NETs in the infarct-related coronary artery – a marker or mediator of adverse outcome?

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In response to cell death or activation, neutrophils extrude modified chromatin, with web-like structure, called neutrophil extracellular traps (NETs).(1) This involves citrullination of the histone tail of positively charged arginine residues by the calcium-dependent peptidyl-arginine deiminase 4 (PAD4), with subsequent nuclear delobulation and breakdown of the nuclear envelope, chromatin decondensation and plasma membrane rupture.(2,3) This process is physiologically beneficial when released during an infection, since NETs can capture and kill bacteria. Yet, excessive NETs formation or reduced NETs breakdown by deoxyribonucleases (DNase) may be harmful and enhance atherothrombosis mechanistically and clinically, in a range of scenarios.(4–7)

Laboratory studies indicate that NETs exhibit potent prothrombotic and proinflammatory properties.(2) The DNA skeleton of NETs can attract and bind circulating proteins, acting as a scaffold for occlusive thrombus formation.(8) Such neutrophil-derived proteins include myeloperoxidase (MPO), serine proteinases such as cathepsin G and neutrophil elastase, and interleukin-1α. NETs also recruit and express non-neutrophil derived proteins, including functional tissue factor, as well as factor XII, histones H3 and H4, and fibrinogen, all of which favour thrombin generation and coagulation.(9) Extracellular NET histones bind von Willebrand factor and fibrin to recruit platelets and erythrocytes, also cause platelet activation via toll-like receptors. In turn, activated platelets induce NETs formation. Furthermore, NETs impair endogenous fibrinolysis, with NET-bound neutrophil elastase cleaving tissue factor pathway inhibitor (TFPI), a factor that inhibits coagulation(10) and histones impair thrombomodulin-dependent protein C activation,(11) resulting in prolongation of clot lysis and enhanced clot stability.(12)

In the current issue of *Thrombosis and Haemostasis*, Blasco and colleagues report on the analysis of coronary thrombi aspirated from 406 patients with ST-elevation myocardial infarction (STEMI) undergoing primary percutaneous coronary intervention (pPCI).(13) Thrombus aspiration was performed for clinical indication, favouring the inclusion of those with large thrombus burden at angiography (62% of all pPCI). NETs were detected in 51% of aspirates, based on the colocalization of antibodies to citrullinated histone 3 (citH3) and MPO (i.e. double positivity). Over a median follow-up of 47 months, the presence of NETs was strongly associated with the occurrence of major adverse cardiovascular events (MACE) in the first 30 days after infarction (HR 2.82; 95% CI 1.26–6.35, p=.012), mainly driven by cardiac death and stent thrombosis, especially in the first 7 days (HR 3.23; 95% CI 1.18–8.82, p=.022). The individual components of MACE associated with NETs were reinfarction (OR 2.28; 95% CI 1.08–4.81, p=.03) and urgent revascularization (OR 2.14; 95% CI 1.11–4.13, p=.02). Bacteria were detected in 45% of aspirated thrombi but were not associated with either NET presence or density.

This is the largest cohort study assessing NETs in coronary aspirates during STEMI. However, earlier, smaller studies have also documented the presence of NETs in thrombi aspirated from the infarct-related artery in patients with STEMI and ischemic stroke.(14,15) NETs were associated with more complex coronary plaques (intraplaque haemorrhages, erosions, and ruptures) than simple plaques, and more frequent in fresh rather than older, lytic thrombi.(16) An earlier, smaller study of 253 patients with stent thrombosis, found NETs in only 23% of specimens.(14)

A number of studies have examined the relationship of potential biomarkers of NETs (including MPO-bound to DNA, citrullinated histones, or less specific markers such as ds DNA or neutrophil elastase) in blood or aspirated thrombi and related these to surrogate markers of clinical outcomes. In patients with STEMI, higher NET burden and reduced

DNase activity at the culprit site correlated with thrombus burden, microvascular obstruction and larger infarct size(17–19) and reduced ST-segment resolution.(17,18) In smaller studies in STEMI patients, dsDNA and citH3 were increased at the culprit site and were positively correlated with infarct size and subsequent left ventricular dysfunction(20) and MACE.(21) Following STEMI, there appears to be an early increase in nucleosomes and MPO levels peaking at 3 hours after pPCI.(22) Furthermore, in a case-control study of patients with ACS, the composite of peripheral markers of NETs and platelet activation was a good predictor of recurrent MACE.(22)

The importance of the current study is that it adds significantly to the volume of data showing the presence of NETs in the infarct related artery in patients with coronary thrombosis and shows a significant relationship with hard clinical outcomes beyond the acute presentation, but whether NETs are simply a marker of adverse prognosis or play a functional role is unclear. Laboratory studies indicate a direct functional role of NETs in thrombosis and impaired fibrinolysis. In mice, inhibition of PADs prevented NET formation and decreased atherosclerotic lesion size and carotid artery thrombosis.(23) Furthermore, addition of DNAse to *ex vivo* thrombi accelerated thrombolysis.(24) In a recent murine model, externalised histone H4 was shown to bind to and lyse atherosclerotic lesion smooth muscle cells (SMC), leading to plaque destabilization; conversely, pharmacological (Cl-amidine treatment) or genetic (*PAD4 knockout*) blockade of NETs release prevented SMC death and decreased overall plaque vulnerability.(25)

Two questions naturally arise in response to reading the paper by Blasco and colleagues. First, could NETs markers help identify high risk patients, and second, could NETs inhibition offer a novel avenue to reduce cardiovascular risk? Since thrombus aspiration during pPCI is no longer routinely performed, could peripheral sampling be helpful? A number of earlier

studies indicate that NETs are increased in STEMI at the culprit site but not at remote sites, such as the femoral artery.(20,21) However, peripheral neutrophil count and neutrophil: lymphocyte ratios are well recognised markers of adverse cardiovascular outcome in STEMI.(26,27) Similarly, NET-related markers such as MPO levels correlate with cardiovascular risk.(28) There are also data that suggest that markers of neutrophil activation, extracellular chromatin, DNase activity correlate with CRP and IL-6.(18) Thus, to what extent circulating biomarkers of NETs reflect the NET burden within the thrombus and the relationship between NETs, peripheral markers and outcome has not been fully elucidated in any of the above studies.

As shown by the study of Basco and colleagues, and other STEMI studies, conventionally employed antithrombotic agents such as heparin and tirofiban appear to be ineffective against already established NETs. Novel therapeutic avenues include inhibition of the formation of and/or increasing the consumption of NETs. Whilst some laboratory studies showed that specific or broad PAD inhibition attenuated NETs formation, others have not replicated this.(3) In mice, inhibition of janus kinase signalling with ruxolitinib decreased NETs formation and venous thrombosis.(29) Inhibition of MPO, colchicine, complement, or phosphodiesterase 4 are other possible strategies.(8,30) Enhanced NETs breakdown with DNase 1 has been shown to accelerate tPA-mediated lysis of coronary thrombi *ex vivo*, improve coronary microvasculature patency, and attenuate infarct size and long-term left ventricular remodeling in rodents.(31) Whilst recombinant DNase 1 has been used in cystic fibrosis patients to improve mucociliary clearance,(32) it has not been used to target NETs associated with cardiovascular thrombosis. Although there was no difference in pain-to-PPCI time between NETs positive and NETs negative samples, amongst those who were NETs positive, the timeline for peak NETs formation is not known. This would be important to

appropriately time the administration of any possible treatment to ameliorate the effects of NETs.

In conclusion, the study by Blasco and co-workers significantly strengthens the link between NETs, coronary thrombosis and adverse outcome. Whether NETs are a marker or an active contributor to adverse outcome in humans, remains unclear. Future studies are required to identify peripheral NETs markers, the peak time-window of NETs formation, and to explore potential pharmacological strategies to inhibit NETs to improve cardiovascular outcome.

Figure legend

During coronary atherothrombosis, neutrophils release prothrombotic extracellular traps and initiate a process termed immunothrombosis. In the study by Blasco and colleagues (13), the presence of neutrophil extracellular traps in aspirated coronary thrombi was associated with the occurrence of major adverse cardiovascular events in the first 30 days in patients with ST-elevation myocardial infarction. MACE, major adverse cardiovascular events; NETs, neutrophil extracellular traps. Figure adapted from BioRender.

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