Rhinovirus infection induces degradation of antimicrobial peptides and secondary bacterial infection in COPD

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Authors’ Contributions

SLJ, PM, JF, PJB, and IMA designed the study.


PM, JF, and SLJ analysed the data.

PM, JF, AP, and SLJ contributed to the writing of the paper.

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At a glance commentary

Scientific knowledge on the subject

Secondary bacterial infections are reported with influenza infection but it is not known whether bacterial infection is associated with other respiratory viruses such as rhinoviruses that are the most common viral cause of COPD exacerbations.

What this study adds to the field

We report that experimental rhinovirus infection is followed by secondary bacterial infections in COPD subjects but not in smokers or non-smokers without COPD. Bacterial infections were associated with reduced levels of antimicrobial peptides suggesting that rhinovirus infection leads to impaired innate immune responses that predispose to bacterial infection.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org
Abstract

**Rationale:** COPD exacerbations are associated with both virus (mostly rhinovirus) and bacterial infections but it is not known whether rhinovirus infections precipitate secondary bacterial infections.

**Objectives:** To investigate relationships between rhinovirus infection and bacterial infection and the role of antimicrobial peptides in COPD exacerbations.

**Methods:** We infected subjects with moderate COPD and smokers and non-smokers with normal lung function with rhinovirus. Induced sputum was collected before and repeatedly following rhinovirus infection and virus and bacterial loads measured with quantitative PCR and culture. The antimicrobial peptides secretory leukoprotease inhibitor (SLPI), elafin, pentraxin, LL-37, α-defensins and β-defensin-2, and the protease neutrophil elastase were measured in sputum supernatants.

**Measurements and main results:** Following rhinovirus infection, secondary bacterial infection was detected in 60% of COPD subjects, 9.5% of smokers and 10% of non-smokers (P<0.001). Sputum virus load peaked on days 5-9 and bacterial load on day 15. Sputum neutrophil elastase was significantly increased and SLPI and elafin significantly reduced following rhinovirus infection exclusively in COPD subjects with secondary bacterial infections, and SLPI and elafin levels correlated inversely with bacterial load.

**Conclusions:** Rhinovirus infections are frequently followed by secondary bacterial infections in COPD and cleavage of the antimicrobial peptides SLPI and elafin by virus-induced neutrophil elastase may precipitate these secondary bacterial infections. Therapy with these anti-microbial peptides, or with neutrophil elastase inhibitors may be useful novel therapies for prevention of secondary bacterial infections in virus-induced COPD exacerbations.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a growing global epidemic and its prevalence is expected to increase markedly (1). Acute exacerbations are the major cause of morbidity and mortality in COPD and are associated with impaired quality of life (2), accelerated loss of lung function (3) and enormous healthcare costs(4). Respiratory infections cause most exacerbations (5, 6) with the relative contributions of viruses and bacteria still debated (7), and it is not known whether bacterial infections in COPD exacerbations occur de novo or secondary to an initial virus infection. Dual virus/bacterial infection has only been reported in a minority of COPD exacerbations (5, 6, 8, 9), consistent with the hypothesis that one may follow the other and therefore detection is frequently separated in time rather than simultaneous. The majority of viruses detected are rhinoviruses. Rhinovirus infection increases bacterial adherence to respiratory epithelial cells (10, 11), and impairs macrophage responses to bacterial stimuli (12) in vitro. A temporal association between rhinovirus infection and invasive pneumococcal disease in children has been reported (13). These data suggest that rhinovirus infections may precipitate secondary bacterial infection but no in vivo data investigating this hypothesis exist. We have developed a unique human model of COPD exacerbation using experimental rhinovirus infection that induces the clinical features of an exacerbation and permits intensive repeated sampling of the lower airways (14, 15). The aims of this study were to determine whether rhinovirus infection can precipitate secondary bacterial infection in vivo and to investigate temporal relationships between viral and bacterial infection. As there is known to be a lower respiratory microbiome in both health and disease (16, 17) and as antimicrobial peptides are known to be important in anti-bacterial host defence (18), we also hypothesised that secondary bacterial infections may be due to perturbations in this host defence mechanism as a consequence of rhinovirus infection. We therefore assessed levels of the major antimicrobial peptides (pentraxin 3, LL-37, alpha and beta defensins, SLPI
and elafin) in sputum before and during rhinovirus infections to determine whether levels were altered from baseline and how these may relate to secondary bacterial infections. Some of the results of this study have been previously reported in abstract form (19).
Methods

Study subjects

Ethical approval was obtained from UK Research Ethics Committees (study numbers 00/BA/459E, 07/H0712/138 and 11/LO/0400) and informed consent obtained from all subjects. The participants were recruited for two studies, the first included 13 subjects with COPD and 13 smokers without airway obstruction and initial findings relating to virus infection and clinical outcomes have been reported (15). The second study (so far unreported) included 18 COPD subjects and 15 smokers with identical inclusion criteria as the first study, and an additional control group of 19 non-smokers. Further details regarding the study participants and inclusion criteria are in the online data supplement.

Study protocol

Subjects underwent clinical assessment including symptom diaries, lung function and sputum induction prior to experimental infection with human rhinovirus 16, performed on day 0 as previously reported (15). Subjects kept daily diary cards of symptoms and sputum sampling was repeated on days 5, 9, 12, 15, 21, and 42 after virus inoculation. The protocols for the two studies were identical other than 3 time points for sputum sampling (days 3, 28, and 35 post-inoculation) that were discordant between the two studies, so these were not included in the present analysis. The study timeline is outlined in Table E2 in the online data supplement.

Clinical procedures

Virus inoculation and detection

Details regarding the preparation and safety testing of the rhinovirus 16 inoculum have been published (20). 10 tissue culture infective doses 50% of the virus were diluted in a total volume of 1mL of 0.9% saline and inoculated in both nostrils using an atomizer (No. 286;
DeVilbiss Co., Heston UK). Rhinovirus infection was confirmed with a combination of virus culture, serology and polymerase chain reaction (PCR) according to previously established protocols (21). The criteria for successful infection are provided in the online data supplement. Infection with viruses other than rhinovirus was excluded by testing nasal lavage samples at baseline and at the peak of upper respiratory symptoms with PCR (details in the online data supplement).

**Sputum induction**

Sputum was induced by inhalations of hypertonic saline according to European Respiratory Society guidelines (22) and processed according to standard protocols (15). Details are provided in the online data supplement.

**Bacterial culture**

Quantitative bacterial culture was performed on induced sputum samples on blood agar, chocolate agar, CLED agar and Sabouraud agar in the Microbiology Laboratory at Imperial College Healthcare NHS Trust(5). Bacterial infection was defined as a colony count of $\geq 10^5\, \text{cfu/mL}$ of a potentially pathogenic microorganism (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Haemophilus parainfluenzae*). However all the sputum cultures were reviewed and there were no potentially pathogenic organisms detected at a load lower than $10^6\, \text{cfu/mL}$. The total bacterial load for each subject was defined as the sum of $\log_{10} \text{cfu/mL}$ counts of all individual bacterial positive cultures detected for that subject.
Antimicrobial peptides and inflammatory mediators

The antimicrobial peptides secretory leukoprotease inhibitor (SLPI), elafin, pentraxin 3, human beta defensin-2 (HBD-2), α-defensins, and the cathelicidin LL-37 and the protease neutrophil elastase were measured in sputum supernatants using commercially available enzyme-linked immunosorbent assays (ELISA) – details are provided in the online data supplement.

Statistical analysis

Data are presented as mean (±SEM) values for normally distributed data or median (interquartile range) for non-parametric data. Changes from baseline were analysed with repeated measures ANOVA (Friedman test for non-parametric data) and, if significant, paired t-tests or Wilcoxon matched pairs test. Differences between groups were analyzed using unpaired t-tests or Mann-Whitney tests. Correlations between data sets were examined using Pearson’s correlation or Spearman’s rank correlation coefficient. Differences were considered significant for all statistical tests at P values of less than 0.05. All reported P values are two-sided. Analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA).
**Results**

**Frequencies of successful rhinovirus infection**

31 COPD subjects, 28 smokers and 19 non-smokers were inoculated with low dose rhinovirus 16 and 77/78 subjects completed the study through to day 42 – one COPD subject withdrew due to ill health believed unconnected to the study. Rhinovirus infection was confirmed in 20/30 COPD subjects (66.7%), 22/28 smokers (78.6%) and 11/19 non-smokers (58%). There were no significant differences in frequencies of successful virus infection between groups (P=0.53).

**Bacterial infection during experimental rhinovirus infection**

Of the remaining 77 subjects, one smoker had a positive bacterial culture in the baseline sputum sample and one non smoker was unable to provide sputum samples - these subjects were excluded from further analyses. The clinical characteristics of the rhinovirus-infected subjects included in the final analysis are shown in Table 1. Following successful rhinovirus infection a positive bacterial culture was detected in 12/20 (60%) of the COPD subjects, 2/21 (9.5%) of the smokers and 1/10 (10%) of the non-smokers (P<0.001). The time course of bacterial infection is shown in Figure 1A. Only COPD subjects had significant increases in sputum bacterial load between baseline and post-virus-infection samples (on days 9, 12, and 15, P<0.05 in each case) and bacterial load in COPD subjects was significantly greater than both smoking and non-smoking groups on day 9 (P<0.05) and than the non-smoking group on day 12 (P<0.05). The individual bacterial species detected are listed in Table E4 and the bacterial loads in Table E5 in the online data supplement.
**Frequency of bacterial infections in the absence of rhinovirus infection**

A number of subjects were inoculated with rhinovirus and went through the entire study protocol but were subsequently found to have no evidence of successful rhinovirus infection. We investigated bacterial detection in these subjects also to determine whether the increased bacteria detected in the rhinovirus-infected COPD subjects were possibly related to sputum induction and/or bronchoscopy. In the non-rhinovirus infected subjects bacterial infection was detected in 2/10 (20%) COPD subjects, 1/6 (16.7%) smoking controls and 1/8 (12.5%) non-smoking controls. Only in the COPD group was the incidence of bacterial infection significantly higher in rhinovirus-infected subjects compared to non-infected subjects (60% vs. 20%, *P*=0.038, Table E6 in the online data supplement). The bacterial loads in the non-rhinovirus infected subjects are depicted in Figure 1B – there were no significant increases in bacterial load above baseline at any time point.

**Temporal and severity relationships between rhinovirus infection and bacterial infection**

There was no clear pattern in the temporal distribution of bacterial detections in the rhinovirus-infected smokers and non-smokers (Figure 1A), nor in the non-rhinovirus infected subjects (Figure 1B). In the rhinovirus-infected COPD subjects bacterial infection first appeared on day 9 and bacterial load peaked on day 15 (Figure 1A), in contrast sputum virus load was maximal on days 5 and 9 (Figure 1C) consistent with bacterial infection occurring secondary to virus infection. Peak rhinovirus loads in sputum were positively correlated with sputum bacterial loads (*r*=0.47, *P*=0.039), providing support for a causal relationship between the two.
**Secondary bacterial infections and underlying COPD severity**

Mean baseline FEV$_1$ and % predicted FEV$_1$ were both significantly lower in the COPD subjects with secondary bacterial infection, compared to those without bacterial infection (1.76±0.12L vs. 2.18±0.10L, P=0.026 and 63.17±1.45% vs. 68.75±2.2%, P=0.04 respectively). There were no significant differences in baseline FEV$_1$/FVC ratio, age, gender or smoking history between bacteria positive and negative COPD subjects (Table E7).

**Bacterial infections and clinical and inflammatory outcomes**

All COPD subjects had similar increases in breathlessness during the viral infection, however breathlessness scores were significantly greater in the bacteria-positive subjects compared to the bacteria-negative subjects on days 20-22 and 25 (Figure 2A). These clinical symptoms were accompanied by physiologic changes, as PEF fell significantly from baseline on days 5–21 (Figure 2B), and FEV$_1$ on days 9 and 21 (data not shown) in the bacteria-positive subjects but there was no significant change in either measure in the bacteria-negative subjects. Similarly, total sputum inflammatory cells (days 9 and 15) and sputum neutrophil elastase (day 9) increased significantly from baseline in the bacteria-positive COPD subjects (Figures 2C and 2D) but not in the bacteria-negative subjects. Supporting a relationship between severity of bacterial infection and pathologic responses, sputum bacterial load correlated significantly with peak inflammatory cell numbers and peak neutrophil numbers in sputum (r=0.75, P=0.0001 and r=0.68, P=0.0014 respectively).

**Antimicrobial peptides in rhinovirus infections**

Following rhinovirus infection sputum levels of pentraxin 3 increased significantly over baseline in the COPD subjects on days 5 – 21 (Figure 3A). In the smoking subject group levels were significantly increased on day 9 and in the non-smokers there was no significant
induction (Figure E1A in the online data supplement). Pentraxin 3 levels were significantly higher in the COPD group compared to the non-smokers on days 5-21 and compared to the smokers on day 15 (Figure 3A). Peak sputum pentraxin 3 levels in the COPD subjects correlated with peak sputum virus load ($r=0.45$, $P=0.046$), peak sputum inflammatory cells ($r=0.63$, $P=0.0029$), peak sputum neutrophils ($r=0.66$, $P=0.0022$) and with sputum bacterial load ($r=0.52$, $P=0.018$).

Sputum LL-37 levels were increased significantly from baseline in the COPD group on day 12 (Figure 3B) but there was no change from baseline in either control group (Figure E1B in the online data supplement). Peak sputum LL-37 levels in COPD subjects correlated with peak sputum virus load ($r=0.53$, $P=0.017$), peak sputum inflammatory cells ($r=0.85$, $P<0.0001$), peak sputum neutrophils ($r=0.85$, $P<0.0001$), sputum bacterial load ($r=0.49$, $P=0.03$) and peak sputum pentraxin 3 levels ($r=0.59$, $P=0.0061$).

Sputum α-defensin increased above baseline in the COPD group on days 9-21 (Figure 3C), but there were no significant changes from baseline in the control groups (Figure E1C in the online data supplement). There were no correlations between α-defensin levels and clinical, virologic or bacterial parameters.

Sputum levels of HBD-2 were very low and there were no significant changes from baseline the COPD group (Figure 3D) or the smoking controls (Figure E1D in the online data supplement). In the non-smokers levels were significantly increased on day 21 compared to baseline (Supplementary Appendix Figure 1D). There were no correlations between HBD-2 levels and clinical, virologic or bacterial parameters.

There were no increases above baseline in SLPI or elafin levels at any time point in the COPD group (Figures 3E and 3F), nor in either control group (Figures E1E and E1F in the online data supplement), and there were no significant differences between the groups.
Increases in neutrophil elastase and reductions in elafin and SLPI following rhinovirus infection were exclusive to COPD subjects with secondary bacterial infections

Since neutrophil elastase has been reported to degrade both SLPI and elafin (23, 24) and strong induction of neutrophil elastase on day 9 was limited to the COPD subjects who developed secondary bacterial infections (Figure 2D), we hypothesized that high levels of rhinovirus-induced neutrophil elastase may degrade these two antimicrobial peptides. Consistent with our hypothesis, in the bacteria-positive COPD subjects sputum elafin levels were significantly reduced compared with baseline levels on day 9 (-68.47ng/mL, P=0.042). In contrast, in the bacteria-negative COPD subjects there was a non-significant increase from baseline in sputum elafin on days 9 and 12 (39.17ng/mL, P=0.28; 159.2ng/mL, P=0.078, Figure 4A). Sputum elafin levels were significantly lower in the bacteria-positive compared to the bacteria-negative COPD subjects on days 9 (-2013ng/mL vs. 775.2ng/mL, P=0.015) and 12 (-46.93 vs. 159.2ng/ml, P=0.023; Figure 4C) and on day 9 correlated inversely with bacterial load (r=-0.71, P=0.004) and peak sputum neutrophils (r=-0.55, P=0.016).

Similar non-significant trends were observed for sputum SLPI (Figure 4B). Sputum SLPI levels were lower in the bacteria-positive compared to the bacteria-negative COPD subjects on day 9 (-1542ng/mL vs. 383.4ng/mL, P=0.07) and on day 12 (-68.47ng/mL vs. 39.17ng/mL, P=0.023; Figure 4D) and on day 12 correlated inversely with bacterial load (r=-0.51, P=0.023). There were no significant differences between bacteria-positive and bacteria-negative COPD subjects in pentraxin 3, LL-37, α-defensin, or HBD-2 levels (data not shown).
Discussion

We have used our human experimental rhinovirus infection model to demonstrate that secondary bacterial infections occur in 60% of COPD subjects following a rhinovirus infection and that this occurs significantly more frequently than in both smoking and non-smoking subjects and in COPD subjects who underwent the same sampling protocol but did not develop rhinovirus infections. We report relationships between virus load and secondary bacterial infection, and that breathlessness, airflow obstruction and airway inflammation were more severe or more prolonged in COPD subjects with secondary bacterial infection. Secondary bacterial infection in COPD subjects was associated with high levels of rhinovirus-induced neutrophil elastase and with reductions the antimicrobial molecules SLPI and elafin.

Respiratory infections are the commonest causes of COPD exacerbations with both viruses and bacteria frequently detected (5, 9), but it is not known whether bacterial infections occur de novo or follow an initial virus infection. Patients frequently report colds prior to exacerbations (6, 25) and in vitro mechanisms linking rhinovirus infections to increased susceptibility to bacterial infection have been reported (10-12). However rates of dual virus/bacterial infection in COPD exacerbations are relatively low (5, 6, 8, 9), consistent with one infection following the other, so that only relatively infrequently are the two detected together. When patients are sampled at the onset of exacerbation and again 5-7 days later 36% of exacerbations in which a virus was detected at onset developed secondary bacterial infection (6), but 71% of bacterial exacerbations had reported symptoms of a viral upper respiratory tract infection prior to onset so the true association may be even higher (6).

Experimental infection studies uniquely allow examination of temporal relationships between viral and bacterial infection in a manner difficult to achieve in naturally-occurring exacerbations. We report that 60% of COPD subjects developed bacterial infection following rhinovirus infection and virus load in sputum peaked on days 5-9 post-inoculation, whereas
bacterial load peaked on day 15. Further evidence providing support for a causal link was the positive correlation between virus and bacterial loads. Therefore these are the first *in vivo* data directly linking rhinovirus infection to secondary bacterial infections in COPD and suggest that studies of naturally-occurring exacerbations have underestimated the rates of dual infection due to virus and bacterial infections occurring at different times (5, 26).

Bacteria cultures should be accompanied by appropriate clinical symptoms and physiologic and pathologic changes to fulfil accepted definitions of infection. We therefore examined the effect of bacterial infection on clinical and inflammatory outcomes and report that the peak of breathlessness, airflow obstruction and airways inflammation occurred on day 9 post-inoculation, coinciding with peak virus load. However in the bacteria-positive subjects symptoms, airflow obstruction and airways inflammation persisted on day 15, when virus load was falling, and day 21 when virus load was undetectable, suggesting that secondary bacterial infections are likely to prolong the duration of initially virus-induced COPD exacerbations.

Following rhinovirus infection sputum levels of neutrophil elastase were higher and levels of the antimicrobial peptides SLPI and elafin lower in bacteria-positive COPD subjects compared to those in whom no bacteria were detected. Low levels of SLPI in bacterial infections in COPD have been reported (27-29), but these studies did not establish whether these were a cause or consequence of infection. We report that neutrophil elastase was elevated on day 9 and SLPI and elafin fell on days 9 and 12, prior to the peak in bacterial load on day 15, and both SLPI and elafin correlated inversely with bacterial load, suggesting that their deficiency increases susceptibility to secondary bacterial infection. SLPI and elafin are cleaved by neutrophil elastase (23, 24) and an inverse relationship between neutrophil elastase and SLPI has been reported in cystic fibrosis (23) and COPD (27). Neutrophil elastase levels were higher in bacteria-positive COPD subjects so low levels of SLPI and elafin may be due
to their cleavage by rhinovirus-induced neutrophil elastase. In contrast, rhinovirus infection in COPD was associated with consistently high sputum levels of pentraxin 3, LL-37 and α-defensins, suggesting that these molecules may have less potent antimicrobial activities than SLPI and elafin. HBD-2 levels in sputum were very low in sputum and increased following rhinovirus infection in the non-smokers only, suggesting that this defensin is unlikely to be important in this context and consistent with a report that smoking suppresses HBD-2 levels in pneumonia (30).

This study has a number of important implications for our understanding of the pathogenesis and aetiology of COPD exacerbations. These data suggest that a substantial proportion of exacerbations attributed to bacterial infection alone may have been preceded and precipitated by viral infection, but the virus is no longer detectable at the time of presentation. Secondary bacterial infections occurred in 60% of subjects with moderate COPD and no bacterial colonisation, and in patients with more severe COPD in whom exacerbations are more frequent (31) and bacterial colonisation more prevalent (32), secondary bacterial infections are likely to be even more frequent and of greater functional significance. Our finding of relationships between secondary bacterial infections and the underlying severity of COPD within our GOLD stage II COPD patients supports this hypothesis.

These findings strengthen the case for trials of antiviral therapies as early interventions at the onset of cold symptoms in COPD subjects, as they raise the prospect that they may not only reduce severity of or prevent virus-induced COPD exacerbations, but they also have potential to prevent secondary bacterial infections. In addition administration of exogenous inhaled SLPI and/or elafin, or inhibitors of neutrophil elastase are now identified as potential novel therapeutic approaches to prevent secondary bacterial infections in COPD exacerbations.

The main limitation of our study is the small numbers of subjects and the limitation of experimental infection to subjects with moderate COPD. However these results are only
achievable in experimental infection studies that, due to the inherent difficulties of such an intensive study design, will always be small. We believe that such studies are a powerful and unique tool to investigate the role of infection in COPD and generate important data that complement equally important information gained from naturally-occurring exacerbations. These data will need replicating in future experimental studies as well as where possible in naturally-occurring viral infections in COPD patients.

In conclusion secondary bacterial infection is common following rhinovirus infection in COPD and is associated with high levels of neutrophil elastase and with reduction in levels of the antimicrobial peptides elafin and SLPI. Treating respiratory virus infections in COPD patients holds promise as a novel therapeutic approach for COPD exacerbations, as do administration of SLPI/elafin or elastase inhibitors.
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Figure Legends

Figure 1. Time course of bacterial load. Panel A. Time course of bacterial load in subjects successfully infected with rhinovirus. Panel B. Time course of bacterial load in all subjects in whom rhinovirus infection was not confirmed but who underwent the same study procedures as the infected subjects. Panel C. Time course of sputum virus load and bacterial load in COPD subjects. All data mean±SEM, *P<0.05 compared to baseline. ***P<0.001 compared to baseline. †P<0.05 compared to NS, #P<0.05 compared to SMK. SMK – smokers, NS – non-smokers.

Figure 2. Clinical and inflammatory parameters in COPD subjects with and without bacterial infection. Panel A. Breathlessness scores. Panel B. Peak expiratory flow. Panel C. Total sputum inflammatory cells. Panel D. Sputum neutrophil elastase levels. All data mean±SEM, *P<0.05. **P<0.01 compared to baseline. †P<0.05, ††P<0.01 compared to bacteria negative COPD subjects.

Figure 3. Levels of antimicrobial peptides in sputum in all subject groups. Panel A. Pentraxin 3. Panel B. LL-37. Panel C α-defensins. Panel D. Secretory leukoprotease inhibitor (SLPI). Panel E. Elafin. Panels A, B and C are mean±SEM, Panels D and E are median±IQR. *P<0.05, **P<0.01 and ***P<0.001 compared to baseline.

Figure 4. Change from baseline of levels of elafin and SLPI in sputum in the COPD subjects with and without bacterial infection. Panel A. Time course of sputum elafin. Panel B. Time course of sputum SLPI. Panel C. Change from baseline of sputum elafin levels on days 9 and 12. Panel D. Change from baseline of sputum SLPI levels on days 9 and 12. All data
median±IQR. *P<0.05 compared to baseline, †P<0.05 compared to subjects without bacterial infection.
| TABLE 1 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Non-smokers (N=10) | Smokers (N=21) | COPD (N=20) | P value COPD vs smokers | P value COPD vs non-smokers |
| Age (years)     | 62.2 (53-71)     | 50.81 (40-66)  | 59.74 (44-72) | P<0.01             | NS              |
| Sex – M:F       | 4:6              | 10:11           | 13:7          | NS                 | NS              |
| Smoking history (pack years) | 0 | 33.86 (20-60) | 44.15 (20-109) | P<0.001          | P<0.001         |
| Current smokers (current/ex) | 0 | 16/5          | 16/4          | NS                | N/A             |
| Chronic bronchitis | N/A         | N/A             | 17/20         | N/A               | N/A             |
| FEV₁ - % predicted mean | 100.3 ± 3.36 | 96.20 ± 3.45 | 68.11 ± 1.58 | P<0.001           | P<0.001         |
| FEV₁ – Litres mean | 2.7 ± 0.18     | 3.26 ±0.16     | 1.93 ± 0.09  | P<0.001           | P<0.01          |
| FEV₁/FVC mean   | 77.98 ± 1.09    | 78.05 ± 1.34   | 58.60 ± 1.87 | P<0.001           | P<0.001         |
| Influenza vaccination | 3/10         | 4/21           | 9/20          | NS                | NS              |
| Antibiotics in the previous year | 2/10       | 2/21           | 7/20          | NS                | NS              |
| Exacerbations in the previous year | N/A | N/A            | 4/20 (20%)   | N/A               | N/A             |
| >2 exacerbations in the previous year | N/A | N/A            | 0             | N/A               | N/A             |
| Hospitalisations in the previous year | 0/10     | 0/21           | 1/20          | NS                | NS              |

Table 1. Clinical characteristics of study subjects included in the final analysis. NS=not significant. Plus-minus values are mean ± SE, values in brackets are ranges. FEV₁ forced expiratory volume in I second, FVC forced vital capacity, N/A not applicable.
Methods

Study design

The study was a non-randomised study of experimental rhinovirus infection in three groups – COPD subjects, smokers without airflow obstruction, and non-smokers also with normal lung function. The study was designed to allow for within group comparisons by comparing post-infection parameters with baseline samples and between group comparisons, and was based on previous experimental infection studies in asthma (1, 2).

The study subjects were recruited for two consecutive studies with the same inclusion criteria (Table E1). The first study ran from 2004-2007 and the second from 2007-2010. Subjects were recruited from a number of sources including newspaper advertisements, primary care, spirometry clinics, smoking cessation clinics, and outpatient hospital clinics. In all 78 subjects were inoculated with rhinovirus (31 COPD subjects, 28 smokers and 19 non-smokers). Only one subject (COPD) did not complete the study following rhinovirus inoculation due to a hospital admission with an acute coronary syndrome believed unrelated to the study. Acute samples were not collected from this subject but he did not develop respiratory symptoms and had no rhinovirus 16 antibodies detected on a serum sample collected six weeks post-inoculation. The timings of the study time points are detailed in Table E2. Bronchoscopy was carried out at baseline and day 7 in all subjects, and on day 42 in the first study only. No results from the samples collected at bronchoscopy were used in this report.

Symptom scores. Symptoms were assessed using diary cards that were completed on a daily basis from screening until six weeks post-inoculation. Upper respiratory symptoms were measured using the Jackson scale assessing eight symptoms – sneezing, headache, malaise,
chilliness, nasal discharge, nasal obstruction, sore throat, and cough – graded 0 (absent) to 3 (severe) (3). The daily cold score was summated from the individual scores and a clinical cold was defined using the Jackson criteria (3). The scoring system for the lower respiratory symptoms of shortness of breath, cough, wheeze, sputum quantity and sputum quality is shown in Table E3 (4). The daily lower respiratory score was summated from the individual scores and a COPD exacerbation was defined as an increase in the lower respiratory score of at least two points over baseline for at least two consecutive days (5, 6). For both upper and lower respiratory daily symptom scores the mean scores on days -6 to 0 were calculated and subtracted from subsequent daily scores to correct for baseline symptoms.

**Induced sputum.** Sputum was induced and processed using standard protocols(7). Briefly subjects were pre-medicated with 200µg salbutamol via metered dose inhaler and large volume spacer and baseline FEV\textsubscript{1} measured. 4% saline was administered with a DeVilbiss UltraNeb99 ultrasonic nebuliser until an adequate sputum sample was obtained. Sputum was processed within 2 hours of induction. Sputum plugs were selected from saliva by macroscopic inspection of the sample. An aliquot was selected and stored unprocessed at –80°C for qRT-PCR for virus load. Another aliquot was selected, weighed and 0.1% Dithiothreitol (DTT) added in the ration 1mL DTT to 1g sputum. This aliquot was vortexed and mixed and transported immediately to the Microbiology Laboratory at Imperial College Healthcare NHS Trust for bacterial analysis. The remaining sample was weighed, DTT added in the ratio 4mL DTT to 1g sputum and the mixture agitated and filtered. The same volume PBS was added, the filtrate centrifuged and the supernatant aliquotted and stored at –80°C. The cell pellet was washed and resuspended and the cells counted to obtain total cell counts and derive total numbers of inflammatory cells per gram of sputum. Cytospins were prepared and stained using Shandon Diffquick kit (Thermo Shandon Ltd, Cheshire, UK), coded and
counted blind to study status to obtain differential cell counts. Cell counts were expressed as a percentage of at least 400 inflammatory cells.

**Confirmation of rhinovirus 16 infection.** Rhinovirus infection was confirmed by at least one of the following: positive nasal lavage, sputum or BAL standard or qPCR for rhinovirus, positive culture of rhinovirus 16, or seroconversion defined as a titre of serum neutralizing antibodies to rhinovirus 16 of at least 1:4 at 6 weeks. Serology was performed at screening and 6 weeks post-infection by microneutralization test for neutralizing antibody to rhinovirus 16 (8). Virus was cultured by adding 250µL of nasal lavage (from the day of peak virus load by qPCR) to semiconfluent HeLa cells that were cultured for up to five passages. Cultured virus was confirmed as rhinovirus 16 by microneutralization assay with rhinovirus 16-specific antisera (ATCC; titre 1:600)(8). RNA was extracted from samples (QIAamp viral RNA minikit; Qiagen Ltd, Crawley, UK) and reverse-transcribed (omniscript RT kit, Qiagen) with random hexamers. Standard rhinovirus PCR (PerkinElmer 9600 GeneAmp) was performed from 2µL of cDNA(9). To differentiate rhinoviruses from other picornaviruses *BglII* enzyme restriction digestion was carried out on the amplicons generated by RT-PCR (10). qPCR was performed on 2µL of cDNA to detect picornavirus in nasal lavage, an unprocessed plug of induced sputum, and unprocessed BAL, using AmplitaqGold DNA polymerase (PE Biosystems ABI Prism 7700) (11). A standard curve was produced by using serially diluted cloned product and results expressed as copies/mL. The sensitivity of this assay was $10^4$ copies/mL. Virus load was measured with a real-time quantitative RT-PCR assay (12).

**PCR for other respiratory viruses.** Infection with viruses other than rhinoviruses was excluded by testing nasal lavage by PCR on random hexamer primed cDNA for *Mycoplasma*
and *Chlamydia pneumoniae*, adenoviruses, respiratory syncytial virus, influenza AH1/AH3/B, parainfluenza 1–3, human metapneumoviruses (HMPV), and coronaviruses 229E and OC43 as described(13), except HMPV which was adapted from (14).

**Enzyme Linked Immunosorbant Assays.** The ELISAs for detection of antimicrobial peptides and neutrophil elastase were carried according to the manufacturers’ instructions. Plates were read on a Spectramax Plus 384 plate reader and the results read using SoftMax Pro software. Initial experiments were carried out to determine whether sample dilution was required. Experiments were carried out to determine the recovery of antimicrobial peptides from sputum. A sputum sample and PBS were spiked with the relevant protein at the same concentration and the levels detected compared. For all the proteins measured the recovery in sputum was >80% that of the PBS sample. The sensitivities and sources of the individual ELISAs were as follows: neutrophil elastase (0.12ng/mL) (Immunodiagnostik, Bensheim, Germany); α-defensins 1-3 (156pg/mL) (Hycult Biotech, Cambridge UK), LL-37 (0.1ng/mL) (Hycult Biotech, Cambridge UK); SLPI (25pg/mL) (R&D Systems, Abingdon UK); elafin (31.5pg/mL) (R&D Systems, Abingdon UK); pentraxin 3 (0.025ng/mL) (R&D Systems, Abingdon UK); HBD-2 (8pg/mL) (PeproTech, London UK).
### Supplementary Tables

**All subjects**
- Age 40-75 years.
- No history of asthma or allergic rhinitis and not atopic on skin testing.
- Absence of a current or previous history of bronchiectasis, carcinoma of the bronchus or other significant respiratory disease (other than COPD).
- Absence of significant systemic disease.
- No COPD exacerbation or respiratory tract infection within the previous eight weeks.
- Serum antibodies to rhinovirus 16 at screening in a titre <1:2.
- No treatment with antibiotics, oral, inhaled or nasal topical steroids, long-acting β-agonists or tiotropium in the previous three months.

**COPD group**
- FEV₁ 50% - 79% predicted normal value and β-agonist reversibility <12%.
- FEV₁/FVC<70%.
- Current or ex-smokers with at least 20 pack years cumulative smoking

**Smokers**
- FEV₁≥80% predicted normal value.
- FEV₁/FVC>70%.
- Current or ex-smokers with at least 20 pack years cumulative smoking

**Non-smokers**
- FEV₁≥80% predicted normal value.
- FEV₁/FVC>70%.
- Non-smokers

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Table E1. Inclusion criteria for study subjects. COPD denotes chronic obstructive pulmonary disease, FEV₁ forced expiratory volume in one second and FVC forced vital capacity
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Study Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>0</td>
<td>5</td>
<td>7</td>
<td>9</td>
<td>12</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Spirometry</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bronchoscopy</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x*</td>
</tr>
<tr>
<td>Induced sputum</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Rhinovirus inoculation</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptom diaries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table E2. Study design, clinical data and sample collection. Diary cards of upper and lower respiratory symptoms were commenced at screening and completed daily throughout the study. Clinical samples were collected at baseline visits prior to inoculation and experimental inoculation with rhinovirus 16 carried out on day 0. After inoculation repeat clinical measurements and sampling were carried out on the time points indicated. *Bronchoscopy was carried out on day 42 in the first study only.
<table>
<thead>
<tr>
<th>SYMPTOM</th>
<th>SCORE</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SHORTNESS OF BREATH</td>
<td>Not breathless</td>
<td>On moderate exertion</td>
<td>On mild exertion</td>
<td>On minimal exertion</td>
<td>At rest</td>
</tr>
<tr>
<td>WHEEZE</td>
<td>No wheeze</td>
<td>On moderate exertion</td>
<td>On mild exertion</td>
<td>On minimal exertion</td>
<td>At rest</td>
</tr>
<tr>
<td>COUGH</td>
<td>No cough</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td>/</td>
</tr>
<tr>
<td>SPUTUM QUANTITY (PER 24 HRS)</td>
<td>None</td>
<td>Minimal (&lt;30mL)</td>
<td>Moderate (30-100mL)</td>
<td>Large (&gt;100mL)</td>
<td>/</td>
</tr>
<tr>
<td>SPUTUM QUALITY</td>
<td>None</td>
<td>Mucoid (clear)</td>
<td>Mucopurulent (yellow)</td>
<td>Purulent (green)</td>
<td>/</td>
</tr>
</tbody>
</table>

Table E3. Scoring system used to quantify lower respiratory symptoms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Non-smokers</th>
<th>Smokers</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Haemophilus parainfluenzae</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table E4. Bacterial species detected in sputum samples. Values are the numbers of sputum samples positive for the organism.
### Table E5. Bacterial loads in rhinovirus-infected subjects. Values refer to number of samples of each organism detected.

<table>
<thead>
<tr>
<th>Bacterial load (cfu/mL)</th>
<th>$10^6$</th>
<th>$10^7$</th>
<th>$10^8$</th>
<th>$10^9$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>2</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong></td>
<td></td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td><strong>Haemophilus parainfluenzae</strong></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table E6. Bacterial infection frequencies in subjects infected and non-infected with rhinovirus.

<table>
<thead>
<tr>
<th></th>
<th>Rhinovirus infected</th>
<th>Rhinovirus non-infected</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-smokers</strong></td>
<td>1/10 (10%)</td>
<td>1/8 (12.5%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Smokers</strong></td>
<td>2/21 (9.5%)</td>
<td>1/6 (16.7%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>COPD</strong></td>
<td>12/20 (60%)</td>
<td>2/10 (20%)</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Table E6. Bacterial infection frequencies in subjects infected and non-infected with rhinovirus.
<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Bacterial status</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (N=12)</td>
<td>Negative (N=8)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>62.67 ± 1.93</td>
<td>55.25 ± 3.39</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/5</td>
<td>6/2</td>
</tr>
<tr>
<td>FEV₁ (litres)</td>
<td>1.76 ± 0.12</td>
<td>2.18 ± 0.10</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>63.17 ± 1.45</td>
<td>68.75 ± 2.2</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>57.83 ± 2.72</td>
<td>59.75 ± 2.46</td>
</tr>
<tr>
<td>Current/ex smoker</td>
<td>10/2</td>
<td>6/2</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>40.42 ± 2.67</td>
<td>49.75 ± 10.83</td>
</tr>
</tbody>
</table>

Table E7. Baseline clinical characteristics of the rhinovirus infected COPD subjects according to bacterial status. Plus-minus values are mean ± SE. FEV₁ forced expiratory volume in one second, FVC forced vital capacity.
Figure E1. Sputum levels of antimicrobial peptides in smokers and non-smokers. Panel A. Sputum pentraxin 3 levels. Panel B. Sputum LL-37 levels. Panel C. Sputum α-defensin levels. Panel D. Sputum HBD-2 levels. Panel E. Sputum secretory leukoprotease inhibitor levels. Panel F. Sputum elafin levels. Panels A, B and C are mean±SEM, Panels D, E and F are median±IQR.*P<0·05 compared to baseline.

Figure E2. Total lower respiratory scores in the bacteria positive and negative COPD subjects.
References


