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Pregnancy Zone Protein is Associated with Airway Infection, Neutrophil Extracellular Trap Formation and Disease Severity in Bronchiectasis

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At a Glance Commentary

Scientific knowledge on the subject: Bronchiectasis is associated with chronic bacterial infection and neutrophilic inflammation. The underlying pathogenesis of the disease is poorly understood. Patients with chronic neutrophil inflammation have impaired innate and adaptive immunity but the mechanisms by which neutrophilic inflammation links to impaired responses to infection are poorly characterized. In a proteomic study we identified pregnancy zone protein (PZP), previously identified in the serum of pregnant females, in the airway of patients with severe bronchiectasis and *Pseudomonas aeruginosa* infection. PZP is a powerful T-cell immunosuppressant thought to prevent rejection of the fetal allograft during pregnancy. We hypothesized that PZP may be associated with airway infection susceptibility in bronchiectasis. In this study we aimed to determine the source of PZP release in the airway and its association with chronic infection, airway inflammation and disease severity.

What this study adds to the field: We demonstrate for the first time that elevated airway levels of PZP are associated with disease severity, frequent exacerbations and airway infection in patients with bronchiectasis. PZP was found in the

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cytoplasm of neutrophils and was released during acute and chronic pulmonary inflammation. In-vitro, PZP was found to be associated with neutrophil extracellular traps (NETS) and in-vivo correlated with NETs detected in bronchiectasis patient sputum. Our findings implicate NETosis in the pathophysiology of bronchiectasis and given its known immunosuppressive effects, PZP may therefore provide a novel link between chronic neutrophilic inflammation and impaired host immunity to infection.

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Abstract

Background: Pregnancy zone protein (PZP) is a broad-spectrum immunosuppressive protein believed to suppress T-cell function during pregnancy to prevent fetal rejection. It has not previously been reported in the airway.

Objectives: To characterise PZP in the bronchiectasis airway including its relationship with disease severity.

Methods: Label free liquid chromatography/mass spectrometry was performed for sputum protein profiling between HRCT confirmed bronchiectasis patients with and without *P. aeruginosa* infection. Sputum and serum PZP was measured by validated ELISA. Airway infection status was established by culture and 16S rRNA sequencing. Immunofluorescence, ELISA and electron microscopy was used to identify the cellular source of PZP in neutrophils treated with multiple stimuli.

Results: Elevated PZP was identified by LC/MS as being associated with *P. aeruginosa* infection. In a validation study of 124 patients, sputum, but not serum levels of PZP were significantly associated with the bronchiectasis severity index, the frequency of exacerbations and symptoms. Airway infection with Proteobacteria such as *P. aeruginosa* was associated with higher levels of PZP. PZP in sputum was directly related to airway bacterial load. Neutrophils induced

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to form neutrophil extracellular traps (NETs) with phorbol myristate acetate (PMA) released high concentrations of PZP in-vitro, and fluorescence microscopy confirmed the presence of PZP in NETs, while fluorescence and electron microscopy localised PZP to the cytoplasm and nuclei of neutrophils. Effective antibiotic therapy reduced sputum PZP.

Conclusion: PZP is released into NETs. We report a novel link between airway infection, NET formation and disease severity in bronchiectasis during chronic airway inflammation.

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Introduction

Bronchiectasis is a chronic respiratory disease characterised by lung inflammation, impaired mucociliary clearance and recurrent airway infection leading to permanent tissue destruction and airway dilatation. There is no licensed treatment for bronchiectasis and therapeutic development has been severely limited by our poor understanding of the pathogenesis of the disease(1). Bronchiectasis is a heterogeneous disease in terms of aetiology, inflammatory profile, patient characteristics, co-morbidities and background treatments. Bronchiectasis is a global health problem and further heterogeneity is added by differences in the above factors between different geographical regions (2-4). An apparent paradox in bronchiectasis is the persistence of pathogens in the airway despite the presence of a robust inflammatory response. During acute inflammation with Gram-negative pathogens such as *Pseudomonas aeruginosa* or Gram-positive pathogens such as *Staphylococcus aureus,* neutrophil recruitment is followed by phagocytosis of pathogens and clearance of both bacteria and neutrophils through apoptosis and efferocytosis by macrophages. During chronic inflammation, this process may be impaired with reduced phagocytosis, reduced neutrophil apoptosis and a switch to tolerance and containment of infection

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through neutrophil extracellular trap (NET) formation. The mechanisms leading to

this immunological tolerance remain largely unexplored in bronchiectasis(5).

Pregnancy zone protein (PZP) is a high molecular weight glycoprotein which was

originally described as being elevated in the serum of women during

pregnancy(6). The synthesis of PZP is oestrogen dependent and it is detectable in

serum a few weeks post-conception and is reported to return to near

undetectable levels immediately postpartum(7). PZP is a broad spectrum

immunosuppressant(8) with anti-proteinase activity. Its role in pregnancy is

thought to be suppression of cell mediated immunoreactivity(9, 10) to prevent

rejection of the fetus. PZP has been shown to depress T-lymphocyte

immunoreactivity, T-cell recruitment, migration, proliferation and IL-2

production(9). These immunosuppressive effects are profound, illustrated by a

study showing that intravenous infusion of PZP was sufficient to prevent rejection

of heart allografts in mice(11). Recent experiments using PZP knockout mice have

shown that PZP also increases susceptibility to viral infection (12). PZP has,

however, never been previously found in the lung or studied in the context of

chronic respiratory disease.

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Chronic infection with *P. aeruginosa* is consistently associated with disease severity and poor outcomes in bronchiectasis. We therefore performed a proteomic study to profile sputum from patients with bronchiectasis and *P. aeruginosa* infection compared to those without chronic *P. aeruginosa* infection. Unexpectedly, PZP was identified as being elevated in patients with *P. aeruginosa* infection. We therefore hypothesised that, given the established immunosuppressive role of PZP, elevated sputum PZP would be associated with increased susceptibility to chronic airway infection and more severe disease.

Our results demonstrate for the first time that PZP is released from neutrophils during degranulation and NET formation. PZP is associated with airway infection in bronchiectasis patients and may be an unexpected mechanism through which NETs modulate T cell function leading to increased susceptibility to respiratory infection.

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Methods

For detailed methods please refer to the online supplement.

Patients and clinical assessments

Patients were recruited at a specialist bronchiectasis clinic at Ninewells Hospital, Dundee, UK. Inclusion criteria were: age ≥ 18 years, bronchiectasis confirmed by high resolution CT scan, chronic expectoration with ability to provide a sputum sample at the study visit and providing written informed consent. Exclusion criteria were: bronchiectasis due to cystic fibrosis, active allergic bronchopulmonary aspergillosis, active non-tuberculous mycobacterial infection, chronic use of oral corticosteroids, a primary clinical diagnosis of another respiratory disease (COPD or asthma) and inability to provide informed consent. Ethical approval for the study was given by the East of Scotland Research Ethics Committee, approval number 12/ES/0059.

Severity of disease was evaluated using the bronchiectasis severity index (BSI), as previously described(13). Exacerbations were defined as administration of antibiotics for increasing respiratory symptoms as defined by the British Thoracic Society(14). Sputum was obtained from all subjects during a period of clinical stability and split into whole (unprocessed) sputum for microbiology and sputum

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which was diluted 1:8 with PBS and then centrifuged at 3000g for 15 minutes at

4°C. All sputum processing took place within 2 hours of expectoration and freeze-

thaw cycles were avoided.

Sputum protein profiling

Sputum protein profiling was performed using nano-flow LC-MS/MS as previously

described (15).

Protein identification and label-free quantification were carried out using

Maxquant (version 1.4.1.2) against Uniprot-human database (version 2014-07-

09). FDR for protein identification was set to 1% at protein level. Data

visualisation was carried out using SIMCA P (version 13.0.3). For statistical

analysis, the dataset was log2 transformed before subjecting to t test using

Perseus (version 1.5.4.1). The Benjamini-Hochberg false discovery rate method

was used and corrected p values of $p<0.05$ is considered significant.

Pregnancy zone protein ELISA

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PZP was measured using a commercial ELISA kit for Human and Mouse (Cloud-

Clone Corp). Validation was performed according to published

recommendations(16).

Quantification of sputum NETs

Measurement of histone-elastase and DNA-elastase complexes provide a semi-

quantitative assessment of neutrophil extracellular traps in sputum and assays

were performed as previously described(17).

Leukocyte studies in healthy volunteers

Neutrophils and peripheral blood monocytes were isolated from healthy

volunteers using Percoll Gradient Density Centrifugation as previously

described(18).

Immunofluorescence was used to confirm and localize PZP within neutrophils and

NETs and identify co-localisation with other neutrophil proteins. NET formation

was induced by treatment for 4 hours with 600nM PMA.

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Co-localisation of PZP with neutrophil granule proteins was quantified using Manders overlap coefficient which calculates the proportion of overlap of each channel with the other, with a value of 1 indicating perfect co-localization and 0 indicating no co-localization.

Electron microscopy was used to identify the cellular location of PZP after staining with anti-PZP antibody and nanogold secondary. Appropriate negative controls were included.

Murine model of acute inflammation

Female 10-12 week-old C57/B6 mice were infected with *S. aureus* strain RN6390 at an infecting dose of 3 x 10^8 cfu. At 24 hours post infection the trachea was carefully dissected, intubated and bronchoalveolar lavage performed with 3 x 0.4ml PBS. BAL supernatant was used for PZP quantification and cells for cytospins to quantify neutrophils.

Sputum bacteriology

Quantitative bacterial culture was performed as described in the supplementary material.

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Microbiota sequencing

PCR and sequencing of the 16S rRNA gene was performed on an Illumina MiSeq as previously described(17). Analysis was performed in QIIME (detailed method in supplemental information). Alpha diversity was evaluated using the Shannon-Wiener diversity index and the Berger Parker Index. To compare groups patients were split into those with predominant (>50% OTUs) Proteobacteria and those with predominant Firmicutes at the phylum level as previously described.

Antibiotic response study

Patients were asked to attend the research centre if they developed symptoms of an exacerbation. Patients were reviewed by a physician and those who were prescribed antibiotics for a protocol defined exacerbation were included in the study. Patients received treatment for 14 days based on their previous sputum microbiology. Spontaneous sputum samples obtained as baseline and after 14 days were used for PZP measurement.

COPD cohort study

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To compare sputum PZP levels in bronchiectasis to those from patients with COPD, 40 patients with COPD without underlying bronchiectasis were enrolled while clinically stable (4 weeks free from antibiotic or corticosteroid therapy). Spontaneous sputum samples were obtained and processed in the same way as the bronchiectasis samples with sputum PZP and sputum NETs measured by ELISA.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 8. Categorical variables are presented by frequencies and percentages and statistical differences were analysed using χ^2 test or Fisher exact test when required. Continuous variables are presented as mean and standard deviation (SD) or median and interquartile range (IQR) when data are not normally distributed. Sputum PZP was not normally distributed and so data were log transformed and then analysed using t-test for comparisons of two groups and one way ANOVA for more than two groups. Paired t-test was used to compare changes in sputum PZP with antibiotic therapy. Linear variables were correlated by Spearman correlation. Principal component analysis (with the dataset log transformed, mean-centred

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and unit variance scaled) was performed using SIMCA-P (Version 13.0.3, Umetrics). We defined statistical significance as a two-tailed p<0.05 for all analyses.

Results

Sputum protein profiling was carried out in 20 bronchiectasis patients (9 with *P. aeruginosa* infection and 11 without) to explore potential biomarkers relevant to disease severity in bronchiectasis. Characteristics of the patients included are shown in Supplement (Table S1). Principle component analysis of sputum protein profiles reveals two distinct clusters where sputum profiles of patients with *P. aeruginosa* are well separated from those without (Figure 1A). A total of 80 proteins were found to be significantly associated with *P. aeruginosa* in sputum. Among differentially expressed proteins many were previously identified biomarkers of bronchiectasis lung disease including neutrophil elastase, myeloperoxidase, S100-A8 and S100-A9 as shown in the loadings plot (Figure 1B).We identified pregnancy zone protein (PZP), not previously described as present in sputum or as a neutrophil associated protein, to be differentially expressed between samples with *P. aeruginosa* infection and those without.

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Direct comparison of PZP in the *P. aeruginosa* infected and uninfected groups is shown in figure S1 online. The raw proteomics dataset has been uploaded as supplementary Table S4. We therefore explored whether PZP was associated with neutrophilic inflammation and bronchiectasis disease severity.

Patient cohort

124 patients were included in the study. There was a slight female predominance and a mean age of 67 years typical of European bronchiectasis cohorts (19-21). The clinical characteristics of our cohort are shown in Table 1.

Association of sputum PZP with disease severity in bronchiectasis

Median serum levels of PZP were 4.1 µg/ml (interquartile range 2.2-9.9), consistent with published data describing levels expected in healthy men and women ((<10 µg/ml in men and postmenopausal women, 10-30 µg/ml in premenopausal women(22, 23)). The median sputum level was 65.9 µg/ml (interquartile range 36.9-205.8). There was no significant difference in sputum PZP between male and female patients, median 65 µg/ml (IQR 39.1-215) vs 66.8

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(IQR 34.7-198.5) µg/ml (p=0.8). There was no significant correlation between

serum and sputum PZP levels (see the online supplement Figure S2).

Using the validated BSI we observed a clear relationship between sputum PZP and

bronchiectasis severity. There was a significant elevated median sputum PZP in

patients with severe disease was 163 µg/ml (IQR 64.6-854.1) compared to mild

58.6µg/ml (IQR 25.3-163.8), or moderate disease 52.6 (IQR 24.1-97.3), (Figure

2A) (p<0.001). It was observed that sputum PZP levels were not normally

distributed and so data are displayed as $\log^{10}$ values.

Sputum PZP was also higher in patients with frequent exacerbations (≥3/year)

compared to patients with less frequent exacerbations, as shown in figure 2B.

Relationships were also observed with quality of life using the Quality of Life

Bronchiectasis (QoL-B) Respiratory Symptom Score (p<0.0001) and a weaker

association was observed with FEV1 % predicted (r=-0.21, p=0.02). Patients with

higher PZP sputum levels also had a higher daily sputum volume (r=0.2, p=0.02),

while hospitalization for a severe exacerbation was also predicted by higher

sputum PZP (p<0.0001). No significant associations were observed between

serum PZP and severity of disease (online supplement, figure S3)

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PZP is a marker of airway chronic infection

Airway infection is linked to disease severity and we next validated the previous observation that PZP levels are higher in patients with chronic airway infection. Using standard microbial culture the most frequently isolated pathogens were *H. influenzae* (n=29) and *P. aeruginosa* (n=16). Overall 75 patients had chronic infection with pathogens while 49 did not. As shown in Figure 3A, patients with *P. aeruginosa* infection had significantly higher levels of sputum PZP compared to patients with no growth of pathogens. *H. influenzae, Moraxella catarrhalis* and Enterobacteriaceae were also associated with higher levels of sputum PZP.

When bacterial diversity was assessed by 16S rRNA sequencing we observed no relationship between sputum PZP and alpha-diversity using either the Shannon diversity index or the Berger-Parker index. PZP levels were, however, significantly different in patients with different microbiota profiles at the phylum level – with those with Proteobacteria dysbiosis (defined as >50% reads) having significantly higher sputum PZP (p=0.01, figure 3B). Patients with PZP levels above the median of the population had a higher average % of OTUs classified as Pseudomonas and lower % of OTUs classified as Streptococcus and Veillonella (Figure 3C).

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Sputum PZP is related to airway bacterial load and is reduced by antibiotic therapy

Bacterial load was quantified in 60 patients with bronchiectasis. The characteristics of this subgroup are shown in the online supplement (table S2). PZP levels were significantly associated with increased bacterial load ($p<0.0001$ by ANOVA, figure 4A). Significant differences were observed between those with bacterial loads above 10(7) and all other subgroups ($p<0.05$ for all pairwise comparisons). Excluding patients infected with *P. aeruginosa* resulted in similar results (figure S4 online).

Reduction in bacterial load with antibiotic treatment of exacerbations was associated with reduced sputum PZP levels. 20 patients were treated, of which 18 had detectable bacterial loads at baseline. Figure 4B shows the changes in sputum PZP from baseline to end of treatment after 14 days. PZP was significantly reduced ($p=0.0014$ by paired t-test). 6 patients, 5 of whom had *P. aeruginosa* infection, had detectable bacterial loads despite 14 days of antibiotics. Those patients who remained culture positive after 14 days had higher PZP at the end of treatment compared to those that achieved bacterial clearance ($p=0.01$, figure 4C). Taken together these data show that PZP increases with increasing bacterial

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load regardless of the infecting pathogen, and that reducing bacterial load with antibiotic therapy reduces sputum PZP.

PZP is released from neutrophils during activation and acute infection

We next examined the potential source of PZP detected in the bronchiectasis airway. There was no detectable PZP in the supernatant or cell lysates of primary human bronchial epithelial cells. Mononuclear cells released small amounts of PZP into the supernatant, mean 46.7ng/ml (SD 18.2, n=4), with higher levels on stimulation with 2.5ng/ml PMA (mean 177.8ng/ml, SD 6.4, n=4). Neutrophils secreted large amounts of PZP when stimulated with fMLP and PMA (Figure 5A). There was a dose-dependent relationship noted with all stimulants. There was also PZP released following incubation with bacteria (E.coli and P. aeruginosa) in a dose and time dependent manner (figure 5A and Figure S5 online) Immunofluorescence by confocal microscopy confirmed the presence of PZP in neutrophils (figures 5B and S6+7, supplement video 1+2). Staining was seen in a diffuse, punctate pattern throughout the cytoplasm, possibly suggestive of granules, however significant co-localisation with other granule proteins (neutrophil elastase, lactoferrin, MPO and MMP9) was not observed, Manders overlap coefficient <0.3.

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We studied whether acute pulmonary infection, which provokes a neutrophil
mediated inflammatory response, would result in an increase in PZP in BAL. *S.
aureus* was used as a common bronchiectasis pathogen that provokes a robust
neutrophil response including the formation of NETs. At 24h post infection, *S.
aureus* were detected in lung homogenate of infected mice and this was
associated with BAL neutrophilia. As shown in Figure 5C, infection resulted in an
increase in PZP in BAL which was directly correlated to BAL neutrophil count.

**PZP is found in the nucleus and cytoplasm of neutrophils and released into
neutrophil extracellular traps**

Based on the observation that PMA, an inducer of NET formation, was a strong
stimulus for PZP release from neutrophils we hypothesised that PZP may be a
marker of NETosis. In-vivo in bronchiectasis sputum we identified a strong
correlation between PZP in sputum and NETs measured by histone-elastase or
DNA-elastase complexes (Figure 6A). Experimentally induced NETs from healthy
control neutrophils, treated with PMA, showed staining for PZP in association
with DNA and neutrophil elastase in a "studded" pattern typical of neutrophil

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extracellular traps (figure 6B). To further investigate the localisation of PZP in neutrophils immune electron microscopy was used. This revealed staining within the euchromatin of the nuclei and a diffuse cytoplasmic pattern of PZP within the neutrophil. Our granulocyte extraction method produces neutrophil preparations that are >95% pure, but may contain small numbers of eosinophils. We unexpectedly observed positive staining of eosinophils in a similar pattern to neutrophils with cytoplasmic and nuclear staining.

Bronchiectasis is not alone in causing chronic neutrophilic inflammation of the airway. COPD is characterised by variable degrees of both neutrophilic and eosinophilic inflammation and neutrophil extracellular traps have been reported in the COPD airway. We therefore measured PZP and NETs in sputum from patients with COPD during a period of stability and compared these to samples obtained from our bronchiectasis cohort. Characteristics of the COPD patients are shown in table S3 online. Both PZP and NETs were significantly lower in COPD subjects compared to bronchiectasis (figure 7).

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Discussion

This study has identified pregnancy zone protein (PZP), which was previously described as a serum protein elevated in the blood of pregnant women, as an unexpected component of NETS and which was found to be released into the bronchiectasis airway during chronic infection and is elevated in patients with the most severe disease and frequent exacerbations.

We found levels of PZP in sputum at least 10x higher than levels in serum, suggestive of local production by inflammatory cells in the airway. It is known that patients with more severe bronchiectasis have higher levels of airway neutrophilic inflammation(24) including markers such as neutrophil elastase(25), matrix metalloproteinases (26, 27) and cathelicidin(28). PZP followed a similar pattern with clear associations with the multidimensional BSI, a higher level in the "frequent exacerbator phenotype" and an association with respiratory symptoms.

The major driver of airway neutrophilic inflammation in bronchiectasis is bacterial infection according to the vicious vortex concept of bronchiectasis pathophysiology. We demonstrate that PZP is increased in patients with chronic infection particularly with *P. aeruginosa* and *H. influenzae*, both frequent

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pathogens in this disease. Molecular "microbiome" based analysis of sputum confirmed elevated PZP in patients with Proteobacteria dysbiosis and airway dominance of *Pseudomonas*. This correlates with the clinically observed phenotypes that patients who regularly culture Proteobacteria (predominantly *Pseudomonas* and *Haemophilus*) display clinically more severe phenotypes(29).

We demonstrate that neutrophils are the likely source of PZP, although in common with many immune proteins we found PZP was present in a number of cell types including monocytes and eosinophils. PZP appears to be present throughout the cytoplasm of neutrophils and is not concentrated within primary or secondary granules, it is also consistently visible within the nuclei of both neutrophils and eosinophils. We demonstrate in a mouse model of *S. aureus* pneumonia that PZP is released into BAL following infection in parallel with neutrophil influx.

Neutrophils eliminate pathogens through phagocytosis, a relatively non-inflammatory intracellular process and extracellularly through degranulation. Neutrophil extracellular trap formation is a distinct antimicrobial pathway in which neutrophils can extrude extracellular DNA, histones and bactericidal proteins, intended to trap and neutralise pathogens. Components released in

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NETs include antimicrobial peptides (lactoferrin, defensins, LL37 and bacterial permeability increasing protein); proteases (neutrophil elastase, proteinase 3 and gelatinase); and enzymes responsible for reactive oxygen species generation (myeloperoxidase). NETs have been described in many chronic diseases including chronic respiratory diseases like COPD and cystic fibrosis. It is believed that they serve a beneficial role in preventing spread of bacterial infection but also contribute to tissue damage.

The identification of PZP in NETs in bronchiectasis is intriguing as it has no known antimicrobial effects. The known effects of PZP are as an anti-proteinase and as a powerful T-cell immunosuppressant. We speculate PZP may be involved in immunological tolerance in bronchiectasis patients by interacting with NETS to modulate T-cell functions. The persistence of bacterial infection despite an apparently intact cell mediated immune system is a feature of bronchiectasis that remains unexplained. It is well established that both Gram-positive and Gram-negative organisms can induce NET formation and it has been shown that neutrophils undergo NET formation in circumstances where phagocytosis is prevented, such as with physical barriers preventing phagocytosis as may occur with biofilms or with high bacterial loads. We demonstrated a strong association

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between PZP and elevated airway bacterial load above 10^7 cfu/g, suggesting that

NET formation may be the dominant neutrophil phenotype in patients with high

bacterial loads. Antibiotic therapy was able to reduce PZP levels consistent with a

cause and effect relationship between bacterial infection and elevated PZP.

The positive staining of eosinophils in a similar pattern to neutrophils is of

interest. The inflammation in bronchiectasis is a predominantly neutrophilic

process but eosinophilic phenotypes do exist.

Multiple previous reports indicate that PZP is regulated by oestrogens and other

female reproductive hormones but we found no evidence of a sex based

difference in serum or sputum PZP levels. Bronchiectasis has a female

predominance but our evidence suggests PZP is unlikely to play any role in this sex

disparity. Both male and female patient groups in our cohort are elderly ,it has

been shown that the physiological fluctuations in estrogen levels affect

exacerbations and *P. aeruginosa* mucoid transformation in Cystic Fibrosis(30) but

such fluctuations will not occur in a predominantly post-menopausal cohort.

Testosterone has also been shown to affect the antimicrobial susceptibility of *P.*

aeruginosa and has established roles in host defence(31). Future studies are

required to define how sex hormones may be implicated in the pathogenesis of

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bronchiectasis, but we found no evidence in established disease that PZP was associated with sex differences.

In summary, this study has identified a novel neutrophil protein which is released predominantly from neutrophil cytoplasm into NETs during chronic inflammation. We demonstrate high levels of PZP in the airway in severe disease and patients with high bacterial loads. This data suggests for the first time that NETs are present in the bronchiectasis airway, are associated with disease severity and contain proteins with the potential to have profound effects on the innate and adaptive immune system. The validation of this observation in multiple datasets using proteomics and ELISA suggests that this finding is robust. Bronchiectasis is a heterogeneous disease and there is increasing interest in identifying phenotypes and endotypes with distinct clinical outcomes and treatment responses. The finding that PZP is a marker of neutrophil mediated inflammation may be important for other diseases where neutrophils play a crucial role. Further mechanistic work is required to determine whether PZP is simply a marker of chronic neutrophilic inflammation or if it has a direct role in the pathogenesis of chronic infection in bronchiectasis.

The strength of this study is the use of multiple methods of clinical assessment and two different cohorts to validate our findings, and the confirmation of the finding of PZP in neutrophils using multiple methods including ELISA, immunofluorescence and electron microscopy. Nevertheless the study has limitations. There are no animal models of bronchiectasis and so there is no direct method of testing whether PZP is directly involved in the pathogenesis of bronchiectasis. We used an acute model of pulmonary inflammation with *S. aureus* because this organism promotes a robust neutrophil mediated response but we acknowledge that alternative models such as models of chronic *P. aeruginosa* may provide complementary information.

In conclusion, this study has identified a novel protein in the bronchiectasis airway associated with neutrophil extracellular traps, chronic infection and disease severity.

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References

1. Aliberti S, Masefield S, Polverino E, De Soyza A, Loebinger MR, Menendez R, et al. Research priorities in bronchiectasis: a consensus statement from the EMBARC Clinical Research Collaboration. The European respiratory journal. 2016;48(3):632-47.

2. Chandrasekaran R, Mac Aogáin M, Chalmers JD, Elborn SJ, Chotirmall SH. Geographic variation in the aetiology, epidemiology and microbiology of bronchiectasis. BMC pulmonary medicine. 2018;18(1):83.

3. Chalmers JD, Polverino E, Blasi F, Ringshausen F, De Soyza A, Vendrell M, et al. The heterogeneity of bronchiectasis patient characteristics, management and outcomes across Europe: Data from the EMBARC registry. European Respiratory Journal. 2018;52(suppl 62):PA2676.

4. Saleh AD, Chalmers JD, De Soyza A, Fardon TC, Koustas SO, Scott J, et al. The heterogeneity of systemic inflammation in bronchiectasis. Respiratory medicine. 2017;127:33-9.

5. Whitters D, Stockley R. Immunity and bacterial colonisation in bronchiectasis. Thorax. 2012;67(11):1006-13.

6. Smithies O. Zone electrophoresis in starch gels and its application to studies of serum proteins. Advances in protein chemistry. 1959;14:65-113.

7. von Schoultz B. A quantitative study of the pregnancy zone protein in the sera of pregnant and puerperal women. American journal of obstetrics and gynecology. 1974;119(6):792-7.

8. Stimson WH. Are pregnancy-associated serum proteins responsible for the inhibition of lymphocyte transformation by pregnancy serum? Clin Exp Immunol. 1980;40(1):157-60.

9. Skornicka EL, Kiyatkina N, Weber MC, Tykocinski ML, Koo PH. Pregnancy zone protein is a carrier and modulator of placental protein-14 in T-cell growth and cytokine production. Cellular immunology. 2004;232(1-2):144-56.

10. Stimson WH. Studies on the immunosuppressive properties of a pregnancy-associated alpha-macroglobulin. Clin Exp Immunol. 1976;25(2):199-206.

11. Svendsen P, Stigbrand T, Teisner B, Folkersen J, Damber MG, von Schoultz B, et al. Immunosuppressive effect of human pregnancy zone protein on H-2 incompatible mouse heart allografts. Acta pathologica et microbiologica Scandinavica Section C, Immunology. 1978;86c(4):199-201.

12. Krause K, Azouz F, Nakano E, Nerurkar VR, Kumar M. Deletion of Pregnancy Zone Protein and Murinoglobulin-1 Restricts the Pathogenesis of West Nile Virus Infection in Mice. Frontiers in Microbiology. 2019;10(259).

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13.   Chalmers JD, Goeminne P, Aliberti S, McDonnell MJ, Lonni S, Davidson J, et al. The bronchiectasis severity index. An international derivation and validation study. American journal of respiratory and critical care medicine. 2014;189(5):576-85.

14.   Pasteur MC, Bilton D, Hill AT. British Thoracic Society guideline for non-CF bronchiectasis. Thorax. 2010;65 Suppl 1:i1-58.

15.   Macleod AK, Zang T, Riches Z, Henderson CJ, Wolf CR, Huang JT. A targeted in vivo SILAC approach for quantification of drug metabolism enzymes: regulation by the constitutive androstane receptor. Journal of proteome research. 2014;13(2):866-74.

16.   Corp. CC. [Available from: http://www.cloud-clone.com/products/SEG324Hu.html.

17.   Dicker AJ, Crichton ML, Pumphrey EG, Cassidy AJ, Suarez-Cuartin G, Sibila O, et al. Neutrophil extracellular traps are associated with disease severity and microbiota diversity in patients with chronic obstructive pulmonary disease. The Journal of allergy and clinical immunology. 2018;141(1):117-27.

18.   Ulmer AJ, Flad HD. Discontinuous density gradient separation of human mononuclear leucocytes using Percoll as gradient medium. Journal of immunological methods. 1979;30(1):1-10.

19.   EMBARC. [Available from: https://www.bronchiectasis.eu/.

20.   Quint JK, Millett ER, Joshi M, Navaratnam V, Thomas SL, Hurst JR, et al. Changes in the incidence, prevalence and mortality of bronchiectasis in the UK from 2004 to 2013: a population-based cohort study. The European respiratory journal. 2016;47(1):186-93.

21.   Chalmers JD, Aliberti S, Filonenko A, Shteinberg M, Goeminne PC, Hill AT, et al. Characterization of the "Frequent Exacerbator Phenotype" in Bronchiectasis. American journal of respiratory and critical care medicine. 2018;197(11):1410-20.

22.   Sand O, Folkersen J, Westergaard JG, Sottrup-Jensen L. Characterization of human pregnancy zone protein. Comparison with human alpha 2-macroglobulin. The Journal of biological chemistry. 1985;260(29):15723-35.

23.   Folkersen J, Teisner B, Grunnet N, Grudzinskas JG, Westergaard JG, Hindersson P. Circulating levels of pregnancy zone protein: normal range and the influence of age and gender. Clinica chimica acta; international journal of clinical chemistry. 1981;110(2-3):139-45.

24.   Fuschillo S, De Felice A, Balzano G. Mucosal inflammation in idiopathic bronchiectasis: cellular and molecular mechanisms. European Respiratory Journal. 2008;31(2):396-406.

AJRCCM Articles in Press. Published on 02-July-2019 as 10.1164/rccm.201812-2351OC

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25. Chalmers JD, Moffitt KL, Suarez-Cuartin G, Sibila O, Finch S, Furrie E, et al. Neutrophil Elastase Activity is Associated with Exacerbations and Lung Function Decline in Bronchiectasis. American journal of respiratory and critical care medicine. 2016.

26. Garratt LW, Sutanto EN, Ling KM, Looi K, Iosifidis T, Martinovich KM, et al. Matrix metalloproteinase activation by free neutrophil elastase contributes to bronchiectasis progression in early cystic fibrosis. The European respiratory journal. 2015;46(2):384-94.

27. Taylor SL, Rogers GB, Chen AC-H, Burr LD, McGuckin MA, Serisier DJ. Matrix Metalloproteinases Vary with Airway Microbiota Composition and Lung Function in Non–Cystic Fibrosis Bronchiectasis. Annals of the American Thoracic Society. 2015;12(5):701-7.

28. Chalmers JD, McHugh BJ, Docherty C, Govan JR, Hill AT. Vitamin-D deficiency is associated with chronic bacterial colonisation and disease severity in bronchiectasis. Thorax. 2013;68(1):39-47.

29. Finch S, McDonnell MJ, Abo-Leyah H, Aliberti S, Chalmers JD. A Comprehensive Analysis of the Impact of Pseudomonas aeruginosa Colonization on Prognosis in Adult Bronchiectasis. Annals of the American Thoracic Society. 2015;12(11):1602-11.

30. Chotirmall SH, Smith SG, Gunaratnam C, Cosgrove S, Dimitrov BD, O'Neill SJ, et al. Effect of Estrogen on Pseudomonas Mucoidy and Exacerbations in Cystic Fibrosis. New England Journal of Medicine. 2012;366(21):1978-86.

31. Vidaillac C, Yong Lee V, Kaur Jaggi T, Roizman D, Sankaran J, Wohland T, et al. The impact of testosterone on Pseudomonas aeruginosa pathogenicity and antimicrobial susceptibility. European Respiratory Journal. 2018;52(suppl 62):PA2648.

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Figure legends

Figure 1. Principle component analysis of sputum protein profiles in bronchiectasis. Twenty patients with bronchiectasis including 9 with *P. aeruginosa* infection and 11 without (labelled in black) were included. The scores plot based on the first two components is shown in (A) and the loadings plot in (B). The cumulative R2X = 0.31 and Q2=0.21. R2X represents the fraction of the variation of the X variables explained by the first two components and Q2 indicates the fraction of the variation of the X variables predicted by the model. Sputum PZP levels are associated with samples with *P. aeruginosa* infection.

Figure 2. Association between sputum PZP and clinical outcomes. A: Severity of disease as stratified by BSI. B: Exacerbation frequency in previous year.

Figure 3. Association between sputum PZP and microbiological outcomes. A: Sputum PZP by microorganism growth on standard culture. B: Sputum PZP by dysbiosis (defined as >50% reads of single phylum on 16S sequencing). C: Patients were divided into those with PZP above and below the median value for the population and the %OTU's compared. The 15 genera most strongly associated with lower and 15 genera most strongly associated with higher PZP levels are shown.

Figure 4. Association between bacterial load in sputum and PZP. A: sputum bacterial load is associated with sputum PZP (p-value refers to comparison by ANOVA). B: Changes in PZP at the start (day 0) and end (day 14) of antibiotic therapy for an acute exacerbation of bronchiectasis. The p-value refers to comparison by paired t-test. C: Comparison of patients in (B) who were culture positive or culture negative at the end of antibiotic treatment for an exacerbation. p-value refers to unpaired t-test.

Figure 5. PZP is released from neutrophils in response to fMLP, PMA and bacterial infection in mice and humans. A: 10^7 neutrophils were treated with the indicated stimuli for 30 minutes and PZP measured in cell free supernatants. No stimulation control was PBS only. Statistically significant differences are obtained using t-test (n=4 biological replicates per condition). B: 10-12 week C57/B6 mice were infected with *S. aureus* strain RN6390 (n=18) or PBS control (n=4). BAL PZP was measured by ELISA. Significant differences are determined using T-test. Association between BAL neutrophils and BAL PZP (n=10 mice undergoing BAL, all infected with *S. aureus*). C: Microscopy image of neutrophils showing DNA: DAPI, Blue, neutrophil elastase: Green and Pregnancy zone protein: Red.

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Figure 6. PZP is a cytoplasmic and nuclear protein which is released in neutrophil extracellular traps A: In patients with bronchiectasis, extracellular PZP correlates with markers of neutrophil extracellular trap formation B: Peripheral blood neutrophils induced to undergo NET formation with 600nM PMA for 4 hours, NETs contain PZP. C: Electron microscopy of neutrophils (left) and neutrophils plus eosinophils (right) showing diffuse cytoplasmic and nuclear staining for PZP (black dots).

Figure 7. A) PZP levels in sputum in patients with bronchiectasis N=124 or COPD N=40 B) Neutrophil extracellular traps measured using the histone-elastase complex assay in bronchiectasis and COPD. For both assays comparisons are by t-test.

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Table 1. Patient characteristics. Abbreviations: BSI= bronchiectasis severity index, FEV1= Forced expiratory volume in 1 second, COPD = Chronic Obstructive Pulmonary Disease, ABPA = Allergic Bronchopulmonary Aspergillosis (* these patients had previously treated ABPA, rather than active disease), PCD= Primary ciliary dyskinesia, GORD = Gastro-oesophageal reflux disease.

N		124
Mean age (standard deviation- SD)		69.1 (10.7)
% female		53.2%
Mean BSI (SD)		7.8 (4.2)
	No. Mild	30 (24.2%)
	No. Moderate	56 (45.2%)
	No. Severe	38 (30.6%)
Mean exacerbation frequency/ year (SD)		2.6 (2.1)
Mean FEV1 (% predicted)		78.1% (25.5)
Aetiology		
	Idiopathic	82 (66.1%)
	COPD	16 (12.9%)
	Post-infective	8 (6.5%)
	Inflammatory Bowel Disease	4 (3.2%)
	Immunodeficiency	3 (2.4%)
	ABPA*	2 (1.6%)
	Rheumatoid Arthritis	2 (1.6%)
	PCD	1 (0.8%)
	Aspiration	1 (0.8%)
	Youngs syndrome	1 (0.8%)
	Asthma	1 (0.8%)
	GORD	1 (0.8%)
	Haematological malignancy	1 (0.8%)
	Other	1 (0.8%)
Smoking		
	Current	12 (9.7%)
	Ex	52 (41.9%)
	Never	60 (48.4%)
Antibiotics		

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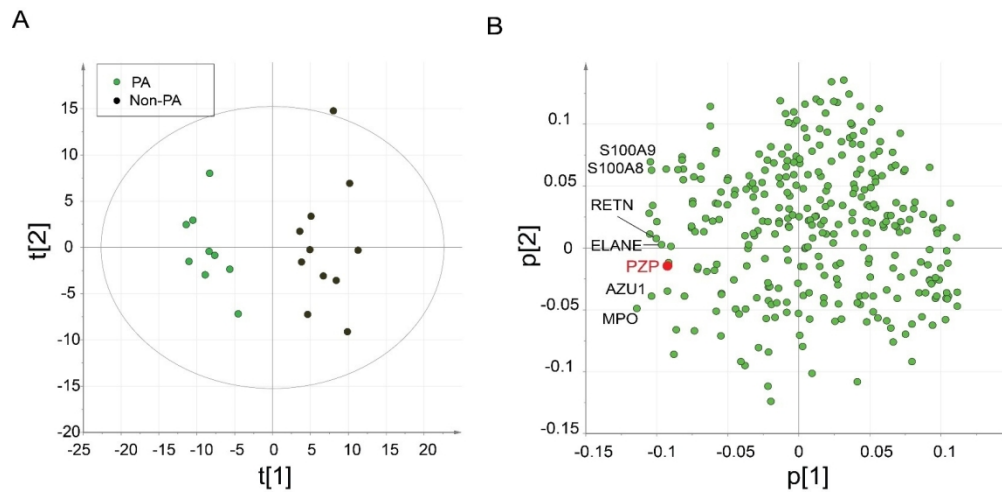| | Long term macrolides | 27 (21.8%) |
| | Inhaled | 5 (4%) |

36

Figure 1. Principle component analysis of sputum protein profiles in bronchiectasis. Twenty patients with bronchiectasis including 9 with P. aeruginosa infection and 11 without (labelled in black) were included. The scores plot based on the first two components is shown in (A) and the loadings plot in (B). The cumulative R2X = 0.31 and Q2=0.21. R2X represents the fraction of the variation of the X variables explained by the first two components and Q2 indicates the fraction of the variation of the X variables predicted by the model. Sputum PZP levels are associated with samples with P. aeruginosa infection.

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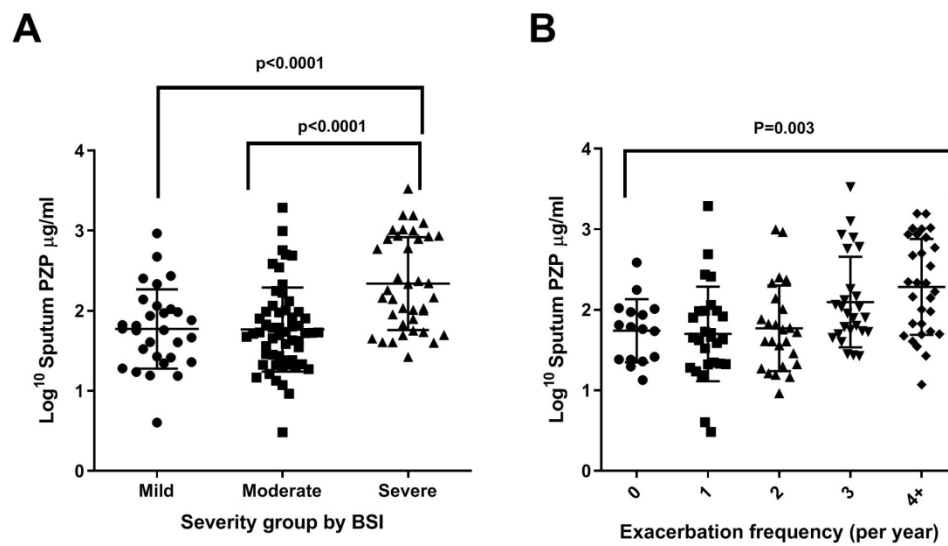
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Figure 2. Association between sputum PZP and clinical outcomes. A: Severity of disease as stratified by BSI. B: Exacerbation frequency in previous year.

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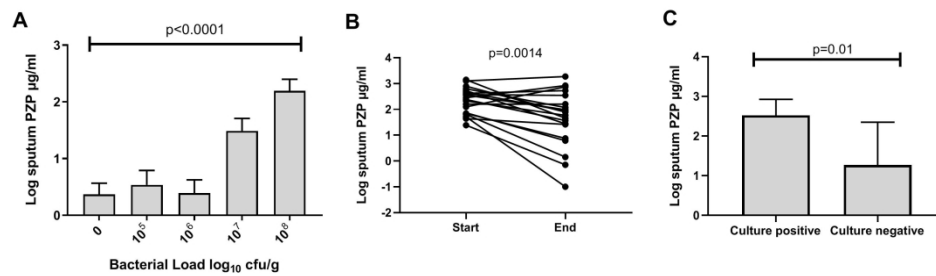
Figure 3. Association between sputum PZP and microbiological outcomes. A: Sputum PZP by microorganism growth on standard culture. B: Sputum PZP by dysbiosis (defined as >50% reads of single phylum on 16S sequencing). C: Patients were divided into those with PZP above and below the median value for the population and the %OTU's compared. The 15 genera most strongly associated with lower and 15 genera most strongly associated with higher PZP levels are shown.

189x216mm (300 x 300 DPI)

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Figure 4. Association between bacterial load in sputum and PZP. A: sputum bacterial load is associated with sputum PZP (p-value refers to comparison by ANOVA). B: Changes in PZP at the start (day 0) and end (day 14) of antibiotic therapy for an acute exacerbation of bronchiectasis. The p-value refers to comparison by paired t-test. C: Comparison of patients in (B) who were culture positive or culture negative at the end of antibiotic treatment for an exacerbation. p-value refers to unpaired t-test.
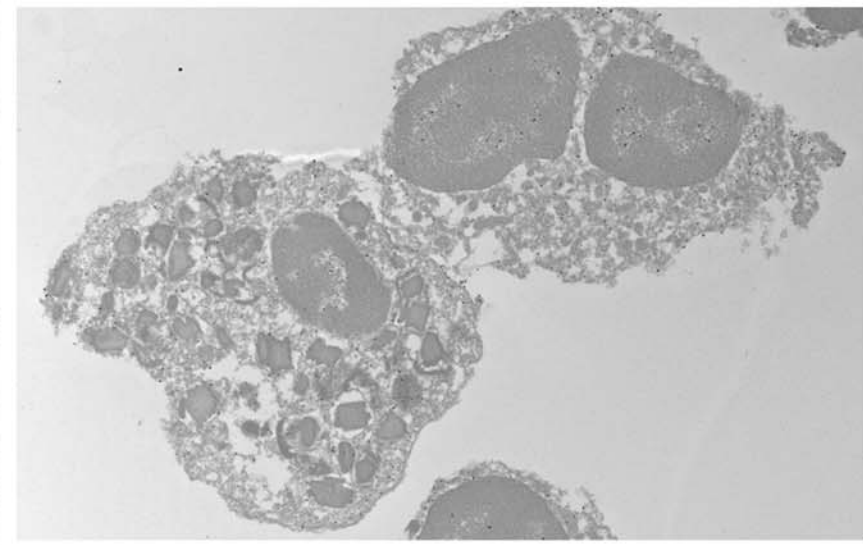
265x81mm (300 x 300 DPI)

Figure 5. PZP is released from neutrophils in response to fMLP, PMA and bacterial infection in mice and humans. A: 107 neutrophils were treated with the indicated stimuli for 30 minutes and PZP measured in cell free supernatants. No stimulation control was PBS only. Statistically significant differences are obtained using t-test (n=4 biological replicates per condition). B: 10-12 week C57/B6 mice were infected with S. aureus strain RN6390 (n=18) or PBS control (n=4). BAL PZP was measured by ELISA. Significant differences are determined using T-test. Association between BAL neutrophils and BAL PZP (n=10 mice undergoing BAL, all infected with S. aureus). C: Microscopy image of neutrophils showing DNA: DAPI, Blue, neutrophil elastase: Green and Pregnancy zone protein: Red.

218x175mm (300 x 300 DPI)

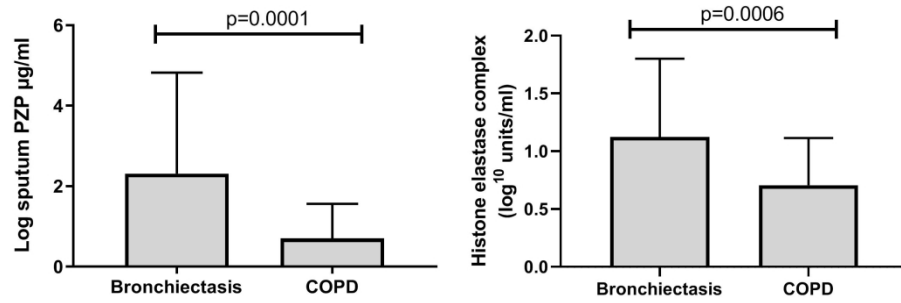AJRCCM Articles in Press. Published on 02-July-2019 as 10.1164/rccm.201812-2351OC
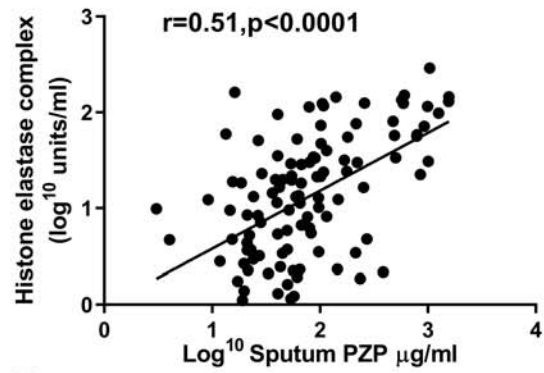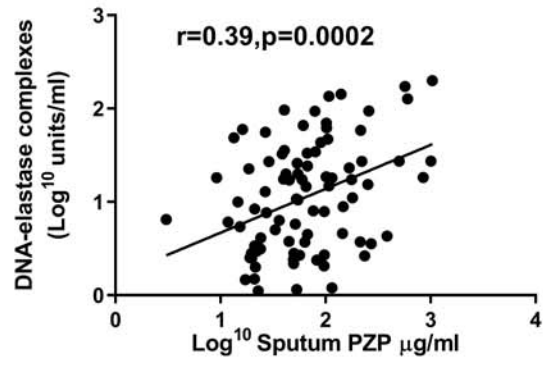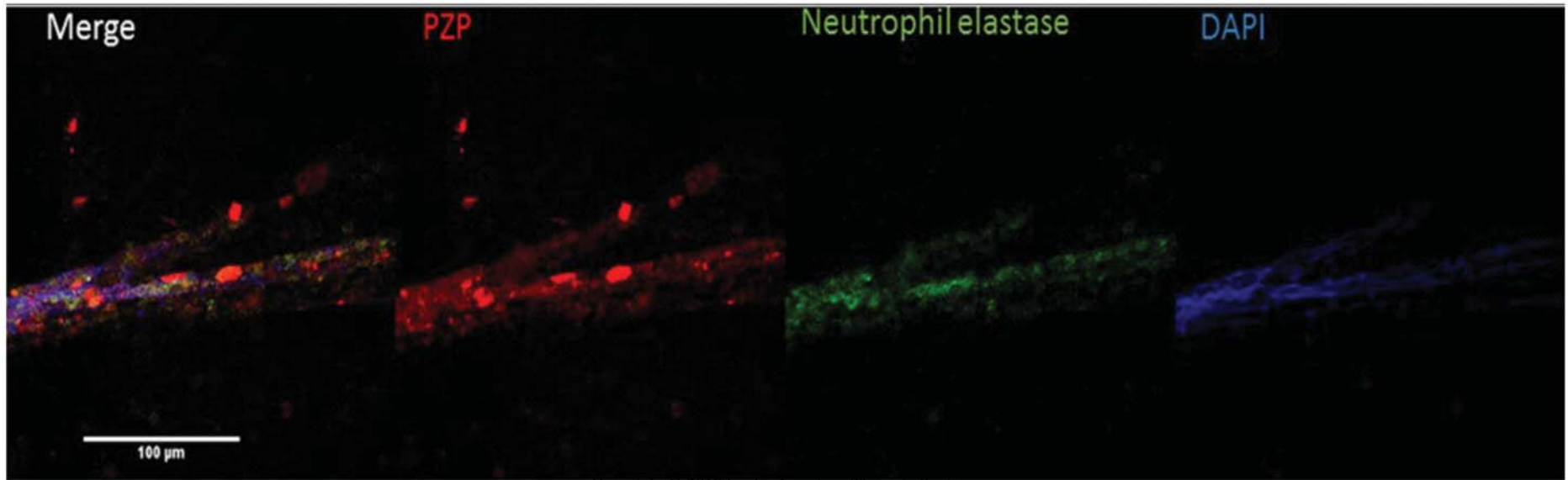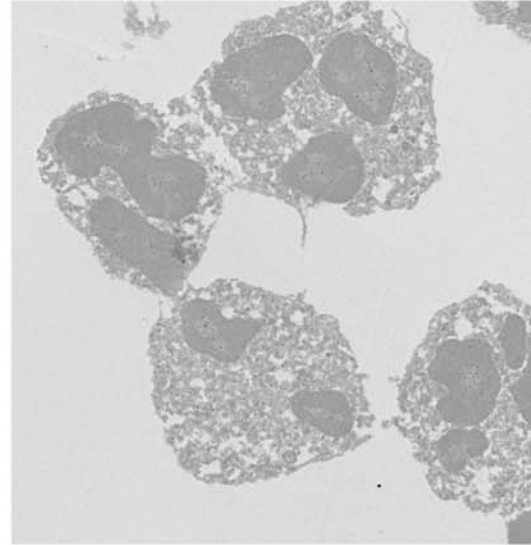
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A

r=0.39,p=0.0002

DNA-elastase complexes (Log^{10} units/ml)

Log^{10} Sputum PZP µg/ml

r=0.51,p<0.0001

Histone elastase complex (log^{10} units/ml)

Log^{10} Sputum PZP µg/ml

C

D

B

Merge

PZP

Neutrophil elastase

DAPI

100 µm

Figure 7. A) PZP levels in sputum in patients with bronchiectasis N=124 or COPD N=40 B) Neutrophil extracellular traps measured using the histone-elastase complex assay in bronchiectasis and COPD. For both assays comparisons are by t-test.

241x86mm (300 x 300 DPI)

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ONLINE SUPPLEMENT FOR:

Pregnancy Zone Protein is Associated with Airway Infection, Neutrophil Extracellular Trap Formation and Disease Severity in Bronchiectasis

Simon Finch, Amelia Shoemark, Alison J Dicker, Holly R Keir, Alexandria Smith, Samantha Ong, Brandon Tan, Jean-Yu Choi, Thomas C Fardon, Diane Cassidy, Jeffrey TJ Huang, James D Chalmers

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jchalmers@dundee.ac.uk

Running head: (50 characters) PZP: a biomarker of bronchiectasis severity

Subject category: 10.16: Non-cystic fibrosis bronchiectasis

Key-words: Neutrophils; bronchiectasis; exacerbations; microbiome, biomarker.

Word count of the manuscript: 3511 (3500)

Supplementary results

	Pseudomonas, n=9	Non-Pseudomonas, n=11
Age (mean)	63	66.91
Sex	66.6% female (n=6)	54.5% female (n=6)
SGRQ score	57.8	45.25
Previous hospitalisations (mean)	0.89	0.27
Exacerbations since last visit (mean)	4.78	1.91
MRC dyspnoea score	3.11	1.91

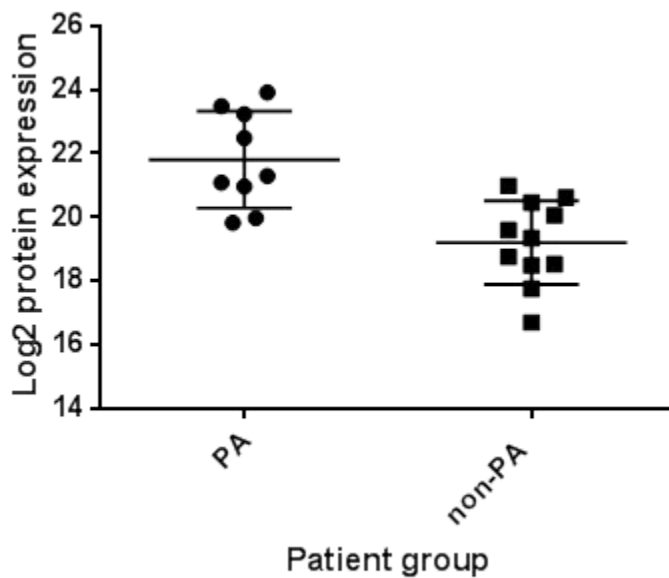Table S1. Characteristics of the patients in original proteomics screening study.

Figure S1 Comparison of Pseudomonas and Non-Pseudomonas groups in original proteomics screen (p=0.007)

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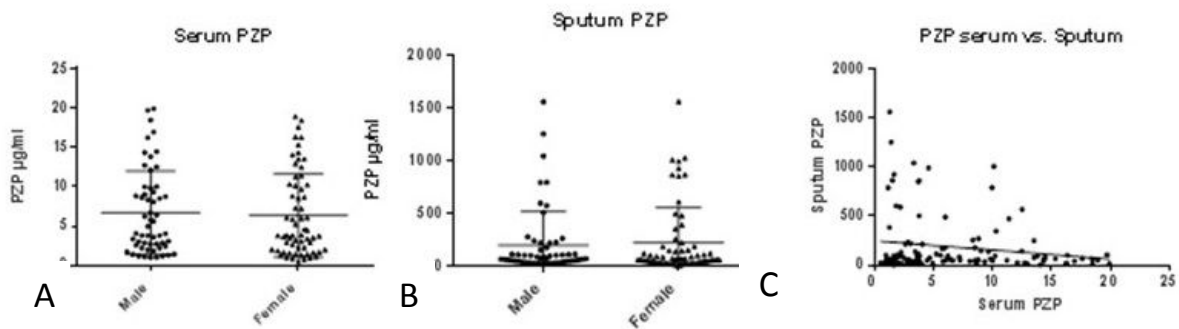
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Figure S2 - A, Serum PZP in male and female patients (error bars show mean with SEM); B Sputum PZP in Male and Female patients (error bars show Mean and SEM); C, Correlation between serum and sputum PZP (p=0.07).
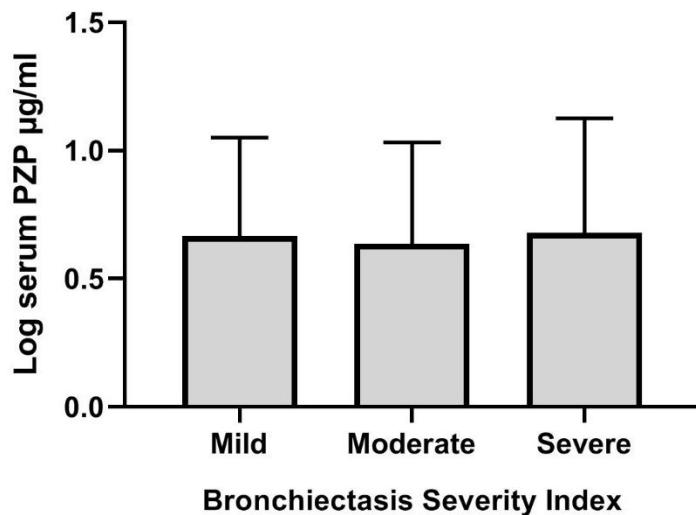

Figure S3 . Serum pregnancy zone protein and the bronchiectasis severity index. No relationship was observed between PZP and BSI (p=0.15 by ANOVA).

Variable	N (%) or mean (SD)
N	20
Mean Age	62 (12.5)
Sex	60% female
Mean FEV1 (% predicted)	60.77% (21.38)
Mean BSI	11.95 (4.53)

Organism cultured	
P. aeruginosa	8 (40%)
H. influenzae	4 (20%)
E. coli	1 (5%)
M. catarrhalis	1 (5%)
H. parainfluenzae	1 (5%)
S. maltophilia	1 (5%)
Achromobacter	1 (5%)
Enterobacter	1(5%)
Mixed flora	2(10%)

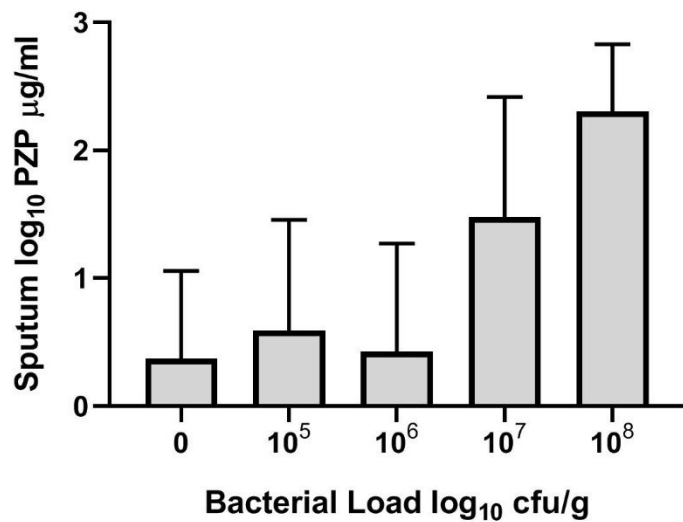Table S2. Characteristics of the bacterial load study participants

Figure S4. The relationship between bacterial load and sputum PZP excluding patients with *P. aeruginosa* infection. The difference across the groups is statistically significant by ANOVA $p < 0.0001$.

AJRCCM Articles in Press. Published on 02-July-2019 as 10.1164/rccm.201812-2351OC
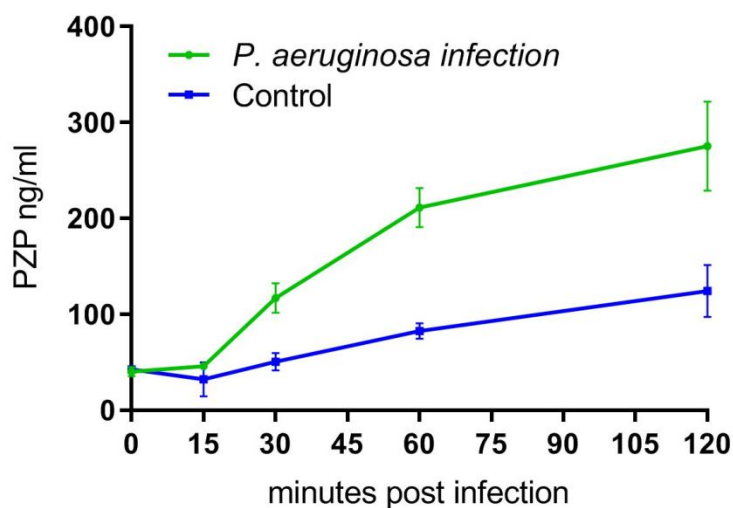
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Figure S5. Release of PZP following *P. aeruginosa* infection. 10(6) neutrophils per time point were infected at a multiplicity of infection of 1:10. Following centrifugation to remove bacteria and neutrophils, PZP was measured in supernatant by ELISA. Results are the mean of 3 biological replicates.
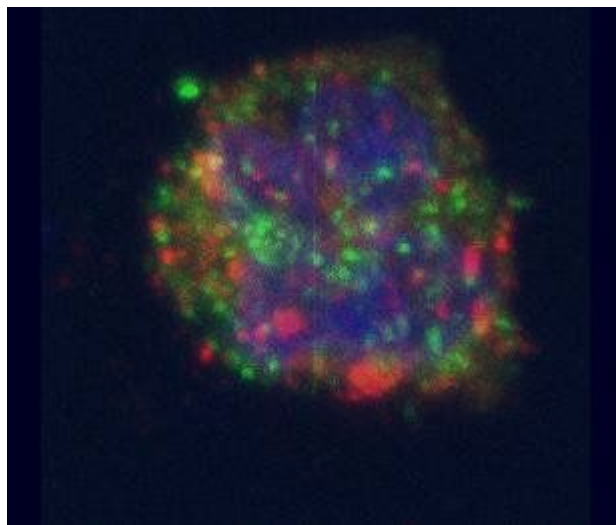

Figure S6 Microscopy image of neutrophils showing DNA: DAPI, Blue, MMP-9: Green and Pregnancy zone protein: Red.
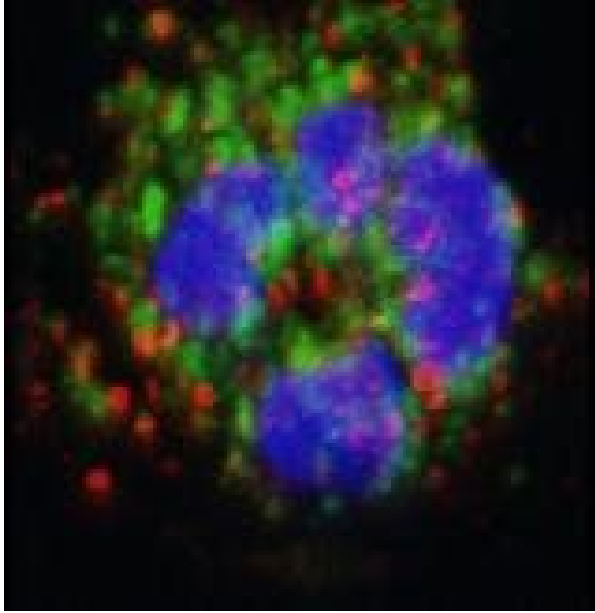
Figure S7 Microscopy image of neutrophils showing DNA: DAPI, Blue, MPO: Green and Pregnancy zone protein: Red.

Video S1 – 3D video of neutrophils stained for PZP and MMP9

Video S2 – 3D video of neutrophils stained for PZP and MPO

N=40	
Mean Age (SD)	72.5 (8.72)
Sex	66.67% male
Mean FEV1 (% predicted)(SD)	68.78% (20)

Table S3. COPD study patient characteristics

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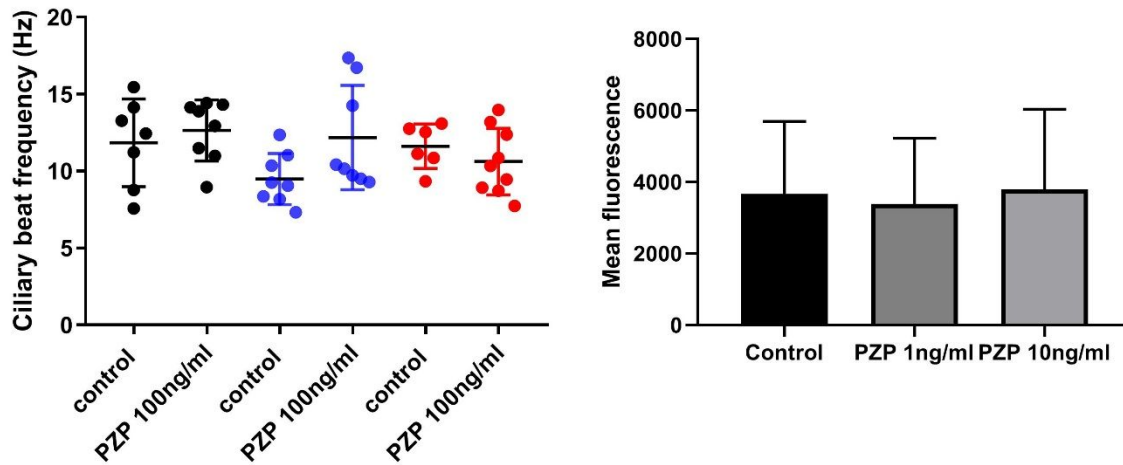
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Figure S8. No effect of pregnancy zone protein at physiological concentrations on ciliary beat frequency (n=3 replicates from different donors, n=8 observations per donor). No significant differences were observed. Right shows no statistically significant difference in neutrophil phagocytosis after exposure to physiological doses of pregnancy zone protein (N=3 biological replicates from different donors, p=0.11).

Supplementary methods

Patients and clinical assessments

Patients were recruited at a specialist Bronchiectasis clinic at Ninewells Hospital,

Dundee, UK. Inclusion criteria were: age $\geq$ 18 years, bronchiectasis confirmed by

high resolution CT scan, chronic expectoration with ability to provide a sputum

sample at the study visit and providing written informed consent. Exclusion

criteria were: bronchiectasis due to cystic fibrosis, active allergic

bronchopulmonary aspergillosis, active non-tuberculous mycobacterial infection,

chronic use of oral corticosteroids and inability to provide informed consent.

Ethical approval for the study was given by the East of Scotland Research Ethics

Committee, approval number 12/ES/0059.

Patients underwent clinical evaluation including microbiology (described below),

assessment of severity of disease using the bronchiectasis severity index, as

previously described(13), and recording of exacerbation frequency. Exacerbations

were defined as administration of antibiotics for increasing respiratory symptoms

as defined by the British Thoracic Society(14). Sputum was obtained from all

subjects during a period of clinical stability and split into whole (unprocessed)

sputum for microbiology and sputum which was diluted 1:8 with PBS and then

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centrifuged at 3000g for 15 minutes at 4°C . All sputum processing took place

within 2 hours of expectoration and freeze thaw cycles were avoided. The

supernatant was stored at -80°C until analysis.

Sputum protein profiling

Protein concentrations of sputum supernatants were quantified using Pierce 660

protein assay. Fifty micrograms of sputum protein from each sample was added

to an equal volume of acetonitrile before incubating at 100°C for 15 min. The

samples were dried down in a centrifugal vacuum and resuspended with 50mM

ammonium bicarbonate (pH8.5) to a final concentration of 1 mg/ml. Samples

were then reduced and alkylated before subjecting to nano-flow-LC-MS/MS

analysis according to the previous report(15).

Protein identification and label-free quantification were carried out using

Maxquant (version 1.4.1.2) against Uniprot-human database (version 2014-07-

09). The fixed modification was carbamidomethylation on cysteine, and variable

modifications include oxidation on methionine and N-terminal acetylation. FDR

for protein identification was set to 1% at protein level. Data visualisation was

carried out using SIMCA P (version 13.0.3). For statistical analysis, the dataset was

log2 transformed before subjecting to t test using Perseus (version 1.5.4.1). The Benjamini-Hochberg false discovery rate method was used and corrected p values of p<0.05 is considered significant.

Pregnancy zone protein ELISA

PZP was measured using a commercial ELISA kit (Cloud-Clone Corp., SEG324Hu and SEG324Mu). Validation was performed according to published recommendations(16). The optimal dilutions were 1:100,000 for sputum and 1:500 for serum and 1:5 for murine Broncho-alveolar lavage (BAL) samples.

All sputum samples used in ELISA were processed within 2 hours of expectoration. We confirmed stability of PZP in sputum by incubated sputum at room temperature for 48 hours with samples taken at 4, 24 and 48 hours as shown below (Figure S9)

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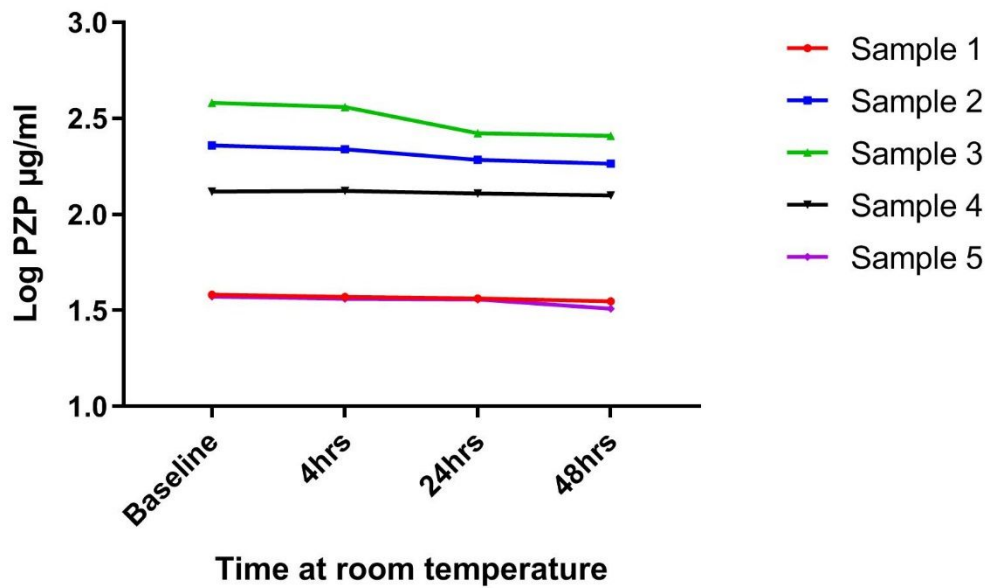
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Figure S9. Stability of PZP in sputum over 48 hours. Samples were incubated in the laboratory at room temperature and aliquots processed for measurement of PZP (as described in the methods) at the indicated time points. No significant differences were observed comparing PZP at baseline and after 4 hours at room temperature (p=0.8)

Quantification of sputum NETs

Measurement of histone-elastase and DNA-elastase complexes provide a semi-quantitative assessment of neutrophil extracellular traps in sputum and assays were performed as previously described(17).

Leukocyte studies in healthy volunteers

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Neutrophils and peripheral blood monocytes were isolated from healthy

volunteers using Percoll Gradient Density Centrifugation as previously

described(18). Neutrophils and monocytes were stimulated with Phorbol

myristate acetate (PMA, 0.1-100µg/ml), N-formylmethionine-leucyl-phenylalanine

(fMLP, 0.1-100µg/ml) and bacteria (*Eschericiacoli* strain BL21 and *P. aeruginosa*

strain PA01, at multiplicity of infection ranging from 10:1 to 1:100. Following

stimulation neutrophils were centrifuged at 1200g for 5 mins. PZP was measured

in the resulting supernatant.

Immunofluorescence was used to confirm and localize PZP within neutrophils and

NETs and identify co-localisation with other neutrophil proteins. Cells were

seeded to glass coverslips ($2*10^5$ cells per slip) in 500µl RPMI (containing 2%

human serum), for 1 hour. NET formation was induced by treatment for 4 hours

with 600nM PMA.

Samples were fixed in 4% paraformaldehyde for 1 hour. Cells were permeabilized

in 0.5% Triton X-100 for 1min before blocking with 5% BSA. Cells were labelled

with primary antibodies, mouse monoclonal antibodies to PZP (Sigma-Aldrich

HPA041471) and a rabbit polyclonal antibody to one of the following: neutrophil

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elastase(R&D MAB91671), matrix metallopeptidase 9 (MMP9) (Thermo-Fisher

MA5-14228), lactoferrin (Thermo-Fisher MA5-18107) or myeloperoxidase (MPO)

(Thermo-Fisher MA1-80878) followed by appropriate secondary antibodies (goat

anti mouse Alexafluor 488 and goat anti rabbit Alexafluor 594). Samples were

stained with DAPI, mounted on clean glass slides and observed by confocal

microscopy (Leica TCS SP5). Co-localisation of PZP with neutrophil granule

proteins was quantified using Manders overlap coefficient which calculates the

proportion of overlap of each channel with the other, with a value of 1 indicating

perfect colocalization and 0 indicating no colocalization.

Electron Microscopy

Granulocytes isolated by Percoll gradient were fixed in 4% paraformaldehyde,

suspended in an agar pellet and stored in 30% wt/vol sucrose until use. Cell

pellets were frozen in cryomoulds using OCT(Tissue-Tek). 10μm cryosections were

cut, blocked with BSA and labelled with anti-PZP antibody (Sigma-Aldrich

HPA041471). To visualise the location of PZP by electron microscopy cells were

labelled with a nanogold secondary (rabbit anti goat 1:200, EMS) followed by gold

enhancement for 20mins (Universal Biologics 2114)

Labelled sections were fixed in 2.5% glutaraldehyde, post fixed in osmium

tetroxide, dehydrated using a gradient of ethanols (70%, 90% and 100%) and

embedded in araldite. 70nm sections were cut using an ultramicrotome (ultracut

E, Leica) and stained post embedding using heavy metals (Uranyl acetate and lead

citrate). Cells were visualised on a transmission electron microscope (Jeol 1400+)

using a digital 4kX4k camera (AMT 16X, Deben Ltd, UK), appropriate negative

controls were conducted.

Murine model of acute inflammation

In-vivo procedures were conducted according to the requirements of the United

Kingdom Home Office Animals Scientific Procedures Act, 1986 and approved by the

University of Dundee ethical review committee. Female 10-12 week old C57/B6

mice. *S. aureus* strain RN6390 was subcultured at 1:100 dilution from an overnight

culture into fresh TSB medium. Cells were grown at 37°C with shaking until an OD_{600}

of 0.5 was reached, before harvesting and washing three times in 1x PBS. Cells were

finally resuspended in 1 x PBS. Mice were anaesthetized and infected intranasally

with 25 μl of the bacterial suspension at an infecting dose of 3 x 10^8 cfu. At 24 hours

post infection the trachea was carefully dissected, intubated and bronchoalveolar

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lavage performed with 3 x 0.4ml PBS. BAL was centrifuged at 1300 rpm for minutes

at 4°C. Supernatant was stored at -80°C and the cell pellet resuspended in 110μl

media containing serum. 10μl cells were mixed with 10μl trypan blue and counted

on a haemocytometer. Cytospins were prepared and stained for differential cell

counts using Wright-Giemsa staining kit. Murine PZP was measured using ELISA.

Sputum bacteriology

Quantitative bacterial culture was performed on sputum samples within 4 hours

of expectoration. Sputum was homogenized in 50:50 volume 0.1% dithiothreitol

and serially diluted in sterile 0.9% sodium chloride for plating on blood agar and

choclate with bacitracin agar. Plates were inoculated with 100ul of diluted sample

and colony forming units of each identified pathogen counted after 48 hours

incubation. Bacterial density is expression as log10 cfu/g.

Microbiota sequencing

The AllPrep DNA/RNA Mini kit was used to extract DNA and RNA from whole

sputum: Sputum was incubated in an equal volume of 1:10 diluted Sputolysin

(Calbiochem) for 30mins at 37°C, mixed with Buffer RLT as per the AllPrep kit protocol, then passed through QIAshredder columns (QIAGEN) with the supernatant undergoing sequential DNA and RNA extraction. extraction on the QIAcube automation platform. Alternatively, 0.1g whole sputum was incubated with Proteinase K for one hour at 55°C then processed as described in Zymo Quick-DNA Miniprep Plus solid tissues protocol, eluting into 50µL elution buffer. We assessed quality and quantity of the DNA and RNA 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.

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, 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

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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 , aligned against

the Greengenes Core reference alignment (Version 13.8) using PyNAST (Version

1.2.2). 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 examined to remove singletons and unassigned OTUs, or OTUs identified as

Eukaryota, Human and Cyanobacteria. The data was normalised to the lowest

number of OTUs and the Shannon-Wiener Species Diversity Index (SWDI) of the

samples determined.

Analysis was performed in QIIME. To compare groups patients were split into

those with predominant (>50% OTUs) proteobacteria and those with

predominant firmicutes at the phylum level as previously described.

Antibiotic response study

Patients were asked to attend the research centre if they developed symptoms of

an exacerbation. Patients were reviewed by a physician and those who were

prescribed antibiotics for a protocol defined exacerbation were included in the

study. Patients received treatment for 14 days based on their previous sputum microbiology. Spontaneous sputum samples obtained as baseline and after 14 days were used for PZP measurement.

COPD cohort study

To compare sputum PZP levels in bronchiectasis to those from patients with COPD, 40 patients with COPD without underlying bronchiectasis were enrolled while clinically stable (4 weeks free from antibiotic or corticosteroid therapy). Spontaneous sputum samples were obtained and processed in the same way as the bronchiectasis samples with sputum PZP and sputum NETs measured by ELISA.

Neutrophil phagocytosis assay

Was evaluated by flow cytometry on a BD Fortessa. FITC-labelled *P. aeruginosa* strain PA01 were opsonised with 10% serum and incubated with neutrophils (multiplicity of infection 10:1) which had been treated with recombinant PZP at the indicated concentrations or vehicle control. Phagocytosis was quantified by the mean fluorescence after gating on the neutrophil population.

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Ciliary beat frequency measurement

Primary nasal epithelial cells from healthy donors were obtained by brushing of the inferior nasal turbinate followed by culture at air liquid interface. Ciliary beating analysis and quantification was performed by high-speed videomicroscopy.