Neutrophil extracellular traps are associated with disease severity and microbiota diversity in patients with chronic obstructive pulmonary disease

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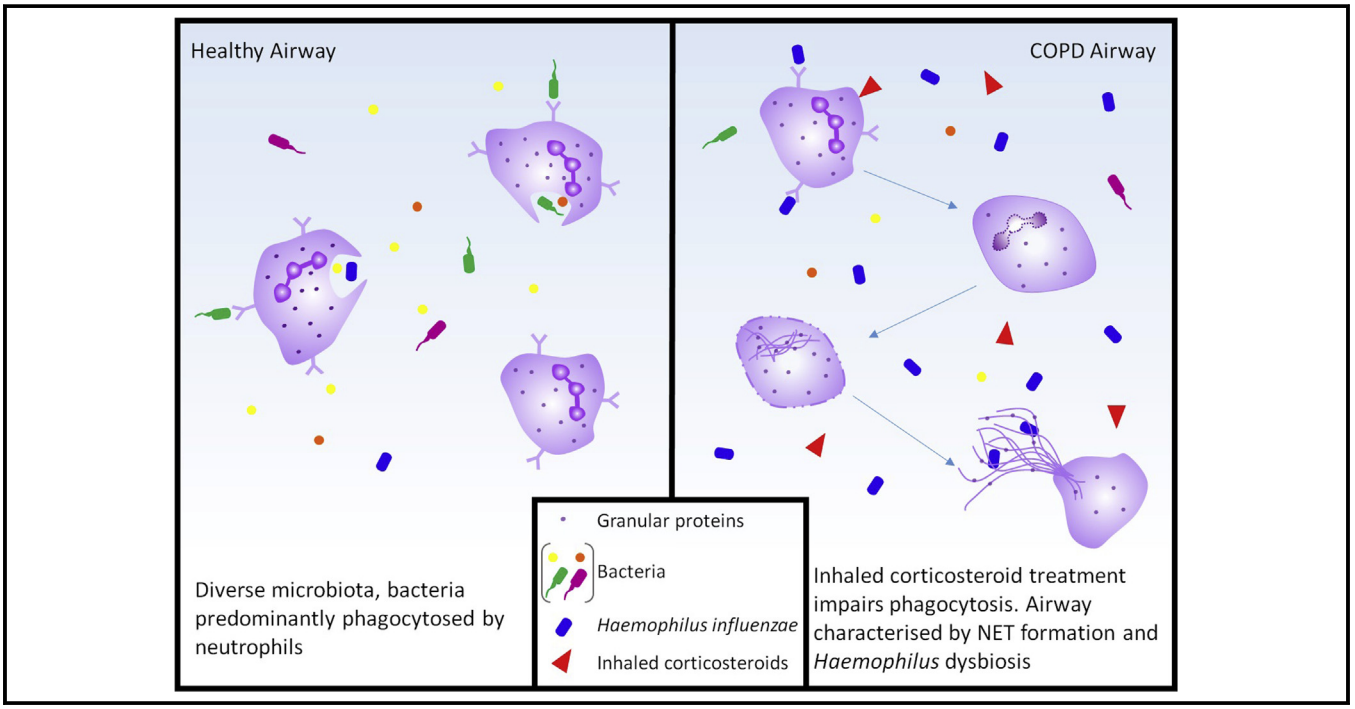
GRAPHICAL ABSTRACT



Background: Neutrophil extracellular traps (NETs) have been observed in the airway in patients with chronic obstructive pulmonary disease (COPD), but their clinical and pathophysiologic implications have not been defined.
Objective: We sought to determine whether NETs are associated with disease severity in patients with COPD and how they are associated with microbiota composition and airway neutrophil function.
Methods: NET protein complexes (DNA-elastase and histone-elastase complexes), cell-free DNA, and neutrophil biomarkers were quantified in soluble sputum and serum from patients with COPD during periods of disease stability and during

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exacerbations and compared with clinical measures of disease severity and the sputum microbiome. Peripheral blood and airway neutrophil function were evaluated by means of flow cytometry *ex vivo* and experimentally after stimulation of NET formation.

Results: Sputum NET complexes were associated with the severity of COPD evaluated by using the composite Global Initiative for Obstructive Lung Disease scale ($P < .0001$). This relationship was due to modest correlations between NET complexes and FEV_1, symptoms evaluated by using the COPD assessment test, and higher levels of NET complexes in patients with frequent exacerbations ($P = .002$). Microbiota composition was heterogeneous, but there was a correlation between NET complexes and both microbiota diversity ($P = .009$) and dominance of *Haemophilus* species operational taxonomic units ($P = .01$). *Ex vivo* airway neutrophil phagocytosis of bacteria was reduced in patients with increased sputum NET complexes. Consistent results were observed regardless of the method of quantifying sputum NETs. Failure of phagocytosis could be induced experimentally by incubating healthy control neutrophils with soluble sputum from patients with COPD.

Conclusion: NET formation is increased in patients with severe COPD and associated with more frequent exacerbations and a loss of microbiota diversity. (J Allergy Clin Immunol 2018;141:117-27.)

Key words: *Neutrophils, phagocytosis, chronic obstructive pulmonary disease,* Haemophilus *species, exacerbations*

Chronic obstructive pulmonary disease (COPD) is a heterogeneous disorder caused primarily by cigarette smoking and with multiple phenotypes and an unpredictable clinical course; drivers of disease progression remain poorly understood.[1-4] Aberrant neutrophilic inflammation is characteristic of COPD, and neutrophils contribute to airway damage through release of proteases and reactive oxygen species,[5] leading to loss of alveoli, increased mucus production, and mucociliary dysfunction. Normally, activated neutrophils rapidly undergo apoptosis and are removed by alveolar macrophages in a noninflammatory manner. This process is essential in resolving inflammation and preventing disease progression, and therefore neutrophil phagocytosis is a crucial defense against bacterial infection but also important in resolving inflammation and limiting disease progression in patients with COPD.[6-8] Cigarette smoke directly promotes neutrophilic inflammation but also impairs this antibacterial defense, leading to disturbance of the resident microbiota, which in turn promotes neutrophil influx and exacerbates inflammation.[9,10]

An alternate method of neutrophil antimicrobial defense, called neutrophil extracellular trap (NET) formation or NETosis, has been described.[11] This is an extracellular method of pathogen trapping in which neutrophils extrude webs of decondensed chromatin studded with histones, neutrophil elastase, and other granule products that ensnare bacteria. Although the ability of NETs to ensnare target microorganisms is not in doubt, their direct role in bacterial killing remains controversial.[11,12] The cellular mechanisms that mediate lytic NET formation are still to be elucidated, but evidence is accumulating that neutrophil elastase plays a central role, initially translocating from cytoplasmic granules to the nucleus, where it instigates chromatin degradation through histone cleavage.[13]

Abbreviations used

CAT:	COPD Assessment Test
cfDNA:	Cell-free DNA
COPD:	Chronic obstructive pulmonary disease
EN-RAGE:	Ligand for the receptor for advanced glycation end products
GOLD:	Global Initiative for Obstructive Lung Disease
ICS:	Inhaled corticosteroid
MPO:	Myeloperoxidase
MRC:	Medical Research Council
NET:	Neutrophil extracellular trap
OTU:	Operational taxonomic unit
PMA:	Phorbol 12-myristate 13-acetate
SWDI:	Shannon-Wiener species diversity index

Recently, NETs have been identified in the sputum of small numbers of patients with stable and exacerbating COPD through the use of confocal fluorescent and electron microscopy.[14-16] In the study by Grabcanovic-Musija et al,[14] COPD disease severity, as measured based on lung function, was associated with a greater amount of NET-associated neutrophil elastase determined by using confocal laser microscopy. However, the clinical and pathophysiologic relevance of NETs in patients with COPD has not been established. In this study we used multiple methods to evaluate airway NET release and correlated them with clinical disease severity, the airway microbiome, and neutrophil function. We demonstrate that NETs are more abundant in patients with severe COPD and are associated with more frequent exacerbations, reduced microbiota diversity, and an abundance of *Haemophilus* species.

METHODS

Patients with COPD enrolled in a community COPD registry (the Tayside Allergy and Respiratory Disease Information System)[17,18] were recruited into this prospective longitudinal cohort study. Patients were included if they were older than 40 years, had an FEV_1/forced vital capacity ratio of less than 70%, and had a clinical diagnosis of COPD. Exclusion criteria included the inability to provide informed consent, previous adverse reaction to nebulized hypertonic saline, asthma, bronchiectasis on high-resolution computed tomographic scanning, cystic fibrosis, active mycobacterial disease, and immunosuppression. Patients receiving long-term antibiotic therapy or maintenance oral corticosteroid therapy at screening were also excluded. Study approval was granted by the East of Scotland Research Ethics Committee (13/ES/0030), and all patients provided written informed consent to participate.

Study design

Patients underwent a comprehensive clinical assessment and sampling of blood and sputum at 2 time points up to 6 months apart while clinically stable. Exacerbations were reported to the research team, who provided standardized treatment with repeat clinical assessment and blood and sputum sampling at the onset of exacerbation and at day 10 after treatment. Exacerbations were defined, as previously described.[19] Relevant medical history was recorded at screening (see the Methods section in this article's Online Repository at www.jacionline.org for details). Sputum was obtained after nebulization of 3% hypertonic saline for up to 20 minutes. Spirometry was performed, and the St Georges Respiratory Questionnaire, COPD Assessment Test (CAT) and Medical Research Council (MRC) Dyspnoea Score were used at each visit. The primary outcome was the association between NET complexes and composite Global Initiative for Obstructive Lung Disease (GOLD) COPD severity classification, which classifies patients into 4 groups, A, B, C, and D, depending on their symptoms (CAT score and MRC Dyspnoea