The Pathophysiological Role of Neutrophil Extracellular Traps in Inflammatory Diseases

Aldo Bonaventura[1,*]   Luca Liberale[1,2,*]   Federico Carbone[1]   Alessandra Vecchié[1]
Candela Diaz-Cañestro[2]   Giovanni G. Camici[2]   Fabrizio Montecucco[1,3,4]   Franco Dallegri[1,3]

[1] Department of Internal Medicine, First Clinic of Internal Medicine, University of Genoa, Genoa, Italy
[2] Centre for Molecular Cardiology, University of Zürich, Schlieren, Switzerland
[3] Ospedale Policlinico San Martino, Genoa, Italy
[4] Centre of Excellence for Biomedical Research (CEBR), University of Genoa, Genoa, Italy

Address for correspondence Aldo Bonaventura, MD, Department of Internal Medicine, University of Genoa, 6 viale Benedetto XV, 16132 Genoa, Italy (e-mail: aldobon85@gmail.com).

Abstract

Neutrophil pathogen-killing mechanism termed neutrophil extracellular traps (NETs) has been recently identified. NETs consist of chromatin and histones along with serine proteases and myeloperoxidase and are induced by a great variety of infectious and non-infectious *stimuli*. NETosis is a kind of programmed neutrophil death characterized by chromatin decondensation and release of nuclear granular contents, mainly driven by peptidylarginine deiminase 4 citrullination of histones. Although classically related to the protection against infectious pathogens, nowadays NETs have been described as a player of several pathophysiological processes. Neutrophil dysregulation has been demonstrated in the pathogenesis of most representative vascular diseases, such as acute coronary syndrome, stroke and venous thrombosis. Indeed, NETs have been identified within atherosclerotic lesions and arterial thrombi in both human beings and animal models. Moreover, an imbalance in this mechanism has been proposed as a critical source of modified and/or externalized autoantigens in autoimmune and inflammatory diseases. Finally, an update on the role of NETs in the pathogenesis of cancer has been included. In the present review, based on papers released on PubMed and MEDLINE up to July 2017, we point to update the knowledge on NETs, from their structure to their roles in infectious diseases as well as in cardiovascular diseases, autoimmunity, metabolic disorders and cancer, with a look to future perspectives and therapeutic opportunities.

Keywords

► NETs
► infectious diseases
► autoimmune diseases
► metabolic disorders
► cancer

Introduction

Neutrophil extracellular traps (NETs) have been recently recognized as a part of the wide defence strategy of neutrophils. In 2004, Brinkmann et al found that the induction of NETs by interleukin (IL)-8 and lipopolysaccharide (LPS) supported their formation during inflammation and bacterial infection, as seen in the course of appendicitis.[1] From this pivotal work, neutrophil alternative death pathway, named NETosis,[2] has been indicated as a further way to act in innate immune defence even after their death. In recent years, NETs have been suggested to play a role also in non-infectious diseases, such as

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* These authors equally contributed to this work as first authors.

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systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), vasculitis, diabetes, atherosclerosis and cancer.

In the present review, based on papers released on PubMed and MEDLINE up to July 2017 (searched terms in combination: NETs, neutrophils, infections, autoimmunity, cancer, diabetes, cardiovascular [CV] diseases; articles have also been retrieved through searches of reference lists and authors' files), we aimed at updating knowledge on NETs, from their structure to their roles in infectious diseases as well as in CV disease, autoimmunity, metabolic disorders, and cancer, finally concentrating on future perspectives in terms of clinical usefulness for therapeutic purposes.

Structure and Formation of NETs

NETs are constituted by extracellular strands of unwound DNA usually in complex with histones and proteins from neutrophil primary, secondary and tertiary granules, including components with inflammatory and bactericidal activity, such as neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, lactoferrin, pentraxin 3, gelatinase, proteinase 3 and peptidoglycan-binding proteins. Generally, most of the neutrophil DNA is transcriptionally inactive and condensed into heterochromatin within the nucleus, with DNA wrapped around histones to form nucleosomes. Chromatin decondensation begins NETosis and is mediated by peptidyl arginase deaminase 4 (PAD4) catalyzing the conversion of histone arginines to citrullines, which weakens histone–DNA binding and consequently unwraps nucleosomes. A central role in

NET regulation is played by intracellular calcium as a second messenger of neutrophil activation,[3] particularly PAD4 is activated by calcium itself.[4] NE is essential for NET production, too, as it cleaves histones during NET formation (►Fig. 1).[5]

Starting from in vitro studies, distinct activation pathways for NET formation have been identified and are believed to be active in vivo as well. These pathways include activation by integrins and toll-like receptors (TLRs)[6,7] as signals triggering NETosis in response to bacterial infections.[8] In this setting, L-selectin-mediated signals have also been described to elicit NETs in vitro.[8] Most in vitro studies to identify mechanisms of formation of NETs used phorbol 12-myristate 13-acetate (PMA), despite this being an artificial trigger bypassing membrane receptors and their specific intracellular pathways. Actually, the widely described critical role of nicotinamide adenine dinucleotide phosphate oxidase (NOX) and MPO for NET formation may be linked to the in vitro activation by PMA. In chronic granulomatous disease (CGD) patients, a critical role of NOX in NET formation was confirmed in vivo.[9]

To date, three different models of NET formation have been identified. The best described one is called "suicidal NETosis" and lasts from 2 to 4 hours.[10] After neutrophil activation, NOX increases its activity via the protein kinase C (PKC)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase ERK kinase (MERK)/extracellular signal-regulated kinase (ERK) complex leading to cytosolic calcium intake, PAD4 activation and chromatin decondensation. Once the increase in cytosolic calcium takes place, PAD4 activation
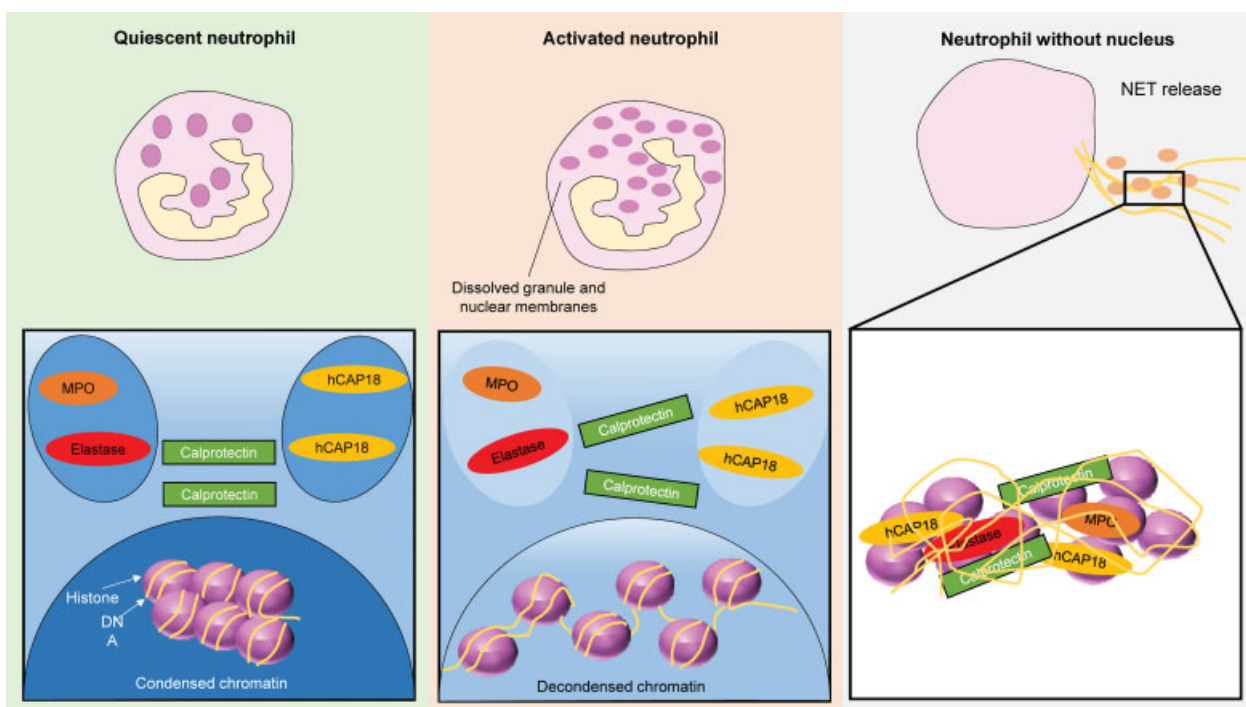

Fig. 1 Viable neutrophil extracellular trap (NET) formation. After neutrophil activation, peptidyl arginase deaminase 4 (PAD4) citrullinates some histone arginines to weaken the tight electrostatic binding between histones and DNA in nucleosomes. The immediate consequence is that both nuclear and granule membranes dissolved. At this moment, DNA is decondensed and meets citrullinated histones and granule proteins, which are all expelled from neutrophils as NETs ready to catch and even kill microbes. The last step sees the surface membrane coming intact leaving a viable neutrophil without nucleus.

and chromatin decondensation occur.[11] Then, ROS acts as second messengers by promoting the loss of the nuclear membrane. In this way, chromatin spreads throughout the cytoplasm mixing with cytoplasmic proteins and granule mediators and is finally released outside the cell through membrane pores and cellular lysis. Differently, during vital NET generation, neutrophils release NETs with no loss of nuclear or plasma membrane within 5 to 60 minutes independently of ROS and Raf/MERK/ERK pathway. This process evolves through different stages characterized by morphological changes: (1) nuclear envelope growth and vesicle release, (2) nuclear decondensation and (3) nuclear envelope disruption.[12] Typically, this type of NET production is stimulated by the recognition of stimuli via TLRs and the C3 complement receptor[6,13] as well as via the interaction between glycoprotein Ib in platelets with β2 integrin in neutrophils by the activation of ERK.[14] Finally, another type of vital, ROS-dependent NET generation has been described, in which mitochondrial DNA is released instead of nuclear DNA with NET formation within 15 minutes by the recognition of C5a or LPS.[15]

By definition, neutrophils are widely recognized as the only producers of NETs, although only a limited part of them (around 20% depending on the stimulus) owns this skill contributing to the rising concept of neutrophils being a heterogeneous class of cells.[16] Anyway, it is not known whether these phenotypic differences could be relevant to the ability of these neutrophils to undergo NETosis. Actually, the formation of extracellular traps (ETs) is far known to be restricted to cells of myeloid origin, even if the classical requirements for NET formation in PMA-stimulated neutrophils—such as respiratory burst, NE and MPO—are mainly met by neutrophils. However, eosinophils have also been described to generate a respiratory burst and their peroxidase can convert hydrogen peroxide into oxidizing halogen derivatives.[17] These eosinophil ETs contain intact eosinophil granules, can catch bacteria and have been demonstrated in vivo in eosinophil-rich secretions.[18,19] Basophils, too, can form ETs killing bacteria, but independently from NOX activity.[20,21] On the contrary, mast cells were shown to release NOX-dependent ETs in vitro when stimulated by either *Staphylococcus pyogenes* or PMA.[22] In mice, monocytes and macrophages can form ETs when stimulated by PMA.[23]

NETs and Infections

NETs have been demonstrated to own a broad effectiveness against different pathogens, such as bacteria, viruses, fungi, and parasites. Anyway, experimental data indicate that NETs trigger is restricted to specific microorganisms. To date, the precise role of NETs in sepsis has not been completely elucidated. NETs have been suggested to reduce bacterial spreading, especially in the early phase of infection,[24] but their role appears to be limited: neither the lack of PAD4 nor the treatment with deoxyribonuclease (DNAse) impacted on bacterial load in animals subjected to sepsis.[25,26] Furthermore, the excessive formation of NETs during sepsis is associated with organ damage. Interactions among platelets,

neutrophils and activated endothelial cells lead to an increased formation of NETs, which interact with vascular endothelium ultimately leading to endothelial damage and organ injury in a histone- and MPO-dependent manner.[27] Besides, histones can stimulate TLR2 and TLR4 to enhance the production of proinflammatory mediators via MyD88 signaling.[28,29] Considering all these findings, it is clear that NETs might have a detrimental role in sepsis. Accordingly, both antibiotic therapy and anti-NETosis conditions, such as DNAse treatment or abrogation of PAD4, have been described to increase the survival rate of animals[26,30,31] and humans[32] with sepsis by reducing NET burden. Microorganisms inducing NET production are indicated in ►**Table 1**.

Bacteria

A great number of Gram-positive and Gram-negative bacteria have been demonstrated to trigger NET formation. Brinkmann et al have used *Staphylococcus aureus* in 2004 in their seminal work as a stimulus to investigate NETosis.[1] Some years later, Pilsczek et al deepened the previous finding by describing a faster, ROS-independent NET production in response to *Staphylococcus*-related infection, named vital NET release.[10] *S. aureus* carries several virulence factors, among which leukotoxin GH and Panton–Valentine leukocidin can promote NET formation via an oxidative mechanism.[33] Despite this, the bacterium has evolved different mechanisms to escape NET killing. For example, *S. aureus* can express pore-forming virulence factors neutralizing neutrophils by inducing necrosis at the expense of NETosis.[34] Besides, its catalase expression blocks the stack of hydrogen peroxide, thus protecting the bacterium from intracellular oxidation and NETs.[35] Furthermore, methicillin-resistant *S. aureus* (MRSA) has been found to express extracellular nucleases for biofilm dispersal and degradation of NETs. As a proof of this, mice infected by MRSA presented with a higher mortality with respect to controls infected with a nuclease-deficient strain that is more susceptible to extracellular killing by neutrophils.[36]

Streptococcus pneumoniae and *S. pyogenes* can induce NETosis, but they also developed some escape mechanisms. *S. pyogenes* virulence factor M1 modulates NET formation via an association with fibrinogen ultimately leading to a complex, which stimulates neutrophils.[37] Furthermore, overexpression of M1 in susceptible strains of *S. pyogenes* confers resistance to extracellular killing because of the sequestration and neutralization of the LL37 (also known as cathelicidin), which is a neutrophil antimicrobial peptide significantly decreasing bacterial colonization.[38] Mutant forms of M1 have been found with a decreased ability of NET induction and deletion of M1 increases the tendency toward NET killing.[7] Similarly, α-enolase from *S. pneumoniae* can increase neutrophil-migrating activity and induce their death by releasing NETs; however, genetic ablation of α-enolase has not blocked NETosis.[39] *S. pneumoniae* can escape NETs in a passive manner through its polysaccharide capsule reducing NET binding[40] or trough active strategies. *S. pneumoniae* can express the DNase EndA, which facilitates the escape from NETs increasing the virulence in vivo.[41] In a similar way, the nuclease Sda1 in *S. pyogenes* degrades NETs

Table 1 Microorganisms inducing NETosis

Authors	Microorganism	Microbe peptides inducing NETs	Effects	Type of NETosis
Bacteria				
Pilsczek et al[10]	*S. aureus*	Leukotoxin GH; Panton-Valentine leukocidin	ROS-independent induction with nuclear DNA liberation, TLR2- and C3-dependent	Vital
Mori et al[39]	*S. pneumoniae*	EndA; α-enolase	ROS-independent induction	Suicidal
Carestia et al,[14] Marin-Esteban et al,[226] Pieterse et al[227]	*E. coli*	Unknown	Platelet-free or platelet-dependent induction, TLR4-dependent or independent, and ROS-dependent or independent	Suicidal (in absence of platelets) or vital (in presence of platelets)
Brinkmann et al[1]	*S. flexneri*	IcsA and IpaB	Induction	Suicidal
Brinkmann et al[1]	*S. typhimurium*	Unknown	Induction	Suicidal
Möllerherm et al,[49] Gillenius and Urban[228]	*Yersinia* spp.	Yops proteins and invasion protein	ROS-dependent induction, PI3K signaling, β-integrin pathway	Suicidal
Braian et al[51]	*M. tuberculosis*	Adhesins, ESAT/6; hsp72 is released after *M. tuberculosis* phagocytosis	Phagocytosis-, elastase, and ROS-dependent induction	Suicidal
Seper et al[17]	*V. cholerae*	Dns and Xds	ROS-dependent induction	Suicidal
Viruses				
Tripathi et al[54]	Influenza virus	Unknown	ROS- and PAD4-dependent induction	Suicidal
Moreno-Altamirano et al[55]	Dengue virus	Unknown	NET inhibition and Glut-1 decreasing glucose capture	Unknown
Saitoh et al[52]	HIV	Unknown	ROS-dependent induction	Suicidal
Raftery *et al*[57]	Hantavirus	Viral particles, Src kinase, and β2 integrin	ROS-dependent induction	Suicidal
Fungi				
Byrd et al[229]	*C. albicans*	Unknown	C3R- and fibronectin-dependent and ROS-independent induction	Vital
Bruns et al[60]	*A. fumigatus*	RodA	ROS-dependent induction; spores containing RodA do not induce NETs	Suicidal
Rocha et al[61]	*C. neoformans*	Unknown	ROS and NETs inhibition	Unknown
Parasites				
Waisberg et al[230]	*P. falciparum*	Agaphelin	Induction through *P. falciparum* and inhibition through agaphelin	Suicidal
Abi Abdallah et al[65]	*T. gondii*	Unknown	MEK-ERK-dependent induction	Suicidal

Abbreviations: C3R, C3 receptor; ESAT, early secretory antigen target; HIV, human immunodeficiency virus; hsp, heat shock protein; MEK-ERK, mitogen-activated protein kinase/extracellular signal regulated kinase; NET, neutrophil extracellular trap; PAD, protein arginine deiminase; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; TLR, toll-like receptor.

and confers high virulence in vivo.[42] Some strains of *Streptococci* can express the protease SpeB, which degrades Sda1 blocking the possibility to escape NETs, as found in mouse models.[43] Interestingly, Sda1 has been demonstrated to degrade bacterial DNA, thus preventing the alert of the immune system via TLR9.[44] This witnesses the virulence attributed to Sda1 which does not depend merely on NET escape, but probably on an intrinsic capacity.

NETs have been described to be significantly induced when neutrophils are stimulated with the serum of patients suffering from septic shock by *Escherichia coli* and this is likely to depend on TLR or complement receptor activation.[45] The enteropathogenic strain WS2572 of *E. coli* can trigger NET formation in neutrophils from the bone marrow of wild-type mice. Interestingly, NET synthesis is abolished in neutrophils from glutathione reductase (GSR)-deficient mice suggesting a

role for oxidative levels in the formation of NETs.[46] As LL37 is associated with NET synthesis, it may play a relevant role in pathogen elimination by cooperating with NETs.[47]

Clostridium difficile is likely to stimulate NET formation, which may act to reach the injured areas of the intestinal epithelium and effectively hinder bacterial dissemination.[48] Brinkmann et al demonstrated that *Shigella flexneri* is trapped by NETs in vitro and described the ability of DNA-associated elastase to abolish virulence factors IcsA and IpaB.[1] *Salmonella typhimurium* has been shown to trigger NETs. *S. typhimurium* is usually trapped and eliminated by components of NETs, including granular proteins and H2A histone.[1] In 2015, Möllerherm et al demonstrated that some serotypes of *Yersinia enterocolitica* could induce NETs in vitro within 1 hour of incubation, but induction diminished as the incubation time increased, maybe related to the effects of calcium- and magnesium-dependent nucleases.[49] *Vibrio cholerae* can induce NETs in vitro after the contact with neutrophils. However, it is able to release the nucleases Dns and Xds as an evasion mechanism, thus feeding the infectious process.[17] Finally, the facultative intracellular bacterium *Mycobacterium tuberculosis* has also been demonstrated to induce NETs when co-cultured with neutrophils. Although NETs effectively trap and hinder the spread of *M. tuberculosis*, NET-derived components do not kill them.[50] Braian et al proposed a role for the heat shock protein 72 sequestered in NETs in the interaction between neutrophils and macrophages during the early innate immune phase of an infection by *M. tuberculosis*.[51]

Viruses

Neutrophils are slightly involved in viral infections and only few studies investigated this issue. After neutrophils recognize human immunodeficiency virus (HIV)-1 nucleic acids through TLR7 and TLR8, they release ROS inducing NETs, which capture and neutralize HIV virions by MPO and α-defensins.[52] At the same time, HIV-1 is also able to suppress NET formation being recognized by CD209 on dendritic cells (DCs) and leading them to the production of anti-inflammatory IL-10.[52] Influenza A virus stimulates NETs via PAD4, while NET-associated α-defensin-1 blocks its replication by abrogating protein kinase C pathway.[53] Also LL37 is involved in NET production in response to influenza A virus in vitro, while arginine-rich H3 and H4 histones are important for viral aggregation and neutralization.[54] A role for NETs has also been demonstrated for dengue virus[55] and respiratory syncytial virus, although for the latter NETs can contribute to airway obstruction, thus exerting a dual protective and pathogenic role.[56] NETs have also been detected in kidney biopsies and sera of patients infected by hantavirus.[57] Hantaan virus (HTNV), the prototype member of the genus *Hantavirus*, uses the β2 integrin complement receptor (CR) 3 and CR4 as entry receptors, but can concurrently induce both ROS production and NET formation through the same β2 integrin signaling. This systemic NET overflow is accompanied by the production of autoantibodies towards nuclear antigens. Moreover, HTNV has been found to stimulate human NETs in a more efficient way and at lower titres compared with vaccinia virus or LPS.[57]

Fungi

Neutrophils play a crucial role in the control of fungal infections, with NETs behaving as a precious weapon for this purpose. *Candida albicans* can change from yeast to hyphae; since hyphae are too big to be phagocytosed, extracellular killing by NETs is a perfect strategy to block this form as well as an efficient way to kill *C. albicans* as a single cell.[58] Calprotectin has been identified as a major antifungal agent toward *C. albicans* and in fact is found to be associated with it in NETs; anyway, direct contact with the fungus is not required as calprotectin can chelate magnesium and zinc ions requested for *Candida* growth.[59]

Aspergillus fumigatus induces NET release in vitro requiring NOX.[35] Although NETs are fundamental for the elimination of *A. fumigatus* hyphae, they are not induced by spores because of the presence of RodA in the wall of spore cells.[60] RodA-deficient *A. fumigatus* conidia induce NETs better than wild-type hyphae, so that RodA may have an inhibitory effect on NET formation by shielding yet unidentified NET-inducing elements in conidia. As a further proof of it, *A. fumigatus* conidia are killed primarily by phagocytosis and not by NETs.[60]

Cryptococcus neoformans can modulate NET production. Particularly, neutrophils incubated with strains whose capsules contained glucuronoxylomannan and galactoxylomannan have been demonstrated to produce neither ROS nor NETs, but they did in non-capsulated strains. NET-associated antimicrobial peptide, such as MPO, elastase and collagenases, are needed to kill the fungus.[61]

Parasites

The number of studies investigating the role of NETs in immune responses toward protozoan parasites is increasing, although most of them have been conducted using animal-derived (e.g. goat or seal) neutrophils. *Plasmodium falciparum* induces NET formation. NETs entangle parasitized red blood cells and trophozoites together with anti-nuclear antibodies are involved in the pathophysiology of malaria in children and in the development of autoimmune phenotypes.[62]

The two main parasite stages of *Leishmania*, amastigotes and promastigotes, have been found to induce NETs ex vivo, which acts as a mechanism of defence against the infection. The induction of NETs is independent of NOS activity and ROS release. The entanglement of parasites within NETs decreased the viability of parasites, even if some authors state that the main role for NETs is immobilization of parasites and control of the infection.[63,64]

Toxoplasma gondii can stimulate NET formation. The presence of the parasite in the bloodstream is not necessary to trigger NET release. NETs kill around 25% of the entangled parasites, thus controlling the infection. In human blood-derived neutrophils, the production of NETs has been demonstrated by the activation of the Raf/MEK/ERK pathway in response to *T. gondii*.[65]

Finally, some animal parasites, such as *Eimeria bovis and Besnoitia besnoiti*, were reported to participate to NET formation.[66–69]

NETs in Cardiovascular Diseases

Dysregulation of neutrophil function has been recently indicated to play a pivotal role throughout the pathogenesis of most representative vascular diseases, such as acute coronary syndrome (ACS), stroke and venous thrombosis. Among these disorders, ACS and stroke are dramatic complications of advanced atherosclerosis. In the earliest phases of atherogenesis, neutrophils are recruited by upregulated adhesion molecules on the dysfunctional endothelial surface, where they exacerbate the oxidative stress and invade the vessel wall. After extravasation, and later in advanced atherosclerotic lesions, these cells sustain a vicious cycle leading to chronic inflammation and increased plaque vulnerability by releasing oxidative enzymes, ROS and chemokines.[70] On the contrary, during venous thrombosis, neutrophils are largely recruited from activated endothelium through von Willebrand factor (vWF)-, tissue factor (TF)- and adhesion molecule-mediated pathways accounting for the large part of inflammatory cells found in thrombus during the early stage of the disease.[71] Thus, after the description of NETosis in 2004, questions raised about its specific contribution to the pathogenesis of CV disease and, consequently, its feasibility as marker of disease or therapeutic target.

NET Activity in CV Risk Factors and Early Endothelial Dysfunction

Supporting the pathophysiological link between NETosis and vascular diseases, some known CV risk factors are associated with exacerbated or dysfunctional NET production. Diabetic patients showed high plasma levels of NET-associated proteins (e.g. elastase, mono-oligonucleosomes and double-strand DNA)[72] and impaired NET release in response to bacterial infections.[73] The constitutive NET formation described during hyperglycaemia can blunt their formation in response to proper *stimuli*.[74] Interestingly, this phenomenon does not appear as a consequence of an impaired glycaemic control, rather it seems related to the chronic proinflammatory environment accompanying diabetes.[75] Recently, age-related CV dysfunction has also been directly linked to NETosis in mice. While wild-type old mice showed a high rate of heart fibrosis and a decline in systolic and diastolic ventricular function, these features are considerably reduced in age-matched PAD4$^{-/-}$ animals, in which NETosis is abolished.[76] Similarly, hypertension has been demonstrated to induce NETosis and platelet recruitment in wild type, but not in PAD4$^{-/-}$ or DNase-treated myocardium.[76] On the contrary, endothelial dysfunction is widely described as *primum movens* of atherogenesis. NET-associated proteins have already shown their ability to induce endothelial toxicity and increase its thrombogenicity. Both circulating cell-free ds-DNA and histones are released by NETs upon the effect of inflammation and, acting as danger-associated molecular patterns (DAMPs), they can exacerbate inflammation itself, thus creating a deleterious vicious cycle at the endothelial level.[77,78] In particular, histones have been shown to stimulate the exocytosis of proinflammatory and procoagulant Weibel-Palade bodies by endothelial cells.[79]

Moreover, matrix metalloproteinase (MMP)-9 contained within the NET web can reduce aortic endothelium-dependent vasorelaxation and trigger endothelial dysfunction through the activation of MMP-2.[80] Interestingly, interactions between NET and dysfunctional endothelium are not unidirectional: activated endothelial cells are able to induce NET formation in a C-X-C motif chemokine (CXCL)8-dependent way and extensive neutrophil co-culture with endothelial cells results in endothelial damage, which could be abrogated by DNase or through the inhibition of NOX.[81]

The Role of NETs in Atherosclerosis and Atherothrombosis

NETs have been identified within atherosclerotic lesions and arterial thrombi in both human samples and atherosclerotic animals.[82,83] The pathophysiological relevance of NETosis in atherosclerosis has been underscored by inhibiting PAD4 in ApoE$^{-/-}$ mice with chloramidine.[84] In particular, chloramidine-treated mice showed reduced vessel inflammation (e.g. interferon [IFN]-α levels), plaque size and thrombotic attitude.[84] Recently, interesting insights in this field came from a research by Warnatsch et al.[85] In this study, cholesterol crystals were able to induce NETosis in vivo and NETs could prime macrophage to produce pro-IL-1β, thus activating a strong pro-inflammatory Th17 response.[85] In atherosclerotic-prone mice fed with high cholesterol diet, intraplaque NETs co-localized with cholesterol crystals and macrophages near the necrotic core, while they did not in more stable zones. The same atherosclerosis model, when lacking NE and proteinase-3, developed dramatically smaller and less inflamed atherosclerotic lesions with respect to ApoE$^{-/-}$ control mice, together with lower plasma IL-1β levels.[85] Another mechanism by which NETs are supposed to increase atherogenesis has been described by Döring et al and involves plasmacytoid DC.[86] In this paper, complexes formed by extracellular DNA and neutrophil-derived proteins have been shown to increase the plaque burden by stimulating a DC-driven strong type I IFN response. On the contrary, the absence of these cells leads to decreased atherogenesis and reduced inflammatory response.[86]

Preclinical studies specifically investigating the effect of NET modulation on atherothrombosis are scarce and the current knowledge largely derives from immunohistochemical analysis of human specimens. The blockage of neutrophil-derived nucleosomes by anti-histones antibodies treatment has been demonstrated to prolong the time to occlusion in the ferric chloride arterial injury model.[87] This effect was completely reversed in elastase/cathepsin G double knockout mice; under a mechanistic point of view, nucleosomes are able to facilitate thrombosis by allowing NE to digest the TF pathway inhibitor.[87] Moreover, neutrophil-derived serine proteases and nucleosomes, two main components of NETs, may contribute to arterial thrombosis in the context of sterile inflammation supporting dramatic atherosclerosis complications, such as myocardial infarction and ischemic stroke. In a mouse model of myocardial ischaemia/reperfusion (I/R) injury, NETosis targeting via DNase I injection has shown cardioprotective features by reducing both plasma levels of nucleosomes and citrullinated histone 3 (citH3) presence at the site of injury.[88]

Similar features have been found after cardiac ischemic damage in PAD4^{−/−} mice.[88] Finally, concurrent DNase I and recombinant-tissue plasminogen activator (r-TPA) therapy has been demonstrated to reduce infarct size, no-reflow area and post-ischaemic left ventricle remodelling in an animal myocardial I/R injury model, whereas these beneficial effects have not been observed in rats treated with DNase and r-TPA alone,[89] thus suggesting a potential role for anti-NET agents as therapeutic strategy during ischaemic heart diseases.

In human coronary specimens from infarcted patients, NETs were most frequently found in the early stages of thrombus evolution (e.g. fresh and lytic thrombi), while these cells missed in advanced, organized thrombi.[83] Recently, a study analysing coronary thrombectomy from patients with ST-elevated myocardial infarction showed that the thrombus NET burden correlates in a positive fashion with the infarct size and negatively with ST-segment resolution.[90] In the same work, the plasma collected from the culprit lesion site contained higher concentrations of NET-related biomarkers when compared with femoral samples from the same patients as well as DNase activity at the culprit lesion site negatively correlated with thrombus NET burden and infarct size. Moreover, recombinant DNase accelerated the coronary thrombi lysis in vitro.[90] In particular, Stakos et al recently demonstrated that neutrophils at culprit lesion site can release thrombogenic signals through NET formation and subsequent delivery of active TF.[91]

Even if most of the currently available evidence links NETs to atherothrombosis, they may also participate to earlier superficial erosion of the plaque by inducing endothelial cell apoptosis.[92] Of importance, biomarkers of NETosis already showed to be positively associated with the severity of atherosclerosis and to predict future CV events (►Table 2). Under this point of view, Borissoff et al gave fundamental insights by correlating the disease severity in patients with coronary disease assessed by computed tomographic angiography and markers of NETosis.[93] Although these data underline the importance of such death mechanism in atherosclerosis-related diseases, more studies are needed to overcome the low specificity of some NET-related biomarkers (e.g. histones and double strain [ds]DNA).

NETs have also been investigated in the setting of acute ischemic stroke (AIS) highlighting the role of thrombo-inflammation as a pivotal player in the pathophysiology of ischaemic stroke[94] along with thrombosis and inflammation, which constitute a loop of bidirectional regulation contributing to ischaemic damage in brain or other tissues.[95] Recently, Vallés et al described for the first time that three markers of NETs—dsDNA, nucleosomes and citH3—are significantly elevated in patients with AIS when compared with healthy subjects; in particular, the greatest proportional increase was found for the most specific NET marker citH3.[96] Levels of the above-mentioned NET markers have been correlated with stroke severity at onset and discharge by the clinical National Institutes of Health Stroke Scale and modified Rankins scale scores as well as by significant elevations of citH3, dsDNA and nucleosomes. Authors also found that citH3 and dsDNA levels were higher in patients with cardioembolic stroke, this being related to a higher inflammatory activation. This issue was in touch with the significant increase in NET markers for patients with a history of atrial fibrillation (AF), too. Finally, citH3 has been found elevated especially in older patients, with higher fasting glucose, and with prior AF and independently associated with all-cause mortality at 1-year follow-up.[96] These results indicate that citH3 might represent a useful prognostic marker in patients with AIS, warranting new researches for neuroprotective therapies in this field. The results of other studies investigating NETs in AIS are listed in ►Table 3.

NETs and Venous Thrombosis

Although arterial and venous thromboses are different syndromes with different main causes, they found in NETs a common pathogenic pathway. Several key components of NETs, such as nucleic acids, histones and enzymes, have shown venous procoagulant features.[97–99] Histone infusion leads to vWF release and has been found to accelerate the thrombus formation in inferior vena cava (IVC) stenosis model of deep venous thrombosis (DVT).[100] Thrombi from the same animal models are characterized by an important presence of NETs associated with vWF, especially in the earliest stages.[100,101] NETs interact with vWF through histone A1 domain,[102] but they can also bind other thrombosis-related proteins (e.g. fibronectin) containing several DNA-binding domains.[101] Not only vWF but also TF sustains the strong relation between venous thrombosis and NETs. During the generation of NET, neutrophils produce both TF and NE. NE is critical to further increase TF activity by the cleavage of TF inhibitory molecules.[71,91] Thus, after platelet and neutrophil activation by procoagulant factors, the latter generates NETs acting as trap and framework for thrombus elements, such as red blood cells, leukocytes, platelets and activated coagulation factors.[71,103] The crucial contribution of NETosis in venous thrombosis has been underlined by PAD4^{−/−} mice, in which the lack of NETs results in fewer thrombi after IVC stenosis compared with wild-type ones; similar effects have also been demonstrated by the administration of DNase I.[104] Although some controversies still remain,[105] these results encouraged scientists to shift their attention from preclinical setting to the bedside. In 2013, two papers showed an association between increased plasma markers of NETs and DVT (►Table 2). The first article stated that in patients with DVT circulating nucleosomes and elastase-α1-antitrypsin complexes, levels are increased with respect to patients with first clinical suspicion of DVT, which has been then excluded by ultrasonography.[106] The second study focused on plasma DNA level showing higher concentrations of this marker in DVT patients and its correlation with D-dimer, Wells score and MPO, the third indicating neutrophil as the source of the nucleic acid.[107] Recently, the immunohistochemical analysis of thrombi at different stages of development from human surgery or autopsy showed high presence of NETs (indicated by the association of citrullinated histone H3 with MPO and DNA) in thrombi during the phase of organization.[108]

Table 2 Recent studies investigating NET biomarkers in CV diseases

Author	Year	Patients	Biomarkers	Results
Atherothrombosis				
Shimony et al[231]	2010	16 patients with STEMI and 47 healthy subjects	dsDNA	dsDNA levels were significantly higher in patients compared with controls ($p = 0.001$) and positively correlated with levels of CK and TnT ($r = 0.79$ and 0.65, $p < 0.001$ and $p = 0.006$, respectively)
Borissoff et al[93]	2013	282 patients with suspected coronary heart disease undergoing coronary CTA were grouped according to the presence and severity of CAD	dsDNA, nucleosomes, citH4 and MPO-DNA complexes	dsDNA, nucleosomes and MPO-DNA complex levels were significantly higher in patients with severe CAD ($p = 0.003$, $p < 0.001$ and $p < 0.05$, respectively) or abundant coronary artery calcification ($p < 0.001$ for all) with respect to healthy controls. Their levels correlate with the severity of luminal stenosis ($p \leq 0.001$ for all) and with number of diseased coronary artery segments ($p \leq 0.001$ for all). Baseline values higher than the total group median of dsDNA (OR, 3.12; 95% CI, 1.27–7.63; $p = 0.013$), nucleosomes (OR, 2.59; 95% CI, 1.09–6.14; $p = 0.030$) and MPO–DNA (OR, 3.53; 95% CI, 1.38–9.03; $p = 0.009$) were significantly associated with the occurrence of MACEs
Cui et al[232]	2013	137 ACS patients (51 with unstable AP, 37 with NSTEMI, and 49 with STEMI), 13 stable AP patients, and 60 healthy controls	dsDNA	ACS patients showed higher dsDNA levels compared with stable AP patients and control group ($p < 0.05$ for both). Significant differences in dsDNA concentrations were observed in ACS group among unstable AP, NSTEMI, and STEMI sub-groups ($p < 0.05$ for all). dsDNA levels were different among ACS patients divided into three groups according to Gensini score with increasing levels of dsDNA concurrent with increasing Gensini score ($p < 0.05$, for all)
Mangold et al[90]	2015	111 patients with STEMI undergoing PCI	Nucleosomes and dsDNA	Nucleosomes and dsDNA levels were significantly higher at the culprit lesion site than to the femoral artery ($p = 0.0002$ and $p < 0.0001$, respectively)
Helseth et al[233]	2016	30 patients with CAD undergoing PCI (20 with STEMI and 10 with stable AP)	dsDNA and nucleosomes	dsDNA and nucleosome levels were higher in patients with STEMI compared with patients with AP ($p < 0.03$ for both). dsDNA significantly correlated with peak TnT and CK-MB at day 5 ($p = 0.03$) and with lesion size assessed by MRI at days 5 and 7 ($p = 0.01$ and 0.04, respectively). Nucleosomes correlate with infarct size at day 5 ($p = 0.02$)

(*Continued*)

Table 2 (*Continued*)

Author	Year	Patients	Biomarkers	Results
Venous thrombosis				
van Montfoort et al[106]	2013	150 patients with symptomatic DVT and 195 patients with clinical suspicion of DVT in whom the pathology was excluded by US examination	Nucleosomes	Nucleosome levels were significantly higher in patients with DVT ($p < 0.001$) and positively correlated with neutrophil activation in both case and control samples ($p < 0.001$ for both). An increase in nucleosome levels >80th percentile carried an increased risk of DVT than levels ≤80th percentile after adjustment for potential confounders (OR: 3.0, 95% CI: 1.7–5.0)
Diaz et al[107]	2013	47 patients with symptomatic DVT, 28 patients with clinical suspicion of DVT not confirmed by US examination, and 19 healthy volunteers	dsDNA	dsDNA levels were higher in DVT group with respect to both negative control groups ($p < 0.01$ for both). dsDNA levels showed a positive correlation with CRP ($p < 0.01$), D-dimer ($p < 0.01$), and vWF ($p < 0.01$) and the Wells score ($p < 0.01$). A negative correlation was found with ADAMST13 ($p < 0.01$)

Abbreviations: ADAMST13, *a d*isintegrin *a*nd *m*etalloproteinase with *t*hromboSpondin-1 motifs (13th member of the family); CAD, coronary artery disease; citH4, citrullinated histone H4; CK-MB, creatine kinase, muscle and brain; CRP, C-reactive protein; CTA, computed tomography angiography; CV, cardiovascular; ds, double strain; DVT, deep venous thrombosis; HR, hazard ratio; LV, left ventricle; MACEs, major cardiovascular events; MI, myocardial infarction; MPO, myeloperoxidase; MRI, magnetic resonance imaging; NSTEMI, non–ST-elevated myocardial infarction; N/L, neutrophil to lymphocyte; OR, odds ratio; PCI, percutaneous coronary intervention; RR, relative risk; SINTAX, Synergy between Percutaneous Coronary Intervention with Taxus and Cardiac Surgery; STEMI, ST-elevated myocardial infarction; TnT, troponin T; US, ultrasound; vWF, von Willebrand factor.

NETs in Autoimmune and Autoinflammatory Diseases

Systemic autoimmune diseases develop as multistep processes through a complex interplay between genetic and environmental factors leading to cellular damage and the consequent exposure of immune cells to autoantigens. The imbalance of different cell death mechanisms (apoptosis, necroptosis, pyroptosis, NETosis and autophagy) has been proposed as a critical source of modified and/or externalized autoantigens.[109] Especially nuclear material released from NETs seems to be more immunogenic than the apoptotic one. Both native and oxidized self-DNA bound to NETs activate DCs to synthetize IFN-α in a TLR-dependent manner.[110,111] In mice, the immunization with NET-loaded DCs promotes the development of autoimmunity better than apoptotic neutrophil debris.[112] NETs also increase T-cell response to antigens and activate B cells to induce immunoglobulin (Ig) class switching and antibody production.[113] Once externalized, oxidized DNA is also more resistant to degradation and this behaviour contributes to sustain a dysregulated immune response.[114] In addition, NET-mediated activation of the inflammasome further amplifies the inflammatory response through a feed-forward loop. The inflammasome stimulation triggers synthesis and release of IL-18 and IL-1β, which in turn induces NET formation.[115] Activation of classic and alternative pathways of complement system as well as coagulation cascade is additional immunogenic mechanisms linking NETs to autoimmune/autoinflammatory diseases.[116] Noteworthy, many of the proteins found in NETs are recognized as major autoantigenic targets in rheumatologic diseases: dsDNA and histones in SLE, vimentin and enolase in RA, MPO and proteinase-3 in vasculitis associated with antineutrophil cytoplasmic antibodies (ANCAs). Different autoantibody profiles may then be influenced by the cargo protein within NETs and detailed analysis revealed differences in protein content and posttranslational modifications associated with different autoimmune diseases.[117–119]

Systemic Lupus Erythematosus

Neutrophils from patients with SLE showed various abnormalities in their phenotype and function.[120] Circulating levels of apoptotic neutrophils, which may provide excess autoantigen such as dsDNA, are increased in patients with SLE and correlate with disease activity.[121] Furthermore, patients with SLE are characterized by a distinct neutrophil subpopulation known as low-density granulocytes (LDGs).[122] Those cells are prone to release proinflammatory cytokines and show enhanced NET formation. NETs released by LDGs contain high levels of autoantigens and immunostimulatory molecules, such as LL37, MMP-9, and α- and β-defensins.[123] LDGs are also enriched of dsDNA and oxidized nucleic acids, which are strong inducers of IFN-α and NOD-like receptor family pyrin domain-containing (NLRP)3 inflammasome.[115,124] Finally, patients with SLE exhibit impaired NET clearance correlating with disease activity.[125] As a result of overproduction and defective clearance of NETs,

Table 3 Studies investigating NET biomarkers in acute ischemic stroke

Author	Year	Patients	Biomarkers	Results
Rainer et al[234]	2003	88 patients with stroke-like symptoms presenting to the ED	nDNA	nDNA concentrations within 3 h of symptom onset were higher in died patients than in those who survived at discharge ($p = 0.03$) as well as in died patients with NIHSS scores >8 who survived to 6 mo ($p = 0.002$). nDNA concentrations correlated with the volume of cerebral hematoma ($p = 0.03$). nDNA concentrations predict 6-mo mortality (OR: 1.6, 95% CI: 1.1–2.4; $p = 0.03$) and 6-mo RS score >2 (OR: 1.8, 95% CI: 1.0–3.3; $p = 0.05$)
Geiger et al[235]	2006	63 patients with stroke observed daily during the first week	Nucleosomes	In patients with BI score ≥50, the increase in nucleosomes is prolonged until day 5. Patients with BI score <50 showed a steeper initial increase with a maximum on day 3. Both days after stroke and BI score significantly influenced nucleosome concentrations ($p < 0.001$ for both). Nucleosome concentration showed a significant correlation on day 3 with infarction volume ($p = 0.001$)
Lam et al[236]	2006	44 patients aged ≥18 y presenting to the ED with a stroke-like syndrome but negative neuroimaging results	nDNA	Patients with post-stroke mRS grades 3–6 have been shown with a nDNA concentration significantly higher than that of patients with post-stroke mRS grades 0–2 ($p = 0.01$). nDNA concentration could predict post-stroke morbidity and mortality in patients with negative neuroimaging
Geiger et al[237]	2007	63 patients with stroke observed daily during the first week	Nucleosomes	Levels of nucleosomes at days 3 and 6 correlated significantly with initial BI ($p = 0.0023$ and 0.0284, respectively) and with infarction volume only at day 3 ($p = 0.0001$). Strong correlations have been shown between BI at admission and BI at discharge and between BI at admission and infarction volume ($p < 0.0001$ for both). In patients with initially severe defects (BI <50), nucleosomes at day 3 have been found to be prognostically relevant ($p = 0.014$). In multivariate analysis, nucleosomes and BI at admission showed independent prognostic relevance ($p = 0.039$)
Tsai et al[238]	2011	50 AIS patients and 50 control subjects	nDNA and mDNA	Levels of nDNA and mDNA were higher in patients with AIS than in controls ($p < 0.05$). Elevated circulating nDNA in plasma persisted until 1 mo after AIS. Levels of nDNA positively correlated with the clinical severity of stroke according to NIHSS score
Hirose et al[239]	2014	49 critically ill patients admitted to ICU, of whom 8 with stroke	DNA, citH3	DNA has been found elevated in patients with stroke with respect to controls. citH3 has been detected in blood smears by immunofluorescence
Thålin et al[240]	2016	31 patients with ischemic stroke	DNA, citH3	Patients with concurrent hsTnT elevation revealed cerebral micro-thrombosis with citH3 in thrombi in a higher rate than controls ($p < 0.001$). citH3 correlated positively with thrombin–antithrombin complex ($p = 0.004$) and soluble P-selectin ($p < 0.001$)

(Continued)

Table 3 (*Continued*)

Author	Year	Patients	Biomarkers	Results
Vallés et al[96]	2017	243 patients with AIS followed up for 12 mo after the event	dsDNA, nucleosomes, citH3	dsDNA, nucleosomes and citH3 were significantly higher among patients with AIS compared with healthy subjects ($p < 0.05$; $p < 0.001$ for the latter). These parameters were increased in patients who were older than 65 y ($p < 0.001$), in those with a history of AF ($p = 0.013$ for dsDNA, $p = 0.007$ for citH3, $p = 0.02$ for nucleosomes), CE stroke ($p < 0.05$ for dsDNA and citH3), high glucose levels ($p < 0.05$ for all markers) and severe stroke scores at admission ($p < 0.001$ for all markers) and discharge ($p < 0.001$ for citH3 and nucleosomes, $p = 0.038$ for dsDNA). In multivariate analysis, elevated levels of citH3 at onset was independently associated with AF (OR: 6.704, 95% CI: 1.4–32.1; $p = 0.017$) and with all-cause mortality at 1-year follow-up (OR: 7.055, 95% CI: 1.631–30.50; $p = 0.009$)
Laridan et al[241]	2017	68 ischemic stroke patients undergoing endovascular treatment	DNA, citH3	citH3 was observed in almost all thrombi and co-localized with extracellular DNA released from neutrophils. citH3 was more abundant in thrombi of CE origin compared with other etiologies ($p < 0.05$). Older thrombi contained significantly more neutrophils and citH3 compared with fresh thrombi ($p < 0.001$ and $p < 0.05$, respectively)

Abbreviations: AF, atrial fibrillation; AIS, acute ischemic stroke; AUC, area under the curve; BI, Barthel index; CE, cardioembolic; CI, confidence interval; citH3, citrullinated histone H3; CRP, C-reactive protein; ds, double strain; ED, emergency department; hsTnT, high sensitive troponin T; mRS, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale; m, mitochondrial; n, nuclear; NET, neutrophil extracellular trap; NSE, neuron-specific enolase; OR, odds ratio.

the presentation of autoantigens to autoreactive B cells is enhanced.[116,126] A leading role in SLE pathogenesis may be played by an alternative form of NETs, called *mitochondrial DNA NETs*. In fact, more severe forms of SLE have been observed in mice after injection of oxidized mitochondrial DNA and in humans carrying NOX-deficient genes (typically in CGD).[124,127] Although the role of NETs in SLE requires further investigations, their high levels found in the skin, kidney and bone marrow support a direct role in SLE-associated organ dysfunction[123] and preliminary clinical studies support a potential association between circulating levels of NETs and disease activity (►**Table 4**).[116,123,125,128–131]

Rheumatoid Arthritis

In RA, activated neutrophils are the most abundant cells in the synovial fluid. In addition, a wide number of anti-citrullinated proteins/peptides antibodies (ACPA) is produced in RA, representing specific disease markers.[132] Both endogenous and exogenous antigens become target of ACPA after deimination (or citrullination), which is a post-translational modification largely catalysed by the PAD2 and 4, usually overexpressed in RA patients.[133,134] A major contribution to the citrullination comes from NET generated from activated neutrophils, especially those belonging to the LDG subpopulation.[118,135]

Furthermore, deiminated histones have been recognized as key mechanism leading to ACPA generation in RA patients, especially those with Felty syndrome.[59,136] Noteworthy, the ectopic lymphoid structures localized in the RA joint synovium may also contribute to the NET generation. They represent functional structures supporting the clonal selection of autoreactive B cells and then their differentiation to plasma cells producing antibodies against citrullinated antigens.[137–139] Therefore, a delay in the clearance of NETs might form a reservoir of citrullinated antigens, which sustain the autoimmune response in RA as already described for SLE.[125]

ANCA-Associated Vasculitis

ANCA-associated vasculitis (AAV) is referred to as a group of pauci-immune vasculitis characterized by neutrophil-rich necrotizing inflammation of small vessels and the presence of ANCAs. In this context, NETs have been recently found at the sites of vasculitic lesions (kidney and skin) and in thrombi, both as co-localizations of DNA and granule proteins and as mere citrullinated histones.[112,140–145] Some cross-sectional studies compared NET levels during remission and active disease, although results remain inconclusive.[146–148] Neutrophils from patients with AAV are less prone to undergo apoptosis and show spontaneous NET formation. Even though the high

Table 4 Clinical evidence linking circulating NETs to clinical activity of autoimmune diseases

Author	Year	Disease	Patients	Outcome	Result
Hakkim et al[125]	2010	SLE	Lupus nephritis vs. healthy controls	Activity index on renal biopsy	Poor NET degradation was associated with lupus nephritis and increased serum levels of anti-dsDNA antibodies
Villanueva et al[123]	2011	SLE	III or IV class GN	Activity index on renal biopsy	Patients with class IV GN had higher activity index (% of netting neutrophils) and higher circulating levels of anti-dsDNA antibodies
Leffler et al[116]	2012	SLE	Patients with disease remission and flare vs. healthy controls	Disease activity	Low NET degradation was associated with complement activation/deposition, high levels of circulating autoantibodies and renal involvement (GN)
Leffler et al[128]	2013	SLE	69 patients followed up for a median of 784 d	Disease activity	Decreased ability to degrade NETs was associated with clinical manifestations in SLE according with the SLEDAI-2K score
Nakazawa et al[129]	2014	MPA SLE	38 patients with MPA vs. 23 SLE vs. 8 control subjects	BVAS SLEDAI-2K score	Both MPA and SLE patients showed reduced NET degradation. In MPA patients, this ability correlated with disease activity, while no correlation was shown between NETs and disease activity (SLEDAI-2K score) in SLE patients
Zhang et al[130]	2014	SLE	54 patients vs. 43 control subjects	Lupus nephritis	High circulating levels of cfDNA (marker of NET dysregulation) was found in patients with SLE and were associated with markers of renal injury, such as 24-h urinary protein content ($r = 0.350$; $p = 0.013$), serum albumin ($r = -0.500$; $p < 0.001$), and creatinine clearance ($r = -0.354$; $p = 0.044$)
Pérez-Sánchez et al[131]	2017	RA	106 patients vs. 40 control subjects	RF, ESR, CRP, NO, cIMT	RA patients exhibited enhanced NET generation and impaired DNase activity. Furthermore, NETosis-derived products, such as cfDNA, correlated with autoimmune parameters, inflammatory mediators, oxidative stress markers as well as early atherosclerosis

Abbreviations: BVAS, Birmingham vasculitis activity score; cfDNA, cell-free DNA; cIMT, carotid intima media thickness; CRP, C-reactive protein; dsDNA, double-stranded DNA; ESR, erythrocyte sedimentation rate; GN, glomerulonephritis; NET, neutrophil extracellular trap; NO, nitric oxide; MPA, microscopic polyangiitis; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

fraction of LDGs observed in AAV may explain this behaviour, normal-density neutrophils have also been found to spontaneously release more NETs as compared with healthy blood donors.[149] A growing body of data indicates ANCAs not only as neutrophil activators but also as promoters of NET generation. As neutrophil activation is epitope specific, epitope specificity and affinity are found increased during active disease.[150–152] In turn, the overproduction of NETs enhanced the exposition of epitopes (MPO end proteinase-3), further perpetuating the generation of ANCAs.[129,140] Alongside, elevated levels of NETs in AAV patients may be explained by a reduced NET clearance as confirmed by in vitro experiments.[129]

Other Autoimmune Diseases

Recent evidence links NETs and antiphospholipid (aPL) antibody syndrome (APS). High levels of dsDNA and NETs have

been found in patients with APS and the serum of those patients display defective NET degradation.[153,154] More specifically, aPL antibodies from patients with APS may induce the release of NETs from control neutrophils and especially LDGs.[154] Also, thrombi from mice treated with APS IgG have been recently found to be enriched of citrullinated histone H3, thus indicating a role for NETs in thrombotic complications of APS.[155] Further supporting a direct role in thrombosis, growing data linked NETs to preeclampsia, also in aPL-negative patients.[156–158] Finally, NETosis has also been observed in psoriatic skin lesions. By inducing the expression of human β-defensin-2 in keratinocytes, NETs amplify the local inflammation leading to DC activation and consequent development of Munro's abscess.[159,160] Finally, a direct correlation between the amount of NETs in the peripheral blood and disease severity has also been demonstrated.[160]

Gout and Inflammatory Bowel Diseases

Actually, very little is known about the link between NETs and gout and inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC). In gout, NETs have been described not only to feed inflammation,[161] but also to regulate the inflammatory process and block gout episodes.[162] NET generation is responsible for the reduction of neutrophil density, then DNA nets encapsulate monosodium urate crystals protecting them from further phagocytosis, and finally NET-derived proteases block cytokines.[163,164]

NET formation has been poorly studied in IBDs. As ROS levels are elevated, it is likely that neutrophils could produce NETs. In UC, NETs have been observed and correlated with inflammation by proteomic studies, but more studies are warranted to clarify the NET involvement in both CD and UC.[165] In 2016, He et al evaluated the role of NETs in IBDs. The authors found that NETs were generated by peripheral blood neutrophils from patients with active IBD; however, stimulation with sera from patients with active disease also induced significantly NET release on neutrophils isolated from healthy subjects. Moreover, NETs along with phosphatidylserine (PS) exposure on platelets, leukocytes and endothelial cells are involved in the hypercoagulability characterizing active IBD. The researchers also proved an inhibitory effect of lactadherin and DNase I on PS and NETs, separately, suggesting new targets for IBD drugs in next studies.[166]

NETs and Lung Diseases

In the last decade, the formation of NETs has drawn great attention concerning lung diseases. Components of NETs have been shown to damage both epithelial and endothelial cells as well as connective tissue worsening the lung pathology.[167] Indeed, NETs have been identified in the lung of patients suffering from cystic fibrosis (CF), transfusion-related acute lung injury (TRALI), asthma, chronic obstructive pulmonary disease (COPD), and in lungs infected with bacteria, virus, or fungi.[168] In these pulmonary diseases, NETs have been described as emerging pathophysiological players of potential therapeutic interest.

Cystic Fibrosis

In CF, patients with high levels of free DNA in the sputum have been shown with a diminished lung function as compared with those with mild disease, because the airway obstruction is mainly a consequence of the amount of NETs and DNA.[161] Marcos et al recently confirmed this finding, showing that free CF airway DNA levels correlated with pulmonary obstruction both in CF patients and mice.[162] The importance of NETs in CF is highlighted by the fact that the elimination of free DNA from patient's airways is considered as an important therapeutic option. In fact, in both early and mild stages of the disease, NETs can provide extracellular antibacterial and antifungal host defences. At this time, recombinant inhaled DNase should be used more cautiously, being potentially responsible for the delivery of encaptured pathogens. Differently, in moderate to severe stages of CF, the amount of mucus and DNA was responsible

for airway obstruction, which can be efficiently resolved by DNase by cleaving DNA traps.[162,169] Moreover, isolates of *Pseudomonas aeruginosa* can trigger a great respiratory burst and NET release in CF.[170] *P. aeruginosa*-mediated NET formation was found responsible for the bactericidal permeability-increasing (BPI) protein cleavage by *P. aeruginosa* elastase, suggesting a novel mechanism in the development of autoimmunity to BPI.[171] Moreover, the authors also provided a role for autoimmunity in CF disease severity, as autoantibody levels have been associated with diminished lung function.

Chronic Obstructive Pulmonary Disease

Neutrophil elastase has been detected in the airway mucosa of COPD patients during severe exacerbations showing a proinflammatory role via the secretion of CXCL8, which is a powerful NET inducer.[172] In the sputum from acutely exacerbated COPD patients, NETs and NETotic neutrophils have been found in a great amount by confocal laser microscopy and electron microscopy; an abundance of NE and citH3 has also been demonstrated.[173] These findings witness that NETosis can be considered as a part of COPD pathology, relevant for new therapeutic options. Anyway, NETs have been described in the airways of stable COPD patients as a marker of neutrophils in the sputum.[174,175] In particular, a positive correlation between the abundance of NETs in the sputum of COPD patients and disease severity has been described; in fact, more than 90% of exacerbated COPD patients presented with a higher number of NETs in their sputum with respect to stable COPD patients. Indeed, the NET amount directly correlated with the disease severity in terms of airflow limitation.[176] Recently, levels of NETs in the sputum of COPD patients have been directly associated with the severity of the disease and the number of exacerbations as well as with the loss of microbiota diversity and impaired ex vivo neutrophil phagocytosis.[177] In this view, NETs could be an interesting therapeutic target in the future, considering that the only effective drugs are currently long-acting bronchodilators, which do not affect inflammation.

Asthma

Asthma has been classically considered as an eosinophilic disease. However, recent data claim that some asthmatic patients show an important neutrophilic inflammation in the lungs. Patients with "neutrophilic" asthma have shown a reduced response to the classical therapy with glucocorticoids, which in turn aggravate local inflammation by increasing neutrophil survival.[178]

In addition, glucocorticoid administration to neutrophilic asthmatics could aggravate lung inflammation, since glucocorticoids can prolong neutrophil survival. Since the hypothesis of autoimmune involvement in asthma has gained great interest recently, NETs have been demonstrated as key players in the stimulation of airway epithelial cells to produce autoantigens, especially in severe asthma, as antibodies against NE or MPO attenuated these effects.[179] Dworski et al have shown that eosinophils in the airways of atopic asthmatic individuals could release eosinophil ETs and co-

localized with eosinophil granule proteins, such as major basic protein and eosinophil cationic protein.[180] In this case, DNA was of mitochondrial origin, and not nuclear.[181] Interestingly, allergens did not show any increase in eosinophil ET or NET formation in the airways of asthmatic patients.[180] Recently, eosinophils from asthmatic mice have been demonstrated to release eosinophil ETs co-localizing with eosinophil peroxidase aggravating pulmonary impairment, which was reversed by DNase therapy.[182] Recombinant human DNase treatment improved resistance and decreased oxidative stress in the lungs of asthmatic mice.[183] In light of this, a combined use of recombinant human DNase therapy along with inhaled glucocorticoids may provide a reduction in sputum viscosity and improve the quality of life and prognosis of these patients.

Transfusion-Related Acute Lung Injury

TRALI is the leading cause of blood transfusion-related death developing within 6 hour of transfusion and presenting with hypoxemia, respiratory distress, and pulmonary infiltrates. In 2012, Thomas et al demonstrated that NETs form during TRALI both in humans and in mice and that their degradation by DNase 1 inhalation improved the condition of mice with TRALI.[184] Also, platelets can accumulate in the lungs of mice with TRALI and have been described to induce NET formation.[185] In turn, histones expressed by NETs may activate platelets, thus feeding a vicious cycle.[186] As a further proof of it, the pre-treatment of mice with a histone-blocking antibody decreased NET generation as well as lung oedema, lung vascular permeability, and mortality.[185]

NETs and Cancer

Neutrophils are known to be present inside and around solid cancers since long time, thus being the most representative tumour-infiltrating immune cells. Anyway, controversies have been raised about their role in this setting and two subsets of tumour-associated neutrophils (TANs) have been found to develop depending on the influences of the tumour microenvironment: the N1 phenotype displays proinflammatory and antitumourigenic functions, while the N2 phenotype has protumorigenic activity with transforming growth factor-β produced by the local tumour microenvironment playing a central role in polarizing mature neutrophils to adopt a pro-tumour N2 phenotype.[187] Many questions are open about NET's function in tumour growth modulation, even if a role for NETs has been recognized in the local tumour development and especially in tumour-associated thrombosis.

The first finding of a role for NETs in tumours dates back to 2013 based on a small number of Ewing's sarcoma samples suggesting that patients with intratumoral NETs experienced a poorer prognosis.[188] A stronger suggestion for a role of NETs in tumour progression comes from studies investigating NET-associated proteins, such as NE and MPO. NE can directly impact on tumour growth, progression, and cell migration by inducing cell proliferation in both human and mouse adenocarcinoma cell lines.[189] In addition, NE deficiency has been found to blunt tumour burden in mice.[189] Although these results mainly referred to the soluble NE, they suggest a potential role for NETs, which need to be clarified by targeted study. Sangaletti et al found that NETs could stimulate the proliferation and malignant transformation of B cells toward malignant lymphoma via the NF-κB signaling.[190] A role for NETs has also been tested in pancreatic ductal adenocarcinoma.[191] Another NET-associated protein involved in the pathophysiology of cancers is MMP-9, which contributes to carcinogenesis, tumour growth and progression, and metastasis. Apart from its role in different cancer types, it is still controversial whether NET-bound MMP-9 conveys these effects or NETs can even protect from MMP-9 functions; the reason of this controversy can be found in the fact that MMP-9 has been shown to be inactive when bound to NETs.[192] Since IL-8 has been demonstrated to play a role in NET generation and angiogenesis, a clear clinical relevance for IL-8 in tumour progression has been shown for many tumours, too.[193,194] The cascade including IL-8 can be probably considered a vicious cycle started by NETs themselves recruiting additional neutrophils, which in turn produce NETs, all this ultimately concluding with tumour growth and angiogenesis. Demers et al have recently linked tumour progression and NETosis by showing that priming of neutrophils by tumour-associated granulocyte-colony stimulating growth factor (G-CSF) is able to promote tumour growth. Indeed, PAD4-deficiency was protective only against tumour progression when the implanted tumour cell line produced G-CSF.[195]

Various studies have confirmed a role for TANs in the enhancement of cancer cell survival, migration and poor prognosis, but none of this has focused on tumour-associated NETs.[196–198] Interestingly, in vivo evidence from cutaneous melanoma has shown that it becomes more aggressive and metastatic after ultraviolet radiation because of neutrophil recruitment. In fact, neutrophil recruitment has been associated with a more migratory phenotype, local angiogenesis and angiotropism of melanoma cells,[199] with a possible responsibility for NETosis, although NETs have not been specifically focused.

CXCL8 can also play a role in tumour-associated thrombosis as it is contained in microparticles. In the granulocytic subset of myeloid-derived suppressor cells (considered an important T-cell immunosuppressive component in cancer-bearing hosts), IL-8 has been shown to stimulate the formation of NETs.[200] Interestingly, tumour-derived microparticles can interact with macrophages, activating them, and promote the production of tumour necrosis factor-α favouring the recruitment of other inflammatory cells and NETs.[201]

vWF is a known actor in metastasis and tumour growth and the interaction with NETs can result deleterious. In mice models, vWF null mice or mice treated with antiglycoprotein Ibα experienced increased experimental lung metastasis.[202,203] NETs have been demonstrated to bind to vWF on the vessel wall and this is important when considering that melanoma cells can stimulate endothelial cells to produce vWF contributing to thrombosis.[204]

In 2016, Guglietta et al investigated about the role of complement in NET induction and tumour growth. In a spontaneous small intestine cancer model, hypercoagulation can directly affect neutrophil effector function and is linked to complement activation, in particular C3a, showing an increased number of TANs and low-density neutrophils. LDGs displayed features of N2 neutrophils and spontaneously underwent NETosis, which was dependent on the involvement of the complement receptor C3aR. To block this reaction, the immune system triggers the development of neutrophils with an N2 phenotype, which are responsible for tumour growth in a mutation-dependent protumorigenic *milieu*.[205]

A role for NETs has been hypothesized for metastasis, too, even if specific studies are still lacking. Neutrophils per se promote the blocking of circulating tumour cells, especially under inflammatory conditions, and NETs are very important in this setting.[206] Anyway, even considering other studies with DNase I suppressing tumorigenesis, the activation of peripherals cells as a result of tumour-induced intravascular NET formation, which predisposes for metastasis, is still a matter of debate. Platelet–granulocyte complexes with tumour cells not clearly identified as NETs have been proved to create early metastatic niches, which are pivotal for later metastatic progression.[207] Upon the secretion of CXCL5 and CXCL7 by platelets due to contact with platelets and the release of IL-8 from tumour cells recruiting neutrophils, the tethering of tumour cells to endothelium takes place, leading to transendothelial migration first and the following development of metastasis.[208] Main roles of NETs in cancer are summarized in ►**Fig. 2**.

Future Perspectives

Accumulating data on the role of NETs in highly prevalent inflammatory conditions (such as sepsis, CV diseases, autoimmune and inflammatory diseases) pave the way to novel biomarkers and treatments, which might readily become available for patient care. However, their translation into the clinical practice requires further investigations specifically focused on NET biology and measurement assay. First, molecules integrated in NETs may vary according to environmental factors, so that the characterization of NET proteome represents an exciting challenge for the next future. Second, the low
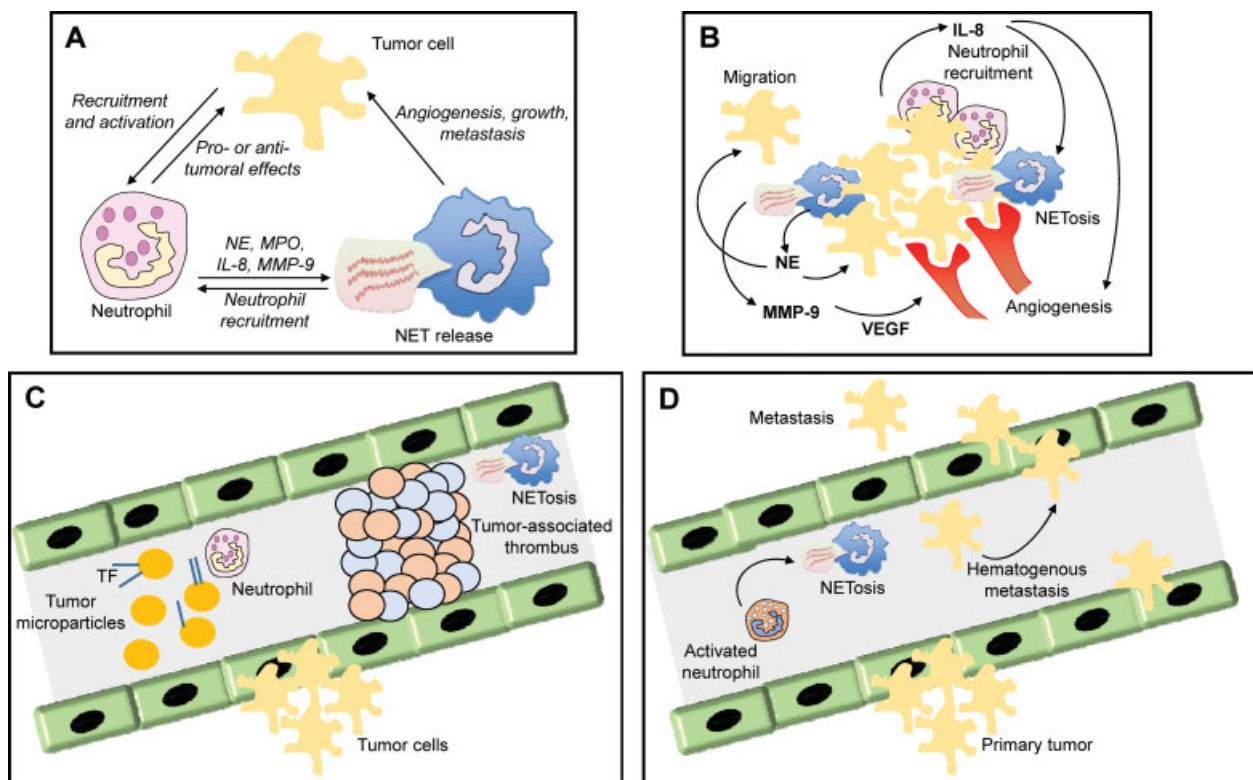

Fig. 2 The complex interplay between neutrophils and cancer. (**A**) Interactions between neutrophils and tumour cells lead to neutrophil extracellular trap (NET) production, which influence tumour growth and progression, angiogenesis and metastasis. In fact, tumour cells can recruit and activate neutrophils, acquiring pro- and antitumoural properties. Neutrophils are also responsible for cytokine secretion promoting the formation of NETs, which present a variety of antimicrobial and cytotoxic substances relevant for tumour growth and progression. (**B**) NETs may influence the local growth and progression of cancer. Neutrophil elastase (NE) is known to stimulate tumour growth, progression and spreading. Interleukin (IL)-8 is responsible for angiogenesis, which in turn is fed by the vascular endothelial growth factor (VEGF) from the extracellular matrix enhanced by matrix metalloproteinase (MMP)-9. NETs themselves can recruit other neutrophils amplifying local inflammatory reaction. (**C**) NETs are known to trigger tumour-associated thrombosis. Indeed, cancer stimulates the release of NETs via the production of granulocyte colony-stimulating factor (G-CSF) or the so-called tumour microparticles with tissue factor (TF) bound on their surface. Since NETs are prothrombotic, their increased production fuels this vicious circle along with the interaction with von Willebrand factor anchored to the vessel wall. Besides, NET-bound NE can inactivate TF pathway inhibitor, ultimately leading to TF-dependent coagulation. (**D**) NETs have been described to have a role in haematogenous metastasis. NET formation within capillaries may provide a scaffold for metastasizing tumour cells, although early adhesive events represent only a part of known mechanisms.

activation threshold of neutrophils allows a ready availability of NETs, but this limits the development of strong, easy and cheap diagnostic assay.[209] The lack of validation of NET assay methods might then explain the contrasting results observed so far in clinical studies. So far, (1) analytical assays (either fluorimetry or enzyme-linked immunosorbent assay [ELISA]) for NET products, (2) confocal microscopy of neutrophil enzymes and extracellular DNA networks and (3) flow cytometry based on nuclear morphology or citrullinated histones and DNA are the main approaches used for NET detection and (semi)-quantification.[209] Furthermore, a standardization of methods and thresholds identifying an increased NET formation is needed. ELISA of plasma samples is likely the method meeting robustness, reproducibility, and easiness criteria for NET quantification. However, ELISA is not yet able to discriminate between increased generation and defective clearance of NETs. Furthermore, caution should be paid to avoid post-sampling NET generation, as many physical and chemical *stimuli* may impact on neutrophil activation.[210]

On the contrary, many concerns exist on considering NETs as a selective therapeutic target. This is largely due to the critical role of NETs as first-line response to infectious agents. Nonetheless, in experimental PAD4$^{-/-}$ mouse, blocking NETosis is indicated as a relatively safe approach, characterized by normal survive in septic shock and better outcome after severe sterile inflammation.[26] According to experimental data, many conventional therapies used for inflammatory diseases revealed inhibitory properties on NET cascade. Reduced NET generation may occur during treatment with ROS scavengers, such as N-acetyl cysteine.[211] Two small studies reported a clinical benefit of treatment with N-acetyl cysteine in patients with SLE.[212,213] Similarly, other conventional treatment used for the treatment of SLE, such as vitamin D and chloroquine, have been found to prevent NET generation.[214,215] Alongside known agents, NET cascade might be inhibited by monoclonal antibodies targeting B cells (rituximab),[216,217] complement system (eculizumab)[218–220] and potentially IFN-α (sifalimumab and rontalizumab).[221] More recently, Cl-amidine, a PAD inhibitor, has been tested in mice. The suppression of NET generation induced by Cl-amidine was associated with improvement of immune complex deposition and organ injury in mouse models of SLE and RA.[222–224] Similarly, the inhibitory effect of Cl-amidine on NET formation has been demonstrated to reduce atherosclerosis burden, arterial thrombosis and ischaemia/reperfusion injury in mice.[84,225]

Conclusion

As depicted in the present narrative review, a growing body of evidence is accumulating on NETs. Although initially investigated in infectious diseases, research studies have expanded their activity in other settings, such as CV diseases, autoimmunity and cancer. Available findings on NETs let us think that they may become new biomarkers of disease activity, prognosis and potentially therapeutic targets. Although a shroud of mystery still surrounds NETs, we now have few answers to some topic questions concerning NET behaviour and a wide overview on their significant roles. However, still many efforts are requested

to clarify different aspects of NETs in both infectious and non-infectious diseases, as the mighty neutrophil surprises every day for its unique features and protean biology.

Authors' Contributions
A.B. and L.L. equally contributed to this work as first authors. A.B. and L.L. conceived the review and designed its structure. A.B., L.L., F.C., A.V. and C.D-C. collected data and prepared the manuscript. F.M., F.D. and G.G.C. gave suggestions to enhance the manuscript. All authors approved the final version of the manuscript.

Disclosure of interest
All authors declare that they have no competing interest.

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