Online Data Supplement

Experimental Rhinovirus Infection as a Human Model of Chronic Obstructive Pulmonary Disease Exacerbation

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Supplementary Methods

Symptom scores. Symptoms were assessed using diary cards that were completed on a daily basis from screening until 6 weeks post-inoculation. Upper respiratory symptoms were measured using the Jackson scale assessing 8 symptoms – sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat and cough – graded 0 (absent) to 3 (severe) (1). The daily cold score was summated from the individual scores and a clinical cold was defined using the Jackson criteria (1). The scoring system for the lower respiratory symptoms of shortness of breath, cough, wheeze, sputum quantity and sputum quality was that used in the pilot study and shown in Supplementary Table 6 (2). 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 2 points over baseline for at least 2 consecutive days (3,4). 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.

Nasal lavage. Nasal lavage was performed by instilling 2.5mL of 0.9% saline into each nostril, holding for 5 seconds and then expelling into a sterile container. The lavage fluid obtained was homogenized, aliquotted and stored at -80^{o}C.

Induced sputum. Sputum was induced and processed using standard protocols (5). Briefly subjects were pre-medicated with 200μg salbutamol via metered dose inhaler and large volume spacer and baseline FEV_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^{o}C for qRT-PCR for virus load. The remaining sample was weighed, 0.1% Dithiothreitol (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. 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.

Bronchoscopy. All bronchoscopies were carried out in the endoscopy unit at St Mary's Hospital, Imperial College Healthcare NHS Trust by an experienced operator. Subjects were administered nebulised salbutamol (2.5mg) and ipratropium bromide (0.5mg) prior to the procedure and intravenous midazolam was used to provide sedation. Bronchoalveolar lavage (BAL) was performed by instillation of sterile 0.9% saline into the left upper lobe bronchus in 30ml aliquots to a total of 240ml. An aliquot of unfiltered BAL was stored for qRT-PCR for virus load and the remaining BAL fluid filtered, centrifuged and the supernatant stored.

The BAL cell pellet was resuspended, a cell count was obtained and cytospins made for differential cell counting using the same procedure as for induced sputum. Viability was assessed with trypan blue exclusion. Cells from the BAL obtained at baseline bronchoscopy were washed and resuspended in RPMI-1640 medium with Glutamax (Invitrogen, UK) containing 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin), at a final concentration of $2x10^6$ cells per mL. Cells were incubated with rhinovirus 16 at a MOI of five, with inoculum from which the virus had been removed by molecular weight filtration or with medium alone. After 48 hours supernatants were harvested and stored at –80°C (6).

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 (7). 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) (7). 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 (8). To differentiate rhinoviruses from other picornaviruses *BglI* enzyme restriction digestion was carried out on the amplicons generated by RT-PCR (9). 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)(10). 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 (11)

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 (12), except HMPV which was adapted from (13).

ELISA. The ELISAs for detection of soluble mediators 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. The sensitivities and sources of the individual ELISAs were as follows: IL-6 (3.9pg/mL), IL-8 (3.9pg/mL), CXCL10 (50pg/mL), IFN-λ (29pg/mL) (R&D Systems, Abingdon, UK); neutrophil elastase (0.12ng/mL) (Immunodiagnostik, Benshein, Germany); IFN-α (12pg/mL), IFN-β (<4 IU/mL) and TNF-α (15pg/mL) (Biosource, USA).

Supplementary Tables

All subjects

- Age 40-75 years.
- No history of asthma or allergic rhinitis.
- Not atopic on skin testing.
- Current or ex-smokers with at least 20 pack years cumulative smoking.
- 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 8 weeks.
- Serum antibodies to rhinovirus 16 at screening in a titre <1:2.
- No treatment with oral, inhaled or nasal topical steroids, long-acting β-agonists or tiotropium in the previous 3 months.

COPD group

- Post-bronchodilator FEV_1 ≤80% and ≥50% predicted normal value and β-agonist reversibility <12%.

- Post-bronchodilator $FEV_1/FVC<70\%$.

Control group

- Post-bronchodilator $FEV_1>80\%$ predicted normal value.
- Post-bronchodilator $FEV_1/FVC>70\%$.

COPD denotes chronic obstructive pulmonary disease, FEV_1 forced expiratory volume in one second and FVC forced vital capacity

Supplementary Table 1. Inclusion criteria for study subjects.

Procedure	Study Day																
	Baseline	0	1	2	3	4	5	6	7	8	9	12	15	21	28	35	42
Spirometry	x						x				x	x	x	x	x	x	x
Transfer factor	x											x					x
Nasal lavage	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Blood count/C-reactive protein	**x**						**x**				**x**	**x**	**x**	**x**	**x**	**x**	**x**
Induced sputum	**x**						**x**				**x**	**x**	**x**	**x**	**x**	**x**	**x**
Bronchoscopy	**x**								**x**								**x**
Rhinovirus inoculation		**x**															
Symptom diaries	→																

Supplementary Table 2. 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.

Inflammatory mediators/virus load		**STUDY DAY**									**ANOVA/ Friedman**
		BASE LINE	**5**	**9**	**12**	**15**	**21**	**28**	**35**	**42**	
Blood neutrophils ($x10^9$/L)	COPD (N=11)	**4.03 ±0.43**	**4.42 ±0.55**	**3.90 ±0.56**	**4.71 ±0.43**	**4.72 ±0.66****	**5.56 ±0.45**	**4.53 ±0.4**	**4.41 ±0.38**	**4.14 ±0.42**	**P=0.014**
	Controls (N=12)	**4.05 ±0.60**	**4.86 ±0.74**	**4.88 ±0.57**	**4.32 ±0.47**	**4.51 ±0.66**	**5.01 ±0.79**	**5.05 ±0.56**	**4.61 ±0.61**	**4.45 ±0.59**	P=0.23
Blood CRP (mg/L)	COPD (N=11)	**0 (0-0)**	**5.00 (0-10)***	**6.00 (0-11)***	**0 (0-6)**	**0 (0-0)**	**0 (0-0)**	**0 (0-0)**	**0 (0-0)**	**0 (0-0)**	**P=0.0001**
	Controls (N=12)	**0 (0-0)**	**5.5 (0-22.5)***	**0 (0-10)**	**0 (0-7.5)**	**0.58 ±0.58**	**0 (0-0)**	**0 (0-0)**	**0 (0-0)**	**0 (0-0)**	**P<0.0001**
Sputum neutrophils	COPD (N=10)	**31.74 ±2.60**	**40.14 ±5.62**	**48.55 ±3.61****	**47.92 ±5.73***	**47.42 ±4.11***	**38.32 ±3.64**	**37.80 ±3.73**	**34.06 ±5.14**	**34.66 ±6.38**	**P=0.013**

(%)	Controls (N=11)	**25.71 ±3.63**	**21.92 ±5.81**	**35.80 ±6.81**	**30.65 ±5.49**	**28.32 ±6.14**	**24.08 ±3.98**	**24.05 ±4.74**	**23.45 ±4.46**	**29.14 ±4.50**	P=0.14
Sputum IL-6 (pg/mL)	COPD (N=10)	**18.98 ±9.40**	**179.8 ±115.7**	**103.4 ±41.77**	**125.3 ±52.36**	**73.95 ±35.6**	**47.12 ±27.44**	**75.37 ±61.66**	**31.92 ±18.07**	**12.54 ±9.76**	P=0.224
	Controls (N=11)	**19.51 ±7.73**	**86.89 ±49.6**	**126.2 ±72.36**	**36.86 ±25.89**	**87.04 ±57.57**	**7.32 ±4.60**	**13.53 ±7.08**	**2.20 ±2.20**	**5.79 ±2.98**	P=0.058
Sputum TNF-α (pg/mL)	COPD (N=10)	**0 (0-27.25)**	**3.32 (0-403.8)**	**177.5 (0-2338)**	**254.2 (0-950.7)**	**194.3 (0-748)**	**278.7 (0-889.7)**	**382.5 (0-724.2)**	**54 (0-634.6)**	**124.7 (0-1017)**	P=0.24
	Controls (N=11)	**167.5 (0-2003)**	**18.65 (0-2256)**	461.1 **(0-1825)**	25.18 **(0-4160)**	334.7 **(13.5-5257)**	2470 **(1.52-5638)**	1074 **(0-5563)**	345.2 **(0-1090)**	216.3 **(0-3989)**	P=0.85
Sputum IL-8 (pg/mL)	COPD (N=10)	**36.89 ±24.8**	**82.22 ±27.29**	**104.1 ±30.18****	**279.5 ±173.1**	**201.9 ±104.8**	**86.52 ±61.19**	**28.98 ±9.87**	**73.28 ±47.22**	**58.35 ±28.08**	**P=0.049**
	Controls (N=11)	**173.0 ±71.63**	**996.3 ±555.5**	**468.4 ±122.6**	**287.2 ±78.10**	**247.5 ±90.97**	**121.7 ±49.55**	**190.0 ±80.74**	**186.3 ±77.31**	**128.3 ±64.88**	P=0.063
Sputum Neutrophil Elastase (µg/mL)	COPD (N=10)	**0.64 ±0.08**	**069 ±0.12**	**0.97 ±0.065****	**0.85 ±0.1**	**0.86 ±0.11**	**0.73 ±0.09***	**06.8 ±0.09**	**0.80 ±0.11**	**0.72 ±0.09**	**P=0.012**
	Controls (N=11)	**0.4 ±0.08**	**0.48 ±0.11**	**0.48 ±0.09**	**0.52 ±0.1**	**0.42 ±0.082**	**0.37 ±0.12**	**0.41 ±0.095**	**0.42 ±0.079**	**0.37 ±0.069**	P=0.175
Sputum virus load (Log_{10} copies/mL)	COPD (N=11)	**1.0 ±0**	**9.04 ±1.64****	**7.72 ±1.20****	**5.44 ±1.71***	**5.11 ±1.48***	**1.0 ±0**	**1.93 ±0.93**	**1.0 ±0**	**1.0 ±0**	**P<0.0001**
	Controls (N=12)	**1.0 ±0**	**6.52 ±1.76***	**6.52 ±1.93***	**4.76 ±1.69**	**5.49 ±1.43***	**3.39 ±1.51**	**2.91 ±1.21**	**1.0 ±0**	**1.0 ±0**	**P=0.0027**

COPD – chronic obstructive pulmonary disease, CRP – C-reactive protein, IL – interleukin, TNF-α – tumour necrosis factor-alpha, ANOVA – analysis of variance.

Supplementary Table 3. Inflammatory cells and soluble mediators in induced sputum and blood and virus load in sputum. Data is expressed as mean±SEM or median (IQR).

BAL parameters		STUDY DAY			
		BASELINE	7	42	ANOVA/ Friedman
Neutrophil Elastase (ng/mL)	COPD (N=10)	6.78 (2.22-7.34)	4.29 (2.18-50.68)	6.95 (4.39-8.56)	P=0.44
	Controls (N=12)	2.94 (0.31-7.38)	2.20 (1.0-10.79)	6.45 (1.88-7.63)	P=0.71
TNF-α (pg/mL)	COPD (N=10)	6.64 ±1.22	15.13 ±6.77	5.75 ±1.13	P=0.22
	Controls (N=12)	6.97 ±1.64	8.73 ±1.59	7.43 ±1.37	P=0.65
IL-8 (pg/mL)	COPD (N=10)	31.52 ±13.45	139.0 ±69.81	30.44 ±12.70	P=0.15
	Controls (N=12)	18.50 ±5.16	31.95 ±10.23	15.43 ±4.41	P=0.13
Neutrophils (%)	COPD (N=10)	3.28 ±1.54	6.26 ±4.05	2.97 ±1.39	P=0.1
	Controls (N=12)	0.50 ±0.13	0.67 ±0.25	0.45 ±0.14	P=0.92
Lymphocytes (%)	COPD (N=10)	2.06 ±0.65	4.44 ±0.89*	2.29 ±0.54	**P=0.0086**
	Controls (N=12)	2.13 ±0.42	2.79 ±0.85	1.68 ±0.40	P=0.13
Virus load (Log_{10} copies/mL)	COPD (N=10)	1	7.26 ±0.63***	1	**P<0.0001**
	Controls (N=12)	1	6.09 ±0.24***	1	**P<0.0001**

BAL – bronchoalveolar lavage, COPD – chronic obstructive pulmonary disease, IL – interleukin, TNF-α – tumour necrosis factor-alpha, ANOVA – analysis of variance.

Supplementary Table 4. Inflammatory cells, soluble mediators and virus load in BAL.

		STUDY DAYS								
		BASE LINE	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Nasal lavage virus load (Log_{10} copies/mL)	**COPD (N=11)**	1.00 ±0	1.38 ±0.38	4.33 ±0.94**	4.96 ±1.14**	6.80 ±0.84***	5.94 ±0.97***	6.54 ±0.77***	5.72 ±0.89***	5.33 ±0.79***
	Controls (N=12)	1.00 ±0	1.47 ±0.32	4.14 ±0.85**	3.51 ±0.81**	4.74 ±0.89**	4.35 ±0.92**	3.84 ±0.88**	3.86 ±0.90**	4.69 ±0.85**
		9	**12**	**15**	**21**	**28**	**35**	**42**	**ANOVA**	
	COPD (N=11)	5.77 ±0.87***	4.99 ±0.84**	3.16 ±0.83*	1.72 ±0.49	1.00 ±0	1.00 ±0	1.00 ±0	**P<0.0001**	
	Controls (N=12)	4.43 ±0.78**	3.24 ±0.69**	3.16 ±0.66**	2.25 ±0.54*	1.61 ±0.41	1.19 ±0.19	1.00 ±0	**P<0.0001**	

COPD – chronic obstructive pulmonary disease, ANOVA – analysis of variance.

Supplementary Table 5. Nasal lavage virus load.

SYMPTOM	SCORE				
	0	1	2	3	4
SHORTNESS OF BREATH	Not breathless	On moderate exertion	On mild exertion	On minimal exertion	At rest
WHEEZE	No wheeze	On moderate exertion	On mild exertion	On minimal exertion	At rest
COUGH	No cough	Mild	Moderate	Severe	/
SPUTUM QUANTITY (PER 24 HRS)	None	Minimal (<30mL)	Moderate (30-100mL)	Large (>100mL)	/
SPUTUM QUALITY	None	Mucoid (clear)	Mucopurulent (yellow)	Purulent (green)	/

Supplementary Table 6. Scoring system used for lower respiratory symptoms.

References

(1) Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. *Arch of Int Med* 101, 267-278. 1958.

(2) Mallia P, Message SD, Kebadze T, Parker HL, Kon OM, Johnston SL. An experimental model of rhinovirus induced chronic obstructive pulmonary disease exacerbations: a pilot study. *Respir Res* 2006;7:116.

(3) Calverley PM. Combined salmeterol and fluticasone for COPD. *Lancet* 2003 May 10;361(9369):1652-3.

(4) Seemungal TA, Donaldson GC, Paul EA, Bestall JC, Jeffries DJ, Wedzicha JA. Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157(5 Pt 1):1418-22.

(5) Efthimiadis A, Spanevello A, Hamid Q, Kelly MM, Linden M, Louis R et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. *Eur Resp J* - Supplement 2002;37:19s-23s.

(6) Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW et al. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12(9):1023-6.

(7) Johnston S.L, Tyrrell DA. Rhinoviruses. Lennette EH SNe, editor. Diagnostic procedures for viral, rickettsial and chlamydial infections, 553-563. 1995. American Public Health Association, Washington DC.

(8) Johnston SL, Sanderson G, Pattemore PK, Smith S, Bardin PG, Bruce CB et al. Use of polymerase chain reaction for diagnosis of picornavirus infection in subjects with and without respiratory symptoms. *J Clin Micro* 1993;31(1):111-7.

(9) Papadopoulos NG, Hunter J, Sanderson G, Meyer J, Johnston SL. Rhinovirus identification by BglI digestion of picornavirus RT-PCR amplicons. *J Vir Methods* 1999 July;80(2):179-85.

(10) Pevear DC, Tull TM, Seipel ME, Groarke JM. Activity of pleconaril against enteroviruses. *Antimicrob Agents Chemother* 1999;43(9):2109-15.

(11) Hayden FG, Herrington DT, Coats TL, Kim K, Cooper EC, Villano SA et al. Efficacy and safety of oral pleconaril for treatment of colds due to picornaviruses in adults: results of 2 double-blind, randomized, placebo-controlled trials. *Clin Infect Dis* 2003;36(12):1523-32.

(12) Seemungal T, Harper-Owen R, Bhowmik A, Moric I, Sanderson G, Message S et al. Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164(9):1618-23.

(13) Maertzdorf J, Wang CK, Brown JB, Quinto JD, Chu M, de Graaf M et al. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. *J Clin Micro* 2004;42(3):981-6.