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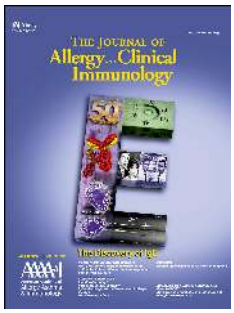

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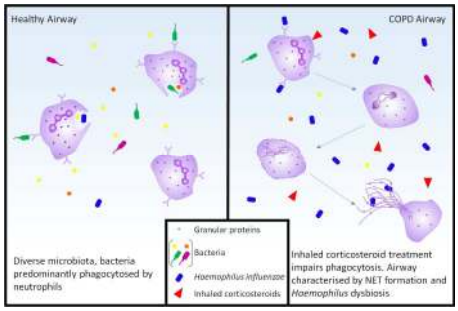

Neutrophil Extracellular Traps are associated with disease severity and microbiota diversity in Chronic Obstructive Pulmonary Disease

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Contribution: Conception and design: JDC, SEM, SS and CNAP. All authors participated in data analysis and interpretation of the data. All authors were involved in writing and revising the article prior to submission.

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ABSTRACT

Background: Neutrophil extracellular traps (NETs) have been observed in the airway in COPD, but their clinical and pathophysiological implications have not been defined.

Objective: To determine if NETs are associated with disease severity in 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 COPD patients during periods of disease stability and during exacerbations, and compared to clinical measures of disease severity and sputum microbiome. Peripheral blood and airway neutrophil function was evaluated by flow cytometry *ex vivo* and experimentally following stimulation of NET formation.

Results: Sputum NET complexes were associated with the severity of COPD evaluated using the composite GOLD scale ($p<0.0001$). This relationship was due to modest correlations between NET complexes and FEV_1, symptoms evaluated by the COPD assessment test and higher levels of NET complexes in patients with frequent exacerbations ($p=0.002$). Microbiota composition was heterogeneous, but there was a correlation between NET complexes and both microbiota diversity ($P=0.009$) and dominance of *Haemophilus* spp operational taxonomic units. ($P=0.01$). *Ex vivo* airway neutrophil phagocytosis of bacteria was reduced in patients with elevated 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 COPD soluble sputum.

Conclusion: NET formation is increased in severe COPD and is associated with more frequent exacerbations and a loss of microbiota diversity.

Abstract word count: 250

Key messages:

- Neutrophil extracellular traps (NETs) have been observed in the lungs of patients with chronic obstructive pulmonary disease; their significance in terms of clinical outcomes and their impact on bacterial clearance in the airway has not been established.
- We show that NETS in sputum are associated with loss of microbiota diversity and impaired *ex vivo* neutrophil phagocytosis suggesting a possible role in disease progression.
- Consistent with this, measurement of NETs in sputum identifies patients with worse lung function, poorer quality of life and a higher risk of future exacerbations.

Capsule summary: Neutrophil extracellular traps are associated with disease severity and loss of microbiota diversity in COPD, suggesting a role in disease pathogenesis and progression.

Keywords: Neutrophils, phagocytosis, COPD, *Haemophilus,* exacerbations

72 **Abbreviations:**

ACE	Angiotensin converting enzyme
ARB	Angiotensin receptor blocker
BAL	Bronchoalveolar lavage
BMI	Body mass index
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft
CAT	COPD assessment test
CCF	Congestive cardiac failure
cfDNA	Cell-free Deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protien
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
ELISA	Enzyme linked immunosorbent assay
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
GOLD	Global initiative for obstructive lung disease
HRCT	High resolution computed tomography scan
HRP	Horseradish peroxidase
ICS	Inhaled corticosteroids
LABA	Long acting beta agonist
LAMA	Long acting muscarinic antagonist
LPS	Lipopolysaccharide
LTOT	Long term oxygen therapy
MPO	Myeloperoxidase
MRC	Medical research council
NET	Neutrophil extracellular trap
OTU	Operational taxonomic unit
PMA	Phorbol 12-myristate 13-acetate
QIIME	Quantitative insights in microbial ecology
ROC	Receiver operating characteristic
SGRQ	St. Georges respiratory questionnaire
StDev	Standard deviation
SWDI	Shannon-Wiener species diversity index
TARDIS	Tayside Allergy and Respiratory Disease Information System
TMB	3,3'5,5'-Tetramethylbenzidine

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INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a heterogeneous disorder, primarily caused by cigarette smoking, 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 the 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 non-inflammatory manner; this process is essential in resolving inflammation and preventing disease progression; neutrophil phagocytosis is therefore a crucial defence against bacterial infection but also important in resolving inflammation and limiting disease progression in COPD (6-8). Cigarette smoke directly promotes neutrophilic inflammation but also impairs this antibacterial defence, leading to disturbance of the resident microbiota which, in turn, promotes neutrophil influx and exacerbates inflammation (9, 10).

An alternative method of neutrophil antimicrobial defence, called neutrophil extracellular trap formation or NETosis has been described (11). This is an extracellular method of pathogen trapping in which neutrophils extrude webs of de-condensed chromatin studded with histones, neutrophil elastase and other granule products that ensnare bacteria. While 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).

NETs have recently been identified in the sputum of small numbers of stable and exacerbating COPD patients through the use of confocal fluorescent and electron microscopy (14-16). In Grabcanovic-Musija *et al* (14), COPD disease severity, as measured by lung function, was associated with a greater amount of NET-associated neutrophil elastase determined by confocal laser microscopy. However, the clinical and pathophysiological relevance of NETs in 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 severe COPD, and are associated with more frequent exacerbations, reduced microbiota diversity and abundance of *Haemophilus* species.

METHODS

COPD patients enrolled in a community COPD registry (Tayside Allergy and Respiratory Disease Information System (TARDIS) (17, 18) were recruited into this prospective longitudinal cohort study. Patients were included if >40 years; a FEV_1/FVC ratio <70% and with a clinical diagnosis of COPD. Exclusion criteria included the inability to give informed consent; previous adverse reaction to nebulised hypertonic saline; asthma; bronchiectasis on HRCT 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); all patients gave written informed consent to participate.

Study Design

Patients underwent a comprehensive clinical assessment and sampling of blood and sputum at two time points up to 6 months apart whilst clinically stable. Exacerbations were reported to the research team who provided standardized treatment with repeat clinical assessment, 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 online supplement for details). Sputum was obtained following nebulisation of 3% hypertonic saline for up to 20 mins. Spirometry, St Georges Respiratory Questionnaire (SGRQ), COPD assessment test (CAT) and MRC dyspnoea scoring was performed at each visit. The primary outcome was the association between NET complexes and composite GOLD COPD severity classification. This classifies patients into four groups, A, B, C and D depending on their symptoms (CAT score and MRC dyspnoea score), lung function

(FEV_1 % predicted) and exacerbation frequency (high risk defined as 2 or more per year or a hospitalisation for a severe exacerbation) (20).

NET Assays

There is no agreed high throughput method of quantifying NETs in biological fluids, consequently, this study utilised multiple methods. Firstly, primary NET constituents including cell free DNA (cfDNA), Myeloperoxidase and neutrophil elastase and EN-RAGE, were quantified (21). These assays are not specific as these components are also released during neutrophil degranulation or necrosis but are commonly used as surrogates of NET release. Subsequently, three specific methods of NET quantification were used: A MPO-DNA ELISA that has been extensively published (21-23) and two assays developed and validated in house for use in sputum, based on the detection of DNA-Elastase and Histone-Elastase complexes. For the DNA-Elastase complex assay, Anti-DNA (HYB331-01, Abcam) capture antibody, was incubated on plates overnight at 4°C, following by washing with PBS + 0.05% Tween 20 (wash buffer). Plates were blocked with 1% Bovine Serum Albumin (BSA) in PBS and washed with wash buffer. Samples were diluted in 1% BSA in PBS. A standard curve was generated by titrating concentrations of heathy human blood-derived neutrophils treated with phorbol 12-myristate 13-acetate (PMA). Plates were washed 3 times with wash buffer after incubation of standards and samples. DNA-elastase complexes were detected with sheep anti-neutrophil elastase-HRP (PA1-74133, Thermo Scientific) and developed with 3,3'5,5'-Tetramethylbenzidine (TMB). For the Histone-Elastase assay, plates were coated for 1h with Anti-Histone H1 (ab71594, Abcam), washed and blocked as above, and incubated for 1h with Rabbit anti-neutrophil elastase (ab21595, Abcam). Anti-Rabbit-HRP (ab6721, Abcam) was used for detection and the plate was developed as above. The assays were validated

against other known NET components (citrullinated histone H3 and DNA) for the effects of sample preparation methods and for passive interactions between DNA and elastase (Figures E1-E3 online).

Sputum Microbiome

DNA was extracted from whole sputum using the AllPrep DNA/RNA Mini kit on the QIAcube automation platform (QIAGEN) using a modified protocol, followed by 16S rRNA gene sequencing on the Illumina MiSeq platform. Bioinformatic analysis and quality checking of the resulting sequences was performed using QIIME (version 1.9.0) (24). Shannon-Wiener Species Diversity Index (SWDI) was used as a measure of alpha diversity of samples. See online supplement for full methods.

Neutrophil studies

Peripheral blood neutrophils were isolated by percoll-gradient density centrifugation as previously described (6). Phagocytosis by peripheral blood and airway neutrophils was assessed using a flow cytometry based assay (25); see online supplement. Sputum neutrophil platelet aggregates were investigated by flow cytometry, while cytospins were obtained for differential sputum cell counts; see online supplement.

Statistical Analysis

Details of all statistical analyses carried out are shown in the online supplement.

RESULTS

99 patients were included in the study. Patient characteristicsare shown in Table 1.

NETs are associated with clinical disease severity in stable COPD

Sputum NETs were measured on expectorated sputum from all individuals. NETs quantified using the Histone-Elastase complex assay were associated with multiple markers of COPD severity. Sputum NETS were highest in those in 2011 GOLD group B and D, the most severe groups using this composite index of COPD severity c(consisting of lung function (%predicted FEV_1), symptoms (MRC dyspnoea and CAT score) and exacerbation frequency), (Figure 1A, P<0.0001). (26) We explored the individual contributors to the GOLD classification and found that Sputum NETs were also independently correlated with annual exacerbation frequency (Figure 1B, P=0.002), % predicted FEV_1 (Figure 1C, P<0.0001and CAT score (Figure 1D, P=0.005),, Patients hospitalised with severe exacerbations also had higher sputum NETs, P=0.002). Very similar results were obtained with the DNA-elastase assay (Figure E4 online). The MPO-DNA assay had a limited dynamic range and was not considered further.

Soluble sputum NET concentrations were not correlated with age, smoking pack years, BMI or use of the anti-platelet agents aspirin or clopidogrel using any assay. In multivariable analysis, sputum NETs were independently associated with % predicted FEV_1 in multiple linear regression (estimate -0.19, per 100 unit change in NET concentration, P=0.03). Histone-elastase levels were also independently associated with % predicted FEV_1 (p=0.01). Sputum NET concentrations using both DNA-elastase and Histone-elastase assays correlated with sputum neutrophils identified on cytospins (P<0.0001), sputum EN-RAGE (P<0.0001), cfDNA (P<0.0001), MPO (P<0.0001) and neutrophil elastase (P<0.0001). These results indicate that the abundance of NETs in sputum correlate with disease severity. To determine

193 if this was simply a reflection of systemic inflammation, the quantity of NETs in sputum were
194 compared with the concurrent presence of NETs in peripheral blood. Circulating DNA-
195 elastase concentrations were on average 10,000 fold lower than in sputum, and did not
196 correlate with any markers of disease severity (data not shown).

197 To determine whether sputum NET concentration could be used as a predictive biomarker,
198 we used ROC analysis. This showed the optimal cut-off to identify frequently exacerbating
199 patients (≥2 per year) was >0.98units/mL DNA-elastase complexes. Using this cut-off,
200 sputum NET concentration predicted time to next exacerbation (P<0.0001) by Kaplan-Meier
201 survival analysis. Similarly, using a cut-off of >0.34 units/mL Histone-elastase complexes
202 predicted time to next exacerbation (P<0.0001). In multivariable analysis, DNA-elastase and
203 Histone-elastase complexes were associated with exacerbation frequency even after
204 adjustment for confounders: (1.03 95% CI 1.01-1.06, per 0.1 unit increase, P=0.02 and 1.04
205 (95% CI 1.02-1.07 per 0.1 unit increase, P=0.007 respectively). Included confounders were
206 age, gender, smoking status, BMI, FEV_1% predicted, MRC dyspnoea score and use of inhaled
207 corticosteroids (ICS).

208 **Other sputum markers of severity**

209 cfDNA was not associated with exacerbation frequency or GOLD score but was associated
210 with sputum colour (Table E1 online). Other NET markers, including elastase, MPO and EN-
211 RAGE were associated with severity markers including exacerbations, % predicted FEV_1 and
212 GOLD score, but generally the relationships were weaker for these non-specific assays than
213 for the NET assays (Figure 2 and Table E1 online). Neutrophil elastase activity was associated
214 with GOLD stage but not significantly with exacerbations.

215 **Sputum NET concentration is associated with microbiota composition**

Results of 16S rRNA sequencing of DNA from whole sputum from stable and exacerbating COPD patients is shown in Figures 3 and 4 respectively. The SWDI was used as a measurement of the richness and evenness of the bacteria population found in the sputum; a lower index indicates fewer species and more un-evenness within a sample. Increasing sputum NET concentration was associated with a decreasing SWDI in stable patients using both DNA-elastase and Histone-elastase assays (Figure 3). In patients with stable disease and also during exacerbations, *Haemophilus* was most frequently the dominant pathogenic genus in patients with reduced species diversity. When stratified by the presence of >40% *Haemophilus* spp. OTUs at genus level (based on Figure E5 online), there was a clear relationship between *Haemophilus* spp dominance and NET formation as measured by Histone-elastase complexes $P<0.0001$ and DNA-elastase complexes $P=0.01$ (Figure 3C).

We investigated microbiota dynamics over the study period to determine if antibiotic therapy may be responsible for reductions in SWDI or *Haemophilus* spp. dysbiosis. Comparing patients who did and did not received antibiotic therapy during 6 months follow-up there were no significant differences in change in SWDI, Chao index or % of *Haemophilus* spp. OTU's (Figure E6 online). High variability in NET concentration between Baseline and Follow-up was observed but this was not statistically significant (Figure E7 online).

Neutrophil extracellular traps during exacerbations of COPD

63 exacerbations requiring antibiotic and corticosteroid treatment occurred during the study period in 39 patients. We studied a convenience sample of 24 exacerbations where patients could be reviewed and sampled prior to administration of treatment. We quantified NETs in induced sputum at onset and after treatment of exacerbation. (Figure 4A). Exacerbations were heterogeneous with microbiota profiling demonstrating some exacerbations were

associated with loss of bacterial diversity while others showed no change overall microbiota profile (Figure 4B). There was an association between DNA-elastase complexes and SWDI during exacerbations (R=0.48, P=0.02). There was a significant association between sputum NETs and severity of exacerbation as measured by CAT (R=0.35, P=0.005) and SGRQ (R=0.25, P=0.01). Similar results were observed with the Histone-elastase assay. NETs were significantly elevated in exacerbations where *Haemophilus* spp. was dominant (P=0.01 for DNA-elastase and P=0.0005 for histone-elastase, Figure 4C). Classifying exacerbations as eosinophilic or non-eosinophilic, as described by Bafadhel *et al* (27); there was a clear excess of NETs in sputum in non-eosinophilic exacerbations compared with low levels during eosinophilic exacerbations (Figure 4D, P=0.01 for both assays), consistent with the premise that the underlying pathologic disease process in these exacerbations may be different.

Investigating potential mechanisms of NET formation in COPD implicates failure of phagocytosis

We investigated a number of recognised NET triggers of relevance to COPD such as CXCL8 (28), complement component C5a, bacterial infection (described above) and activated platelets (23). We observed no relationship between sputum CXCL8 and NETs (Figure 5C). Complement component C5a was not detectable in the majority of sputum samples by ELISA (data not shown). Markers of platelet activation were significantly elevated in COPD sera (CD40L, (Figure 5B) and P-selectin); analysis of sputum by flow cytometry showed the presence of neutrophil-platelet aggregates (Figure 5A), but we found no evidence of a correlation between the degree of platelet activation and NET formation (correlation between NETs and CD41a positive neutrophils, P=0.053). A sub-analysis in patients with and

without treatment with anti-platelet drugs confirmed these findings. Similar results were observed with the Histone-elastase assay (not shown).

Branzk *et al* demonstrated that NET formation in bacterial infection only occurred when phagocytosis was inhibited (29). The phagocytic capacity of neutrophils, monocytes and alveolar macrophages in COPD have been extensively studied (30), and there is abundant evidence that phagocytosis is compromised in this disease. We therefore evaluated airway neutrophil phagocytosis of FITC labelled *P. aeruginosa* to test the hypothesis that impaired phagocytosis in the presence of airway bacteria may contribute to NET formation. We observed a failure of neutrophil phagocytosis in patients with high sputum DNA-elastase complex concentrations (Figure 6B, P=0.002 and P=0.007). Near identical results were obtained using the histone-elastase assay (Figure E8 online). The relationships between cfDNA, free sputum CXCL8, IL-1beta, TNF-alpha and EN-RAGE and phagocytosis were not statistically significant.

We excluded an effect of neutrophil viability on phagocytosis by demonstrating no correlation between caspase positive cells and phagocytosis. We also observed a correlation between NET formation and daily beclomethasone dose equivalent of ICS. The relationship was explained by a higher level of NET formation in patients receiving fluticasone propionate/salmeterol (P=0.01, Figure E9 online). *In vitro*, we found that fluticasone propionate at therapeutically relevant doses inhibited neutrophil phagocytosis of FITC-labelled *E. coli* (Figure 6C), and that pooled sputa from COPD patients similarly reduced phagocytosis (this pool was formed of patients not receiving ICS to exclude the possibility of inhaled drug in the samples affecting neutrophil function). To test the hypothesis that neutrophil products released into the sputum were responsible for phagocytosis inhibition

we treated blood neutrophils with PMA for 4 hours to induce NET formation and harvested the supernatant. This supernatant also demonstrated a dose dependent inhibition of neutrophil phagocytosis after 30 mins incubation (a time point too early for NET formation in response to PMA to have occurred). The positive control, cytochalasin D, inhibited phagocytosis as expected.

DISCUSSION

This study shows NET formation is present in COPD, and that NET concentrations are associated with disease severity in COPD, with higher levels of NET complexes in patients with more severe disease when classified by the composite GOLD severity score which incorporates % predicted FEV_1, symptoms and the frequency of exacerbations.During exacerbations, elevated NETs were associated with non-eosinophilic exacerbations and reduced bacterial diversity, driven by increased *Haemophilus* species.

NETs therefore appear to be potential biomarkers of disease severity and microbial dysbiosis in COPD. Further work is required to address whether NETs directly contribute to disease progression in COPD or are a reflection of more severe lung damage and associated alterations in the microbiota.

Neutrophil killing is critical to defence against bacterial and fungal pathogens in the lung. It is now known that neutrophils are able to alter their killing method, for example by sensing pathogen size and releasing NETs in response to large pathogens (29). The killing method appears to be binary, as Branzk *et al* (29) showed that NET formation was inhibited by phagocytosis through sequestration of neutrophil elastase, which is required to translocate to the nucleus to initiate NET formation (29, 31). *In vivo* evidence of this dichotomous neutrophil behaviour has not previously been shown during human infections.

Microscopy studies have now demonstrated the presence of NETs in the airways of patients with cystic fibrosis, COPD and asthma (14-16, 32, 33). The questions are therefore not whether NETs are present, but whether they are important in the progression of the disease and what drives NET formation in the COPD airway?

311 The number of recognised triggers for NET formation in *in vitro* systems is vast, and includes
312 pro-inflammatory cytokines (CXCL-8, TNF-alpha), bacterial products (formylated peptides,
313 LPS), bacteria (*P. aeruginosa*, *H. influenzae),* fungi, activated platelets and rheumatoid factor
314 (immunoglobulin) (11, 23, 29, 31, 33-36). Evaluating the drivers of NET formation in COPD is
315 challenging since the majority of these proposed drivers are present in the COPD airway
316 under normal conditions (5). In this study NETs were most strongly correlated, during both
317 stable COPD and exacerbations, with the presence of *Haemophilus* spp. OTUs. Juneau
318 previously demonstrated that *H. influenzae* were able to induce NETs directly, and it has
319 been shown that *H. influenzae* may survive in NETs through the production of nucleases and
320 resistance to NET killing. Our study was not designed to answer whether the association
321 between *Haemophilus* spp. and NETs is due to natural selection owing to *H. influenzae*'s
322 resistance to NET killing (37).

323 Branzk *et al* observed that NET formation in response to Gram-negative bacteria did not
324 occur under normal conditions, where neutrophil bacterial interaction results in
325 phagocytosis and intracellular clearance. When phagocytosis was prevented, through a
326 physical barrier, NET formation resulted (29). We hypothesised therefore that phagocytosis
327 would be impaired in COPD airway neutrophils to explain the exaggerated NET formation in
328 COPD. Our data showed a direct relationship between airway neutrophil phagocytosis and
329 NETs. Experimentally, exposure to soluble sputum from patients with COPD inhibited
330 phagocytosis in healthy neutrophils and could be replicated using supernatants from healthy
331 donor neutrophils that had been induced to undergo NETosis by PMA. We speculate that
332 neutrophil activation and NET formation in COPD may cause the release of mediators that
333 inhibit phagocytosis, creating an airway environment that promotes NET formation (38, 39).
334 This may be exacerbated by ICS, which are widely used in COPD, as we demonstrated that

335 fluticasone propionate *in vitro* and *in vivo* was associated with reduced neutrophil
336 phagocytosis.

337 While the question of whether NETs are able to kill bacteria is controversial, it is clear that
338 NETs are a less effective means of bacterial killing compared to phagocytosis and are
339 associated with greater collateral damage (36). We speculate that this could explain the loss
340 of bacterial diversity and increased abundance of *Haemophilus* spp. OTUs in COPD. Larger,
341 longitudinal studies are needed to determine whether NET formation identifies a specific
342 endotype in COPD, whether NET status fluctuates over time and whether loss of phagocytic
343 ability and subsequent NET formation precedes changes in the lung microbiota.

344 We acknowledge some potential limitations of the study. The majority of data are cross-
345 sectional and we are unable to assess whether the presence of NETs lead to more rapid
346 disease progression, such as long term decline in FEV_1. We performed a large number of
347 correlations in this study, increasing the possibility that some of the weaker correlations may
348 be statistically significant by chance. For this reason and due to relatively small sample sizes
349 in some of our analyses there is a need for independent replication of our findings. Sputum
350 was selected for microbiota analysis due to the less invasive method of collection compared
351 to bronchoalveolar lavage; whilst it is accepted that a protected brush bronchoscopy would
352 be preferred for monitoring the lower airway microbiota, sputum is more practical in a
353 routine clinical environment. The results of our study are similar to previously published data
354 acquired using various sample types from both COPD and healthy lungs as reviewed in
355 Dickson *et al* (40). It is not feasible to perform and quantify microscopy images in very large
356 number of patients and it is unlikely to be translated into a point of care clinical test,

whereas a NET ELISA, such as those described here, are potentially applicable in clinical practice.

Simplified *in vitro* systems do not necessarily reflect the complex lung environment, and so, although we can demonstrate inhibitory effects of COPD lung fluids and drugs like fluticasone propionate on neutrophil functions such as phagocytosis, we acknowledge that such assays are highly simplified. Nevertheless, we have identified a correlation between reduced phagocytosis *ex vivo* in airway neutrophils which may be more reflective of their true *in vivo* function.

The majority of patients with COPD are treated with ICS and bronchodilators (38). ICS target eosinophilic inflammation and effectively reduce exacerbations in eosinophilic COPD (38, 41). Patients who do not have eosinophilic COPD have neutrophilic airway inflammation and, to date, we have limited therapies capable of targeting neutrophilic inflammation (5). Drugs targeting NETs are in development; inhibition of NET formation has been shown to be beneficial in experimental models of diverse clinical diseases from psoriasis to lupus (42, 43). Our data suggests that NETs should be further evaluated as a therapeutic target in COPD.

Conclusions

NETs are associated with disease severity and exacerbation frequency in this COPD cohort. NETs are associated with microbial dysbiosis and further longitudinal studies are needed to determine if modulation of NETs may affect airway microbial dysbiosis and clinical outcomes.

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529 **TABLES**

530 Table 1: Demographic and clinical characteristics of the cohort at study entry.

	Cohort – N(%) or mean (StDev)
Demographics and major comorbidities	
N	99
Age (StDev)	71.3 (8.3)
Age at diagnosis (StDev)	59.8 (11.5)
Male gender (%)	66 (66.7)
Active smokers (%)	24 (24.2)
Ex-smokers (%)	71 (71.7)
Pack years (StDev)	42 (29)
BMI (StDev)	28.3 (5.7)
Myocardial Infarction (%)	15 (15.2)
CABG (%)	14 (14.1)
Angina (%)	24 (24.2)
Stroke (%)	9 (9.1)
Diabetes (%)	19 (19.2)
Cancer (%)	3 (3.0)
CCF (%)	5 (5.1)
Lung surgery (%)	4 (4.0)
Kidney disease (%)	1 (1.0)
COPD Severity	
% Predicted FEV_1 (StDev)	70.3% (21.7)
MRC Dyspnoea Score (StDev)	2.8 (1.4)
Exacerbations per year (StDev)	2.1 (2.0)
GOLD Score	
A (%)	7 (7.1)
B (%)	36 (36.4)
C (%)	5 (5.1)
D (%)	51 (51.5)
SGRQ (StDev)	44.5 (22.1)
On LTOT	5 (5.1)
Medications	
Statins (%)	54 (54.6)
ICS (%)	61 (61.6)
LABA (%)	13 (13.1)
LAMA (%)	53 (53.5)
Theophylline (%)	8 (8.1)
Mucolytic (%)	13 (13.1)
Aspirin (%)	26 (26.3)
Beta Blocker (%)	13 (13.1)
ACE-inhibitor (%)	27 (27.3)
ARB (%)	6 (6.1)
Clopidogrel (%)	8 (8.1)

531 Abbreviations: ACE= angiotensin converting enzyme, ARB= angiotensin receptor blocker,
532 BMI= body mass index, CABG= coronary artery bypass graft, CCF= congestive cardiac failure,
533 FEV_1= forced expiratory volume in 1 second, ICS= inhaled corticosteroid, LABA= long acting
534 beta agonist, LAMA= long acting muscarinic antagonist, LTOT= long term oxygen therapy,
535 MRC= Medical Research Council, SGRQ= St. Georges Respiratory Questionnaire, StDev=
536 standard deviation.

FIGURE LEGENDS

Figure 1. Histone-elastase complex concentrations in soluble sputum of COPD patients are associated with clinical markers of COPD disease severity. **A:** NET concentration in stable samples compared to GOLD score (n=99). **B:** NET concentration compared to number of exacerbations reported by study patients in previous year (n=99). **C:** NET concentration in stable samples compared to percent predicted forced expiratory volume in 1 second (% predicted FEV_1) (n=99). **D:** NET concentration in stable samples compared to COPD assessment test (CAT) (n=99).

Figure 2. Sputum biomarkers and severity of COPD. **A:** Sputum myeloperoxidase activity is associated with GOLD stage and with the frequency of exacerbations (n=99). **B:** Sputum cell free DNA (cfDNA) is not significantly associated with GOLD stage or exacerbations (n=99). **C:** Neutrophil elastase activity is associated with GOLD stage but not significantly with frequency of exacerbations (n=99).

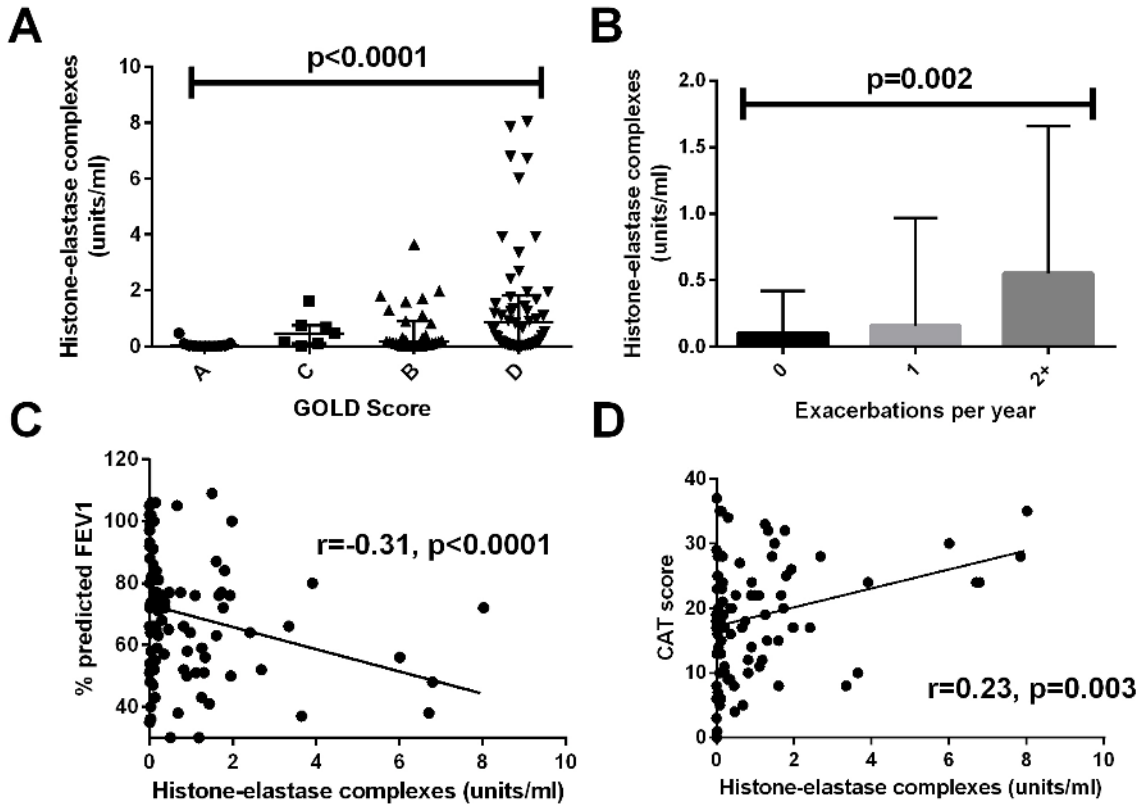
Figure 3: Microbiota composition and NET formation in stable COPD. In the above bar figure, each bar represents an individual patient. **A:** Microbiomes of COPD patients when clinically stable with 14 of the most commonly identified genera per patient highlighted. Each patient is only represented once. **B:** Correlation of all stable samples Shannon-Wiener Diversity Indexes (SWDI) against NET complexes (n=89). **C:** NET formation in stable soluble sputum samples stratified by % of *Haemophilus* spp. OTUs present (n=82).

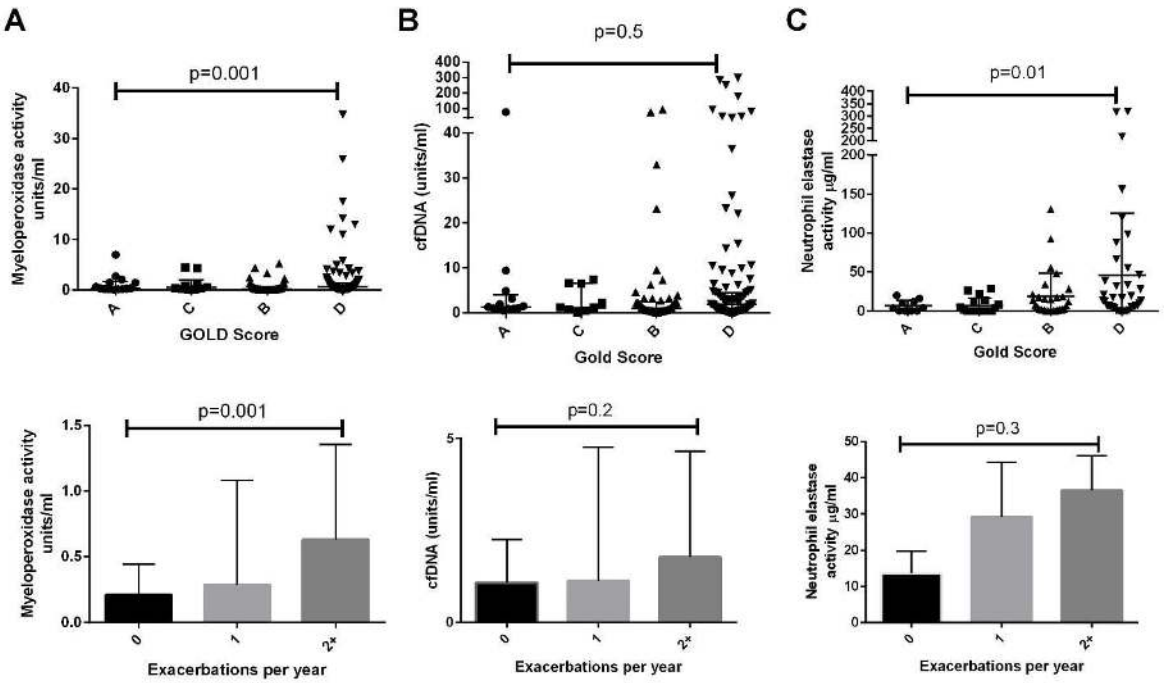
Figure 4: Changes in microbiota and NET formation at exacerbation of COPD. **A**: Individual microbiome profiles of all exacerbation samples (n=37 from 24 separate exacerbation events) with corresponding start and end exacerbation samples adjacent to each other, exacerbation number denotes start of exacerbation sample with end of exacerbation sample positioned to its right. Some patients were unable to produce sputum at both visits. **B:** Examples of longitudinal changes in microbiomes over time, showing two individual patients. StartEx refers to the onset of exacerbation before treatment, EndEx refers to 10 days following exacerbation treatment once clinical recovery has occurred. **C**: *Haemophilus* spp. OTU dominance at exacerbation is associated with significantly higher NET formation (n=24). **D**: Based on blood eosinophilia (26), NETs were elevated in non-eosinophilic exacerbations and not in eosinophilic exacerbations (n=24).

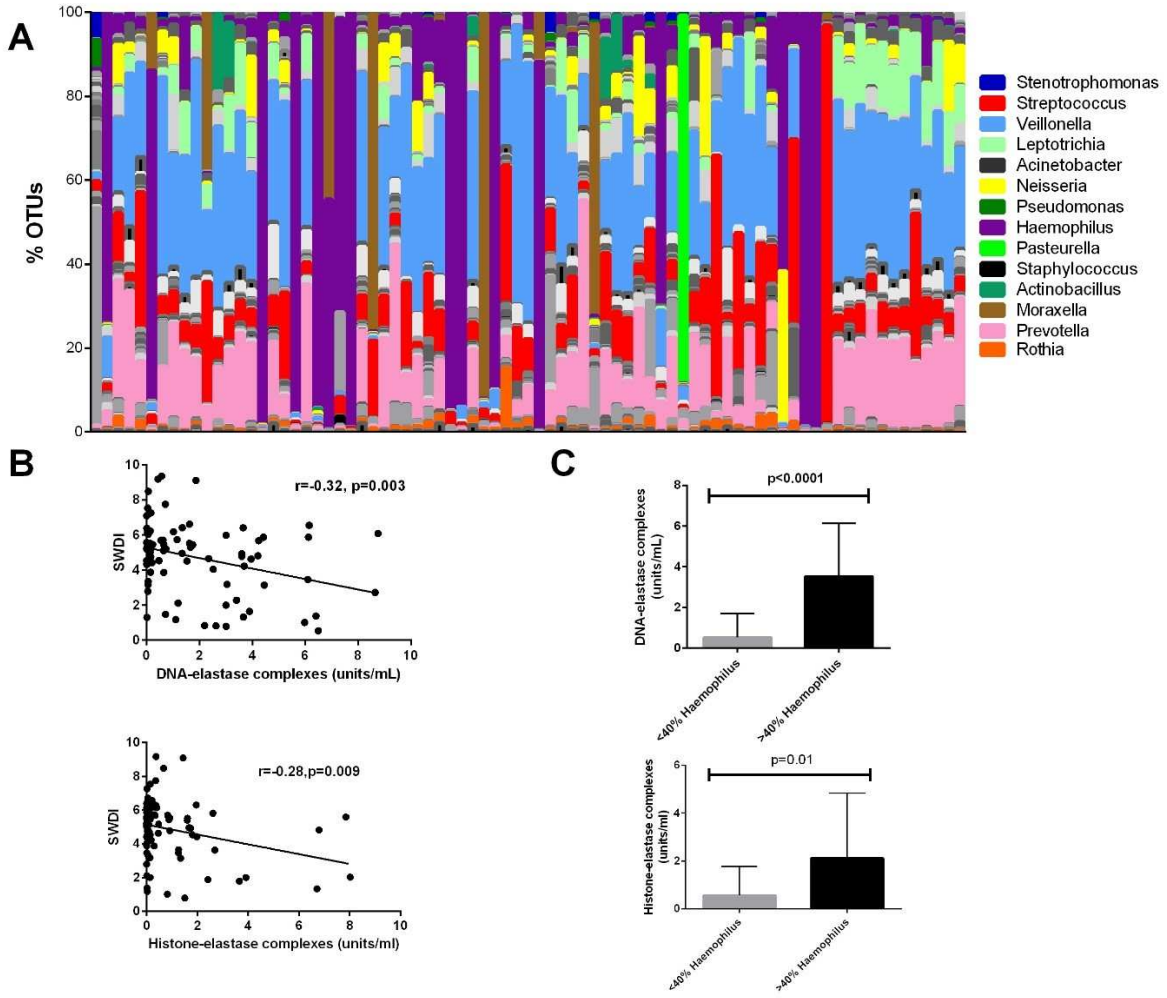
Figure 5: Platelet-neutrophil aggregates are present in COPD airways **A:** Neutrophils were gated based on CD16 and side scatter (top left), then the quadrants for positive and negative CD41a set (bottom left) in the isotype control then these gates applied to the test sample (right panels). The example shows positive staining for the platelet marker CD41a PE. **B**: No relationship between DNA-elastase complexes and soluble CD40 ligand, a marker of platelet activation (n=72). **C**: No relationship between sputum NETs and CXCL8 in sputum (n=72).

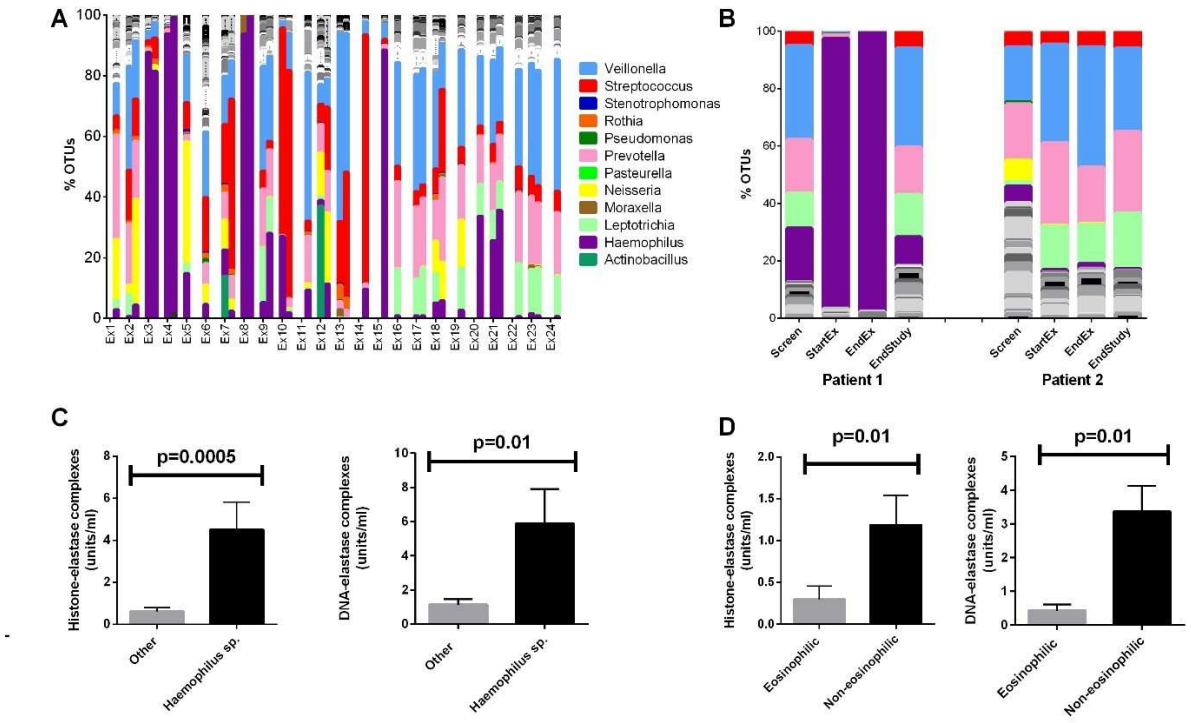
Figure 6: Direct relationship between sputum DNA-elastase complexes and *ex vivo* phagocytosis assessed by flow cytometry. **A**: Representative image of phagocytosis flow cytometry showing isotype control (top image) used to set gates for test sample (Bottom image); test sample results were normalised using isotype control to account for background fluorescence. **B:** Phagocytosis of FITC-labelled *P. aeruginosa* was evaluated by the % of positive cells and the mean fluorescence (which quantifies the number of fluorescent bacteria ingested per cell) (n=40). **C**: Dose dependent inhibition of phagocytosis of FITC-labelled *E. coli* by healthy donor neutrophils in response to pre-treatment for 30mins

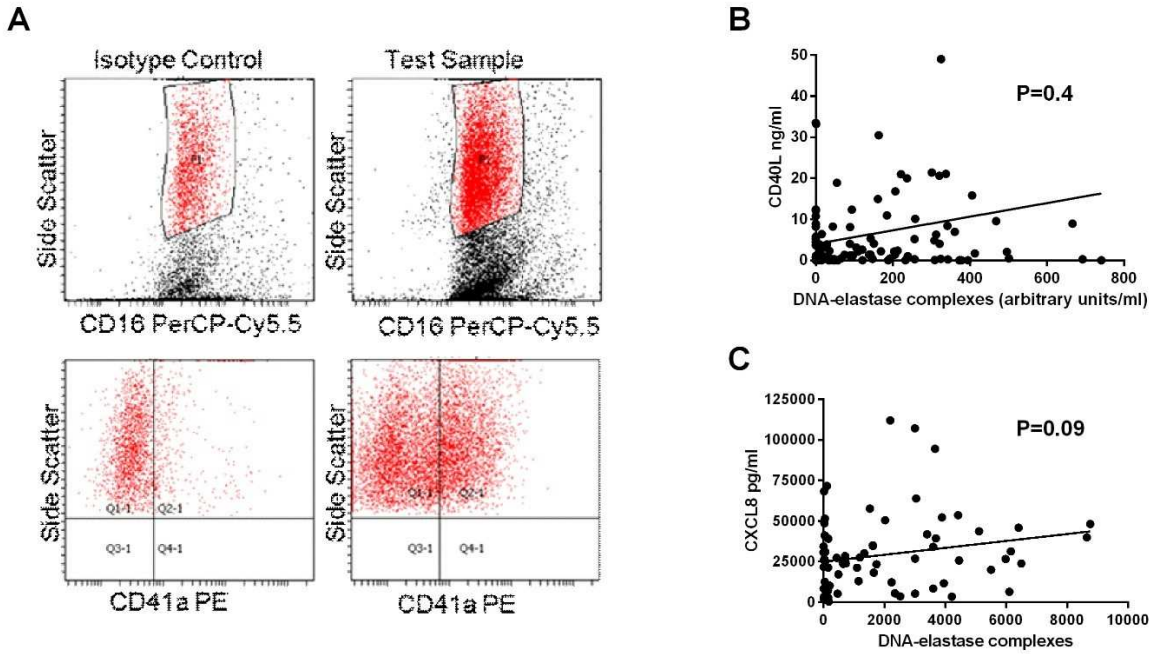
fluticasone propionate, pooled soluble sputum (N=7 COPD donors), or supernatant from neutrophils induced to undergo NETosis by incubation for 4 hours with 20nM PMA. N=4 replicates with different donors for each experiment, (statistical significance $p<0.05$ is denoted by *) **D:** Individual soluble sputum from n=24 patients with COPD used to pre-treat healthy donor neutrophils followed by phagocytosis of FITC-labelled *E. coli* for 30 mins. Data shows a direct relationship between the sputum DNA-elastase complex concentration and subsequent neutrophil phagocytosis, suggesting that samples with high NET concentrations inhibit phagocytosis.

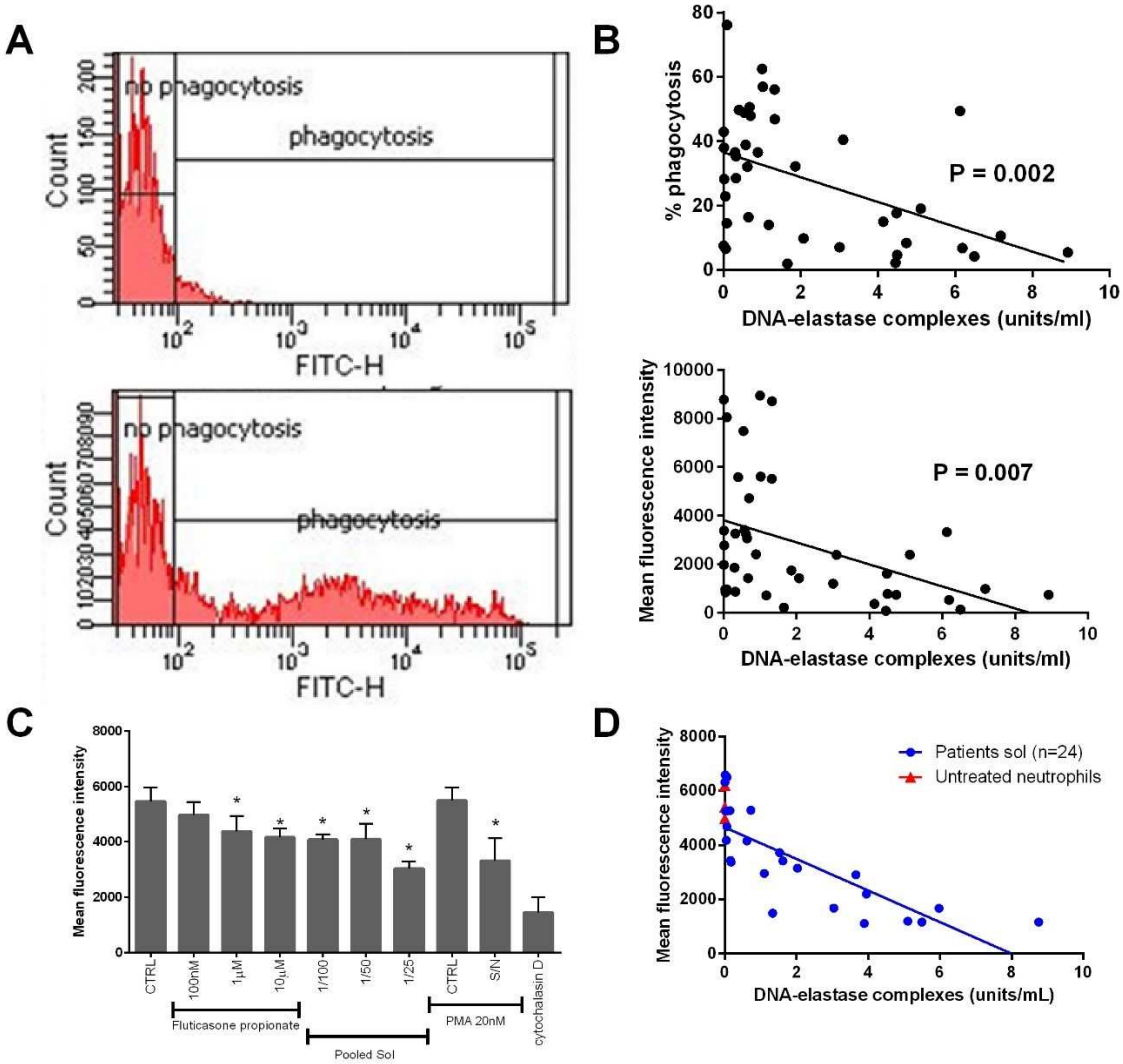











A
no phagocytosis
phagocytosis
Count
FITC-H
B
% phagocytosis
P = 0.002
DNA-elastase complexes (units/ml)
Mean fluorescence intensity
P = 0.007
DNA-elastase complexes (units/ml)
C
Mean fluorescence intensity
CTRL
100nM
1µM
10µM
1/100
1/50
1/25
CTRL
S/N
cytochalasin D
Fluticasone propionate
PMA 20nM
Pooled Sol
D
Patients sol (n=24)
Untreated neutrophils
Mean fluorescence intensity
DNA-elastase complexes (units/mL)

Neutrophil Extracellular Traps are associated with disease severity and microbiota diversity in Chronic Obstructive Pulmonary Disease

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Running title: Neutrophil extracellular traps in COPD

Keywords: Neutrophils, phagocytosis, COPD, *Haemophilus*, exacerbations

METHODS

Inclusion and Exclusion Criteria

Patients were included if >40 years; a FEV_1/FVC ratio <70% and with a clinical diagnosis of COPD. Exclusion criteria included the inability to give informed consent; previous adverse reaction to nebulised hypertonic saline; asthma; bronchiectasis on HRCT scanning; cystic fibrosis; active mycobacterial disease; and immunosuppression. Patients receiving long term antibiotic therapy or maintenance oral corticosteroid therapy at screening were also excluded; additionally, patients needed to be clinically stable and free of antibiotic or corticosteroid therapy for 4 weeks prior to enrolment.

Data Collection

All relevant medical history (comorbidities, current medications, significant past conditions, operations and diagnostic procedures) was recorded at screening. Inhaled corticosteroid (ICS) dose was converted to Beclomethasone daily dose equivalent for analysis

Systemic and Airway Inflammation

Sputum IL-1β, IL-8 and TNFα, Serum CD40L and P-selectin were measured using commercially available kits (R&D Systems, Abingdon, UK) according to manufacturers' instructions. Sputum was processed by ultracentrifugation to obtain soluble sputum as previously described (E1). Soluble sputum was stored at -80°C until analysis. All sputum ELISAs were validated by spike and recovery experiments (E2). EN-RAGE was measured as follows: Plates we coated with 1 in 2000 rabbit polyclonal antibody to EN-RAGE (Abcam Ab37657) and washed 3 times with PBS 0.05% tween 20. Plates were blocked with 1% BSA in PBS for 1 hour and then diluted sputum samples or recombinant EN-RAGE standards

(diluted in 1% BSA PBS) were added and incubated for 2 hours at room temperature. Plates were incubated with 1ug/ml mouse monoclonal detection antibody (MAB10522, R+D systems) in 1% BSA PBS, detected with anti-mouse HRP (R+D systems) and developed with TMB substrate with values read at 450nm in a microplate reader. Neutrophil elastase was measured using a kinetic assay employing the substrate N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide as previously described (Sigma-Aldrich) (E3). Cell free DNA was measured by diluting sputum samples 1 In 100 in PBS and adding SYTOX green at a final concentration of 6µM. Fluorescence was measured at 538nm emission and 450nm excitation and results were compared to a standard curve made from pure DNA of known concentration.

Fluorescein isothiocyanate (FITC) labelling of bacteria

Pseudomonas aeruginosa strain PA01 and *Escherichia coli* strain (ATCC 25922) were grown on *Pseudomonas* isolation agar and nutrient agar plates respectively. Freshly isolated colonies were inoculated into 10mls of Luria-Bertani broth at cultured overnight at 37^{o}C with gentle shaking. Cultures were sub cultured 1 in 10 and grown for 3 hours to enter the logarithmic growth phase. Cultures (OD_{600} = 0.1) were labelled with FITC as described below: Bacterial suspensions were serially diluted and plated out on agar plates to determine colony counts. Overnight cultures of bacteria were heat inactivated 60°C for 1 hour before centrifugation at 3000 × g for 15 mins and OD brought to 1 with cold PBS. Samples were washed with 1% BSA-Hanks' balanced salt solution (HBSS) containing Ca and Mg (Thermo Fisher). 100µL of FITC solution (0.5mg/ml in PBS) was added and the bacteria placed on a rotary mixer at 4^{o}C for 30 mins. 900µl of ice cold 1%BSA-HBSS was added and samples were centrifuged at 10000rpm for 2 minutes, then re-suspended in 1mL 1%BSA-HBSS. Labelling was confirmed by flow cytometry and microscopy. Labelled bacteria were stored at -80°C until use.

Phagocytosis assays

Phagocytosis of peripheral blood and airway neutrophils was assessed using a standard flow cytometry based assay (E4): FITC labelled *Pseudomonas aeruginosa* (strain PA01) and *Escherichia coli* (ATCC 25922) were opsonised with 25% pooled healthy donor serum at 37^{o}C for 1 hour. Opsonised bacteria were then added to patient neutrophils at a multiplicity of infection 10:1 to neutrophils (0.5 x 10^6 per experiment). Phagocytosis was permitted at 37^{o}C for up to 30 mins and then terminated by placing the samples on ice. Excess bacteria were removed by washing with PBS and cells subsequently analysed by flow cytometry. To differentiate phagocytosed (intracellular) bacteria from adherent (extracellular bacteria), cells were incubated with 0.1% trypan blue to quench extracellular fluorescence. A minimum of 10,000 events were counted. Results were expressed as the normalised rate of phagocytosis and mean fluorescent intensity for each sample. For some of these experiments, neutrophil phagocytosis was assessed after treatment with COPD patients' soluble sputum (at a final concentration ranging from 10% soluble sputum to 0.01% in PBS), supernatant from phorbol 12-myristate 13-acetate (PMA) treated neutrophils or controls (PBS or Cytochalasin D) at 37^{o}C.

For analysis of sputum neutrophils, the protocol was modified as follows; whole sputum was incubated at room temperature in PBS with 5% normal human serum, centrifuged at 20 x g for 10 mins then filtered through 48µM nylon gauze. 1 x 10^6 sputum neutrophils were incubated with FITC labelled PA01 bacteria or control for 30 mins at 37°C at a multiplicity of infection of 10:1 as described above, washed in PBS and analysed as above.

Sputum neutrophil platelet aggregates

Sputum neutrophil platelet aggregates were measured by flow cytometry. Sputum neutrophils were isolated from sputum as for the phagocytosis assay above, aggregated were visualised by co-staining the neutrophils with CD16 and CD41a (BD biosciences), gating of the neutrophils based on forward scatter, side scatter and CD16 then the normalised rate of platelet binding (CD41a) determined against an isotype control. A minimum of 10,000 events were counted.

Sputum Neutrophil Cytospins

Sputum neutrophils were isolated from sputum by filtering through a 48µM nylon gauze as described above, the concentration of cells in 100µL PBS adjusted to 30,000 cells per cytospin. The microscope slides were assembled with filter paper and cytofunnels then pre-wetted with 50µL PBS by centrifugation at 1000 rpm for 1 min in a cytocentrifuge (Shandon). Cells were added to cytofunnel and centrifuged for 1200 rpm for 3 mins. Slides were removed, allowed to air dry, stained with DiffQuik, dried, fixed and mounted before differential counts were determined.

NET Assay with purified neutrophils

NETs were studied using a fluorescent assay (E5) as follows: In 96 well plates, 5 x 10^4 isolated blood neutrophils were added per well in HBSS containing Ca and Mg (ThermoFisher) and 20mM Hepes. After adhering for 30 mins, cells were treated with PMA at concentrations 1-100nM to induce NET formation. In some of these experiments, diphenyleneiodonium (DPI), a NADPH oxidase inhibitor that blocks NETosis, was added at 100nM for 30 mins prior to stimulation with PMA. After 4 hours at 37°C, NETs were stained with SYTOX green (10uM final concentration). Extracellular DNA was quantified by mean

fluorescence and shown to correlate (r^2 >0.95) with NET quantification by fluorescence microscopy (E5).

Sputum microbiota sample preparation

DNA and RNA was extracted from whole sputum using the AllPrep DNA/RNA Mini kit on the QIAcube automation platform (QIAGEN) as follows: Whole sputum was incubated in an equal volume of 1 in 10 diluted Sputolysin (Calbiochem) in a shaking incubator for 30mins at 37°C, mixed with Buffer RLT as per the AllPrep kit protocol, then passed through QIAshredder columns (QIAGEN) with the resulting supernatant undergoing sequential DNA and RNA extraction on the QIAcube. Quality and quantity of the DNA and RNA was determined by Nanodrop and Qubit machine, using the Qubit dsDNA broad range kit (Thermo Scientific). Metagenomic sequencing of the bacterial 16S rRNA gene was performed following the protocol in the Illumina library prep guide (https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf), using primers targeting the V3 and V4 region (E6). Nextera XT Indices were added to each sample to allow multiplexing and the libraries sequenced using 2 x 300 paired end sequencing on the MiSeq platform using a MiSeq V3 kit (Illumina). Following sequencing on the Illumina MiSeq platform, FastQ files were imported into QIIME (version 1.9.0) and quality of reads checked; any reads with a Phred quality score less than Q20 were excluded when paired end reads were joined together for each sample. Un-joined reads were excluded from subsequent analysis. Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity using the UCLUST algorithm (E7), aligned against the Greengenes Core reference alignment (Version 13.8) (E8)

using PyNAST (Version 1.2.2) (E9). Taxonomy of the OTUs was assigned using the Ribosomal Database Project Classifier (Version 2.2) with the *de novo* OTU picking option (E10). OTUs were filtered to remove singletons and unassigned OTUs, or OTUs identified as Eukaryota, Human and Cyanobacteria. The dataset was normalised to the lowest number of OTUs and the Shannon-Wiener Species Diversity Index (SWDI) of the samples determined. All sequence data generated in this project can be found on the NCBI Sequence Read Archive, accession number SRP073159, a table of OTUs identified is shown in this supplement (Table E2).

Validation of NET assays

A series of controls were performed to ensure that the assays used in this study were measuring NETs. Citrullinated histones are regarded as one of the most specific markers of NET formation and their presence is frequently used as evidence that NET formation has occurred (E11). We used a semi-quantitative ELISA for citrullinated histone H3 (CITH3) (Cayman Chemical) to identify sputum samples with NETs present. Levels of DNA-elastase or histone-elastase complexes were compared between samples with detectable CITH3, defined as above the lower limit of detection of the ELISA, and those with undetectable CITH3 (at 1 in 10).

To exclude the possibility that sample preparation methods may affect NET formation we compared the detection of NETs in samples from patients prepared by ultracentrifugation at 50,000g for 90 minutes with samples diluted in 4xPBS followed by standard centrifugation, and also tested the correlation of samples obtained from paired sputum and BAL from the same patient taken on the same day. Agreement was determined by linear regression and by the Bland-Altman method.

Finally, we investigated possible passive interaction between DNA and elastase or between DNA and other components in sputum (E12). Fish sperm DNA (Sigma Aldrich) was mixed with purified neutrophil elastase (Sigma Aldrich) at 37^{o}C for 1 hour. Neutrophil elastase was added at a concentration of 3ug/ml based on the mean concentration of total elastase present in 10 sputum samples (measured by ELISA, Total neutrophil elastase, Assaypro EE1001-1). Increasing concentrations of DNA were added at 4, 20 and 40ug/ml based on concentrations of DNA measured in sputum samples. Passive association was measured by DNA-elastase ELISA as described in the main manuscript text.

To evaluate passive association *in vivo*, soluble sputum contained 120ng/ml neutrophil elastase was incubated at 37^{o}C for 1 hour with DNA at 4, 20 and 40ug/ml and the effect of increasing excess DNA on the presence of DNA-elastase complexes was measured by ELISA.

To validate the Histone-elastase assay, we performed a degradation experiment using DNAse. If histones and elastase are indirectly associated as part of DNA based traps, then the levels of histone-elastase complexes should be reduced by treatment of sputum samples with DNAse. Conversely, if histones and elastase were passively associating and directly bound, then DNase treatment should have no effect on sputum levels of histone-elastase complexes. Sputum containing 10ug/ml of DNA was incubated with 0 to 5 units of DNAse, where 1 unit is the amount of DNAse required to degrade 1ug of DNA.

Statistical Analysis

Statistical analysis of data was carried out using SPSS 21 and GraphPad Prism 6.07. Multivariable analysis was conducted using logistic regression for categorical outcomes with

model fit evaluated with the Hosner-Lemeshow goodness of fit test. Multiple linear regression was used for continuous outcomes and negative binomial models for analysis of exacerbations. Pre-specified confounders were age, gender, smoking status, BMI, FEV_1% predicted, MRC dyspnoea score and use of inhaled corticosteroids (ICS). Biomarker method agreement was evaluated by linear regression and Bland-Altman plots. Statistical significance was set at $P<0.05$.

RESULTS

Validation of NET ELISA

DNA-elastase complexes accurately quantified NETs induced in healthy control neutrophils by treatment with PMA whilst release of DNA-elastase complexes was inhibited by diphenyleneiodonium (DPI), which prevents NET formation through NADPH oxidase. Importantly, neutrophils lysed with 0.1% Triton X100 released negligible quantities of DNA-elastase complexes (Figure E1A). There was a direct correlation between the DNA-elastase and SYTOX fluorescence in healthy control blood neutrophils treated with PMA (Figure E1B). In patient samples, there was a strong correlation between MPO-DNA and DNA-elastase ELISAs, and between the DNA-elastase and Histone-elastase ELISAs (Figure E1C, $r=0.81$, $p<0.0001$ and figure E1D $r=0.66$, $p<0.0001$ respectively). Samples containing citrullinated histones (N=30) contained more DNA-elastase complexes and histone-elastase complexes ($p<0.0001$ for all comparisons) compared to samples without detectable citrullinated histones by ELISA (N=30) (Figures S1E and S1F respectively).

(FIG E1)

We found no evidence that sample preparation affected the formation of NETs, with a strong linear correlation demonstrated between samples split for ultracentrifugation and PBS dilution methods, and between sputum and BAL samples taken from the same patient on the same day (Figure E2).

(FIG E2)

We found little evidence of non-specific interaction between DNA and elastase. Incubation of increasing concentrations of DNA with elastase resulted in only low levels of DNA-

elastase complexes detectable by ELISA, at levels far below those detected in patients. Adding increasing concentrations of free DNA to sputum samples did not result in significant increases in DNA-elastase complexes over the levels present prior to addition of DNA (Figure E3A). As a specific control for the histone-elastase complex assay, we treated sputum with DNAse to determine if this could disrupt NET complexes. We demonstrated a dose dependent decrease in histone-elastase complexes with DNAse treatment ($p=0.01$) (Figure E3B).

(FIG E3)

NETs are associated with clinical disease severity in stable COPD

(FIG E4)

(TABLE E1)

Sputum NET concentration is associated with microbiota composition

(FIG E5)

Impact of antibiotic therapy on diversity and Haemophilus dominance in COPD

Sequential samples were studied in a subgroup of patients over the 6 month follow-up period. 28 patients had paired samples and received no antibiotic therapy between baseline and follow-up. 38 patients had paired samples and received at least one course of antibiotics between baseline and follow-up.

Changes between the two time-points in the Shannon-Wiener Species Diversity Index (A), Chao index (B) and % of *Haemophilus* OTU's (C) are shown in Figure E6. During follow-up, mean change in SWDI was 0.93 for those not receiving antibiotics and 0.46 for those receiving antibiotics. Mean difference 0.47 95% CI -0.66 to 1.61, p=0.4. The proportion of patients experiencing a reduction in SWDI was also not significantly different between groups 36% for those not receiving antibiotics vs 50% for those receiving antibiotics, p=0.2.

(FIG E6)

In terms of the Chao index, mean change during follow-up was -416 for those not receiving antibiotics and -157 for those receiving antibiotics (mean difference -258 9% CI -635 to 117,p=0.2). The proportions showing a reduction in Chao index, were 67% vs 52%, p=0.2.

There was also no significance difference in Haemophilus OTUs between those treated with antibiotics and those not receiving antibiotics. Mean change was -8% for those receiving antibiotics and -12% for those not receiving antibiotics (mean difference -4% 9%CI -28 to 20, p=0.7).

We conclude from this analysis that antibiotic therapy does not have a large impact of diversity in the short term, but that our study was not powered to show small short term effects and was not designed to show longer term effects with repeated antibiotic courses over time.

In a second longitudinal analysis we compared DNA-elastase and histone-elastase complexes measured at baseline and follow-up visits to examine the stability of the marker when patients are clinically stable. We observed high variability as shown below in figure E7. There were, however, no significant differences between baseline and follow-up for either the histone-elastase (pairwise comparison p=0.6) and DNA elastase (p=0.2).

(FIG E7)

(FIG E8)

(TABLE E2)

Table E1. Comparison of different methods of measuring NET components, NET complexes, and cytokines hypothesised as being important in lung inflammation and their association with clinical markers of COPD disease severity*p<0.05, **p<0.001, ***p<0.0001. In view of multiplicity of testing, all p-values should be interpreted with caution.

Assay	Age	Exacerbations	MRC Dyspnoea Score	Long term Oxygen treatment	Sputum colour	% predicted FEV_1	CAT	SGRQ	GOLD score
Assays specifically targeting NETS									
Histone-elastase NET	0.13	0.27**	0.20*	0.28**	0.31**	-0.31**	0.23**	0.27**	0.30**
DNA-elastase NET	0.05	0.28**	0.27***	0.19*	0.25**	-0.30**	0.33***	0.37***	0.31***
Non-specific NET components									
cfDNA	0.06	0.12	0.07	0.17*	0.41***	-0.15	0.06	0.14	0.14
Elastase activity kinetic	0.11	0.23*	0.05	0.10	0.39***	-0.20*	0.15	0.19*	0.21*
MPO activity	-0.03	0.21**	0.02	0.17	0.43***	-0.11	0.06	0.12	0.16*
EN-RAGE	0.03	0.08	0.11	0.09	0.11	-0.26**	0.21**	0.27***	0.23**
Cytokines									
IL-1beta	0.14*	0.09	0.02	0.18*	0.37***	-0.14	0.10	0.14	0.19**
CXCL8	0.10	0.03	0.04	0.05	0.39***	-0.14	0.14	0.16	0.20**
TNF-alpha	0.10	0.15	0.07	0.28**	0.40***	-0.15	0.13	0.15	0.20**
Cells									
Neutrophil cell count	0.18	0.06	0.25*	0.18	0.30**	-0.13	0.08	0.13	0.16

Table E2: List of OTUs identified in stable and exacerbating COPD sputum samples, classified at the genus level. If identification was not possible at genus level, the OTUs were classified at a higher taxonomic level. OTUs identified in less than 10 samples and with a maximum representation in a sample of 0.5% are excluded from this list.

Stable OTUs	**Exacerbation OTUs**
[Prevotella]	*[Prevotella]*
Acholeplasma	*Achromobacter*
Achromobacter	*Acinetobacter*
Acidocella	*Actinobacillus*
Acinetobacter	*Actinomyces*
Actinobacillus	*Aggregatibacter*
Actinomyces	*Agrobacterium*
Aeromocrobium	*Anaerococcus*
Aggregatibacter	*Arsenicicoccus*
Agrobacterium	*Atopobium*
Alloiococcus	*Bacillus*
Anaerococcus	*Bacteroides*
Anaerovorax	*Bifidobacterium*
Atopobium	*Bulleidia*
Bacillus	*Burkholderia*
Bacteroides	*Butyrivibrio*
Beijerinckia	*Campylobacter*
Bifidobacterium	*Capnocytophaga*
Bilophila	*Cardiobacterium*
Bradyrhizobium	*Carnobacterium*
Bulleidia	*Catonella*
Burkholderia	*Chryseobacterium*
Butyrivibrio	*Corynebacterium*
Campylobacter	*Cryocola*
Capnocytophaga	*Curvibacter*
Cardiobacterium	*Delftia*
Catonella	*Dermacoccus*
Chryseobacterium	*Devosia*
Chthonomonas	*Dialister*
Clostridium	*Dokdonella*
Comamonas	*Eikenella*
Corynebacterium	*Elizabethkingia*
Curvibacter	*Enhydrobacter*
Delftia	*Enterococcus*
Desulfobulbus	*Ethanoligenens*
Desulfovibrio	*Filifactor*
Devosia	*Finegoldia*
Dialister	*Fusobacterium*
Dokdonella	*Gemella*

Eikenella	*Granulicatella*
Elizabethkingia	*Haemophilus*
Enhydrobacter	*Kingella*
Erwinia	*Klebsiella*
Exiguobacterium	*Kocuria*
Filifactor	*Lactobacillus*
Finegoldia	*Lactococcus*
Flavisolibacter	*Lautropia*
Fluviicola	*Leptotrichia*
Fusobacterium	*Megasphaera*
Gemella	*Methylobacterium*
Gemmata	*Microbacterium*
Geobacillus	*Micrococcus*
Granulicatella	*Mogibacterium*
Haemophilus	*Moraxella*
Hymenobacter	*Moryella*
Jonquetella	*Mycoplasma*
Kaistobacter	*Neisseria*
Kingella	*Nevskia*
Lactobacillus	*Novosphingobium*
Lactococcus	*Ochrobactrum*
Lautropia	*Oribacterium*
Leptotrichia	*Paludibacter*
Lysinibacillus	*Parachlamydia*
Megasphaera	*Paracoccus*
Methylobacterium	*Parvimonas*
Microbacterium	*Pasteurella*
Mogibacterium	*Pedobacter*
Moraxella	*Peptococcus*
Moryella	*Peptoniphilus*
Mycobacterium	*Peptostreptococcus*
Mycoplasma	*Phyllobacterium*
Neisseria	*Porphyromonas*
Ochrobactrum	*Prevotella*
Oribacterium	*Propionibacterium*
Paenibacillus	*Pseudomonas*
Paludibacter	*Pseudoramibacter_Eubacterium*
Parachlamydia	*Psychrobacter*
Paracoccus	*Ralstonia*
Parvimonas	*Rhodococcus*
Pasteurella	*Rothia*
Pedobacter	*Ruminococcus*
Peptococcus	*Schwartzia*
Peptoniphilus	*Selenomonas*

Peptostreptococcus	*Slackia*
Phyllobacterium	*Sneathia*
Porphyromonas	*Sphaerochaeta*
Prevotella	*Sphingobacterium*
Propionibacterium	*Sphingomonas*
Propionivibrio	*Staphylococcus*
Proteus	*Stenotrophomonas*
Pseudomonas	*Streptococcus*
Pseudoramibacter_Eubacterium	*Tannerella*
Pyramidobacter	*Thermus*
Ralstonia	*Treponema*
Rheinheimera	Unknown [*Mogibacteriaceae*]
Rhodococcus	Unknown [*Mogibacteriaceae*]
Roseateles	Unknown [*Paraprevotellaceae*
Roseburia	Unknown *Acetobacteraceae*
Rothia	Unknown *Actinomycetaceae*
Scardovia	Unknown *Aerococcaceae*
Schwartzia	Unknown *Aeromonadaceae*
Segetibacter	Unknown *Alphaproteobacteria*
Selenomonas	Unknown *Bacilli*
Sharpea	Unknown *Bacteria* CW040
Slackia	Unknown *Bacteria* EW055
Sneathia	Unknown *Bacteria* F16
Sphaerochaeta	Unknown *Bacteria* Rs-0445
Sphingobacterium	Unknown *Bacteria* SR1
Sphingomonas	Unknown *Bacteria* TM7-3
Sphingopyxis	Unknown *Bacteria* WPS-2
Spirosoma	Unknown *Bacteroidales*
Staphylococcus	Unknown *Bacteroidales* S24-7
Stenotrophomonas	Unknown *Bifidobacteriaceae*
Streptococcus	Unknown *Bradyrhizobiaceae*
Sutterella	Unknown *Cardiobacteriaceae*
Tannerella	Unknown *Caulobacteraceae*
Treponema	Unknown *Chitinophagaceae*
Unknown [*Chloracidobacteria*] DS-100	Unknown *Clostridiales*
Unknown [*Chloracidobacteria*] Ellin6075	Unknown *Clostridiales*
Unknown [*Chloracidobacteria*] RB41	Unknown *Comamonadaceae*
Unknown [*Mogibacteriaceae*]	Unknown *Coriobacteriaceae*
Unknown [*Paraprevotellaceae*]	Unknown *Dethiosulfovibrionaceae*
Unknown [T*issierellaceae*]	Unknown *Enterobacteriaceae*
Unknown [*Weeksellaceae*]	Unknown *Flavobacteriaceae*
Unknown *Acetobacteraceae*	Unknown *Gemellaceae*
Unknown *Acidimicrobiales* C111	Unknown *Gemellaceae*

Unknown *Acidobacteria*	Unknown *Intrasporangiaceae*
Unknown *Actinomycetaceae*	Unknown *Lachnospiraceae*
Unknown *Actinomycetales*	Unknown *Lachnospiraceae*
Unknown *Aerococcaceae*	Unknown *Lactobacillales*
Unknown *Alcaligenaceae*	Unknown *Moraxellaceae*
Unknown *Alphaproteobacteria*	Unknown *Neisseriaceae*
Unknown *Anaerolinaceae* SHD-231	Unknown *Oxalobacteraceae*
Unknown *Aurantimonadaceae*	Unknown *Oxalobacteraceae*
Unknown *Bacilli*	Unknown *Pasteurellaceae*
Unknown *Bacteria* BD1-5	Unknown *Peptococcaceae*
Unknown *Bacteria* CW040	Unknown *Peptostreptococcacea*
Unknown *Bacteria* EW055	Unknown *Phycisphaerales*
Unknown *Bacteria* F16	Unknown *Propionibacteriaceae*
Unknown *Bacteria* Rs-045	Unknown *Rickettsiales*
Unknown *Bacteria* SR1	Unknown *Rickettsiales*
Unknown *Bacteria* TM7-3	Unknown *Solirubrobacteraceae*
Unknown *Bacteroidales*	Unknown *Streptococcaceae*
Unknown *Bacteroidales* BE24	Unknown *Veillonellaceae*
Unknown *Bacteroidales* S24-7	Unknown *Vibrionaceae*
Unknown *Bifidobacteriaceae*	Unknown *Weeksellaceae*
Unknown *Caldilineaceae*	Unknown *Xanthomonadaceae*
Unknown *Campylobacterales*	Unknown *Xanthomonadaceae*
Unknown *Caulobacteraceae*	Unkown Bacteria BD1-5
Unknown *Chitinophagaceae*	*Variovorax*
Unknown *Clostridiales*	*Veillonella*
Unknown *Clostridiales*	
Unknown *Comamonadaceae*	
Unknown *Coriabacteriaceae*	
Unknown *Dethiosulfovibrionaceae*	
Unknown *Enterobacteriaceae*	
Unknown *Enterobacteriaceae*	
Unknown *Firmicutes*	
Unknown *Flavobacteriaceae*	
Unknown *Gaiellaceae*	
Unknown *Gaiellales*	
Unknown *Gaiellales* AK1AB1_02E	
Unknown *Gemellaceae*	
Unknown *Geodermatophilaceae*	
Unknown *Intrasporangiaceae*	
Unknown *Koribacteraceae*	
Unknown *Lachnocpiraceae*	
Unknown *Lactobacillaceae*	
Unknown *Lactobacillales*	
Unknown *Leptotrichiaceae*	

Unknown *Methylobacteriaceae*	
Unknown *Microbacteriaceae*	
Unknown *Microbacteriaceae*	
Unknown *Micrococcaceae*	
Unknown *Moraxellaceae*	
Unknown *Myxococcales* OM27	
Unknown *Nacillaceae*	
Unknown *Neisseriaceae*	
Unknown *Neisseriaceae*	
Unknown *Nocardioidaceae*	
Unknown *Oxalobacteraceae*	
Unknown *Oxalobacteraceae*	
Unknown *Paenibacillaceae*	
Unknown *Pasteurellaceae*	
Unknown *Pasteurellaceae*	
Unknown *Peptostreptococcaceae*	
Unknown *Phycisphaerae* WD2101	
Unknown *Phyllobacteriaceae*	
Unknown *Planococcaceae*	
Unknown *Prevotellaceae*	
Unknown *Propionibacteriaceae*	
Unknown *Pseudomonadaceae*	
Unknown *Rhizobiaceae*	
Unknown *Rhodobiaceae*	
Unknown *Rickettsiaceae*	
Unknown *Rickettsiales*	
Unknown *Rickettsiales*	
Unknown *Rikenekkaceae* Blvii28	
Unknown *Ruminococcaceae*	
Unknown *Sinobacteraceae*	
Unknown *Solibacterales*	
Unknown *Solirubrobacteraceae*	
Unknown *Solirubrobacterales*	
Unknown *Solirubrobacterales*	
Unknown *Sphingomonadaceae*	
Unknown *Sporichthyaceae*	
Unknown *Tenericutes* ML615J-28	
Unknown *Thermomicrobia* JG30-KF-CM45	
Unknown *Veillonellaceae*	
Unknown *Veillonellaceae*	
Unknown *Verrucomicrobiaceae*	
Unknown *Xanthomonadaceae*	
Veillonella	

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FIGURE LEGENDS

Figure E1. Validation of an ELISA to measure neutrophil extracellular trap (NET) formation through detection of DNA-elastase and histone-elastase complexes. **A:** Quantification of NETs in neutrophils induced by phorbol 12-myristate 13-acetate and inhibited by diphenyleneiodonium (values shown are the mean and SEM of 3 independent experiments). **B:** Correlation of DNA-elastase complexes with SYTOX green quantification of extracellular DNA after neutrophils were induced to undergo NET formation with PMA, both of which are accepted proxies for NETs (n=7 samples). **C and D:** Correlation of DNA-elastase complexes with Histone-elastase complexes (n=162 samples) and MPO-DNA complexes (n=82 samples). **E and F:** citrullinated histone H3 positive samples contain DNA-elastase and Histone-elastase complexes, indicative of NET formation.

Figure E2: NETs are not induced due to sample preparation method. **A:** Correlation between different sputum samples methods. **B:** Bland Altman plots show acceptable agreement between sputum samples from the same patient prepared using ultracentrifugation and PBS dilution methods. **C:** Agreement between ultracentrifuged sputum and BAL. **D:** Bland Altman comparison between sputum and BAL in the same patient. Data are presented for the DNA-elastase assay. n=10 patient samples per comparison.

Figure E3: A: Limited evidence of passive interaction between DNA and elastase. **B:** DNAse treatment reduces the association between histone and elastase consistent with these being contained within NETs. Experiments shown are the mean (standard error of the mean) of 3 independent experiments.

Figure E4. DNA-elastase concentrations in soluble sputum of COPD patients are associated with clinical markers of COPD disease severity. **A:** NET concentration in stable samples compared to GOLD score (n=99). **B:** NET concentration compared to number of exacerbations reported by study patients in previous year (n=99). (SGRQ) (n=99). **C:** NET concentration in stable samples compared to percent predicted forced expiratory volume in 1 second (% predicted FEV_1). **D:** NET concentration in stable samples compared to COPD assessment test (CAT) (n=99).

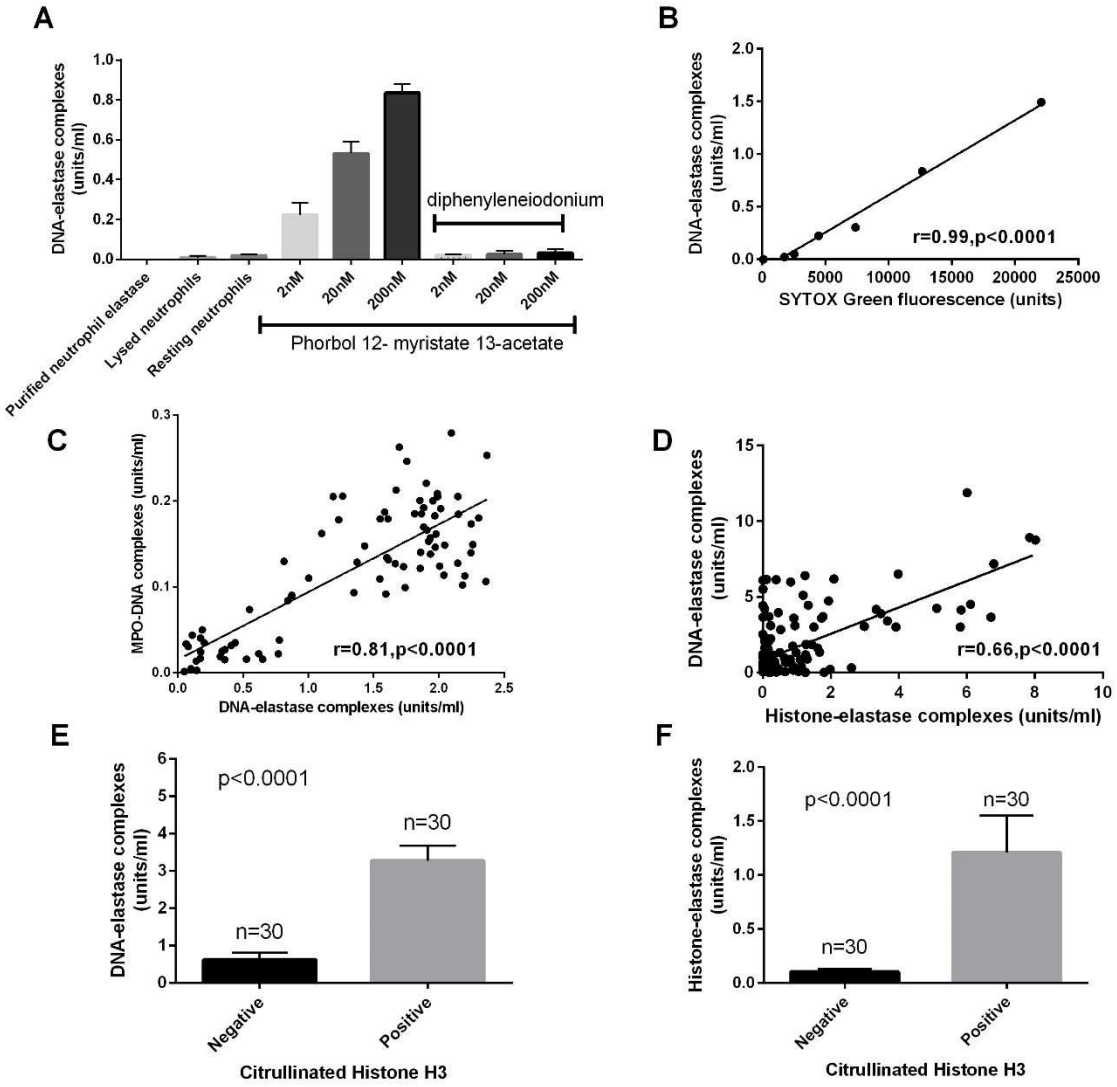
Figure E5: Two distinct clusters of sputum samples are apparent; the arbitrary cut-off of 40% *Haemophilus* spp. OTUs was chosen based on this data.

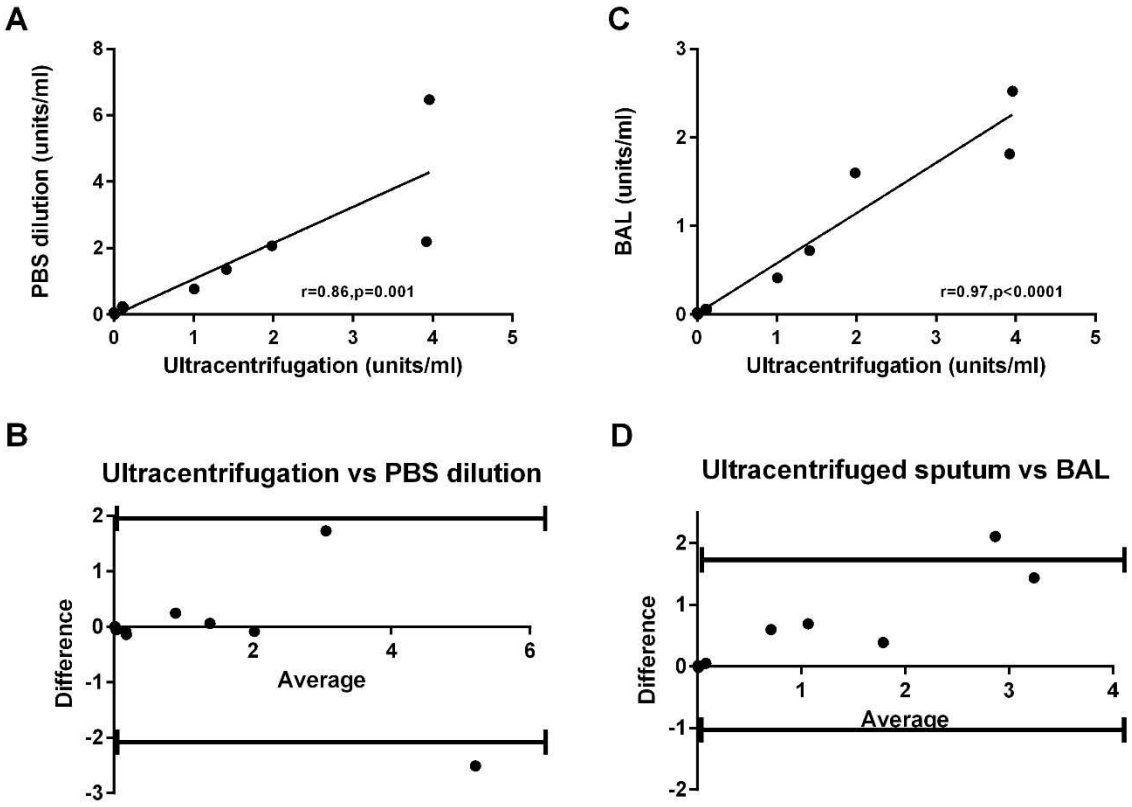
Figure E6: Impact of antibiotic therapy during the study on diversity **A:** Shannon Wiener Diversity Index. **B:** Chao evenness index. **C:** % *Haemophilus* spp OTUs.

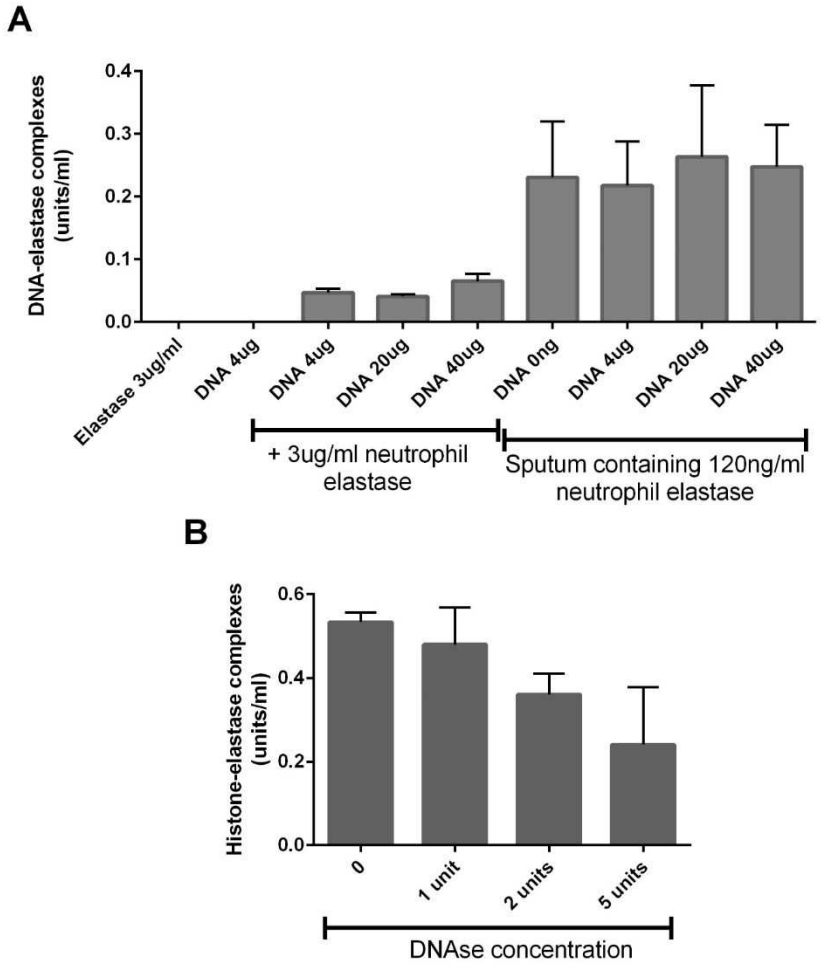
Figure E7. Changes over time in levels of NET complexes.

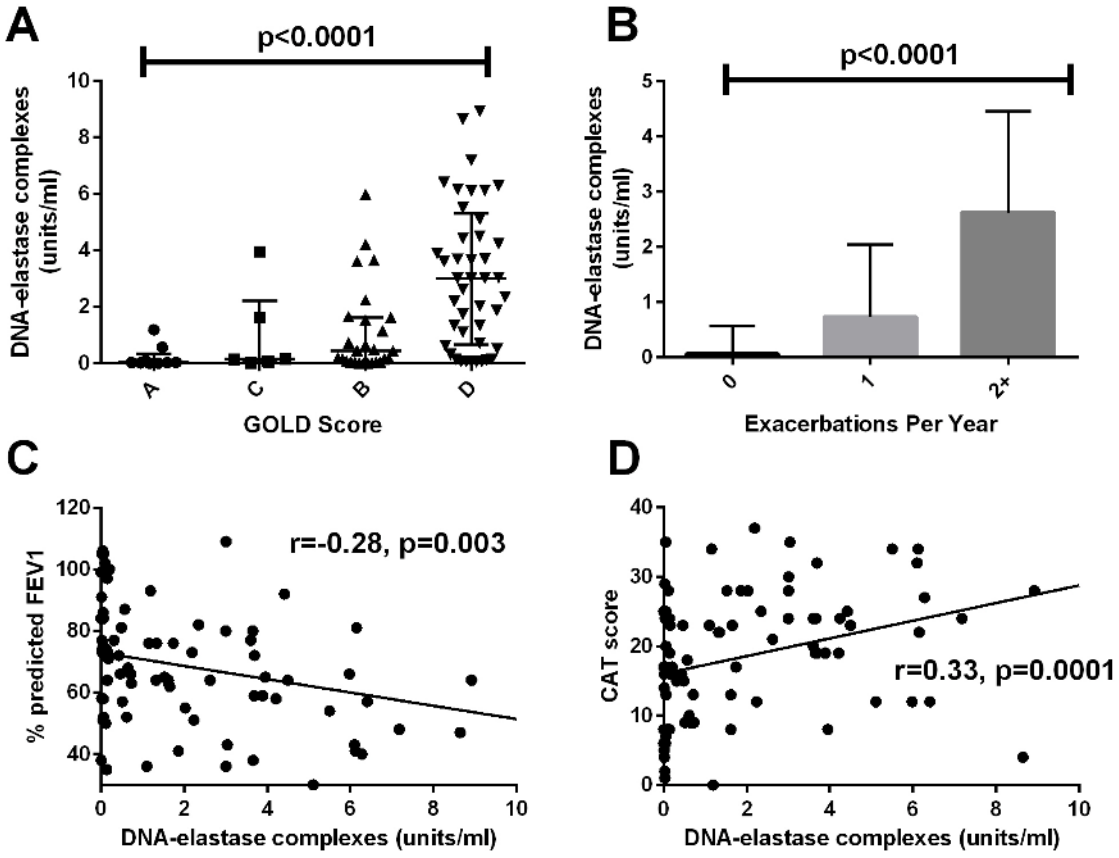
Figure E8. Relationship between neutrophil assays and phagocytosis. The top 2 panels show Histone-elastase complexes, the middle panels caspase positive cells on flow cytometry, and the bottom panels cfDNA concentrations in sputum.

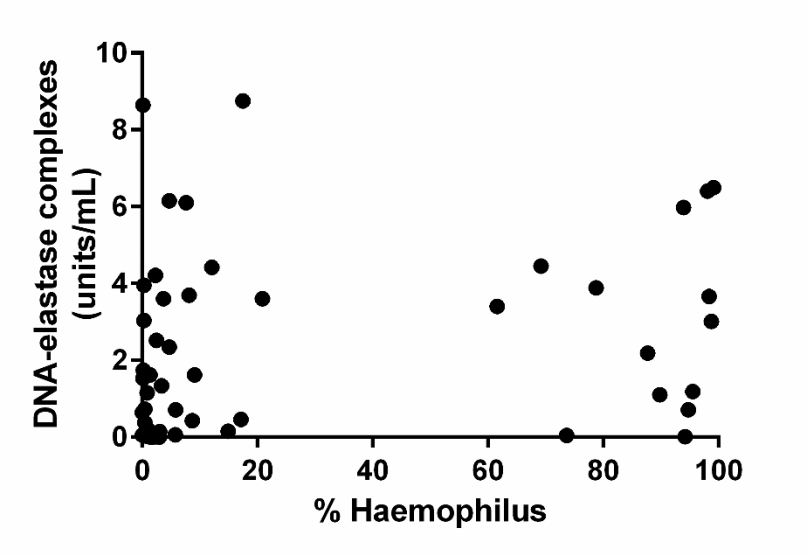
Figure E9. Relationship between inhaled corticosteroid regime and NET formation (P=0.01 by Kruskal Wallis test). 45% of study participants were receiving fluticasone, 6% budesonide containing regimes and 13% beclomethasone contained regimes.

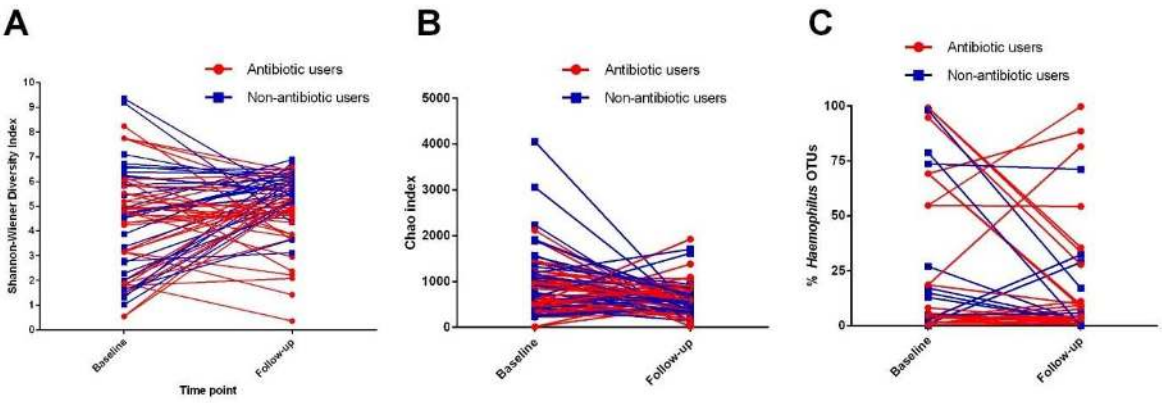

A
DNA-elastase complexes (units/ml)
1.0
0.8
0.6
0.4
0.2
0.0
diphenyleneiodonium
Purified neutrophil elastase
Lysed neutrophils
Resting neutrophils
2nM
20nM
200nM
2nM
20nM
200nM
Phorbol 12- myristate 13-acetate
B
DNA-elastase complexes (units/ml)
2.0
1.5
1.0
0.5
0.0
r=0.99,p<0.0001
0
5000
10000
15000
20000
25000
SYTOX Green fluorescence (units)
C
MPO-DNA complexes (units/ml)
0.3
0.2
0.1
0.0
r=0.81,p<0.0001
0.0
0.5
1.0
1.5
2.0
2.5
DNA-elastase complexes (units/ml)
D
DNA-elastase complexes (units/ml)
15
10
5
0
r=0.66,p<0.0001
0
2
4
6
8
10
Histone-elastase complexes (units/ml)
E
DNA-elastase complexes (units/ml)
6
5
4
3
2
1
0
p<0.0001
n=30
n=30
Negative
Positive
Citrullinated Histone H3
F
Histone-elastase complexes (units/ml)
2.0
1.5
1.0
0.5
0.0
p<0.0001
n=30
n=30
Negative
Positive
Citrullinated Histone H3

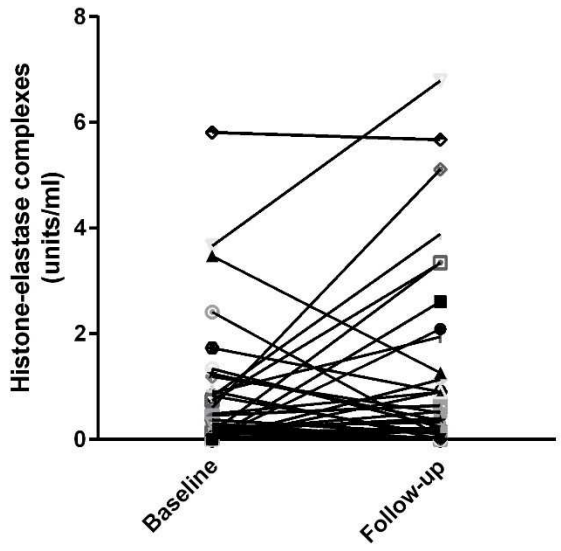


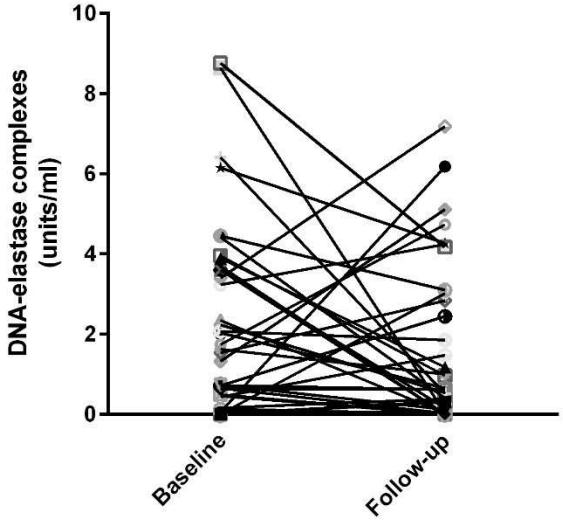


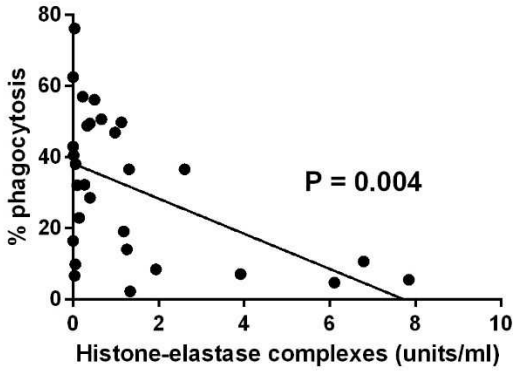


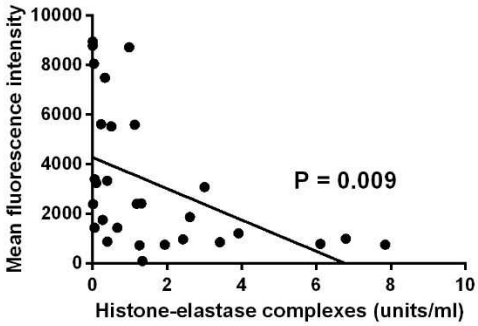


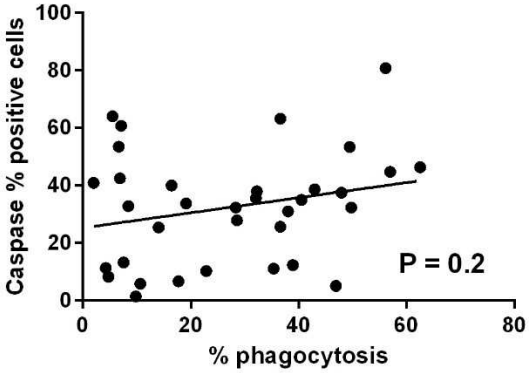


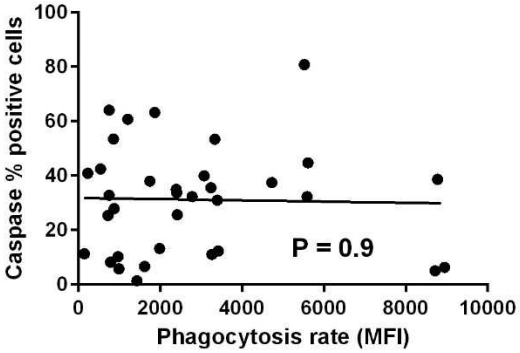


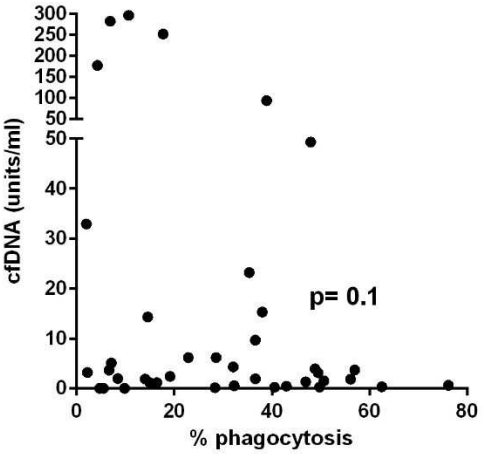


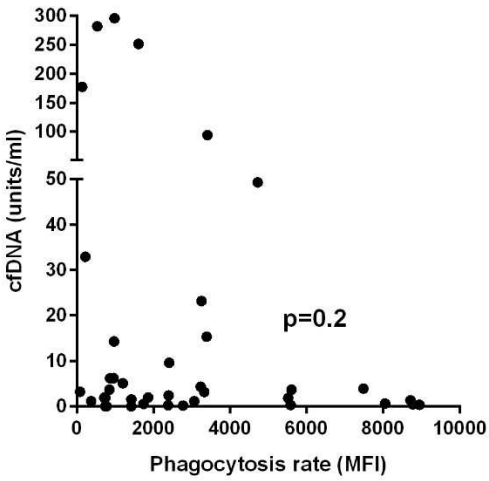

% phagocytosis
Histone-elastase complexes (units/ml)
P = 0.004


Mean fluorescence intensity
Histone-elastase complexes (units/ml)
P = 0.009


Caspase % positive cells
% phagocytosis
P = 0.2


Caspase % positive cells
Phagocytosis rate (MFI)
P = 0.9


cfDNA (units/ml)
% phagocytosis
p= 0.1


cfDNA (units/ml)
Phagocytosis rate (MFI)
p=0.2