Experimental rhinovirus infection as a human model of Chronic Obstructive Pulmonary Disease exacerbation

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At a glance commentary

Scientific knowledge on the subject

Respiratory virus infections are associated with COPD exacerbations but a causative relationship has not been established and mechanisms of virus-associated exacerbations are undetermined.

What this study adds to the field

We report that experimental rhinovirus infection in COPD subjects reproduced the clinical, physiologic and inflammatory features of COPD exacerbations and implicates neutrophilic inflammation and impaired interferon production as important mechanisms of virus-induced COPD exacerbations.

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

Abstract

Rationale: Respiratory virus infections are associated with COPD exacerbations but a causative relationship is not proven and mechanisms are poorly understood. Studies of naturally occurring exacerbations are difficult and have yielded little mechanistic insight. We hypothesized that experimental rhinovirus infection in COPD subjects would reproduce the features of naturally occurring COPD exacerbations and is a valid model of COPD exacerbations.

Objectives: To evaluate experimental rhinovirus infection as a model of COPD exacerbation and investigate the mechanisms of virus-induced exacerbations

Methods: We used experimental rhinovirus infection in 13 subjects with COPD and 13 non-obstructed controls to investigate clinical, physiologic pathologic and anti-viral responses and relationships between virus load and these outcomes.

Measurements and Main Results: Clinical data, inflammatory mediators in blood, sputum and bronchoalveolar lavage and viral load in nasal lavage, sputum and bronchoalveolar lavage were measured at baseline and following infection with rhinovirus 16. Following rhinovirus infection COPD subjects developed lower respiratory symptoms, airflow obstruction and systemic and airways inflammation that were greater and more prolonged compared to the control group. Neutrophil markers in sputum related to clinical outcomes and virus load correlated with inflammatory markers. Virus load was higher and interferon production by bronchoalveolar lavage cells impaired in the COPD subjects.

Conclusions: We have developed a new model of COPD exacerbation that strongly supports a causal relationship between rhinovirus infection and COPD exacerbations.

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Impaired interferon production and neutrophilic inflammation may be important mechanisms 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 in the future (1). The major cause of morbidity and mortality in COPD are acute exacerbations that are associated with impaired quality of life (2;3), accelerated loss of lung function (4) and enormous health care costs (5). Current therapies are only partially effective, have significant side effects and may not address the true underlying mechanisms (6;7). Exacerbations are associated with increased airways inflammation; however studies of cellular and molecular inflammatory mediators in COPD exacerbations have yielded conflicting results (8-13). This is due to factors such as heterogeneous exacerbation aetiologies, variations in time from onset to investigation, effects of treatment and difficulties with both baseline and acute sampling that are inherent in studies of naturally occurring exacerbations. Respiratory virus infections, most commonly rhinoviruses, can be detected in COPD exacerbations but detection rates vary (11;13-16) and viruses are found in stable COPD also (14;17). Therefore a causal relationship between respiratory virus infection and acute exacerbations in COPD patients has not been established. In healthy individuals rhinovirus infections cause a predominantly upper respiratory tract illness with little evidence of lower respiratory tract involvement (18;19). In COPD the association of rhinovirus infection with exacerbations is suggestive of increased susceptibility to infection and lower respiratory tract involvement, however this has not been proven and mechanisms involved are unknown.

Experimental rhinovirus infection studies in asthma have yielded important insights into disease mechanisms, and have identified candidates for development of new therapies (19-21). No similar experimental model has existed for studying

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mechanisms of COPD exacerbations. Such a model could overcome the sources of variation in naturally occurring exacerbations and provide a tool with which to investigate the relationship between virus infection and COPD exacerbations. We previously reported an uncontrolled pilot study investigating experimental infection with low dose rhinovirus in 4 COPD patients; infection induced symptoms consistent with COPD exacerbation and no adverse safety signal was observed (22). In the present study we aimed to demonstrate that this model is valid in larger numbers of COPD subjects and perform intensive lower airway sampling to investigate airways inflammation in virus-induced COPD exacerbations. In addition we also infected a group of smokers without airflow obstruction to investigate mechanisms of susceptibility to virus infection in COPD. Here we report that rhinovirus infection induces the symptomatic, physiologic and inflammatory features that have been reported in naturally occurring exacerbations, and that infection is associated with neutrophilic inflammation and deficient production of interferon-β in COPD subjects. Some of the results of this study have been previously reported in abstract form (23-25)

Methods

Participants

Subjects were recruited from Imperial College Healthcare NHS Trust (St Mary's Hospital), the Royal Brompton Hospital, local General Practices and by advertisement. Ethical approval was obtained from the Local Research Ethics Committee (study number 00/BA/459E) and informed consent obtained from all subjects. The inclusion/exclusion criteria for the COPD subjects were identical to

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those used in our pilot study (22) and a group of control subjects with a similar smoking history but with normal lung function was also recruited (Supplementary Table 1).

Study design and experimental infection protocol

We aimed to recruit and infect 13 subjects in each group as such numbers had yielded significant findings in similar studies in asthma (19;20;26). At initial screening visits suitability for the study was assessed, informed consent obtained and serum neutralising antibodies to rhinovirus 16 measured (19). For those entering the study baseline clinical sampling was performed 1–4 weeks prior to virus inoculation, which was on study day 0. Subjects were seen daily on the 9 days immediately post-inoculation, on days 12 and 15 and then weekly until 6 weeks post-inoculation. The timeline for clinical measurements and sampling is outlined in Supplementary Table 2.

Clinical procedures

Upper and lower respiratory symptom scores

Daily diary cards of upper and lower respiratory symptoms were commenced at screening and continued until 6 weeks post-inoculation. The symptom scores and cold and exacerbation criteria were the same as those used in the pilot study and are described in the Supplementary Methods and Supplementary Table 3.

Pulmonary function

Spirometry was performed on a Micromedical MicroLab spirometer (MicroMedical, Rochester, UK) according to British Thoracic Society guidelines (27) before and 15 minutes after administration of 200µg salbutamol via metered dose inhaler and large

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volume spacer for pre- and post-bronchodilator values. Carbon monoxide diffusion capacity corrected for alveolar volume (K_{CO}) was measured on a Vmax 229 (Viasys) in the pulmonary function laboratory of St Mary's Hospital, Imperial College Healthcare NHS Trust according to established protocols.

Virus inoculation

Details regarding the preparation and safety testing of the rhinovirus 16 inoculum used in this study have been published (28). 10 $TCID_{50}$ of the virus was diluted in a total volume of 1 mL of 0.9% saline and inoculated in both nostrils using an atomizer (No. 286; DeVilbiss Co., Heston UK).

Nasal lavage, induced sputum and bronchoscopy

Sputum was induced by inhalations of hypertonic saline according to European Respiratory Society guidelines (29) and processed according to standard protocols (30). Bronchoscopies were performed according to British Thoracic Society guidelines for research bronchoscopies (31). Details of nasal lavage, sputum and bronchoalveolar lavage (BAL) processing are provided in the Supplementary Material.

Virus detection

Detection of rhinovirus and other respiratory viruses was performed using polymerase change reactions (PCR) according to previously established protocols (19). Infection with viruses other than rhinoviruses was excluded by testing nasal lavage with PCR. Details are provided in the Supplementary Methods.

Inflammatory mediators

Interleukin (IL)-6, IL-8, neutrophil elastase and tumour necrosis factor-alpha (TNF)-α were measured in sputum and BAL supernatants. Interferons-beta (IFN-β), alpha (IFN-α) and lambda (IFN-λ) and IFN-gamma-inducible protein 10 (CXCL10) were measured in supernatants from BAL cells cultured *ex vivo*. All soluble mediators were measured using enzyme-linked immunosorbent assays (ELISA) - details are provided in the Supplementary Methods. Serum CRP and peripheral blood cell counts were measured in the Clinical Biochemistry and Haematology laboratories of St Mary's Hospital, Imperial College Healthcare NHS Trust.

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 by using unpaired t-tests or Mann-Whitney tests. Correlations between data sets were examined using Pearson's correlation and 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

Thirteen subjects were recruited in each group and inoculated with low dose rhinovirus 16. In 3/26 subjects (2 COPD and 1 control) rhinovirus was not detected

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after inoculation so they were excluded from further analysis. The clinical characteristics of the 23 successfully infected subjects included in the data analysis are shown in Table 1. Following inoculation there were no adverse events and no subjects withdrew from the study or required corticosteroids, antibiotics or hospital admission.

Clinical responses to rhinovirus infection

Upper respiratory symptoms

Twenty-one of the 23 subjects fulfilled the symptom criteria for a clinical cold (see Supplementary Material). The remaining 2 subjects (1 in each group) had the subjective sensation of a cold but did not fulfil the other 2 symptom criteria however both had virologic evidence of rhinovirus infection. Daily upper respiratory symptom scores were significantly increased above baseline in the COPD group on days 2–14 post-inoculation and on days 1–12 in the controls (Figure 1A) and were significantly greater in the COPD group compared to the controls on days 13–16.

Lower respiratory symptoms

Ten of the 11 infected COPD subjects fulfilled the symptom criteria for a COPD exacerbation. Daily total lower respiratory symptom scores were increased significantly over baseline on days 5–19 in the COPD group and on days 3–9 in the controls (Figure 1B). Daily lower respiratory symptoms were significantly greater in the COPD group compared to controls on days 11, 14, 27, 29, 34 and 35. When the individual symptoms were analysed daily breathlessness scores were increased over baseline on days 5–12 in the COPD group, while breathlessness did not increase significantly in the controls (Figure 1C). Daily breathlessness scores were significantly higher in the COPD group than in controls on days 5 through 25, except days 14 and 15.

Lung function

Post-bronchodilator peak expiratory flow (PEF) fell significantly from baseline in the COPD group but no significant reductions were observed in the controls (Figure 1D) and on day 28 PEF was significantly lower in the COPD group compared to the controls ($92.36\pm2.23\%$ vs. 102.5 ± 2.95, P=0.014). K_{CO} (% baseline) on day 12 fell significantly from baseline in the COPD group (100 vs. 93.82 ± 1.52, P=0.0027) but not in the controls (100 vs. 96.18 ± 2.15, P=0.1) There were small falls from baseline in Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC) and the FEV_1/FVC ratio that were of similar magnitude in both groups but were not statistically significant.

Inflammatory responses to rhinovirus infection

Blood

There was a significant increase in blood neutrophils on day 15 compared to baseline in the COPD group; however no significant change in blood cell counts were observed in the control group. There were significant increases from baseline in serum C-reactive protein (CRP) on day 5 in both the COPD and control groups, while CRP was increased above baseline on day 9 only in the COPD group. There were no significant differences between groups in blood neutrophils or CRP (Supplementary Table 4).

Sputum

Adequate sputum samples (<20% squamous cells) were obtained from 10/11 COPD subjects and 11/12 controls. Percent sputum neutrophils increased significantly over baseline on days 5, 9, 12 and 15 in the COPD subjects, while there was no significant increase in sputum neutrophils in the controls (Figure 2A). Sputum neutrophil

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percentages were significantly higher in the COPD group compared to the controls on days 5, 12, 15, 21 and 28. Sputum supernatant neutrophil elastase levels were significantly increased over baseline on days 9 and 15 and IL-8 levels on day 9 in the COPD subjects, while no significant increases were observed in the control subjects (Figures 2B and 2C). Sputum neutrophil elastase levels were significantly higher in COPD subjects compared to controls on days 9, 12 and 15. There were no statistically significant changes from baseline in either group in sputum supernatant levels of IL-6 or TNF-α and no statistically significant differences between groups in sputum IL-6, IL-8 or TNF-α (Supplementary Table 4).

BAL

BAL was not obtained from 1 COPD subject at the infection bronchoscopy. There was a significant increase in BAL lymphocyte percentage from baseline to infection (Figure 2D) in the COPD group but not in the controls. There was no significant change between baseline and infection in BAL neutrophils, however BAL neutrophils at infection were significantly higher in the COPD group compared to the controls (Figure 2E). There was a significant increase from baseline to infection in BAL fluid IL-6 levels in the COPD group, but not in the controls, while BAL fluid neutrophil elastase, IL-8 and TNF-α did not increase significantly in either group. There were no significant differences between groups in BAL lymphocytes or BAL fluid levels of neutrophil elastase, IL-6, IL-8 or TNF-α (Supplementary Table 5).

Virologic responses

Infection with rhinovirus was confirmed by PCR in all 23 subjects. Following inoculation with rhinovirus 16, nasal lavage virus load (Log_{10} copies/mL) showed a rapid rise in both groups commencing ~48 hours after inoculation in a manner typical

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of an acute infection (Figure 3A and Supplementary Table 6). Virus load peaked on days 4–8 in nasal lavage and in sputum on day 5, remaining significantly elevated over baseline up to day 15. Nasal lavage virus load was 1–2 Logs higher in the COPD group compared to the controls from days 3–12, and this difference was statistically significant on day 6. Sputum virus load was also 1–3 Logs higher in the COPD group on days 5 and 9 but this was not statistically significant (Figure 3B). Peak virus load was 1–2 Logs but non-significantly higher in the COPD group in nasal lavage (7.0±0.58, 5.85±0.47; P=0.14), sputum (9.44±0.95, 8.53±0.34; P=0.41) and BAL (6.26±0.63, 5.09±0.24; P=0.14).

Relationships between virus load, inflammatory markers and clinical outcomes

In the COPD subjects there were strong correlations between sputum neutrophil elastase levels and clinical outcomes: peak increase in sputum neutrophil elastase correlated with peak total lower respiratory symptom scores (r=0.62, P=0.044), peak breathlessness scores (r=0.62, P=0.042) and with maximum fall in PEF (r=-0.8, P=0.003) and FEV_1/FVC ratio (r=-0.68, P=0.021). Peak sputum neutrophil numbers also correlated with fall in PEF (r=-0.77, P=0.0092).

Peak sputum virus load in COPD subjects correlated positively with each of peak serum CRP levels (r=0.89, P=0.0002), peak sputum neutrophil numbers (r=0.74, P=0.009) and peak sputum supernatant levels of IL-8 (r=0.81, P=0.0016), IL-6 (r=0.61, P=0.046), neutrophil elastase (r=0.69, P=0.018) and TNF-α (r=0.63, P=0.037) (Figure 4). BAL virus load in COPD subjects correlated positively with BAL fluid levels of neutrophil elastase (r=0.84, P=0.0024), IL-8 (r=0.65, P=0.042) and IL-6 (r=0.67, P=0.034). There were no significant correlations between virus load and inflammatory markers in the controls.

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Impaired interferon production in COPD subjects

Sufficient BAL cells for *ex vivo* cultures were obtained at the baseline bronchoscopy from 10 controls and 7 COPD subjects. These cells were incubated with live rhinovirus, medium alone or virus inoculum from which virus had been removed by molecular weight filtration (20). In BAL cells infected with live rhinovirus induction of both IFN-α and IFN-λ release into supernatants was impaired by ~50% in the COPD group but differences between groups were not statistically significant (Figures 5A and 5B). In BAL cells from the controls rhinovirus infection induced significant increases in IFN-β compared to filtered virus but not in the COPD subjects and IFN-β levels induced by infection were significantly higher in the controls compared to the COPD group (Figure 5C). The deficient IFN induction by rhinovirus in COPD was also accompanied by deficient induction of the interferon stimulated gene (ISG) CXCL10. Rhinovirus infection induced significant up-regulation of CXCL10 protein levels in BAL supernatants in the controls (1411±777.3 pg/mL vs. 8413±1707, P=0.0022) but not in the COPD group (379.1±150.3 vs. 6040±4069, P=0.26). Virus infection induced significant up-regulation of IL-8 in BAL cells from both groups (data not shown).

Discussion

In this study we report that experimental rhinovirus infection in COPD subjects induced symptoms, airflow obstruction and neutrophilic inflammation of significant severity and duration. Virus load in sputum correlated strongly with inflammatory markers and IFN-β production by airway macrophages was impaired in COPD. These data suggest that experimental rhinovirus infection is a valid human model of COPD

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exacerbations and a novel tool with which to further investigate mechanisms of virus-induced exacerbations.

There is no universally agreed definition of a COPD exacerbation, however, exacerbations are normally defined by acute increases in lower respiratory symptoms above normal daily variability (5) and are associated with increased airflow obstruction (32). Following rhinovirus infection COPD subjects reported significantly increased lower respiratory symptoms above their baseline symptoms and 10/11 fulfilled the predetermined criteria for an acute exacerbation that was based on accepted criteria from studies of naturally occurring exacerbations (2;33-35). The controls also developed lower respiratory symptoms but these were of lesser severity and duration than the COPD subjects. Only the COPD subjects developed significant increases in breathlessness, suggesting this is the key symptom that differentiates a COPD exacerbation from an uncomplicated acute viral bronchitis. This is supported by data from naturally occurring rhinovirus infections (36). Furthermore we documented increased airflow obstruction in COPD subjects with falls from baseline in PEF of ~10%, observations that are consistent with studies of naturally occurring exacerbations (32;37). There was a temporal relationship between virus detection in the respiratory tract and the onset of symptoms and airflow obstruction, and virus clearance was followed by clinical recovery. Therefore this study provides the first direct experimental evidence that rhinovirus infection precipitates the symptomatic and physiological changes in COPD that define an acute exacerbation.

The nature of the inflammatory response in COPD exacerbations remains controversial and there is no single cellular or molecular inflammatory marker that defines an exacerbation (9;11;38). Both neutrophilic and eosinophilic inflammation has been reported related to virus infection (11) but other studies have reported that

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virus infection is not associated with pulmonary or systemic inflammatory markers (12;13;39) and therefore the relationship between virus infection and airway inflammation in COPD exacerbations is unclear. We report that rhinovirus infection was temporally associated with a significant and sustained neutrophil influx and increased levels of neutrophil elastase and IL-8 in sputum in COPD subjects. There were strong correlations between sputum neutrophil elastase levels and severity of symptoms and airflow obstruction exclusively in COPD subjects. Neutrophil elastase has been related to exacerbation severity in bacterial exacerbations (40) and our data implicates it as a key mediator of virus-induced exacerbations also. In addition virus load correlated significantly with neutrophil numbers and inflammatory mediators in serum, sputum and BAL. In BAL lymphocytes and IL-6 were both also significantly increased after rhinovirus infection and further studies will be needed to investigate the importance of these and other inflammatory cells and mediators in COPD exacerbations. Therefore these data are the first to directly link virus infection of the lower respiratory tract to lower airways inflammation in COPD and strongly support a causative role for virus infection.

It is not known whether COPD are more susceptible to respiratory virus infections. We demonstrated increased severity and duration of respiratory symptoms, greater lung function impairment and increased airway inflammation in COPD subjects compared to smokers without airflow obstruction. Daily and peak virus loads in each of nasal lavage, sputum and BAL were consistently 1–3 Logs higher in COPD subjects, and although these differences were not statistically significant other than on day 6 the consistency of these findings throughout the acute infection and in 3 different clinical samples adds weight to their validity. These data confirm increased susceptibility to rhinovirus infection in COPD. A key constituent of innate immune

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responses to virus infection is production of interferons by infected cells. We have previously shown that IFN-β and IFN-λ production is impaired in asthma (20;21) and herein we report impaired BAL cell production of IFN-β in response to virus infection in COPD subjects and trends to reduced IFN-α and IFN-λ. As IFN-β is an early and essential component of anti-rhinoviral immunity (21) this may be an important mechanism underlying increased severity of rhinovirus infection in COPD. A recent report of *in vitro* rhinovirus infection of epithelial cells from COPD patients reported higher virus load but no differences in interferon production (41). Therefore other mechanisms may also be involved in susceptibility to virus infection in COPD.

We have established experimental rhinovirus infection is a valid model of COPD exacerbation and therefore this provides an important new tool for studying exacerbations. This model has major advantages as it permits investigations of exacerbations in a controlled manner with a single aetiogical agent, and facilitates longitudinal sampling allowing investigation of temporal and quantitative relationships between virus infection, inflammatory mediators and biological and physiological markers in a manner not possible with cross-sectional studies of naturally occurring exacerbations. The evidence we provide linking rhinovirus infection to COPD exacerbations should stimulate research into both existing and novel antiviral therapies as potential therapies in COPD (42). Therapies targeting neutrophilic inflammation (43) and neutrophil elastase inhibitors (44) or augmentation of interferon-β may also have potential in treatment or prevention of COPD exacerbations. This model will also be a useful tool to assist in testing efficacy of new therapies.

Limitations of our study include the relatively small numbers of subjects and the higher mean age of the COPD group. As this is the first study to perform experimental

rhinovirus infection in COPD subjects and smokers there was no data on which to base power calculations. The data from this study can be used to design further experimental infection studies and it is possible that with larger subject numbers statistically significant differences in virus load and IFN-α and IFN-λ will become apparent. We examined the data for correlations between age and clinical, inflammatory and virologic outcomes and interferon production and none were found. However further studies are required to replicate these findings in greater numbers of subjects and with subject groups more closely matched for age.

In conclusion we have shown that experimental rhinovirus infection induces the clinical features of COPD exacerbations, that neutrophilic airway inflammation is an important mechanism of virus-induced exacerbations and that deficient production of IFN-β may contribute to increased susceptibility to virus infection in COPD. This model will facilitate research into mechanisms of COPD exacerbations thereby boosting efforts to develop new approaches for the prevention and treatment of exacerbations of COPD.

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Figure Legends

Figure 1. Symptom scores and lung function during experimental rhinovirus infection. The time course for daily symptom scores are shown for total daily upper respiratory symptoms (Panel A), total daily lower respiratory symptoms (Panel B) and breathlessness (Panel C). Panel D shows the time course of post-bronchodilator peak expiratory flow as percentage of baseline (all mean±SEM). *$P<0.05$ vs. baseline, **$P<0.01$ vs. baseline, †$P<0.05$ COPD vs. controls, ††$P<0.01$ COPD vs. controls, †††$P<0.001$ COPD vs. controls.

Figure 2. Airway inflammatory cells and soluble mediators during experimental rhinovirus infection. The time course of percentage neutrophils (mean±SEM) (Panel A), neutrophil elastase (median (IQR) (Panel B) and IL-8 (mean±SEM) (Panel C) in induced sputum are shown. Panels D and E show lymphocytes and neutrophils in bronchoalveolar lavage (both mean±SEM). *$P<0.05$ vs. baseline, **$P<0.01$ vs. baseline, †$P<0.05$ COPD vs. controls, ††$P<0.01$ COPD vs. controls.

Figure 3. Virus load in nasal lavage and sputum. The time course of virus load measured with qPCR is shown in nasal lavage (Panel A) and sputum (Panel B) (both mean±SEM). *$P<0.05$ vs. baseline, **$P<0.01$ vs. baseline, ***$P<0.01$ vs. baseline, †$P<0.05$ COPD vs. controls.

Figure 4. Correlations between sputum virus load and inflammatory markers. Relationships between sputum virus load and inflammatory markers in COPD subjects are shown. There were significant correlations between peak sputum virus load and peak serum C-reactive protein (Panel A), peak sputum neutrophils (Panel B),

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peak sputum IL-8 (Panel C), peak sputum neutrophil elastase (Panel D), peak sputum

IL-6 (Panel E) and peak sputum TNF-α (Panel F).

Figure 5. Interferon responses in BAL cells. Cells obtained by BAL were infected *ex vivo* with rhinovirus 16 and interferon-α (Panel A), interferon-λ (Panel B) and interferon-β (Panel C) measured in supernatants 48 hours post-infection (all mean±SEM). *P<0.05 vs. filtered virus, **P<0.01 vs. filtered virus, †P<0.05 COPD vs. controls. Comparisons between COPD and control groups are indicated above the relevant brackets.

Tables

Subject characteristics	COPD (N=11)	Controls (N=12)	Unpaired t test
Age (years)	59.6 [47 – 70]	48.5 [40 – 58]	P=0.0021
Sex (M/F)	6/5	6/6	P=NS
Smoking history (pack-years)	48 [20 – 109]	34.8 [20 – 60]	P=NS
Current smokers (no.)	8	9	P=NS
FEV$_1$ (litres)	1.94 [1.23 – 2.7]	3.58 [2.8 – 4.76]	P<0.0001
FEV$_1$ (% of predicted normal value)	69.73 [62 – 78]	109.5 [90 – 128]	P<0.0001
FEV$_1$/FVC (%)	55.55 [39 – 69]	80.33 [73 – 86]	P<0.0001
COPD denotes chronic obstructive pulmonary disease, FEV$_1$ forced expiratory volume in one second, FVC forced vital capacity, pack-years the number of cigarettes smoked per day multiplied by the number of years of smoking. Values are mean (range).			

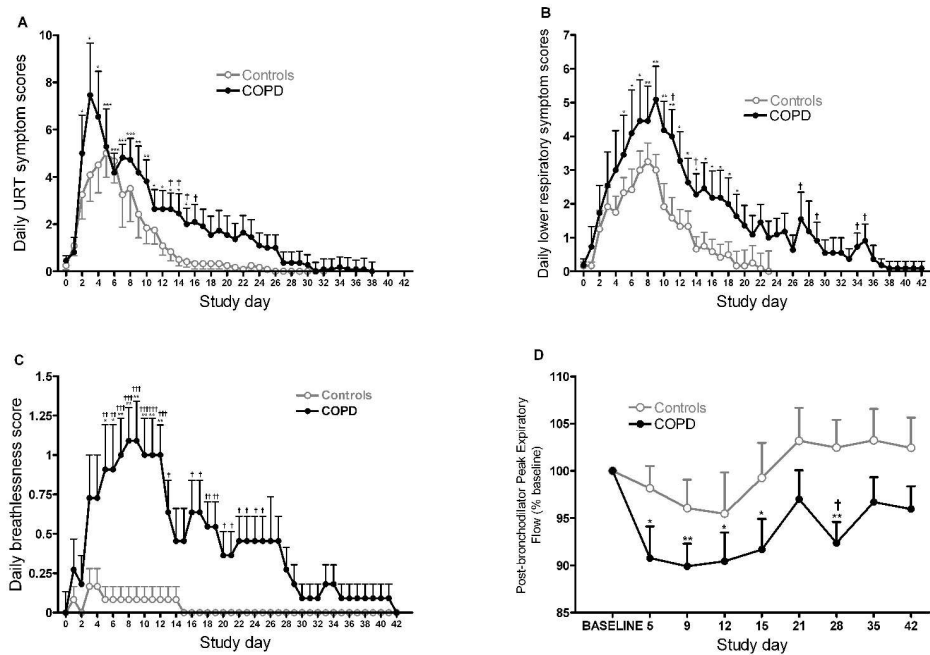Table 1. Clinical characteristics of the 23 study subjects included in the study.

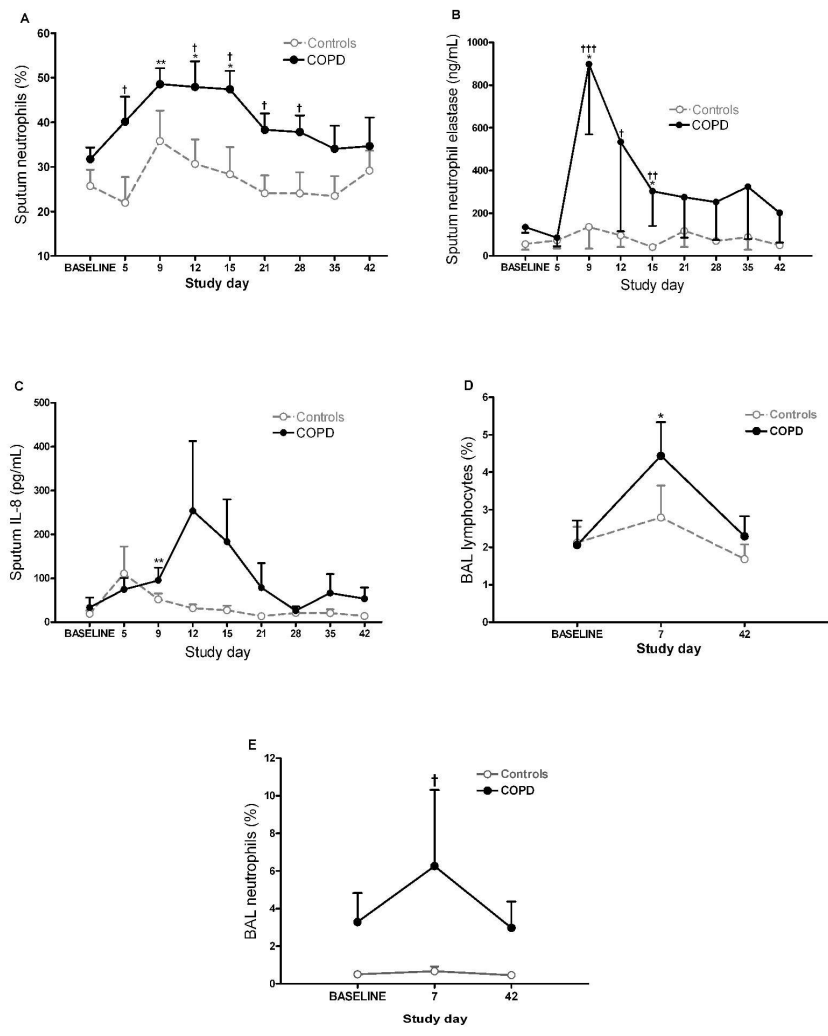Figure 1
268x201mm (600 x 600 DPI)
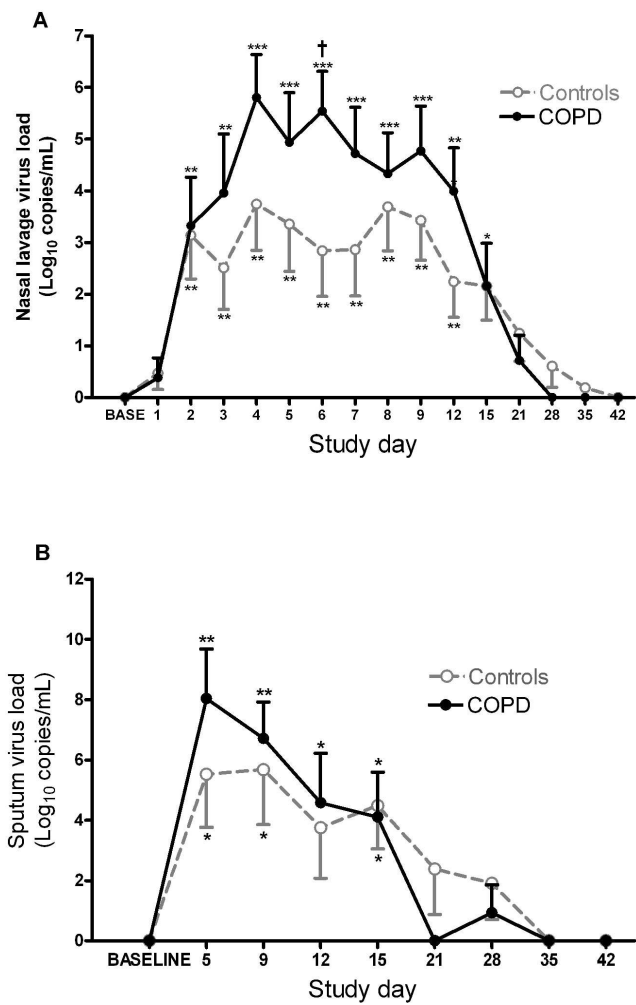
Figure 2
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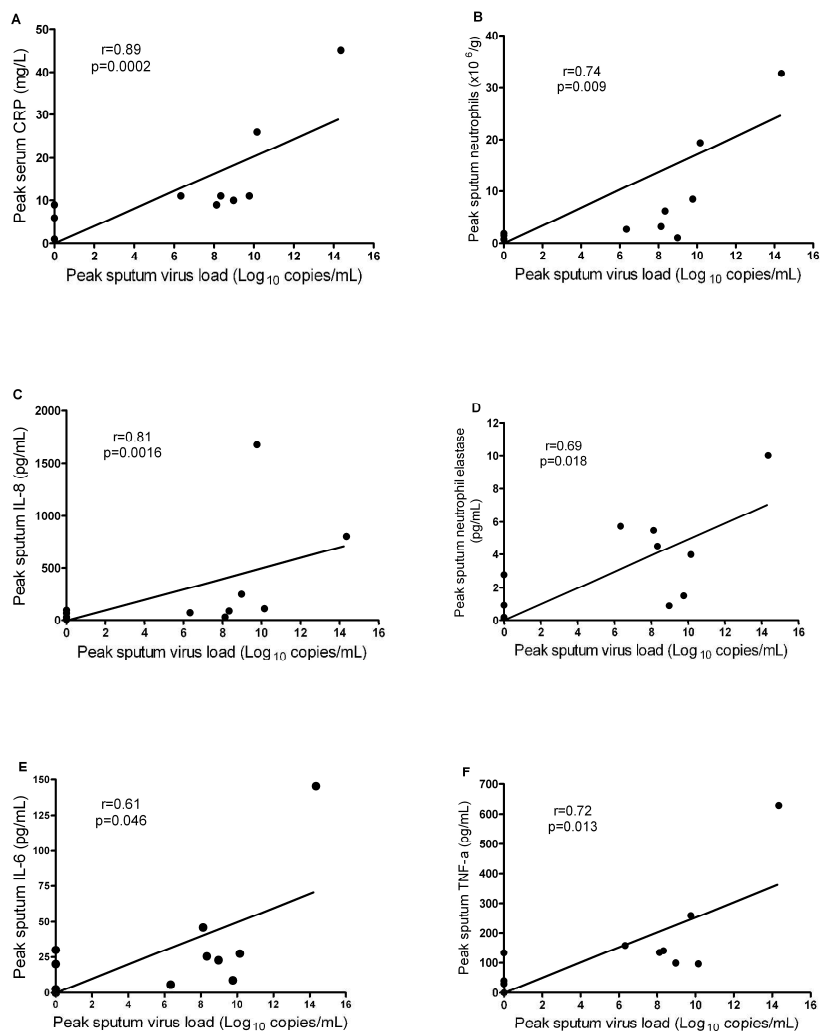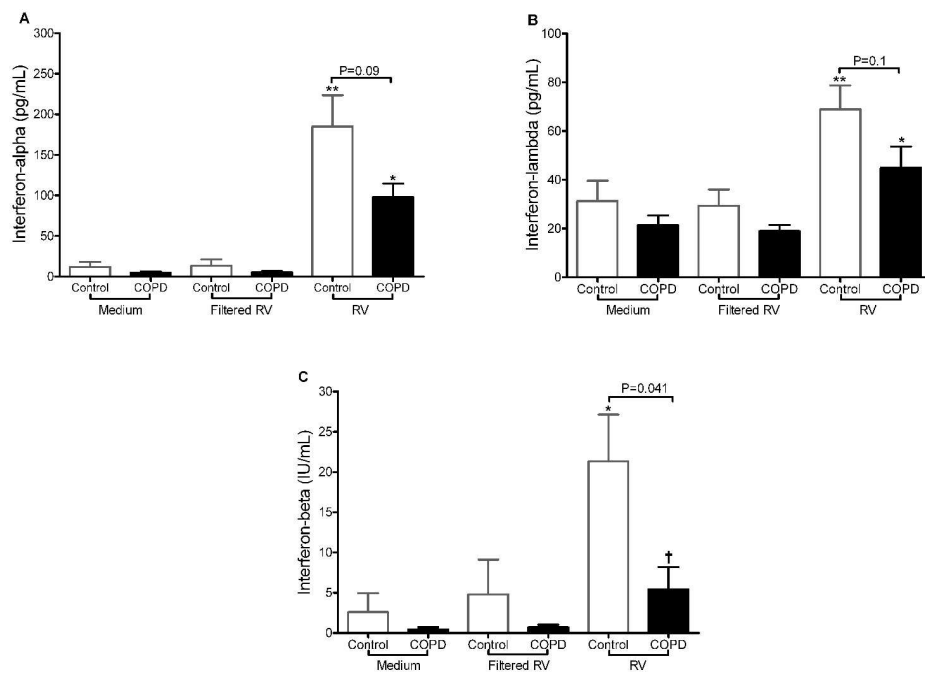
Figure 3
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Figure 4
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Figure 5
267x202mm (600 x 600 DPI)