Narrative review article

Role of neutrophil extracellular traps in the prognosis of respiratory tract infections

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Abstract
Las trampas extracelulares de neutrófilos (NET, por sus siglas en inglés) han surgido recientemente como un vínculo potencial entre la inmunidad y la inflamación, que podría cumplir un papel clave en la patogénesis de las infecciones de vías respiratorias. El objetivo de esta revisión es determinar su rol como marcador pronóstico en enfermedades infecciosas de vías respiratorias. Para la elaboración de este artículo de revisión narrativa se consultaron las publicaciones disponibles a través de una búsqueda automatizada en las bases de datos de PubMed, Scopus y Embase. Las concentraciones elevadas de trampas extracelulares de neutrófilos (cfADN, complejos de mieloperoxidasas-ADN) en pacientes con cuadro clínico grave por infecciones de vías respiratorias, se relacionan con una estancia hospitalaria más larga, periodo prolongado de administración de antibióticos, aumento del riesgo de ingreso a la UCI, necesidad de ventilación mecánica, disfunción orgánica e incluso la muerte ($p \leq 0,05$). A pesar de no contar con un parámetro de medición estandarizado, el exceso de trampas extracelulares de neutrófilos se corresponde con la gravedad del daño tisular observado en pacientes con infecciones de vías respiratorias, esto revela el importante rol pronóstico de la respuesta de los neutrófilos y del proceso de la NETosis en las enfermedades infecciosas pulmonares.

Keywords
Pneumonia, Coronavirus Infections, Neutrophil Extracellular Traps, Prognosis.

Resumen
Neutrophil extracellular traps (NET) have recently emerged as a potential link between immunity and inflammation, which could play a key role in the pathogenesis of respiratory tract infections. This review aims to determine the role of neutrophil extracellular traps as prognostic markers in respiratory tract infectious diseases. For this article a literature review was undertaken, consulting available publications through an automated search in PubMed, Scopus, and Embase databases. High concentrations of neutrophil extracellular traps (cfDNA, Myeloperoxidase-DNA complexes) in patients with severe clinical presentation due to respiratory tract infections are related to a longer length of hospital stay, prolonged period of antibiotic administration and increased risk of admission to the ICU, need for mechanical ventilation, organ dysfunction and even death ($p \leq 0.05$). Despite not having a standardized measurement parameter, the excess of neutrophil extracellular traps corresponds to the severity of tissue damage observed in patients with respiratory tract infections, revealing the important prognostic role of the neutrophil response and NETosis process in pulmonary infectious diseases.

Palabras clave
Neumonía, infecciones por coronavirus, trampas extracelulares de neutrófilos, pronóstico.

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Rol de trampas extracelulares de neutrófilos en el pronóstico de las infecciones del sistema respiratorio

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Introduction

Neutrophil extracellular traps (NET) consist of networks of chromatin granular proteins, and DNA, which are released from the contents of neutrophils as a defense mechanism to trap and destroy microorganisms[1].

Neutrophils are the first line of defense of the immune system, which in response to inflammatory stimuli, uses different mechanisms to eliminate pathogenic microorganisms, including phagocytosis, degranulation[2], and neutrophil extracellular traps composed of extracellular chromatin fibers labeled with antimicrobial proteins that participate in the immune response[3] though, their role in the immune response is clear, the excessive formation can also be detrimental to the host, as they trigger unwanted inflammatory response mechanisms[4,5].

An alternative mechanism developed by neutrophils, which was described by Brinkmann *et al.* in 2004, in which neutrophils carry out a neutrophil defense mechanism where fibrillar DNA structures are released into the extracellular space, and it is evident that one of its particularities, compared to phagocytosis and degranulation, is the efficient elimination of microorganisms[6,7].

Currently, the study of new immunological markers constitutes a relevant area of research with multiple potential applications in the diagnosis, prognosis, and treatment of infectious diseases[8], making it of interest due to the pleiotropic functions of neutrophils, especially in the respiratory tract[9].

On the other hand, respiratory tract infections have been identified as resulting from a varied group of bacterial and viral agents, which cause diseases with similar symptomatology, thus representing one of the leading causes of medical attention worldwide[10]. Recently, the emergence of the SARS-CoV-2 pandemic has highlighted the importance of viral respiratory infections, such as influenza, and other bacterial infections, which are frequently associated with high mortality[11,12].

Respiratory tract infections are a significant cause of morbidity and mortality worldwide[13]. According to the International Forum of Respiratory Societies (FIRS), it is estimated that nearly four million people die from infections each year, 98 % of them due to lower respiratory tract infections[14].

The preparation of this narrative review article included consultation of publications available through an automated search contained in the PubMed, Scopus, and Embase databases. Original articles, cohort studies, and review articles, in Spanish and English, fewer than five years of antiquity, were included in 81.2 % of the references. Boolean connectors and search expression were used: respiratory tract infections AND neutrophil extracellular traps, pneumonia AND neutrophil extracellular traps, respiratory tract infection AND neutrophil extracellular traps, pneumonia, AND neutrophil extracellular traps.

Therefore, this review aims to describe the role of neutrophil extracellular traps (NET) as a prognostic marker in respiratory tract infectious diseases.

Discussion

Neutrophil extracellular traps in respiratory tract infectious diseases

NETs are a biological phenomenon of rupture of the neutrophil nuclear membrane in response to the presence of microorganisms, which allows the mixing of cellular and nuclear components, ending with the rupture of the cell membrane and the release of the compounds to the extracellular matrix, thus trapping the microorganisms and preventing their proliferation.

Neutrophils are highly effective in eliminating pathogens, with minimal adverse effects on the host; however, these effector mechanisms may not be insufficient to control a massive infection[15].

The role of NET has become of interest for their involvement in infectious diseases of the respiratory tract. Before their release, they are composed of a complex mixture of intracellular compounds assembled by neutrophils within the cytoplasm[16]. Between 20 and 30 proteins that form the extracellular traps are known, including antimicrobial proteins and proteases, as well as cytoskeletal proteins and glycolytic enzymes. NET release may depend on neutrophil elastase, reactive oxygen species (ROS), reactive nitrogen species (RNS), and histone citrullination[17].

When released, NET capture and destroy bacteria, fungi, viruses, and protozoa and participate in the host's immune defenses by forming traps to prevent the spread of pathogens in the organism[18]. Although NET formation is a defense mechanism against microorganisms, recent data suggest that their excessive formation contributes to lung injury by causing epithelial, endothelial, and pulmonary cell death, along with intravascular thrombus formation[19,20].

NET production is a regulated form of cell death called NETosis, different from apoptosis or necrosis[21,22]. Vorobjeva *et al.* have described various mechanisms of NETosis. One of them, the classical or suicidal NETosis

ends with cell death, while vital NETosis is a kind of programmed cell death in which neutrophils maintain their viability and function. In addition, it is characterized by the release of granular components into the cytosol and chromatin decondensation associated with histone modification[23].

Dysregulated formation of neutrophil extracellular traps contributes to the pathogenesis of respiratory tract infections; NETosis is associated with disease severity in patients suffering from multiple organ injuries induced by viral infections such as SARS-CoV-2[24].

Some surviving neutrophils are thought to become anuclear and may cause tissue damage. According to Twaddell *et al.*, the formation of NET produces a cytotoxic effect on the pulmonary epithelium and endothelium. Their excessive production is observed in airway diseases such as pneumonia and other pathologies that cause acute lung injuries, as well as in a varied number of chronic lung diseases[25].

By initiating the release of chemoattractant components and the recruitment of neutrophils, a universal response of the individual to viruses or bacteria is generated. Upon such a stimulus, the neutrophil cell membrane expresses receptors and adhesion molecules for various ligands, including immunoglobulins, membrane molecules on other cells, and cytokines[26].

According to Tomar *et al.*, the progressive infiltration of neutrophils at the site of infection and release of NET produces an immune response by releasing cytokines and chemokines in large quantities resulting in a "cytokine storm" that contributes to the development of acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome and sepsis. Through the release of NET in infectious processes histones, DNA and granular proteins, myeloperoxidase complexes, neutrophil elastase, cathepsin G, and proteinase 3 are dispersed, resulting in severe tissue destruction and establishing the self-amplifying cycle of necroinflammation[27].

Inflammation is crucial for immune defense against pathogens. However, when dysregulated, cytokines that usually mediate protective immunity and promote recovery can cause an overactive immune state, resulting in increased lung tissue damage[28,29].

Although various biomarkers of inflammatory response and infection are currently used, specifically interleukins, C-reactive protein (CRP), or procalcitonin because of their reliability for routine use as diagnostic and prognostic markers, they have several limitations. Some show poor stability and a short half-life, and others have slower kinetics or are not elevated in viral infections, which limits their usefulness[30].

Therefore, the usefulness of new molecules, such as NET and their components, as a new prognostic marker in respiratory infections should be explored.

Identification and quantification of neutrophil extracellular traps in respiratory tract infectious diseases

Pérez *et al.* report that the procedures for quantifying neutrophil extracellular traps are diverse, and no consensus has been reached among researchers to establish a particular method that is considered the "gold standard"[31]. Similarly, Plana *et al.* state that, to date, no direct and reliable method to quantify them has been reported; therefore, NETosis measurements are usually based on indirect quantifications of their components separately or in combination[32].

Neutrophil extracellular traps have been studied in great detail, and several components have been described; the most important include decondensed chromatin, nuclear histones, and 30 other granular protein components with bactericidal activity, such as neutrophil elastases (NE), myeloperoxidases (MPO), cathepsin G, and lactoferrin, among others[33,34].

However, not all components are specific to NETosis, but may also be present in other nucleated cells (e.g., eosinophils, macrophages, mast cells) and may be released during other forms of cell death than NETosis[35]. The NET formation is by far best reflected in the release of neutrophil-specific markers, such as histone citrullinated (H3Cit), MPO-DNA complexes, NE, and fragmented circulating DNA (cfDNA), which are the markers measured in most studies[36].

The measurement of total DNA is not NET-specific and also measures DNA from necrotic cells. MPO-DNA complexes are more accurate, because together they measure DNA and MPO specifically from neutrophils[37]. However, quantification of H3Cit by immunohistochemistry is considered the most reliable marker; this process is specific to NETosis[38].

Methods to quantify both NET and their components rely on a wide variety of techniques including enzyme-linked immunoassay absorbance (ELISA), flow cytometry, cell-free DNA-based assays, three-dimensional confocal microscopy and fluorescent microscopy[39,40]. Although each of these techniques and methods has its advantages,

Gupta *et al.* report that there are limitations specific to each of them[22].

Although these measurement methods provide information on changes in neutrophil morphology during infection and in the biochemical processes presented by cells dying from NETosis, they are limited by a series of constraints, such as lack of objectivity, low performance, monotonous, slow and difficult to compare and, or reproduce across laboratories, and high cost[41,42]. However, Rebernick *et al.* state that one of the most commonly used methods to quantify NETs is to make a naked-eye count of the number of decondensed DNA cells and, or NET-associated proteins cells by fluorescence microscopy[43].

Access to tissue samples is generally limited; however, it has been possible to analyze NET in patient samples, which has contributed to the characterization of the role of NET in different diseases and, probably, to outline new lines of biomarkers and therapeutic targets, since blood and plasma parameters of NET formation have been considered as potential prognostic markers[44].

The quantification of NET in plasma and blood allows a better understanding of the pathophysiological processes on human immunity, poorly recapitulating the biology of neutrophils in the lung undergoing inflammatory processes. Inactive neutrophils present in the peripheral circulation to migrate to lung tissue per se, must undergo different maturation processes before reaching an active state. Therefore, bronchioalveolar neutrophils represent a more accurate model for the study of pulmonary pathologies, especially those of infectious origin[45,46].

Given that the existing methods for quantifying NET and their components are not standardized and present some sensitivity and specificity problems, there are no specific values or range to determine "normal" versus "pathological" levels or concentrations of NET and their components in tissue[48].

Usually, in clinical trials, what is known as "cut-off value" is used to establish these values. For this purpose, the levels of NET or its components are determined in plasma, bronchioalveolar lavage, or sputum samples from control patients to obtain a test value and subsequently, compare it with a reference value. The reference value then represents the threshold level of NETs; levels above this indicate risk of mortality, complications, or prolonged hospital stay (Table 1)[49].

Neutrophil extracellular traps in the prognosis of respiratory tract infectious disease

A recent study by Zhu *et al.* carried out among 93 patients hospitalized with influenza A, showed that NET from patients with H7N9 and H1N1 increased epithelial cell permeability and, consequently, cfDNA and MPO-DNA levels correlated positively with the APACHE II scoring system and estimates of mortality ($r = 0.4802$, $p < 0.05$) confirming the damaging effect of NET on lung parenchyma. The singular deterioration of pulmo-

Table 1. Comparison of NETosis marker reference values, detection method and type of sample

Reference	Sample	Measurement method	NET	Reference value
Zhu L *et al.*[50]	Blood	ELISA	cfDNA	248.6 ± 38.5 ng/mL
			MOP-DNA complexes	1.1 fold
Mikacenic C *et al.*[51]	Bronchoalveolar lavage fluid	Colorimetric assays	cfDNA	Indetectable
			MOP-DNA complexes	Indetectable
			Peroxidase	Indetectable
Ng H *et al.*[52]	Blood	ELISA	cfDNA	345 (319 - 486) ng/mL
			H3Cit-DNA complexes	84 (64 - 101) ng/mL
			NE	16 (12 - 23) ng/mL
Huckriede J *et al.*[53]	Plasma	Real time CRP	cfDNA	4.1 (2.8 - 5.3) ng/µL
		ELISA	H3 Histones	0.0 (0.0 - 0.0) µg/mL
			NE	0.0 (0.0 - 0.0) ng/mL
			GAS6	14.4 (11.0 - 19.7) ng/mL
			sAXL	17.3 (13.7 - 18.4) ng/mL

cfDNA: circulating fragmented DNA; ELISA: enzyme immune adsorption analysis; GAS6: growth arrest-specific gene 6 detection; H3Cit: citrullinated histones 3; NE: Neutrophil elastases; NET: neutrophil extracellular traps; MPO: myeloperoxidase; PCR: polymerase chain reaction; sAxl: soluble Axl.

nary distensibility suggests that patients with higher serum NETs in the first days after admission are at higher risk of complications, hospital stay, and even death[50].

Similarly, Zho *et al.* mention that when NET are not adequately regulated, they are associated with severe disease, even in the lungs of patients with acute respiratory distress syndromes under mechanical ventilation, as demonstrated in their study comparing samples from patients with severe COVID-19 requiring mechanical ventilation and patients with mild COVID-19 breathing room air, showing that patients requiring mechanical ventilation had significantly higher levels of cfDNA and MPO-DNA ($p = 0.05$)[54].

These studies suggest that severe COVID-19 may be defined by neutrophilia, in the same manner as seen in other diseases caused by pandemic viruses, including H1N1 influenza, SARS-CoV, and Middle East respiratory syndrome coronavirus, which also show neutrophil infiltration at the site of infection, revealing an association between circulating NET markers and inflammation, dysregulation of homeostasis and endothelial damage, with the consequent development of acute respiratory distress syndrome[55].

Recently, Ebrahimi *et al.* also observed that excessive neutrophil activation and subsequent NET production in patients with pneumonia are associated with increased complications, such as impaired alveolar gas exchange, pulmonary dysfunction, and increased risk of death. Their study included 310 patients with community-acquired pneumonia, in which they noted that the time to clinical stabilization of vital signs was longer in patients with higher NET levels with a median of 5.0 (between 2.6 and 9.0 days), compared to those with lower levels, i.e., those in the lower three quartiles (median of 4.0; between 2.0 and 7.9 days) with an adjusted hazard ratio of 0.97; 95 % CI, 0.94 - 0.99; $p < 0.01$), time to discharge was shorter in subjects with lower NET levels (median 7.0; between 5.0 and 11.0 days compared to 9.0; between 5.0 and 14.0 days: HR 0.90; 95 % CI, 0.82 - 0.99; $p < 0.05$) and longer IV antibiotic administration in subjects with higher concentrations (95 % CI, 0.1 - 0.94; $p < 0.05$)[56].

Therefore, the authors propose that the systemic and local effect of inflammation provides a way to measure changes in serum NET markers to assess disease prognosis and to establish a new therapeutic targets. Recent evidence shows that high serum NET levels on admission are associated with a worse prognosis.

Conclusions

Based on the literature reviewed, the data show that elevated levels of circulating NET markers in patients with respiratory tract infections can be considered a prognostic marker since they illustrate the severity of the cellular damage observed, precisely among patients with a more severe clinical picture, indicated by severity scales. In addition, they are related to a prolonged hospital stay, a prolonged period of intravenous antibiotic administration, a higher risk of admission to the intensive care unit, the need for mechanical ventilation, organ dysfunction and even death.

Quantification of NET markers is difficult to be implemented in clinical practice due to the challenges that exist because of the lack of standardized methods for their measurement; the findings are significant as they reinforce the fact that NETosis may become a helpful severity prognostic biomarker in infectious airway diseases, especially in COVID-19, influenza, and bacterial pneumonia, which have been the most studied and could be the therapeutic target before the development of acute respiratory distress syndrome.

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