed generally more NET formation.<sup>23,98</sup> Histones have been attributed a key role in recruiting platelets and promoting their activation.<sup>22</sup> Histones have been described to induce platelet aggregation, P-selectin expression, phosphatidylserine exposure, and enhanced cell surface binding of FV/Va, which were in part mediated by Toll-like receptor 2 (TLR2) and TLR4.<sup>99</sup> Further, histones enhanced coagulation by inducing prothrombinase activity<sup>99</sup> (at least to some extent) by inhibition of thrombomodulin-dependent generation of the anticoagulant protein C<sup>100</sup> and induced the endothelial release of vWF.<sup>101</sup> Infusion of antihistone antibody (anti-H2A-H2B-DNA) leads to prolonged time to occlusion and lower thrombus stability upon injury of carotid arteries in wild-type mice.<sup>20</sup> In line with this, the presence of NETs was associated with an increased thrombus stability and reduced thrombus resolution in an ex vivo study of human thrombus material.<sup>102</sup>

Additionally to the NET core structure itself, neutrophil-derived molecules presented on NETs could amplify thrombosis involving mechanisms described above. However, NETs might also represent a scaffold for other mediators of thrombosis. Tissue factor represents a highly prothrombotic molecule associating with NETs in venous as well as in arterial thrombi.<sup>93,103</sup> Hematopoietic or intravascular tissue factor plays a critical role in deep vein thrombosis.<sup>104</sup> Though monocytes are likely the major source of intravascular tissue factor, macrovascular thrombosis is also driven by vessel wall-derived tissue factor.<sup>105</sup> Moreover, neutrophils themselves may deliver tissue factor to NETs and thereby stimulate thrombin generation and platelet activation.<sup>106</sup> Nonetheless, the main function of neutrophils and their NETs in coagulation-dependent thrombosis lies in their ability to propagate the thrombotic process.<sup>107</sup>

Another protein which is not predominantly released by neutrophils but interacting with NETs is high-mobility group box 1 (HMGB1). HMGB1 induces NET formation in vitro<sup>108,109</sup> and also contributes to NETs in arterial thrombi in vivo.<sup>110</sup> HMGB1 is a chromatin-binding nuclear protein that stabilizes nucleosomes and stimulates gene transcription.<sup>111</sup> When HMGB1 is released by dying cells or actively secreted by stressed cells, it acts as a damage-associated molecular pattern molecule and shows strong proinflammatory properties in several diseases.<sup>112</sup> HMGB1 has also been found in arterial thrombi of mice with platelets being the major source<sup>110</sup> and is associated with activated platelets in patients with coronary artery disease.<sup>113</sup> HMGB1 induced platelet activation via TLR4 and MyD88-dependent

signaling.<sup>110</sup> Mice deficient in platelet HMGB1 display decreased arterial thrombus formation with prolonged bleeding time but no changes in coagulation parameters.<sup>110</sup>

Taken together, NETs seem to play an important role in atherothrombosis, in part through their composition of nucleic acids and histones as their backbone, serving as scaffolds for cells and plasma proteins but also by their high potential to expose the aforementioned inflammatory and prothrombotic molecules. Therefore, targeting NET generation might represent a powerful tool to treat acute atherothrombosis.

Mutations in the hematopoietic lineage leading to clonal hematopoiesis (CH) represent an emerging field of research in the context of atherosclerosis and thrombosis, and seem to affect both neutrophil and platelet biology. Somatic mutations are common in blood cells and increase with age, that is, the frequency is > 10% of people aged > 70.<sup>114</sup> They result in loss of function in epigenetic modifiers (e.g., *DNMT3A* or *TET2*) or increased hematopoietic signaling (*JAK2*), driving the clonal expansion of hematopoietic stem cells in a process termed CH. Notably, carriers of CH not only exhibit an increased risk for hematologic malignancies but are also prone to develop atherosclerotic cardiovascular disease. Thus, CH represents an important nontraditional risk factor underlying myocardial infarction and stroke.<sup>115</sup> Importantly, in a population study of 10,893 individuals without a known myeloid disorder, *JAK2V617F* positive CH was associated with higher incidence of thrombosis.<sup>116</sup> In transgenic mice expressing *Jak2<sup>V617F</sup>*, platelet reactivity to agonists was enhanced.<sup>117</sup> Further, neutrophils expressed higher levels of peptidylarginine deiminase 4, which catalyzes citrullination of histones, and displayed increased NET formation.<sup>116</sup> These findings suggest that both platelets and neutrophils are players in CH-associated thrombosis. Future work will have to determine in more detail the function and interactions of platelets and neutrophils in atherothrombosis in patients harboring CH-associated mutations.

## Conclusion

Aspects of acute inflammation are intriguingly linked with atherothrombosis and may provide several therapeutic targets in the future. To date, bleeding represents the most relevant side effect of all current pharmacological treatments. Targeting of platelet–neutrophil crosstalk, neutrophil-derived soluble, or surface bound mediators seems to have less impact on hemostasis than established antithrombotic therapies. While anti-inflammatory therapeutic strategies can also be associated with side effects as shown for long-term treatment with canakinumab,<sup>27</sup> short-term inhibition of inflammation in acute thrombosis is likely to be less problematic from an immunologic perspective. Undoubtedly, new avenues of research in platelet biology and immunothrombosis are driving the identification of novel therapeutic targets and their translation into clinical practice.

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## Conflict of Interest

None declared.

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