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IM - REVIEW

An exploratory look at NETosis in atherosclerosis

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Abstract Current evidence suggests the likelihood of a link between venous thromboembolism (VTE) and atherosclerosis, although they have been traditionally considered as different pathological entities. The contribution of neutrophils to human atherogenesis has been underestimated, if compared to their contribution established in VTE. This is due to the major importance attributed to macrophages in plaque destabilization. Nevertheless, the role of neutrophils in atherogenesis deserves increasing attention. In particular, neutrophil extracellular traps (NETs) are net-like chromatin fibres that are released from dying neutrophils. The death of neutrophils with NETs formation is called NETosis. During activation, neutrophils produce reactive oxygen species (ROS), through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The main function of NETs is trapping and killing pathogens. Nevertheless, NETs formation has been observed in various chronic inflammatory diseases, autoimmune diseases, vasculitis, lung diseases, cancer and VTE. Recent studies suggest that NETs formation might contribute also to atherosclerosis progression. New data report the presence of NETs in the luminal portion of human atherosclerotic vessels and coronary specimens obtained from patients after acute myocardial infarction. Programmed death mechanisms in atherosclerosis such as apoptosis, efferocytosis and also NETosis, share common features and triggers. If defective, they can lead the cells to a switch from programmed death to necrosis, resulting in the release of pro-atherogenic factors, accumulation of cell debris and progression of the disease. This review provides evidence on the emerging role of neutrophils focusing on NETosis and oxidative stress burden in orchestrating common mechanisms in atherosclerosis and thrombosis.

Keywords NETosis · Atherosclerosis · Venous thromboembolism · Coronary artery disease · Oxidative stress

Abbreviations

MP	Microparticles
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
Nrf2	Nuclear erythroid-related factor 2
ROS	Reactive oxygen species
TF	Tissue factor
VTE	Venous thromboembolism

The evidence of a link between venous thromboembolism (VTE) and atherosclerosis

Current evidence suggests the likelihood of a link between VTE and atherosclerosis, although they have been traditionally considered as different pathological identities. The

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link between VTE and atherosclerosis was initially hypothesized by Prandoni et al., [1], who demonstrated a relationship between asymptomatic carotid plaques and spontaneous venous thrombosis of the legs. The association was also present after adjustment for risk factors for atherosclerosis and thrombophilic conditions. The study was based on the previous results of a prospective cohort study dealing with a long term follow-up of patients with first episode of acute deep vein thrombosis of the lower extremities [2]. In this cohort, Authors found a surprisingly high proportion of patients who died of acute myocardial infarction or ischemic stroke.

Other confirmations emerged: Schulman et al., find a significantly higher mortality rate associated with myocardial infarction and stroke in patients with acute deep venous thrombosis previously enrolled in the Duration of Anticoagulation Study (DURAC) [3]. Hong et al., find a higher prevalence of coronary artery calcifications in patients with idiopathic deep venous thrombosis than in matched control subjects [4].

It is known that VTE and atherosclerosis share many risk factors, such as obesity, diabetes, smoking, hypertension and hyperlipidaemia, as reviewed by Lowe [5]. He summarized the current evidence and the possible mechanisms responsible for the association between arterial thrombosis and VTE. Thrombotic risk factors (obesity, diabetes, immobility, oestrogens, infections and smoking) may increase the risk of VTE as well as arterial thromboembolism. Atherosclerotic risk factors (diabetes, smoking, hypertension and hyperlipidaemia) may be more relevant in atherothrombosis.

Two important studies [6, 7] tried to further investigate the association between VTE and atherosclerosis. In one study by Reich et al., [6], no associations are found between ultrasound parameters of sub clinical atherosclerosis (intima media thickness) and VTE development. Nevertheless, at multivariate analysis, the occurrence of cardiovascular and cerebrovascular events might increase the risk of VTE and of arterial thromboembolism as well. In another study by Van der Hagen et al., [7], sub clinical atherosclerosis is not associated with increased risk of VTE. These results seem to be in contrast with Prandoni's ones [1], although it has to be mentioned that Prandoni measured sub clinical atherosclerosis parameters at the time of VTE development, while Reich and Van der Hagen measured these parameters before the thromboembolic events.

This topic has been carefully reviewed in [8, 9], leading to the hypothesis that sub clinical atherosclerosis is not predictive of VTE, but the opposite is likely to be true. Prandoni speculates that venous and arterial thrombosis may be two aspects of the same disease. This fact also has several implications in clinical practice. Prandoni et al., propose that patients with VTE of unknown origin should be examined for asymptomatic atherosclerosis, to lower their risk profile.

The discovery of neutrophil extracellular traps (NETs), the NETosis itself and the differences from other modalities of cellular death: an overview

Neutrophils are the main cells of innate immunity. One of the mechanisms of neutrophils actions is the formation of NETs. Brinkman was the first to report the release of NETs, in 2004 [10]. The discovery of NETs has spawned a new field in granulocytes investigation. NETs are composed of nuclear chromatin, associated with nuclear histones and granular antimicrobial proteins. They are a sort of scaffold, ideal to retain microbes. The main function of NETs is the trapping and killing pathogens, as such as bacteria, fungi, viruses and protozoa [10]. The trapping within DNA fibres prevents the spread of pathogens, and facilitates the concentration of antimicrobial factors at the infection site [10].

The process of NETs generation, called NETosis, is a specific type of cell death, different from necrosis and apoptosis. It is a multi-step cell death program: enzymes from granules translocate to the nucleus and facilitate chromatin de-condensation. Then, internal membranes break down, and cytolysis releases NETs. Both the nuclear and granular membranes disintegrate during NETosis, but plasmatic membrane integrity is maintained. This is in contrast to apoptosis or necrosis. NETosis is associated with disintegration of the nuclear envelope and mixing of nuclear and cytoplasmic material, loss of internal membranes and disappearance of cytoplasmic organelles. More precisely, no peculiar signs of apoptosis are observed (membrane blebs, phosphatidylserine exposure, nuclear chromatin condensation and DNA fragmentation). NETosis is characterized by rapid intracellular de-condensation of nuclear chromatin [11]. NETosis is distinct from necrosis as the plasma membrane, in addition to the nuclear membrane, also loses integrity in the latter. In NETosis, DNA is released in the form of strands or meshes.

After intracellular assembly, NETs are released via plasma membrane perforation [11]. NETs release is a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent cellular death process. During activation, neutrophils produce reactive oxygen species (ROS), through the activation of NADPH oxidase [11].

ROS are implicated in NETs release through a neutrophil elastase (NE)-mediated mechanism: it translocated from cytoplasmic granules to the nucleus, and triggers chromatin degradation through histone cleavage [12]. NE

translocates first to the nucleus, where it digests nucleosomal histones and promotes extensive chromatin decondensation. Also myeloperoxidase (MPO) contributes to the nuclear DNA de-condensation [12]. In resting neutrophils, NE and MPO are stored in the azurophilic granules. Upon activation and ROS production, NE escapes the granules and translocates to the nucleus. In the nucleus, NE cleaves histones and promotes chromatin decondensation. MPO binds to chromatin in the late stages of the process. MPO binding promotes further decondensation. NE and MPO cooperatively enhance chromatin decondensation, leading to cell rupture and NET release. ROS may promote the release of NE directly by disrupting the association of NE with the proteoglycan matrix that is thought to down-regulate protease activity in resting cells.

In support of the role of ROS in NETs formation, sickle cell disease mice are found to contain plasma heme to cause NETs formation in a ROS-dependent manner. The abnormal shape of red blood cells leads to the release of an excess amount of heme and an excess of ROS formation [13].

The role of ROS in NETosis has been carefully reviewed [14, 15] but their precise role is not fully understood. It is clear that when neutrophils are activated, they generate a massive amount of superoxide, by activating the NADPH oxidase. It remains unclear how ROS precisely influence the main features of NETosis (chromatin decondensation, binding of enzymes to DNA, membrane rupture). ROS enable the release of NE and MPO from the azurophilic granules in the neutrophil cytoplasm. ROS act to facilitate the citrullination of histone proteins. All these actions promote nucleosome disassembly and chromatin decondensation. It has been supposed that ROS contributing to NETs formation not necessarily derives only from pathways involving NADPH oxidase. The alternative source of ROS may be the mitochondrial oxidative phosphorylation complex. It has been demonstrated that NADPH oxidase-deficient neutrophils of mutant mice and of humans with chronic granulomatous disease are not able to form NETs [16, 17].

Therefore it appears that ROS are essential for the NETosis induction.

The clearance of NETs largely depends on deoxyribonuclease 1 (DNase I) and its impaired activity is involved in the pathogenesis of different diseases, in particular systemic autoimmune diseases [18]. DNase 1 is required for disassembly of NETs. It facilitates NETs subsequent clearance by macrophages. It is a sort of dismantlement before the action of macrophages. NETs are then internalized through an endocytic mechanism. Macrophages are capable of efficient clearance of NETs. The clearance of NETs resembles the clearance of apoptotic cells [11].

An intriguing point about NETosis is that current evidence suggests that it is not only a death pathway: two different mechanisms have been described, and one of them might be considered the "vital" NETosis, as carefully reviewed [19]. This is to differentiate it from the classical "suicidal" form of NETosis, that we have previously described in this paper. Also the "vital" NETosis allows NETs release. The principal differences between the two forms are the nature of the trigger stimulus, the timing of NETs release and the mechanisms employed to make NETs release [19].

More precisely, the "suicidal" form of NETosis requires strong activation of NADPH oxidase. MPO and NE mediate the chromatin decondensation resulting in a mixture of DNA and granule proteins that are extruded out of a perforation in the plasma membrane. Neutrophils that undergo "suicidal" NETosis can no longer be recruited.

"Vital" NETosis requires vesicular trafficking of DNA within the nucleus to the extracellular space (without requiring membrane perforation). It follows microbial-specific molecular patterns recognized by host-receptors, such as toll-like receptors. In particular, lipopolysaccharide, a Gram-negative bacterial stimulus, induces rapid NETs release, which can be triggered also by viruses and fungi. This process occurs in a ROS-independent manner, and more rapidly compared to the "suicidal" classical form.

NETs inference in different diseases: focus on VTE

NETs formation has been observed in various chronic inflammatory diseases, autoimmune disease and vasculitis, lung diseases, cancer and, in particular, in VTE [20–24]. VTE is the third most common vascular disease after myocardial infarction and ischemic stroke [25]. In contrast to arterial thrombosis, which is the result of the atherosclerotic plaque erosion or rupture, the factors that contribute to thrombosis include endothelial or vessel wall damage, stasis and hypercoagulability (Virchow's triad) [26]. Venous thrombi have a laminar structure consisting of layers of platelets, fibrin, blood red cells and neutrophils [27]. The majority of leukocytes in the early thrombus appear to be neutrophils, whereas macrophages predominate in the later stages of resolution [28, 29]. The recruited neutrophils may initiate thrombosis through the formation of NETs [30]. It has been shown that these traps appear in the plasma and in the thrombi following induction of deep venous thrombosis in a baboon model [30]. They provide a scaffold for thrombus consolidation. NETs provide a new link between innate immunity and thrombosis. Stasis leads to platelet deposition, an increase in the concentration of pro-coagulant factors and thrombus formation. Local

hypoxia may activate the endothelium, with the final production of compounds that, upon contact with neutrophils, stimulate NETosis [31].

A previous study in mouse model of VTE confirms that the cleavage of NETs by DNase 1 prevents the cascade of events leading to thrombosis [32]. Thrombosis can also be initiated by the release of tissue factor (TF) that binds factor VII to give TF-Factor VIIa complex that is able to activate the coagulation cascade with clot formation. TF can be produced by neutrophils during NETs formation [33]. The link between NETosis and coagulation is related to the presence of neutrophil elastase on NETs. It inactivates TF pathway inhibitor (TFPI), through cleavage, resulting in increasing pro-coagulant activity [34]. Pro-coagulant activity leads to platelets activation, which enhances NETs formation. Moreover, NETs fibres contain other factors that can render them pro-coagulant, in particular the negatively charged DNA in NETs may provide a scaffold for Factor XII activation which is aided by platelets [35]. Also platelets and red blood cells are involved: in fact, NETs fibres bind them and support their aggregation, contributing to thrombus formation [36]. In this context, is the role of inflammation and its consistent role in the venous thrombotic process [27]. Inflammation increases TF activity, platelets reactivity and fibrinogen, and leads to exposure of phosphatidylserine. These molecules are able to stimulate the recruitment and activation of leukocytes and produce cytokines [27].

Micro-particles (MP) are also involved in the thrombotic process and in the amplification of thrombosis. They are small (0.1–1 μm) membrane fragments shed from platelets, leukocytes and endothelial cells. They may express TF in their surface. MP is pro-coagulant because of the presence of TF and the exposure of negatively charged phospholipids, such as phosphatidylserine. The negatively charged surface facilitates the assembly of positively charged coagulation protein complexes [37]. Exposure of phosphatidylserine not only facilitates the formation of coagulation complexes, but also promotes the ability of TF to initiate the coagulation process [38]. MP is not only prothrombotic, but also appears to inhibit fibrinolysis, delaying thrombus resolution and facilitating its growth [38]. They are present in low plasma concentrations in healthy subjects, but are elevated in patients with cancer and VTE [39, 40]. Of relevance, MP is also found to be elevated in patients with acute coronary syndrome [41] and with diabetes [42, 43]. It has been demonstrated that MP levels are predictive for the presence of coronary artery lesions in diabetic patients, and they are a more significant independent risk factor than the length of the diabetic disease, lipid levels or presence of hypertension [44]. Atherosclerotic plaques contain MP, mostly derived from activated leukocytes and from erythrocytes (indicating the occurrence of intra- plaque haemorrhage). MP could also contribute to plaque instability by mediating the recruitment of inflammatory cells [45]. So, MP might also contribute as linking elements between atherosclerosis and VTE.

NETs inference in different diseases: focus on autoimmune diseases, cancer, chronic obstructive pulmonary disease (COPD) and metabolic diseases

NETs display broad ranges of effectiveness against a variety of different species of Gram-positive and Gram-negative bacteria, fungi, parasites and viruses [10, 46, 47]. These topics are beyond the scope of this review. Nevertheless, a summary of the current evidence about the involvement of NETosis or neutrophil activation in different diseases should be mentioned. Firstly, different groups suggest a role of NETosis in the development of autoimmune diseases, such as lupus erythematous, rheumatoid arthritis, Sjogren syndrome, scleroderma and vasculitis, as recently highlighted in Behcet's disease by Becatti et al., [48]. The complexity of mechanisms and new insight around this topic have been reviewed recently [49]. In particular, studies in patients with lupus have been performed [18, 50]. There is a substantial agreement in considering an insufficient NETs degradation partially due to the insufficient activation or action of the DNase 1. The explanations are not unequivocal: presence of anti-NETs antibodies that prevent DNase access to NETs, or the presence of DNase1 inhibitors or other mechanisms that are not well established. Moreover, patients with lupus develop antibodies against chromatin and neutrophil proteins. So, defects in NETs clearance exacerbate the disease. Interestingly, it has been suggested that anti NETs antibodies and persistent NETs may form NETs immune-complexes, which may be relevant in the exacerbation of the disease. Summarizing, experimental evidence suggests that autoimmune diseases are characterized by an imbalance between NETs formation and NETs clearance.

The role of NETs in cancer biology and tumour progression has been proven [51, 52]. Neutrophils are increasingly being recognized as important elements in tumour progression. They have been shown to exert important effects at nearly every stage of tumour progression with a number of studies demonstrating that their presence is critical to tumour development. Briefly, the finding that tumour-induced neutrophils are more prone to NETs formation than their normal counterparts opens up a new area of research in cancer biology. Readers are invited to investigate further about the topic in dedicated papers [51, 52].

NETs also have a role in patients with chronic obstructive pulmonary disease (COPD). The presence of NETs has been demonstrated in induced sputum of stable COPD patients [53]. NETs formation is markedly augmented in COPD sputum irrespective of its purulence or smoking status. Moreover, [54] the presence of large amounts of NETs is associated with disease severity, and augmented during COPD exacerbation. The sputum structure of most subjects with very severe and severe COPD (GOLD grades 4 and 3) is enriched in NETs, associated with neutrophils in various stages of NETosis. The presence of NETs is also found in the sputum of subjects with mild COPD (GOLD grades 1 and 2), but with a lower prevalence. So NETs may contribute to chronic inflammation in COPD. The mentioned study also indicates that cigarette-smoking induced NETosis affects smokers in a self-perpetuating cycle of inflammation before the airflow limitation. Moreover, this study underlines that NETs may be major contributors, not only to the chronic inflammation in COPD, but also in the lung tissue damage, through oxidative stress mechanisms (because of the production of ROS during NETosis).The close relationship among oxidative stress, chronic inflammation and cancer is well established [55, 56].

Current evidence also underlines the role of NETosis in diabetes. Different studies point out this fact [57, 58]. These studies show that circulating NETosis-related biomarkers are increased in the sera of type 2 diabetic patients, with a correlation with HbA1c levels. The increased susceptibility of diabetic neutrophils to NETosis is at least in part due to high blood glucose. In fact, NETosis metabolically requires glucose [59]. Moreover, high glucose triggers ROS production [57, 60]. Furthermore, in diabetics there is an overexpression of peptidylarginine deiminase 4 (PAD4) that is a calcium-dependent enzyme that is key in mediating NETosis [61]. NETosis has also been considered in the setting of the metabolic syndrome, as recently reviewed [62]. Like diabetes, the metabolic syndrome is characterized by the activation of the innate immune system. NETosis appears to be part of an abnormal response to damage in diabetes and in the metabolic syndrome, which in turn, can promote or aggravate organ complications.

NETosis in atherosclerosis and in acute coronary artery disease: current evidence

Atherosclerotic plaque disruption and intraluminal thrombosis is the hallmark of both acute coronary syndrome and ischemic stroke [63–65]. Extracellular DNA has cytotoxic and pro-thrombotic effects [66], creating a link between inflammation and coagulation.

A large contribution in this area was made by Borissoff. He performed a study [67], with the aim of clarifying the relationships between extracellular DNA formation, coronary atherosclerosis and the presence of a pro-thrombotic state.

282 subjects with suspected coronary artery disease were examined by using coronary computed tomographic angiography and in vivo markers of NETosis were measured. The study reveals that markers of NETosis (double-stranded DNA, nucleosomes, citrullinated histone H4, and MPO-DNA complexes) are independently associated with the severity of coronary artery disease, a pro-thrombotic state and also the occurrence of major adverse cardiac events. The study suggests that NETs formation might contribute to atherosclerosis progression.

Previous histological studies have shown the presence of NETs in the luminal portion of mouse and human atherosclerotic lesions [68]. In particular, human plaques obtained by endarterectomy were fixed and stained and the presence of NETs was confirmed in luminal location at sites of atherosclerosis.

The presence of neutrophils and NETs were observed in coronary specimens obtained from patients after acute myocardial infarction in fresh and lytic thrombi [69]. NETs are most frequently found in lytic thrombus specimens. In organised thrombus tissue NETs are never observed. In this study, NETs can be observed only in the early (fresh and lytic) stages of thrombus evolution. It can be assumed that the formation of NETs precedes the lytic changes that can be observed histologically, and occurs very early in the process of thrombus dissolution. Moreover, coronary thrombi contain NETs that are coated with interleukin-17, a pro-inflammatory cytokine that promotes platelet aggregation.

In a very recent study [70] coronary thrombarterectomies derived from patients with ST-elevation acute coronary syndrome (undergoing primary percutaneous coronary intervention) were analysed. NETs contribute to the scaffolds of the coronary thrombi. Moreover, in the culprit lesion site, NETs burden positively correlate with infarct size and negatively with ST-segment resolution. In fact, nucleosomes, double-strand DNA, neutrophil elastase, MPO, all marker of NETosis, are found to be increased in the culprit lesion site [70]. In addition, Stakos et al., [71] find expression of functional TF by NETs in infarct-related coronary artery of acute myocardial infarction.

An intriguing point about NETs and atherosclerosis has recently been underlined and commented upon [72] on the basis of a recent study [73]. In a mouse model of atherosclerosis, cholesterol crystals act both as priming and danger signals for IL-1β production. Cholesterol crystals trigger neutrophils to release NETs. NETs prime macrophages for cytokines release, amplifying immune cell recruitment in atherosclerotic plaques. Therefore, danger signals may drive sterile inflammation, such as that seen in atherosclerosis, through their interactions with neutrophils.

This study provides new insight as to how neutrophils and macrophages communicate, and introduces a new concept of interaction between inflammasome and NETs. Cholesterol crystal-induced NETosis as a macrophage inflammasome stimulator, is a new concept that enlarges our knowledge about the role of neutrophils in monocyte recruitment. Macrophage activation through NETosis is different from macrophage-neutrophil interaction in inflammation resolution. In fact, the presence of cholesterol crystals highly influences these cells interactions.

For what concerns macrophages, NETs are removed by these cells via phagocytosis. In general, the sub-population M2 rather than M1is considered to be preferentially implicated in the clearance of dead cells [74]. A recent study [75] demonstrates that both M1and M2 can digest NETs, but with different timing. Three or four hours after the interaction with NETs, M2 cells are the most implicated, while later (24 hours), the extracellular DNA is completely degraded by M1 cells.

As supposed [76], NETs can be considered as potential new biomarkers in atherosclerosis.

Borissoff proposes in a clever review that there is cross-talk between coagulation, inflammation and atherosclerosis [77]. Although there is not a well-established clinical evidence of a role for the haemostatic system in the progression of atherosclerosis, it is reasonable that such a link occurs. In particular, platelets are an interface between hemostasis and inflammation in atherosclerosis. The binding between platelets and circulating cells creates aggregates that support continuous leukocyte activation, both in the venous thrombus and on the arterial one. In particular, the binding between platelets monocytes and neutrophils, dendritic cells, and progenitor cells produces co-aggregates that support further leukocyte activation, adhesion, and transmigration. The adhesion of platelets on a compromised endothelium is considered an early step for thrombus formation. Once adherent, they secrete cytokines, adhesion molecules and coagulation factors that enhance the process. TF also has a prominent role, not only in the venous thrombus formation, but also in atherosclerotic plaques.

Figure 1 aims to clarify these concepts and the common features shared in venous and arterial thrombus formation.

Macrophage apoptosis, efferocytosis and defective efferocytosis in the vulnerable plaque: synergistic effects of oxidative and endoplasmic reticulum stress

The role of the macrophage cell in the context of atherosclerotic plaque has been precisely reviewed by our group [78].

The monocytes infiltration into early lesions is one of the first effects to the sub-endothelial apolipoprotein B lipoproteins retention, which is considered the primary event in atherogenesis. The monocytes become macrophages in the sub-endothelium, and start to internalize the retained lipoproteins [79, 80]. Features of macrophage apoptosis start to appear as the lesion progresses. If limited, apoptosis of macrophages occurs without necrosis. Quick removing actions of apoptotic cells might be completed by macrophages by efferocytosis [81, 82]. These characteristics are markers of the early lesions. The continuous enrolment of further macrophages and inflammatory cells and lipid accumulation increases the apoptotic cells amount, and the efferocytosis becomes defective. The apoptotic cells (not phagocytized) are subjected to secondary necrosis, and support higher inflammation. The necrotic core expansion is the consequence of the augmented macrophage apoptosis linked with defective efferocytosis [83]. The necrotic core is the major feature responsible for plaque disruption and acute luminal thrombosis. In particular, Van Vrè et al., examined in detail these death cell modalities in atherosclerosis [84].

As reviewed [78] oxidative stress is implicated in the expansion of the necrotic core: in fact, many oxidized derivatives of polyunsaturated fatty acids promote defective efferocytosis, with the final result of the necrotic core expansion. In the past few years, the role of the endoplasmic reticulum (ER) stress is also under investigation to better define its possible contribution in atherosclerosis [85–87]. It is known that a variety of insults can interfere with ER function, leading to the accumulation of unfolded and misfolded proteins in the ER [85]. The abnormal amount of apoptotic cells in the vulnerable plaque has been demonstrated to be related both to the sustained ER stress and to the expression of survival and protective genes, such as the unfolded protein response (UPR) or the nuclear erythroid- related factor 2 (Nrf2) [88]. Nrf2 is a master transcriptional activator, which regulates many of the antioxidant defence genes. It is an ER-dependent transcription factor, implicated in cellular defence against ROS. All these data reveal that oxidative stress, ER stress and inflammation play a key role in the initiation and progression of atherosclerosis, with increased production of ROS and activation of pro-inflammatory signalling. As explained in this review, neutrophils and ROS are strongly linked, so it is plausible that our knowledge about oxidative and ER stress enriches data in this area of research. It is clear that researchers have attributed the major importance to macrophages in plaque destabilization.

Nevertheless, the role of neutrophils in atherogenesis deserves increasing attention [89–92]. Rapid clearance of NETs by macrophages prevents the induction of an autoimmune response. In the context of the atherosclerotic

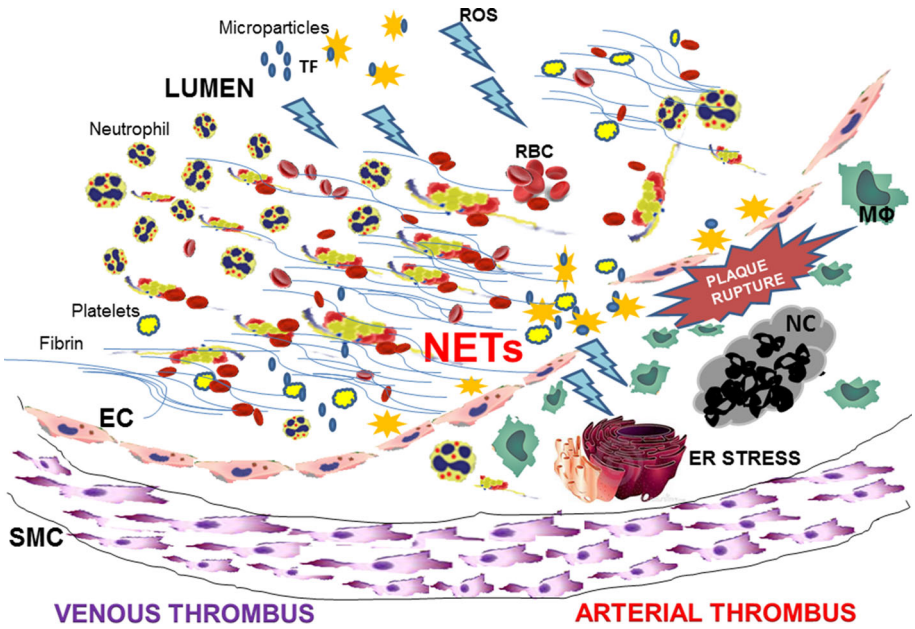

Fig. 1 NETosis interweaves inflammation and coagulation: focus on venous and arterial thrombosis: On the *left* NETs entrap both platelets and red blood cells, creating a scaffold for fibrin deposition and the subsequent stabilization of the venous thrombus. Activated platelets are able to activate neutrophils for further NETs release. Microparticles, originated from different inflammatory cells, are pro-coagulant. On the *right* NETs are involved in the mechanisms that lead to plaque vulnerability. The main cell in this situation is the macrophage. Nevertheless, neutrophils, NETs and microparticles are present in plaque rupture. Oxidative and endoplasmic reticulum (ER) stress trigger and orchestrate the necrotic core expansion. Finally the plaque breaks down. *MΦ* macrophage; *NC* necrotic core; *NETs* neutrophil extracellular traps; *RBC* red blood cells; *ROS* reactive oxygen species; *TF* tissue factor

plaque their excessive presence could determine the expansion of the necrotic core. If the clearance of NETs is insufficient, persistent inflammation and oxidative stress occur. The disruption of the atherosclerotic plaque and the thrombosis in the lumen, which are mostly determined by the expansion of the necrotic core, are driven by various mechanisms, including accelerated macrophage apoptosis and defective phagocytic clearance (defective efferocytosis). Oxidative stress and inflammation are implicated in the expansion of the necrotic core. Therefore, a defective NETs removal might contribute to the expansion of the necrotic core.

Conclusions

This review has tried to summarize the current evidence of the role of neutrophils in different diseases, focusing on VTE.

We have analysed their peculiar death modality, NETosis, in atherosclerosis, as a mechanism shared with VTE.

There are several gaps in the knowledge of NETosis, and there is certainly much more to investigate.

Surely it is not yet time to purpose therapeutic options targeting neutrophils in the context of atherosclerosis, this in agreement with Doring who reviewed this topic [93].

Nevertheless, the contributions of circulating cells to human atherogenesis (initiation and progression) has to be further investigated, focusing attention not only to the macrophage cell, but also the neutrophils.

Compliance with ethical standards

Conflict of interests None.

Ethical approval The study was conducted in accordance with the ethical standards laid down in the Helsinki Declaration of 1975 and its late amendments. The local ethics committee approved the study.

Human and animal rights statement No human nor animal data have been collected in this paper.

Informed consent None.

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