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Acute Exacerbation and Respiratory InfectionS in COPD (AERIS): A prospective, observational cohort study of the dynamics of airway pathogens and the seasonal aetiology of exacerbations in COPD

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Acute Exacerbation and Respiratory InfectionS in COPD (AERIS): A prospective, observational cohort study of the dynamics of airway pathogens and the seasonal aetiology of exacerbations in chronic obstructive pulmonary disease

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Key Questions

What is the key question?

Is there a relationship between chronic bacterial airway infection and viral exposure that might influence the aetiology and seasonality of AECOPD?

What is the bottom line?

In this prospective, observational cohort study, exacerbations were associated with infections with *Moraxella catarrhalis* and NTHi and with respiratory viruses, particularly HRV, and a seasonal peak in exacerbations was associated with a combination of higher incidence of seasonal pathogens, a seasonal interaction between NTHi and viral infection, and greater bacterial loads.

Why read on?

The AERIS study used repeated sampling of a well phenotyped cohort along with sensitive molecular diagnostic techniques to detect airway bacterial and viral pathogens and its results suggest that the seasonal burden of AECOPD is driven partly by the effect of acute HRV infection on a background of NTHi infection, an effect size that has been quantified for the first time.

Conclusion for Twitter feed (140 characters limit)

The AERIS study has shown for the first time that seasonal risk of exacerbation differs depending on bacterial pathogen concerned.

Keywords

Chronic obstructive pulmonary disease, exacerbation, seasonality, non-typeable *Haemophilus influenzae*, rhinovirus

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Abstract

Background

The aetiology of acute exacerbations of chronic obstructive pulmonary disease (AECOPD) is incompletely understood. Understanding the relationship between chronic bacterial airway infection and viral exposure may explain the incidence and seasonality of these events.

Methods

In this prospective, observational cohort study (NCT01360398), patients with COPD aged 40–85 years underwent sputum sampling monthly and at exacerbation for detection of bacteria and viruses. Results are presented for subjects in the full cohort, followed for one year. Interactions between exacerbation occurrence and pathogens were investigated by generalised estimating equation and stratified conditional logistic regression analyses.

Findings

The mean exacerbation rate per patient year was 3.04 (95% CI 2.63–3.50). At AECOPD, the most common bacterial species were non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis*, and the most common virus was rhinovirus. Logistic regression analyses (culture bacterial detection) showed significant odds ratio (OR) for AECOPD occurrence when *M. catarrhalis* was detected regardless of season (5.09 [95% CI 2.76–9.41]). When NTHi was detected, the increased risk of exacerbation was greater in high season (October–March, OR 3.04 [1.80–5.13]) than low season (OR 1.22 [0.68–2.22]). Bacterial and viral coinfection was more frequent at exacerbation (24.9%) than stable state (8.6%). A significant interaction was detected between NTHi and rhinovirus presence and AECOPD risk (OR 5.18 [1.92–13.99]; p=0.031).

Conclusions

AECOPD aetiology varies with season. Rises in incidence in winter may be driven by increased pathogen presence as well as an interaction between NTHi airway infection and effects of viral infection.

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INTRODUCTION

Acute exacerbations of chronic obstructive pulmonary disease (AECOPD) are highly seasonal in incidence,[1, 2] which has important consequences for patients and healthcare services, which are often overstretched during winter seasons.[3] One of the causes of this seasonality may be the increased incidence of respiratory viral infections.[3]

Bacterial pathogens are commonly identified in the lower airway of COPD patients both in stable state and during acute exacerbations, with significant changes in prevalence of airway bacteria during AECOPD.[4, 5] Understanding the interaction between chronic bacterial airway infection and seasonal exposure to viruses may provide important insights into the mechanisms of exacerbation stratified for causal or associated pathogens and point to potential therapies that prevent rather than treat events. Previous studies have identified human rhinovirus (HRV) infection as a key factor and that secondary bacterial infection, most commonly with non-typeable *Haemophilus influenzae* (NTHi), may be an important modulator of consequent inflammation and clinical severity.[6, 7] However, the nature of interactions between acute viral infection and chronic bacterial infection are not fully understood, nor are the effects of seasonality on the characteristics of exacerbation events.

In this prospective study, a well-characterised cohort of COPD patients underwent sputum sampling each month and at exacerbation. Repeated identification of bacterial and viral airway infections with sensitive molecular diagnostic techniques allowed the influences of season and other factors on exacerbation occurrence to be examined over a full year. Insights into the aetiology of these important events were derived by examining the relationship between chronic airway bacterial infection and the associated risk and impact of acute viral infection.

METHODS

Study design

The Acute Exacerbation and Respiratory InfectionS in COPD (AERIS) study is a

prospective, observational cohort study based at University Hospital Southampton (UHS), registered with ClinicalTrials.gov (NCT01360398). The study protocol has been published previously.[8] This two-year longitudinal epidemiological study assessed the contribution of changes in the COPD airway microbiome to the incidence of AECOPD. Patients aged 40–85 years with a confirmed diagnosis of COPD, categorised as moderate, severe, or very severe,[8, 9] were recruited from UHS and referring practices from June 2011 to June 2012. AERIS was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice, and was approved by the Southampton and South West Hampshire Research Ethics Committee. All participants provided written informed consent. The protocol summary is available at www.gsk-clinicalstudyregister.com (study identifier, 114378). Full inclusion and exclusion criteria are listed in the appendix.

We report results for the primary objective (estimation of the incidences of all-cause AECOPD and AECOPD with sputum containing bacterial pathogens detected by culture) for subjects followed over one year. We also describe secondary objective results on the incidences of bacterial and viral pathogens detected in AECOPD by polymerase chain reaction (PCR) and in stable state COPD by culture (bacteria only) and PCR.

Procedures

Patients were followed monthly in the stable state and reviewed within 72 hours of onset of AECOPD symptoms. Exacerbations were detected using daily electronic diary cards. The definition of AECOPD, as described previously,[8] and definitions of severity categories are provided in the appendix.

Sputum samples were obtained by spontaneous expectoration or induced and were processed according to standard methods, as described in the appendix.

Statistical analysis

The sample size calculation was described previously.[8] First year results are presented for subjects included in the full cohort, defined as all patients considered by the investigator as

eligible for study procedures and excluding those who withdrew consent at the first visit.

The percentage of stable-state and exacerbation-state sputum samples containing bacterial or viral pathogens (overall and by species) was calculated with 95% confidence intervals (95% CI). The 95% CI of the incidence rate was computed using the generalised linear model assuming a negative binomial distribution for the response variable with logarithm as link function, and the logarithm of time for follow-up as an offset variable.

Post hoc conditional logistic regression models, stratified by subject, were used to identify the effect of the presence of pathogens in sputum on the odds of experiencing an exacerbation rather than stable COPD. This model does not take into account possible correlations between successive measures within each subject. However, tests of the fit of generalised estimating equations (GEEs) and generalised linear mixed models with the same logit link indicated that models assuming independence between observations within each subject provided a fit similar to those taking correlations into account. By stratifying by subject, the conditional logistic model has the added advantage of taking into account any confounding factors that remain constant over time (such as age, gender, and COPD status) for each subject. Bacterial respiratory pathogens, HRV, and any other virus were entered into the model to identify species associated with AECOPD, assessing new infection occurrences (detection after negative sputum sample at previous visit) as well as any infections (presence) and taking into account the seasonality. For the analysis of the effect of new infection occurrences, if the presence of bacteria or virus was not evaluated at the preceding stable/exacerbation visit, the last value observed before this visit was used. The seasons were divided into two: high season (October to March) and low season (April to September). The final conditional logistic models used were selected by a backward elimination procedure in which only statistically significant main or interaction effects (provided there were more than five observations in each combination of factors included in the interaction) were kept ($p<0.05$). Seasonal rates of exacerbations, with the presence or new occurrence of bacterial or viral pathogens, were estimated post hoc by a GEE model

with a logit link and assuming an exchangeable correlation matrix. Seasonal differences in incidence rates were tested by corresponding likelihood ratio tests. The GEE model with compound symmetry correlation (exchangeable structure) was chosen to detect differences between marginal frequencies. Investigation of the fit of different GEE models, using exchangeable, autoregressive, or independent structure correlation matrices, found that the model assuming compound symmetry provided the best data fit.

Statistical analysis was performed using the SAS Drug Development platform version 4.3.2 (SAS Institute, Cary, NC, USA).

RESULTS

Of 152 patients screened, 25 were excluded from the full cohort for reasons shown in Figure 1 and 105 completed all follow-up visits up to month 12. The last patient visit of the study was in June 2014. Baseline characteristics of the full cohort are shown in Table 1. The patients' age range was 42–85 years and most had moderate (44.9%) or severe (40.2%) COPD. Almost half (48.8%) had more than two documented exacerbations in the year before enrolment. Bronchiectasis, which was assessed at enrolment by high-resolution computed tomography scan, was present in a minority of patients (10 of 127 patients; 7.9%).

Table 1: **Characteristics of the patients at enrolment (full cohort, year 1)**

Characteristic	N = 127
Age (years) at enrolment, mean ± SD	66.8 ± 8.6
Female sex, n (%)	59 (46.5%)
Smoking history pack-years, median (interquartile range)	47.0 (33.7–60.0)
Medication for COPD, n (%)	127 (100%)
Influenza vaccination during previous year, n (%)	114 (89.8%)
Pneumococcal vaccination during previous year, n (%)	12 (9.4%)
COPD status, GOLD stage, n (%)	
Mild	0 (0%)
Moderate	57 (44.9%)
Severe	51 (40.2%)
Very severe	19 (15.0%)
BODE index, median (interquartile range)	4 (2–6)
TL_{CO} predicted/actual (mmol/kPa/min), median (interquartile range)	7.9 (7.2–8.8)/4.5 (3.4–5.8)
Number of subjects reporting exacerbations in preceding 12 months, n (%)	
One exacerbation	28 (22.0%)
Two exacerbations	37 (29.1%)
Three exacerbations	25 (19.7%)
Four or more exacerbations	37 (29.1%)
Number of exacerbations in preceding 12 months, mean ± SD/median (interquartile range)	3.1 ± 2.3/2 (2–4)
Number of exacerbations in preceding 12 months according to severity, mean ± SD	
Mild	0.5 ± 1.2
Moderate	2.3 ± 1.9
Severe	0.4 ± 0.6
FEV_1 after bronchodilator use (% predicted), mean ± SD	46.4 ± 15.2

N = total number of subjects, SD = standard deviation. BODE index = body mass index, airflow obstruction, modified Medical Research Council Dyspnoea Scale, exercise capacity index. COPD = chronic obstructive pulmonary disease. FEV_1 = forced expiratory volume in 1 s. GOLD = Global

Initiative for Chronic Obstructive Lung Disease. TL_{CO} = transfer factor of the lung for carbon monoxide.

During the first year of follow-up, a total of 355 acute exacerbations were recorded and 47.2% of patients had more than two exacerbations (Table S1). The mean exacerbation rate was 3.04 (95% CI 2.63–3.50) per patient-year. Most exacerbations (304, 85.6%) were moderate in severity, 31 (8.7%) were mild, and 20 (5.6%) were severe.

The rate of sputum collection was high at stable (79.1%) and exacerbation states (91.3%) (Figure 1). An antibiotic was administered before sputum collection in 1.1% (11 of 959) stable and 8.6% (28 of 324) exacerbation samples. Most AECOPD samples (71%) were collected within two days of the start of exacerbation symptoms. Overall, 48.9% of stable-state and 58.8% of exacerbation-state samples were positive for bacteria by culture (Figure 2A, Table S2). For PCR-detected bacteria, corresponding proportions were 56.6% at stable state and 67.1% at exacerbation (Figure 2B; Table S2). The most common species isolated were NTHi*, Streptococcus pneumoniae*, and *Moraxella catarrhalis*. For a small proportion of samples (10.6%) with cultured isolates phenotypically identified as *H. influenzae*, the lgtC/P6 PCR did not confirm the presence of true *H. influenzae* (see appendix). Of 230 sputum samples where *S. pneumoniae* was identified phenotypically by culture and optochin sensitivity testing, 147 tested negative by PCR. Preliminary bacterial genome sequencing on a subset of isolated strains identified phenotypically as *S. pneumoniae* showed that the colonies belonged to the pneumococcus-like viridans group and were mainly *S. pseudopneumoniae* (data not shown).

The proportion of samples that were NTHi or *M. catarrhalis*-positive was higher at exacerbation than stable state (Figures 2A and B). NTHi was highly prevalent at exacerbation; 70.3% (95% CI 60.4–79.0) of patients had at least one exacerbation that was NTHi-positive by PCR (56.7% [46.7–66.4] by culture) (Table S3).

The proportion of sputum samples positive for at least one virus increased from 13.6% at stable state to 41.3% at exacerbation (Figure 2C, Table S2). The most common species

isolated was HRV, detected in 6.2% and 23.0% of stable and exacerbation samples, respectively. Nearly half (46.5%; 95% CI 36.5–56.7) of patients had at least one exacerbation that was HRV-positive during the year of follow up (Table S4). There was also increased simultaneous bacterial and viral presence at exacerbation compared to stable state when determined by culture (24.9% versus 8.6%) and PCR (29.2% versus 9.1%) (Figure 3).

The distribution of AECOPD cases with sputum samples showed seasonal variations (Figure 4, Figure S1). The percentage of visits where an exacerbation was recorded increased from 20.6% in low season (April to September) to 29.3% in high season (October to March) ($p<0.001$, Table S5), corresponding to an increase from 126 to 194 exacerbations. A similar effect was observed for exacerbations in which bacterial or viral aetiology was detected (15.9% of all visits at low season, 25.3% at high season; $p<0.001$). For exacerbations where no bacteria or viruses were detected, there was no significant difference between low and high seasons (4.6% versus 3.9%, $p=0.513$).

The risk of AECOPD in relation to pathogen detection was examined with conditional logistic regression models stratified by subject, meaning that there was no need to adjust for time-invariant covariates. No statistically significant relationship was found between the presence (by culture or PCR) of *S. pneumoniae*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* and risk of exacerbation. The odds ratio (OR) of experiencing an AECOPD rather than being in stable state was significant for *M. catarrhalis* detected by culture (5.09 [95% CI 2.76–9.41], Figure 5A) and PCR (3.52 [2.12–5.83], Figure 5B). The percentage of visits in which *M. catarrhalis* was detected increased from low to high season (4.9% versus 8.7%; $p=0.005$, Table S6), suggesting increased *M. catarrhalis* presence during the high season may partly account for the seasonal increase in exacerbations. However, no additional interaction with season was detected, suggesting that the strength of effect of *M. catarrhalis* infection on the likelihood of AECOPD was constant throughout the year.

For NTHi detected by culture, but not by PCR, a statistical interaction was detected with

season, so ORs were calculated for the high and low seasons. The OR for exacerbation was significant (3.04 [95% CI 1.80–5.13]) in high season but not significant in low season (1.22 [0.68–2.22]) (Figure 5A). This suggested that susceptibility to exacerbation in the presence of NTHi is greater in high season, which is when NTHi detection is also higher (35.6% during high season versus 28.6% during low season, p=0.010, Table S6). Interestingly, the effect of NTHi presence detected by PCR (but not by culture) on the likelihood of AECOPD was found to be higher when HRV was also detected (OR 5.18 [95% CI 1.92–13.99]) than when HRV was absent (OR 1.69 [1.10–2.59]; interaction p=0.031) (Figure 5B). The percentage of visits with any virus detected was statistically significantly higher at high season (p<0.001), as was the percentage of visits in which any virus other than HRV was detected (p=0.002), but this was not the case when considering HRV presence only (Table S6). ORs for exacerbation were significant in the presence of HRV or any other virus (Figures 5A and B), with no statistical interaction with season. This suggests that, although viral infection rates varied overall with season, the risk of a particular viral detection event being associated with an exacerbation did not differ with the time of year, except perhaps for HRV because of the higher prevalence of NTHi during high season.

We also explored the effect of bacteria detected as new occurrences on the likelihood of experiencing an AECOPD. The OR of being in an exacerbation rather than stable state was significant for new occurrences of NTHi (detected by culture), *M. catarrhalis* (culture and PCR), and HRV or other viruses (Figures 5A and B). No interaction with season was found. The percentage of visits with new NTHi occurrences detected by culture was increased during high season compared to low season (11.8% versus 8.5%, p=0.043, Table S6).

To test the robustness of these results, analyses were also performed on the cohort of subjects who had a sputum sample taken and measured on each stable and exacerbation visit (complete cases only) or the cohort of subjects that excluded those without a tested sputum sample at the previous visit. The results obtained from these analyses were similar to those of the primary analyses (data not shown).

Bacterial load data suggested that NTHi and *M. catarrhalis* loads at exacerbation tended to be higher during high season compared to low season (p=0.015 and 0.048, respectively), but the average difference was less than a factor of 10 (Figure S2). For stable visits, and for *S. pneumoniae* or HRV load, no statistically significant differences were found.

DISCUSSION

In this prospective, observational cohort study, repeated measures of both bacterial and viral infection were taken over one year from patients with well-characterised COPD. Identification of *M. catarrhalis* and NTHi, but no other bacteria, were associated with a heightened risk of exacerbation. There was an additional risk of exacerbation from October to March associated with NTHi infection driven by both significantly more frequent detection and a greater specific risk of exacerbation in comparison with low season. With *M. catarrhalis,* a significant seasonal pattern of detection was also seen but, in contrast to NTHi, the risk of exacerbation when *M. catarrhalis* was detected did not differ with the time of year. Interestingly, no seasonal effect was detected on AECOPD risk with new occurrences of bacterial pathogens (NTHi or *M. catarrhalis*), suggesting that longer term colonisation with NTHi may contribute to seasonal susceptibility to AECOPD rather than its acquisition. Although viral infection rates overall were highly seasonal, the prevalence of HRV infection was more evenly spread throughout the year. The study also highlighted an interaction between HRV and NTHi infection and the risk of AECOPD, and that bacterial loads at exacerbation were higher in the period that included winter. These results provide novel insights into the dynamics of infection in COPD, suggesting that exacerbations are associated with seasonal and non-seasonal infections. The seasonal burden of infective AECOPD appears to be driven partly by the effect of acute HRV infection on a background of chronic NTHi infection, an effect size that has been quantified for the first time in the AERIS study. This may have more of an impact on the seasonality of AECOPD than acquisition of NTHi or *M. catarrhalis* throughout the year.

The exacerbation frequency for the cohort of 127 patients during the study year was similar

to that in the year before enrolment, suggesting a relatively constant phenotype. This was in line with other studies that identified the number of exacerbations in the previous year as a significant predictive factor.[10-13] Most exacerbations were moderate in severity, possibly because patients were monitored closely via daily electronic diary cards, enabling early capture and rapid treatment of exacerbation events.

At stable state, the most prevalent bacterial species identified were NTHi, *M. catarrhalis*, and *S. pneumoniae*, consistent with other studies.[14-16] The prevalence of NTHi and *M. catarrhalis* increased at exacerbation. Previous studies also reported an association between exacerbation and a shift in the microbiome towards enrichment of Proteobacteria.[17-19] In our study, there was a discrepancy between culture and PCR identification of *S. pneumoniae*, with 64% of colonies identified as *S. pneumoniae* by culture subsequently identified by PCR and molecular methods as predominantly *S. pseudopneumoniae*. This was unexpected but may be explained by the specificity of the target *lytA* gene used in our PCR method; other target genes (*ply* or *psaA*) are less specific in differentiating streptococcal species.[20] We found that *S. pneumoniae* and closely-related species did not contribute to exacerbation occurrence but cannot exclude the possibility of a role at a later stage, since AECOPD events might have been identified and treated early.

Among the viral species identified, HRV was most prevalent, with 23% of sputum samples positive at exacerbation. Almost half of patients had at least one HRV-positive exacerbation. The differential detection rate for enterovirus between stable and exacerbation samples may be related to the biology of infection; enteroviruses other than HRV might also have been present, causing less symptomatic AECOPD than HRV subtypes. Other viruses were detected infrequently but with prevalences that tended to be higher at exacerbation than stable state, as reported previously.[21] This supports evidence suggesting that respiratory viruses initiate a large proportion of acute exacerbations.[22, 23] We also noted a large increase in bacterial and viral coinfection at exacerbation compared to stable state. In various studies, this coinfection has been associated with more marked lung function

impairment and longer hospitalisations than only bacterial or viral infections.[6, 7, 24, 25]

Post hoc logistic regression analyses revealed differences among the pathogens in terms of mechanism of effect in AECOPD. For cases in which pathogens were a new occurrence at exacerbation, *M. catarrhalis* had an effect that was independent of viral and seasonal effects. This suggests an association between acquisition of *M. catarrhalis* strains and exacerbation.[26] In contrast, new NTHi occurrences appear to play a less important role and analyses of its presence showed an interaction with HRV and an effect in AECOPD that is partly explained by season. It is likely that complex mechanisms exist for the associations among NTHi, HRV, and season. Bacterial colonisation is associated with airway inflammation in stable COPD, which is likely to increase the likelihood of exacerbation and may also increase the risk of lower airway viral infection.[16] There is also evidence of interactions between lower airway bacterial and rhinoviral infection in AECOPD and stable COPD.[6, 18, 27] The exact mechanisms by which HRV or other viral infections lead to a change in the balance of microbiota and host immunity may contribute to increased detection rates of NTHi, inflammation, and exacerbation. Acute viral infection can disrupt host immunity by affecting epithelial barrier function,[28] limiting macrophage phagocytosis and directly impacting on innate responses to bacteria.[29] Whilst this may lead to a more permissive niche for outgrowth of more opportunistic pathogens, such as NTHi, it is likely that the interplay of bacterial, viral, and host interactions is more complex than such a description of cause and effect. The airway microbiome has been described in a number of recent studies,[30] with COPD characterised by a loss of bacterial diversity and dominance of certain species. Furthermore, experimental studies highlight the effects of airway bacterial pathogens on responses to subsequent viral infection.[31-33] Indeed, in our own data, viruses were detectable in both stable and exacerbation states, although rates varied. Hence, the dynamics driving the ultimate consequence of this abnormal host pathogen interaction, an exacerbation, will require yet further study using molecular tools and sophisticated modelling to assess the impacts of microbial communities and acute infections.

Few other studies have addressed the complexity of the microbiological components of COPD or employed real-time electronic tracking of symptoms to identify AECOPD and potential aetiological triggers. Moreover, other longitudinal studies have not examined viral and bacterial infection rates over the same period of time.[5, 15, 34-36] Also, the most current technology was used to identify respiratory microorganisms including highly sensitive PCR, increasing both the detection rates and specificity for identifying key pathogens. The selection of patients with a history of exacerbations limits the generalisability of the data to a more heterogeneous population and additional studies are required to determine if our results can be extrapolated to other COPD phenotypes. Moderate exacerbations were largely represented probably because close monitoring and early therapeutic intervention are likely to have led to an attenuation in overall severity. We also acknowledge the potential for missing variable results, although sputum sampling rates were very high for a study of this nature. Moreover, results are only presented for subjects followed for the first year of this two-year study. While further analysis of longer term data is planned, the subject populations of year 1 and year 2 were dissimilar because of the number of drop outs that occurred during the study (105 subjects completed one year and 85 completed two years). The protocol was designed to maintain the cohort, with recruitment continuing up to the beginning of the second year to ensure an adequate number of individual exacerbations was captured. Hence, whilst the cohort was relatively stable for the first year, it changed during the second. Attrition of subjects and sample points from this relatively intensive study affected the cohort make up and hence the second year results. In addition, with the changing pattern of clinical practice in the second year of the study, some of the more frequent exacerbators were commenced on long-term macrolide therapy, which would have altered airway microbial patterns. For this longitudinal analysis of repeated samples within individuals, the first year dataset was therefore selected.

Another limitation of the study is that exact timing of sputum collection was not recorded in relation to initiation of antibiotic or oral corticosteroid treatment following AECOPD onset. As

a consequence, we cannot exclude the possibility that initiation of treatment during AECOPD could have had an impact on the bacterial culture results and, to a lesser extent, PCR results. However, the number of patients concerned is likely to be small because sputum collection occurred within two days of the start of AECOPD symptoms for most patients. Bacterial or viral pathogen detection was not possible in patients who were unable to produce sputum. We therefore cannot exclude the possibility that infection rates in this group were different to those in patients who did provide sputum samples. This could not be adjusted for in the model and highlights the need for non-invasive means of measuring biomarkers which predict infectious aetiology in COPD patients.

We also cannot exclude the possibility of confounding of findings by participant characteristics that varied over time. We consider this unlikely since patient characteristics associated with COPD, such as disease severity, usually do not vary significantly over a one-year period and other characteristics, such as inflammatory factors, are closely related to the aetiology of the disease and cannot be adjusted for in the model. The results presented on seasonality were obtained by post hoc analyses of association. Studies are needed to confirm these results and describe more precisely the effect of airway bacterial infection in relation to viruses on AECOPD during the year.

In conclusion, the AERIS study has identified that NTHi, *M. catarrhalis*, and HRV infections have a key role in AECOPD and for the first time has shown that seasonal risk of exacerbation differs depending on the bacterial pathogen concerned. The association with longer term NTHi infection, alone and in combination with HRV infection, may be key to the seasonality of AECOPD. Understanding the mechanisms driving the complex interactions between chronic bacterial airway infection and seasonal exposure to viruses will provide insights necessary to develop potential therapies to prevent AECOPD.

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J Alnajar, R Anderson, E Aris, WR Ballou, A Barton, S Bourne, M Caubet, SC Clarke, D Cleary, C Cohet, N Coombs, K Cox, J-M Devaster, V Devine, N Devos, E Dineen, T Elliott, R Gladstone, S Harden, J Jefferies, V Kim, S Mesia Vela, P Moris, K Ostridge, TG Pascal, M Peeters, S Schoonbroodt, KJ Staples, A Tuck, L Welch, V Weynants, TMA Wilkinson, AP Williams, N Williams, C Woelk, M Wojtas, S Wootton. All members of the AERIS Study Group were involved in the planning, conduct, and/or reporting of the work described in the article.

Author contributions

JMD, TGP, SB, SW, SS, AT, SCC, AW, and TMAW conceived and designed the study. EA, JMD, TGP, MP, SS, SB, SW, AT, NW, KO, KJS, SCC, VK, AW, and TMAW collected or generated the data. EA, JMD, TGP, MP, SB, SW, NW, KO, KJS, SCC, VK, AW, and TMAW analysed or interpreted the data. TMAW, EA, SB, SCC, MP, and JMD are members of the core writing team. TMAW, EA, SB, SCC, MP, KJS, AW, SW, and JMD are members of the AERIS publication steering committee. All authors contributed substantially to the development of the manuscript and approved the final version.

Declaration of interests

TMAW has received reimbursement for travel and meeting attendance from Boehringer Ingelheim and AstraZeneca, outside of the submitted work. SB received grants and assistance in travel to conferences from GSK outside of the submitted work. SCC received a grant from Pfizer outside of the submitted work. KJS received grants from Asthma UK (08/026) and BMA HC Roscoe Award outside of the submitted work, and he has a patent PCT/GB2010/050821 "Ex Vivo Modelling of Therapeutic Interventions" pending. EA, JMD, SS and TGP are employees of the GSK group of companies. MP was an employee of the GSK group of companies at the time the study was conducted. EA, JMD, SS and TGP hold shares/restricted shares in the GSK group of companies. KJS, VK, NW, KO, SW, and TMAW received an institutional grant from the GSK group of companies to conduct this study. AW and AT declare no conflict of interest.

Funding source

The study funder, GlaxoSmithKline Biologicals SA, designed the study in collaboration with the investigators, and coordinated collection, analysis, and interpretation of data. The investigators obtained data and cared for the study participants. The authors had full access to all data in the study, contributed to the writing of the report, and had final responsibility for the decision to submit for publication.

Figure legends

***Figure 1*: Flow chart of patients and sputum sampling in the study**

***Figure 2*: Percentage of culture-positive or PCR-positive sputum samples at stable state and exacerbation state (full cohort, year 1)**

A. Percentage of sputum samples positive for bacteria by culture

B. Percentage of sputum samples positive for bacteria by PCR*

* Group A streptococcus (*Streptococcus pyogenes*) was not detected.

C. Percentage of sputum samples positive for virus by PCR

***Figure 3:* Percentage of sputum samples that contained more than one bacterial or viral species by culture or PCR at stable state and exacerbation (full cohort, year 1)**

N = Number of samples identified in each category.

***Figure 4*: Seasonal distribution of AECOPD cases with sputum samples: total number and number of cases positive by PCR for NTHi, *M. catarrhalis*, HRV, or any viral species, and cases negative for bacteria and viruses (full cohort year 1; month of follow-up considered regardless of year)**

***Figure 5:* Effect of presence or new occurrence (detection after negative sputum sample at previous visit) of bacteria (NTHi or *M. catarrhalis* [Mcat]) or HRV on the odds of experiencing an AECOPD rather than being in stable state (full cohort, year 1).**

Odds ratios (ORs) for AECOPD occurrence were obtained from conditional logistic models.

A. ORs for AECOPD occurrence obtained from conditional logistic models containing bacteria culture data, HRV, all viruses other than HRV, and season (high season, October to March; low season, April to September), stratified by subject. The effect of NTHi presence is provided for low and high seasons because the interaction between NTHi and season was statistically significant (p=0.010; more than 100 observations in each combination of factors). The effect of presence of Mcat, HRV, or other viruses did not differ between low and high seasons. The effect of new NTHi, Mcat, HRV, or other virus occurrences did not differ between low and high seasons.

B. ORs for AECOPD occurrence obtained from conditional logistic models containing bacteria PCR data, HRV, other viruses, and season, stratified by subject. The effect of NTHi presence is provided in the presence or absence of HRV, and the effect of HRV in the presence or absence of NTHi, because the interaction between NTHi and HRV was statistically significant (p=0.031; more than 50 observations in each combination of factors). The effect of Mcat presence did not differ in the presence or absence of NTHi, HRV, or season. The effect of new Mcat, HRV, or other virus occurrences did not differ between high and low seasons. The effect of new NTHi occurrences was not statistically significant.

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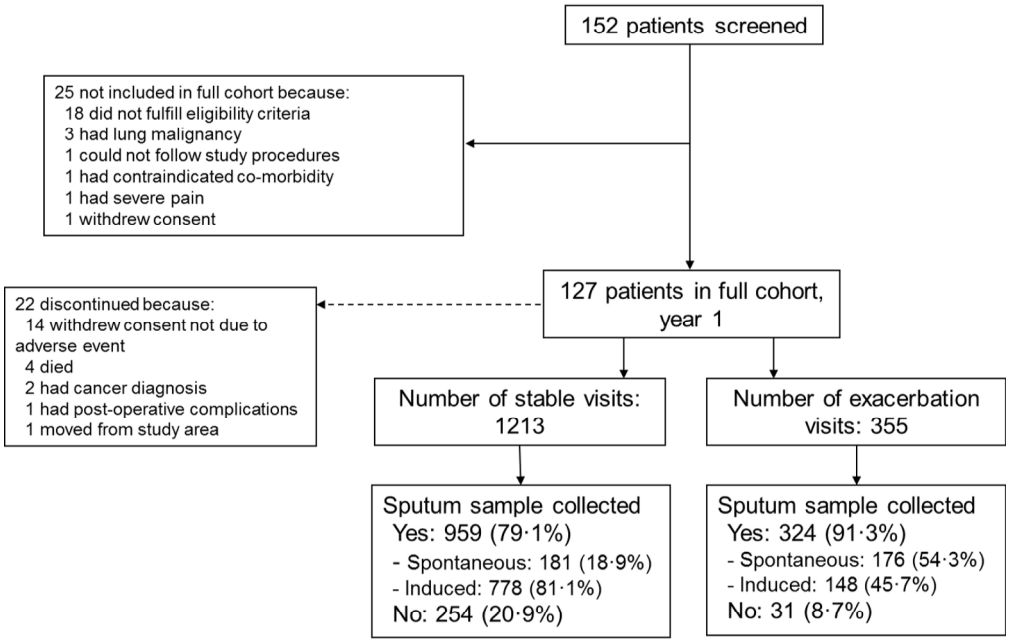

Figure 1: Flow chart of patients and sputum sampling in the study
Figure 1
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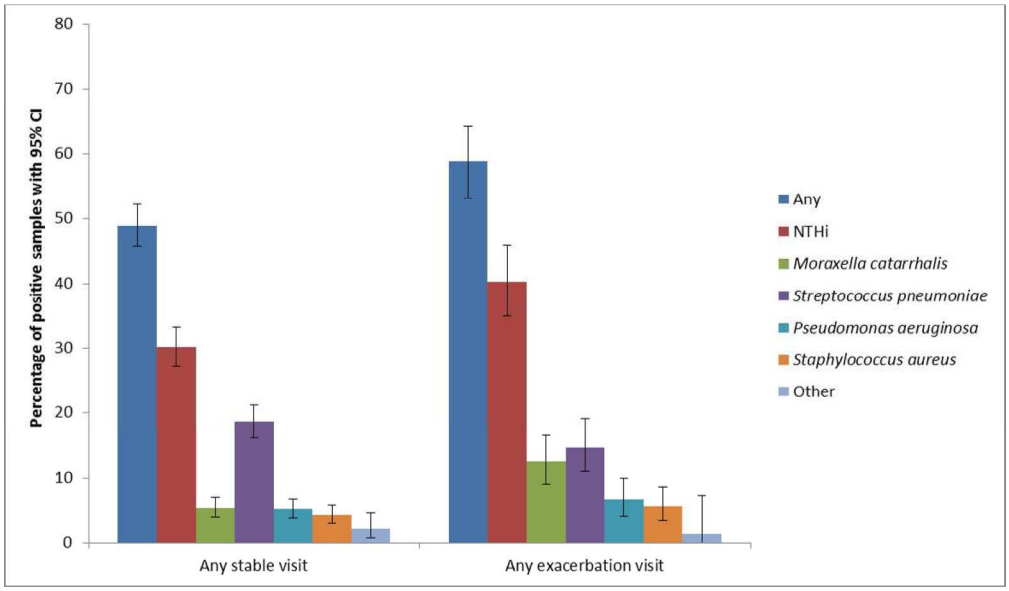
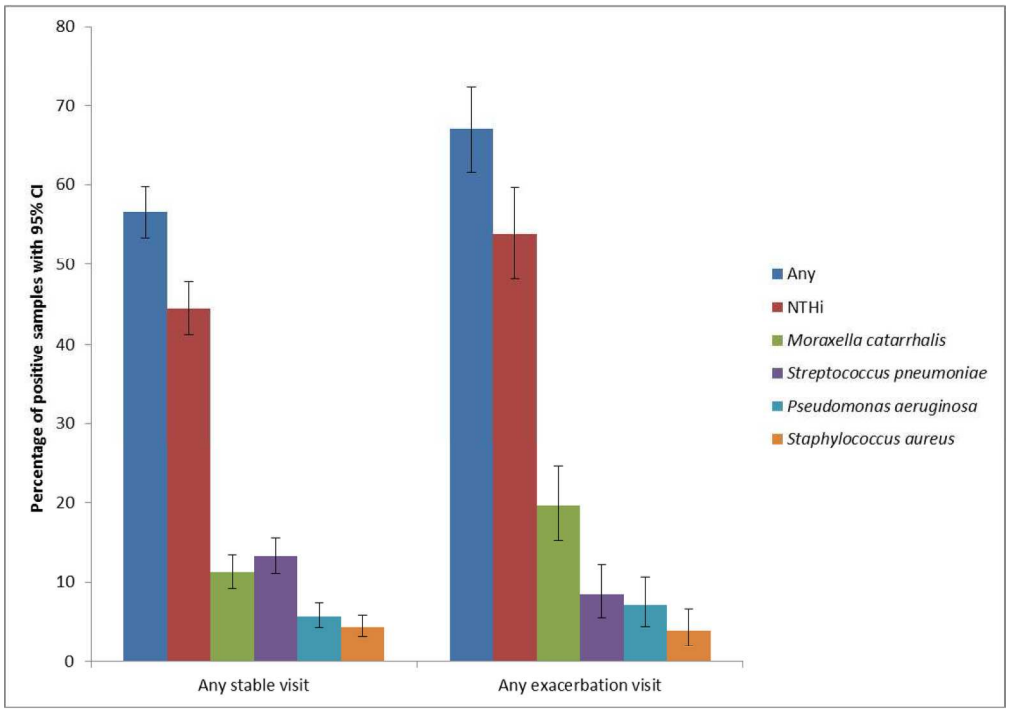


Figure 2: Percentage of culture-positive or PCR-positive sputum samples at stable state and exacerbation state (full cohort, year 1.) ┬ A. Percentage of sputum samples positive for bacteria by culture.
Figure 2A
215x166mm (300 x 300 DPI)



B. Percentage of sputum samples positive for bacteria by PCR*. ‼ ┼ *Group A streptococcus (Streptococcus pyogenes) was not detected.‼ ┼

Figure 2B
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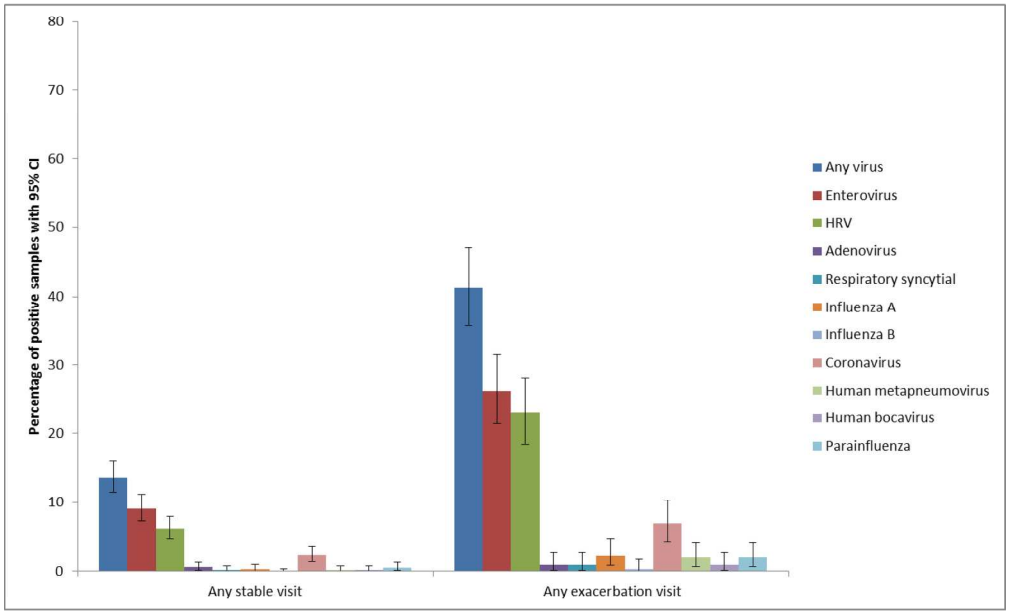

C. Percentage of sputum samples positive for virus by PCR
Figure 2C
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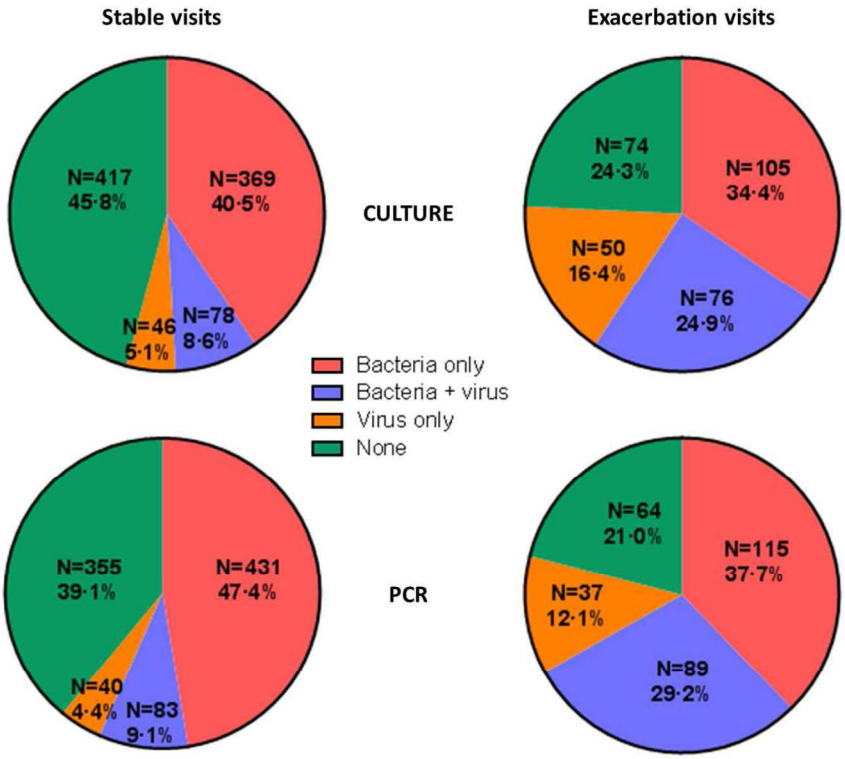

Figure 3: Percentage of sputum samples that contained more than one bacterial or viral species by culture or PCR at stable state and exacerbation (full cohort, year 1) ‼ ┼ N = Number of samples identified in each category. ‼ ┼

Figure 3
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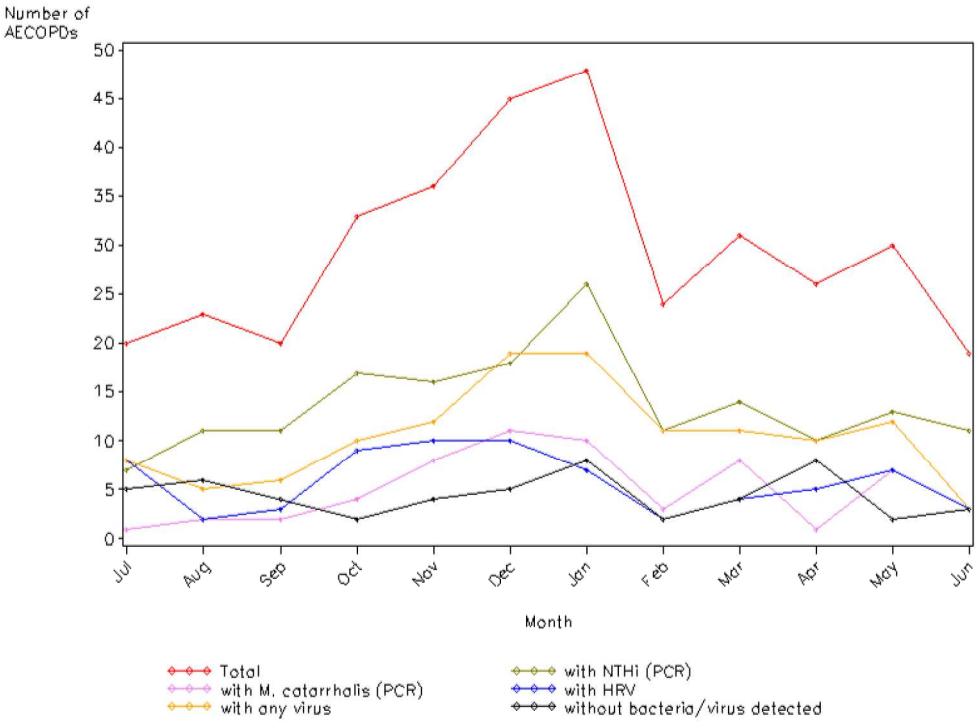

Figure 4: Seasonal distribution of AECOPD cases with sputum samples: total number and number of cases positive by PCR for NTHi, M. catarrhalis, HRV, or any viral species, and cases negative for bacteria and viruses (full cohort year 1; month of follow-up considered regardless of year)
Figure 4
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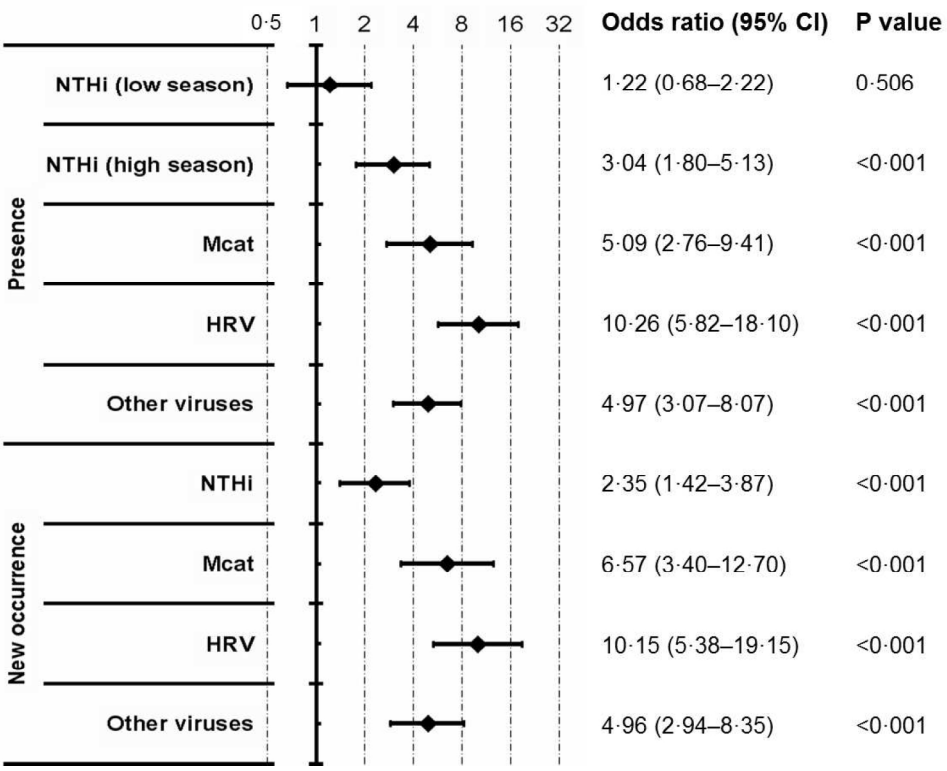
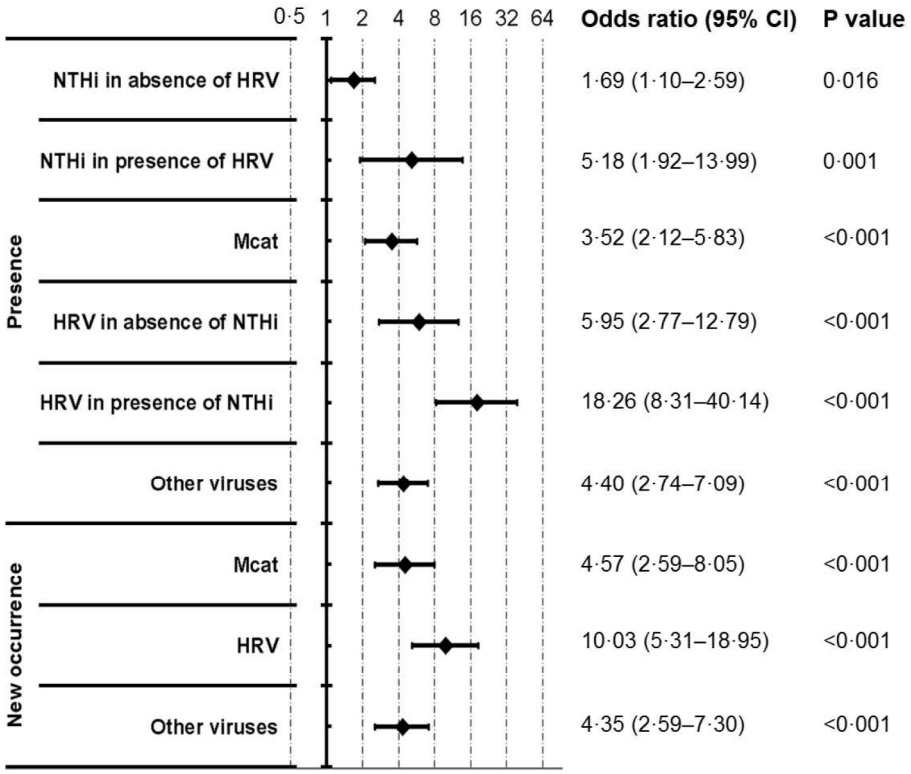


Figure 5: Effect of presence or new occurrence (detection after negative sputum sample at previous visit) of bacteria (NTHi or M. catarrhalis [Mcat]) or HRV on the odds of experiencing an AECOPD rather than being in stable state (full cohort, year 1). Odds ratios (ORs) for AECOPD occurrence were obtained from conditional logistic models. ‼ ┼ 5A. ORs for AECOPD occurrence obtained from conditional logistic models containing bacteria culture data, HRV, all viruses other than HRV, and season (high season, October to March; low season, April to September), stratified by subject. The effect of NTHi presence is provided for low and high seasons because the interaction between NTHi and season was statistically significant (p=0.010; more than 100 observations in each combination of factors). The effect of presence of Mcat, HRV, or other viruses did not differ between low and high seasons. The effect of new NTHi, Mcat, HRV, or other virus occurrences did not differ between low and high seasons.‼ ┼

Figure 5A

215x166mm (300 x 300 DPI)



5B. ORs for AECOPD occurrence obtained from conditional logistic models containing bacteria PCR data, HRV, other viruses, and season, stratified by subject. The effect of NTHi presence is provided in the presence or absence of HRV, and the effect of HRV in the presence or absence of NTHi, because the interaction between NTHi and HRV was statistically significant (p=0.031; more than 50 observations in each combination of factors). The effect of Mcat presence did not differ in the presence or absence of NTHi, HRV, or season. The effect of new Mcat, HRV, or other virus occurrences did not differ between high and low seasons. The effect of new NTHi occurrences was not statistically significant.

Figure 5B
215x166mm (300 x 300 DPI)

Acute Exacerbation and Respiratory InfectionS in COPD (AERIS): A prospective, observational cohort study of the dynamics of airway pathogens and the seasonal aetiology of exacerbations in chronic obstructive pulmonary disease

Tom MA Wilkinson, Emmanuel Aris, Simon Bourne, Stuart C Clarke, Mathieu Peeters, Thierry G Pascal, Sonia Schoonbroodt, Andrew C Tuck, Viktoriya Kim, Kristoffer Ostridge, Karl J Staples, Nicholas Williams, Anthony Williams, Stephen Wootton, Jeanne-Marie Devaster, on behalf of the AERIS Study Group

Supplementary appendix

METHODS

Inclusion and exclusion criteria

All subjects must satisfy all the following criteria at study entry:

- Subjects who the investigator believes can and will comply with the requirements of the protocol.
- Written informed consent obtained from the subject.
- Male or female subjects between, and including, 40 and 85 years of age, at the time of consent.
- Subjects with confirmed diagnosis of COPD (based on post-bronchodilator spirometry)[1] with forced expiratory volume of air expired in 1 second (FEV_1) of ≤80% of predicted normal and FEV_1/forced expiratory vital capacity <0·7.
- Subjects have moderate, severe, or very severe COPD, according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging.[1]
- Subjects have a current or prior history of ≥10 pack-years of cigarette smoking. Former smokers are defined as those who have stopped smoking for at least 6 months. Number of pack years = (number of cigarettes per day/20) x number of years smoked.
- Subjects present a documented history of ≥1 exacerbation requiring antibiotics and/or oral corticosteroids or hospitalization in the previous 12 months. Subjects with recent COPD exacerbations, in stable condition, and having stopped antibiotics, can be enrolled one month post exacerbation.

The following criteria should be checked at the time of study entry. If any exclusion criterion applies, the subject must not be included in the study:

- Subject has a confirmed diagnosis of asthma (as only cause of obstructive respiratory disorder), cystic fibrosis, pneumonia risk factors or other respiratory disorders (e.g. tuberculosis, lung cancer).
- Subjects having undergone lung surgery.
- Subject has a α-1 antitrypsin deficiency as underlying cause of COPD.
- Subject who experienced a moderate or severe COPD exacerbation not resolved at least 1 month prior to enrolment visit and at least 30 days following the last dose of oral corticosteroids (subjects can be enrolled when their AECOPD or pneumonia has resolved).
- Subject using any antibacterial, antiviral, or respiratory investigational drug or vaccine up to 30 days prior to the enrolment visit.
- Subject has other conditions that the principal investigator judges may interfere with the study findings, such as:
  - Subject at risk of non-compliance or unable to comply with the study procedures.
  - Evidence of alcohol or drug abuse.
- Women who are pregnant or lactating or are planning on becoming pregnant during the study.

Procedures

AECOPD was defined as worsening of at least two major symptoms (dyspnoea, sputum volume, and sputum purulence) or worsening of at least one major symptom and one minor symptom (wheeze, sore throat, cold

symptoms, cough, and fever without other cause).[2] An exacerbation was considered mild if self-managed by the patient using inhaled therapy, moderate if it required treatment with oral corticosteroids or antibiotics, and severe if the patient required hospitalisation or a home care intervention.[3]

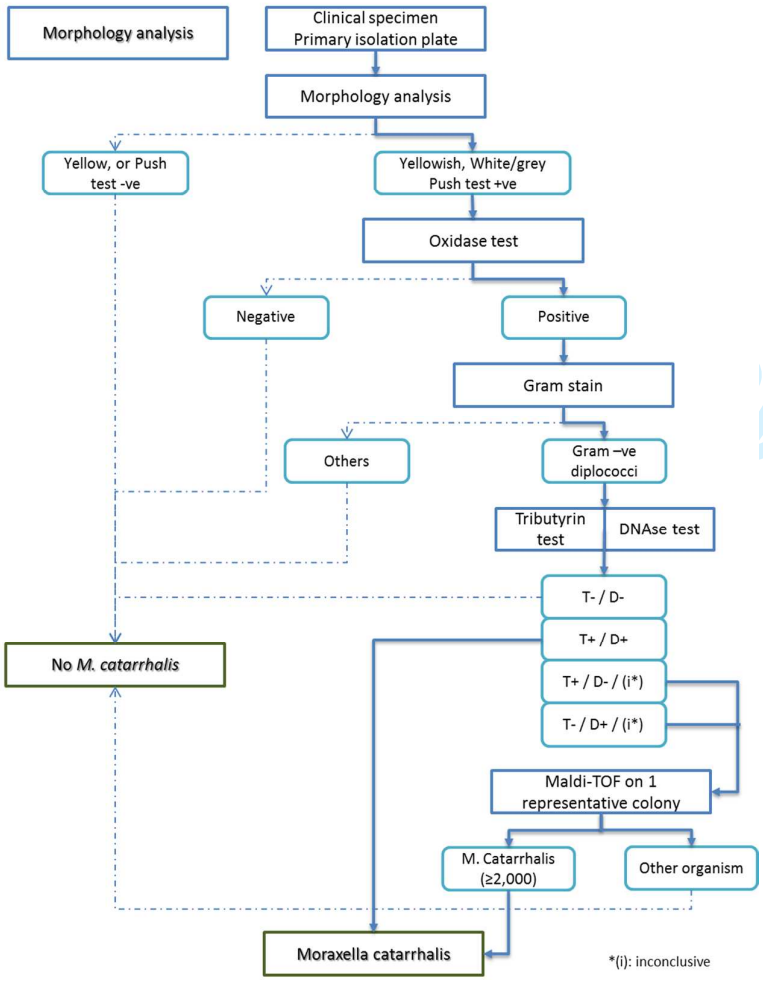
Sputum samples were collected at study entry, monthly, and at exacerbation. Within two hours of expectoration, sputum plugs were separated from saliva using sterile forceps. Samples were kept at room temperature and sent to the Public Health England laboratory for culture-based microbiology.

Sputum samples were also processed for the detection of viruses, including HRV, respiratory syncytial virus, influenza virus, parainfluenza virus, human metapneumovirus, adenovirus, human bocavirus, and coronavirus. The qualitative nucleic acid multiplex test used (xTAG® Respiratory Viral Panel Fast v2; Luminex, Austin, TX, USA) is described below along with methods for calculating bacterial or viral load with the other PCR assays used.

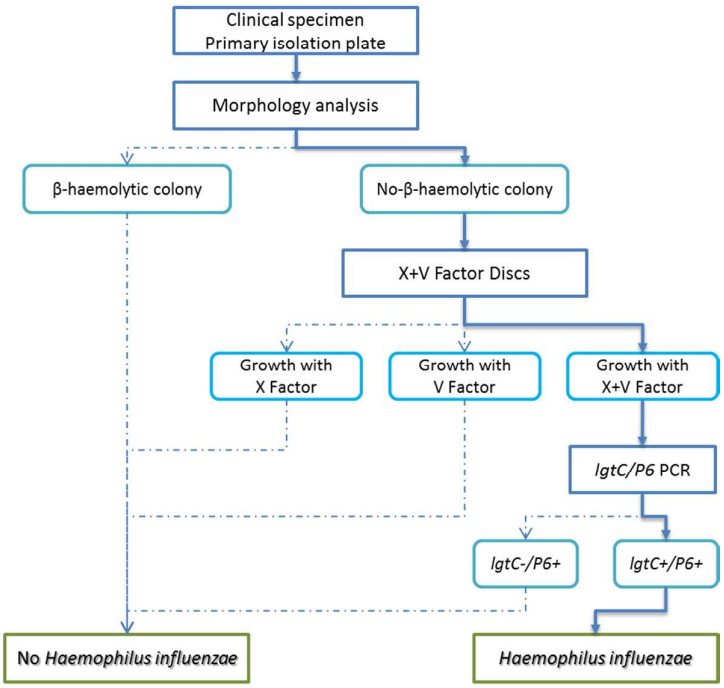
Potential bacterial respiratory pathogens, including *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were identified using conventional culture techniques and by PCR. Both techniques were used because culture detects only viable bacteria and has been the gold standard in the research of bacteria in COPD, while PCR has higher sensitivity but background signals could have a diluting effect.[4]

The method for culture of sputum samples was in accordance with Public Health England's UK Standards for Microbiology Investigations.[5] Bacterial isolates phenotypic identification steps were slightly modified as represented in the following figures for *M. catarrhalis*, *H. influenzae* and *S. pneumoniae.*

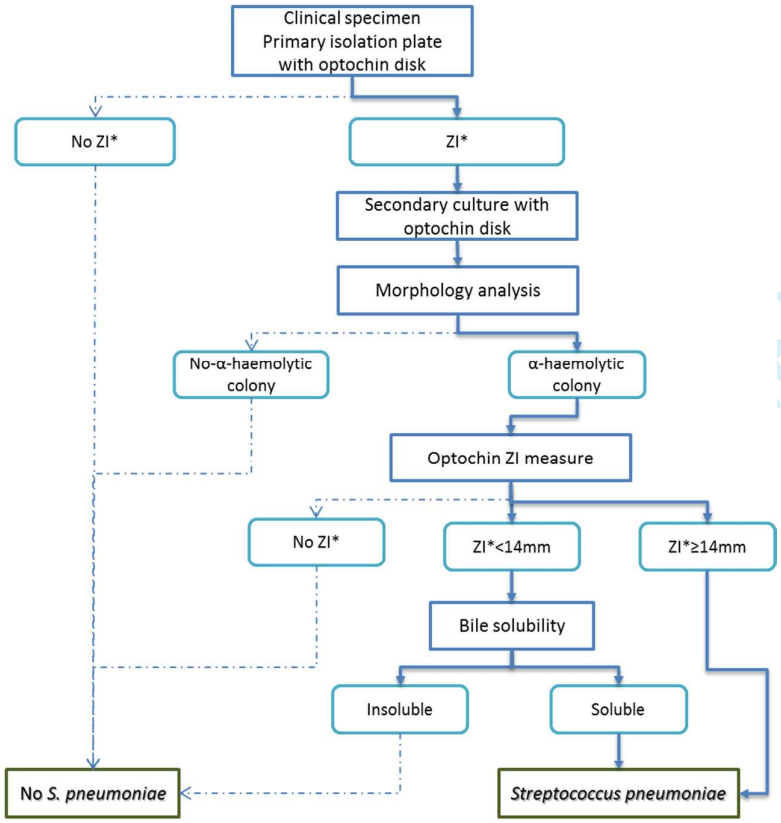
Identification scheme for *Moraxella catarrhalis*



Identification scheme for *Haemophilus influenzae*



Identification scheme for *Streptococcus pneumoniae*



*ZI: zone of inhibition

For PCR, nucleic acids were extracted using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics), as per the manufacturer's instructions. A triplex real-time quantitative PCR assay was used for the detection and quantification of the lipo-oligosaccharide glycosyltransferase encoding gene (*lgtC*) of *Haemophilus influenzae*, the CopB outer membrane protein encoding gene (*copB*) of *M. catarrhalis*, and the autolysin encoding gene (*lytA*) of *S. pneumoniae*. Noteworthy for the latter, the sequences of the primers and probe correspond to the *lytA*-CDC assay.[6]

The presence of *Streptococcus pyogenes*, *S. aureus*, and *P. aeruginosa* was determined using a triplex real-time PCR assay targeting conserved regions of the CDS23 gene, the clumping factor A encoding gene (*clfA*), and the GDP mannose dehydrogenase encoding gene (*algD*), respectively.

The concentration of bacterial DNA in each sample, expressed in copy/mL, was inferred from the calibration curve (made of serial dilutions of a plasmid containing the sequences targeted by the PCR assays) present in each PCR plate and corrected against the dilution factors at each step of the process (DNA extraction and PCR reaction). Positivity thresholds were used for each PCR target. They were set at the limit of detection defined during characterisation of the technical performance of the PCR assays, corresponding to 2000, 15000, 12875, 5000, 3375, and 2750 copies/mL, respectively for *H. influenzae*, *M. catarrhalis*, *S. pneumoniae, S. aureus*, *P. aeruginosa*, and *S. pyogenes*. Further details will be presented in a separate paper.

Isolates initially identified as *H. influenzae* by bacteriological methods were later retested by PCR, targeting the glycosyltransferase (*lgtC*) and outer membrane protein P6 (P6) encoding genes[7] to differentiate *H. influenzae* from *H. haemolyticus*. It became clear that for 10·6% of the samples, the isolates identified as *H. influenzae* by conventional microbiological methods were in fact *H. haemolyticus*.[8,9] Also, genetic analysis of the capsule locus indicated that more than 99% of *H. influenzae* isolates were non-typeable (NTHi).

The xTAG® Respiratory Viral Panel (RVP) Fast v2 (Luminex) is a qualitative nucleic acid multiplex test intended for the simultaneous detection and identification of multiple respiratory virus nucleic acids in respiratory specimens.[10] It detects influenza A, including subtypes of influenza A (H1 and H3), and distinguishes between 2009 H1N1 and other H1N1 (seasonal) strains, influenza B, respiratory syncytial virus, human metapneumovirus, parainfluenza virus 1–4, coronavirus (OC43, 229E, NL63, HKU1), rhinovirus/enterovirus, adenovirus, and bocavirus.

A quantitative real-time PCR (RT-PCR) assay was used for the detection and quantification of a fragment of a conserved region of the 5' noncoding region of rhinovirus[11] in samples displaying a positive signal for rhinovirus/enterovirus by xTAG® RVP Fast v2.

The concentration of rhinovirus RNA in each sample, expressed in copies per mL, was inferred from the calibration curve (made of serial dilutions of an *in vitro* transcript containing the sequence targeted by the RT-PCR assay) present in each RT-PCR plate and corrected against the dilution factors at each step of the process (nucleic acid extraction and RT-PCR reaction).

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Table S1. Number of exacerbations recorded during the study (full cohort year 1).

	Acute exacerbation severity			
	Any	**Mild**	**Moderate**	**Severe**
Number of exacerbations	355	31	304	20
Exacerbation rate per patient-year[a], mean (95% CI)	3·0 (2·6–3·5)	0·3 (0·2–0·4)	2·6 (2·2–3·0)	0·2 (0·1–0·4)
Number of exacerbations	Number of subjects (N=127)			
0	19 (15·0%)	99 (78·0%)	27 (21·3%)	113 (89·0%)
1	30 (23·6%)	25 (19·7%)	29 (22·8%)	11 (8·7%)
2	18 (14·2%)	3 (2·4%)	20 (15·7%)	1 (0·8%)
3	18 (14·2%)	0 (0%)	18 (14·2%)	1 (0·8%)
4	9 (7·1%)	0 (0%)	8 (6·3%)	1 (0·8%)
5	15 (11·8%)	0 (0%)	12 (9·4%)	0 (0%)
6	8 (6·3%)	0 (0%)	4 (3·1%)	0 (0%)
7	5 (3·9%)	0 (0%)	8 (6·3%)	0 (0%)
>7	5 (3·9%)	0 (0%)	1 (0·8%)	0 (0%)

[a] Negative binomial model. Total exposure time was 117 years.

Table S2. Bacterial and viral pathogen incidence in exacerbation-state sputum samples (full cohort year 1).

	Number stable-state samples/total number samples	Number exacerbation-state samples/total number samples	Rate per patient-year of exacerbations with samples containing pathogen[a] (95% CI)
Bacteria: culture-positive			
Any	466/952 (48·9%)	188/320 (58·8%)	1·59 (1·30–1·95)
NTHi	287/952 (30·1%)	129/320 (40·3%)	1·10 (0·84–1·43)
M. catarrhalis	50/952 (5·3%)	40/320 (12·5%)	0·34 (0·24–0·48)
S. pneumoniae	177/952 (18·6%)	47/320 (14·7%)	0·39 (0·27–0·58)
S. aureus	40/952 (4·2%)	18/320 (5·6%)	0·16 (0·07–0·36)
P. aeruginosa	49/952 (5·1%)	21/320 (6·6%)	0·18 (0·09–0·35)
Bacteria: PCR-positive			
Any	515/910 (56·6%)	206/307 (67·1%)	1·75 (1·43–2·14)
NTHi	405/910 (44·5%)	165/306 (53·9%)	1·41 (1·12–1·77)
M. catarrhalis	102/910 (11·2%)	60/306 (19·6%)	0·50 (0·37–0·69)
S. pneumoniae	120/910 (13·2%)	26/306 (8·5%)	0·22 (0·14–0·35)
S. aureus	40/910 (4·4%)	12/307 (3·9%)	0·10 (0·03–0·32)
P. aeruginosa	52/910 (5·7%)	22/307 (7·2%)	0·19 (0·09–0·39)
Virus			
Any	124/910 (13·6%)	126/305 (41·3%)	1·07 (0·86–1·32)
Enterovirus	83/910 (9·1%)	80/305 (26·2%)	0·68 (0·51–0·89)
Human rhinovirus	56/908 (6·2%)	70/305 (23·0%)	0·59 (0·45–0·78)
Human coronavirus	22/910 (2·4%)	21/305 (6·9%)	0·18 (0·11–0·29)
Influenza virus	5/910 (0·5%)	15/305 (4·9%)	0·07 (0·03–0·14)
Human metapneumovirus	2/910 (0·2%)	6/305 (2·0%)	0·05 (0·02–0·11)
Parainfluenza virus	5/910 (0·5%)	6/305 (2·0%)	0·05 (0·02–0·11)
Adenovirus	6/910 (0·7%)	3/305 (1·0%)	0·03 (0·01–0·08)
Respiratory syncytial virus	2/910 (0·2%)	3/305 (1·0%)	0·03 (0·01–0·08)
Human bocavirus	2/910 (0·2%)	3/305 (1·0%)	0·02 (0·00–0·12)

[a] Negative binomial model. Total exposure time was 117 years.

Table S3. Proportion of patients with at least one sputum sample that was positive for a specific bacterial pathogen (by culture or PCR) at stable and exacerbation states (full cohort year 1).

	Culture (95% CI)		PCR (95% CI)	
	Stable (N=119)	**Exacerbation (N=104)**	**Stable (N=118)**	**Exacerbation (N=101)**
Any bacteria	83·2% (75·2–89·4)	76·9% (67·6–84·6)	91·5% (85·0–95·9)	82·2% (73·3–89·1)
NTHi	63·9% (54·6–72·5)	56·7% (46·7–66·4)	82·2% (74·1–88·6)	70·3% (60·4–79·0)
M. catarrhalis	23·5% (16·2–32·2)	28·8% (20·4–38·6)	39·8% (30·9–49·3)	37·6% (28·2–47·8)
S. pneumoniae	47·9% (38·7–57·2)	27·9% (19·5–37·5)	36·4% (27·8–45·8)	20·8% (13·4–30·0)
S. aureus	15·1% (9·2–22·8)	8·7% (4·0–15·8)	13·6% (8·0–21·1)	5·0% (1·6–11·2)
P. aeruginosa	15·1% (9·2–22·8)	11·5% (6·1–19·3)	13·6% (8·0–21·1)	10·9% (5·6–18·7)

N = number of subjects with culture/PCR results available.

Table S4. Proportion of patients (95% CI) with at least one sputum sample that was positive for a specific viral pathogen at stable and exacerbation states (full cohort year 1).

	Stable (N=118)	Exacerbation (N=101)
Any virus[a]	51·7% (42·3–61·0)	68·3% (58·3–77·2)
Enterovirus	39·0% (30·1–48·4)	48·5% (38·4–58·7)
Human rhinovirus	26·3% (18·6–35·2)	46·5% (36·5–56·7)
Human coronavirus HKU1	2·5% (0·5–7·3)	3·0% (0·6–8·4)
Human coronavirus NL63	6·8% (3·0–12·9)	6·9% (2·8–13·8)
Human coronavirus 229E	1·7% (0·2–6·0)	1·0% (0·0–5·4)
Human coronavirus OC43	5·1% (1·9–10·7)	7·9% (3·5–15·0)
Influenza A virus	2·5% (0·5–7·3)	6·9% (2·8–13·8)
Influenza A H1N1 virus	0% (0·0–3·1)	0% (0·0–3·6)
Influenza A H3N2 virus	1·7% (0·2–6·0)	6·9% (2·8–13·8)
Influenza B virus	0% (0·0–3·1)	1·0% (0·0–5·4)
Human metapneumovirus	1·7% (0·2–6·0)	5·9% (2·2–12·5)
Parainfluenza virus 1	0% (0·0–3·1)	0% (0·0–3·6)
Parainfluenza virus 2	0% (0·0–3·1)	0% (0·0–3·6)
Parainfluenza virus 3	0·8% (0·0–4·6)	4·0% (1·1–9·8)
Parainfluenza virus 4	1·7% (0·2–6·0)	2·0% (0·2–7·0)
Adenovirus	5·1% (1·9–10·7)	3·0% (0·6–8·4)
Respiratory syncytial virus	1·7% (0·2–6·0)	3·0% (0·6–8·4)
Human bocavirus	1·7% (0·2–6·0)	2·0% (0·2–7·0)

[a] Virus detection via the xTAG® Respiratory Viral Panel Fast v2 (Luminex) qualitative nucleic acid multiplex test. For samples displaying a positive signal for rhinovirus/enterovirus, a quantitative real-time PCR assay was used to confirm identification.
N = number of subjects with PCR results available.

Table S5. Seasonality of exacerbation visits overall and according to detection or no detection of bacterial or viral aetiology.

	Exacerbations		Exacerbation with aetiology		Exacerbation without aetiology	
Month/season	**n**	**% (95% CI)[a]**	**n**	**% (95% CI)[a]**	**n**	**% (95% CI)[a]**
July	17	16·8% (10·8–25·2)	12	11·8% (6·7–19·9)	5	5·1% (2·3–11·1)
August	21	21·6% (14·5–30·9)	15	15·7% (10·0–23·7)	6	6·5% (2·7–14·6)
September	18	17·0% (11·0–25·5)	14	13·7% (8·2–21·8)	4	3·8% (1·5–9·4)
October	31	28·4% (20·8–37·4)	26	24·9% (17·4–34·3)	2	2·0% (0·5–7·6)
November	33	28·8% (22·2–36·5)	29	26·4% (19·8–34·3)	4	3·7% (1·4–9·3)
December	40	36·8% (29·4–45·0)	34	32·1% (24·8–40·3)	5	4·4% (1·8–10·2)
January	43	36·0% (28·8–43·9)	35	30·0% (23·1–37·9)	8	7·5% (4·0–13·8)
February	19	19·2% (12·8–27·7)	16	16·6% (10·7–24·9)	2	2·0% (0·3–10·4)
March	28	25·1% (19·1–32·4)	23	20·9% (14·8–28·5)	4	3·6% (1·5–8·7)
April	23	24·4% (17·2–33·4)	15	15·8% (10·2–23·7)	8	8·8% (4·5–16·5)
May	29	26·3% (19·2–34·9)	25	23·3% (16·7–31·5)	2	1·9% (0·5–7·2)
June	18	17·4% (11·3–25·8)	15	14·8% (9·6–22·3)	3	2·9% (0·9–8·9)
Monthly effect p value[b]	p=0·0033		p=0·00141		p=0·43529	
High season[c]	194	29·3% (25·5–33·3)	163	25·3% (21·8–29·2)	25	3·9% (2·5–6·1)
Low season[c]	126	20·6% (17·0–24·7)	96	15·9% (12·6–19·9)	28	4·6% (3·0–7·0)
High vs. low season p value[b]	p=0·00033		p=0·00009		p=0·51317	

[a]Percentages and confidence intervals estimated from generalised estimating equation (GEE) regression model with logit link and assuming an exchangeable correlation matrix. Only exacerbation, enrolment, and stable visits were taken into account in the total visit number; not recovered visits (in which the subject was recorded by the physician as not recovered from a previous exacerbation) were excluded.
[b]Tests of the effect of month or season were obtained via likelihood ratio tests.
[c]High season, October to March; low season, April to September.

Table S6. Seasonality of the presence or new occurrence of bacteria (NTHi or *M. catarrhalis* [Mcat]) or viruses.

	Visits with NTHi present in culture		Visits with Mcat present in culture		Visits with any virus present		Visits with HRV present		Visits with any virus other than HRV present		Visits with NTHi new occurrence in culture	
Month/Season	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]
July	32	31·3% (22·9–41·0)	5	4·6% (1·8–11·6)	22	20·7% (13·4–30·6)	16	14·4% (8·4–23·6)	6	6·0% (2·7–12·5)	10	11·0% (6·2–18·7)
August	29	28·2% (20·2–37·9)	6	6·6% (3·1–13·4)	13	14·7% (8·9–23·3)	6	7·6% (3·7–14·8)	7	7·6% (3·4–16·1)	8	9·3% (4·8–17·4)
September	32	29·8% (21·7–39·4)	1	0·8% (0·1–7·7)	15	15·1% (9·4–23·3)	9	9·4% (5·2–16·4)	6	5·9% (2·7–12·6)	7	7·5% (3·6–15·0)
October	44	37·0% (28·5–46·3)	6	5·5% (2·4–12·1)	22	22·2% (15·3–31·0)	15	15·0% (9·5–22·9)	7	7·0% (3·5–13·5)	15	14·3% (8·9–22·1)
November	41	35·8% (26·9–45·8)	13	11·3% (6·8–18·1)	21	21·4% (14·6–30·1)	13	14·5% (9·3–22·0)	8	7·5% (3·8–14·2)	11	10·5% (5·9–17·9)
December	34	32·0% (23·9–41·4)	8	7·4% (3·8–14·1)	32	31·9% (22·9–42·4)	15	15·0% (9·0–24·1)	17	16·6% (10·4–25·4)	8	8·3% (4·2–15·5)
January	47	33·9% (25·1–44·0)	12	9·9% (5·4–17·3)	31	27·0% (19·8–35·6)	12	11·0% (6·0–19·4)	18	16·3% (10·4–24·8)	16	14·3% (9·2–21·6)
February	33	34·2% (25·8–43·6)	8	7·7% (3·7–15·1)	24	25·3% (17·3–35·4)	7	7·4% (3·7–14·2)	16	16·9% (10·6–25·8)	7	7·9% (3·9–15·4)
March	42	40·7% (32·1–50·0)	12	9·7% (5·5–16·5)	22	20·1% (13·9–28·2)	7	6·0% (2·8–12·5)	16	15·3% (9·8–22·9)	15	14·7% (9·2–22·6)
April	28	31·6% (23·2–41·4)	3	2·7% (0·7–9·8)	19	20·2% (12·8–30·4)	8	8·9% (4·6–16·6)	11	11·4% (5·9–21·0)	8	9·1% (4·6–17·0)
May	27	25·5% (18·4–34·2)	11	9·0% (4·8–16·3)	21	19·9% (13·5–28·4)	12	11·9% (6·9–19·6)	10	9·1% (5·1–15·7)	6	6·2% (2·9–12·8)
June	27	26·1% (18·8–35·1)	5	5·2% (2·4–11·2)	8	8·8% (4·8–15·7)	6	7·3% (3·8–13·4)	2	2·0% (0·5–7·8)	7	8·1% (4·0–15·9)
Monthly effect p value[b]		p=0·2748		p=0·00276		p=0·00107		p=0·0814		p=0·0001		p=0·42186
High season[c]	241	35·6% (29·5–42·1)	59	8·7% (6·5–11·6)	152	24·5% (20·5–29·0)	69	11·5% (8·5–15·3)	82	13·2% (10·7–16·2)	72	11·8% (9·6–14·5)
Low season[c]	175	28·6% (22·9–35·0)	31	4·9% (3·0–7·9)	98	16·6% (12·8–21·3)	57	10·1% (7·0–14·3)	42	7·0% (4·9–9·9)	46	8·5% (6·5–11·0)
High vs. low season p value[b]		p=0·0098		p=0·00484		p=0·00098		p=0·42315		p=0·00193		p=0·04309

[a]Percentages and confidence intervals estimated from generalised estimating equation (GEE) regression model with logit link and assuming an exchangeable correlation matrix. Only exacerbation, enrolment, and stable visits were taken into account in the total visit number; not recovered visits (in which the subject was recorded by the physician as not recovered from a previous exacerbation) were excluded.
[b]Tests of the effect of month or season were obtained via likelihood ratio tests.
[c]High season, October to March; low season, April to September.

Figure S1. Seasonal distribution of AECOPD cases with sputum samples: total number and number of cases positive for NTHi or *M. catarrhalis* (detected by culture or PCR), HRV, or any viral species, and cases negative for bacteria and viruses (full cohort year 1; month of follow-up considered regardless of year).

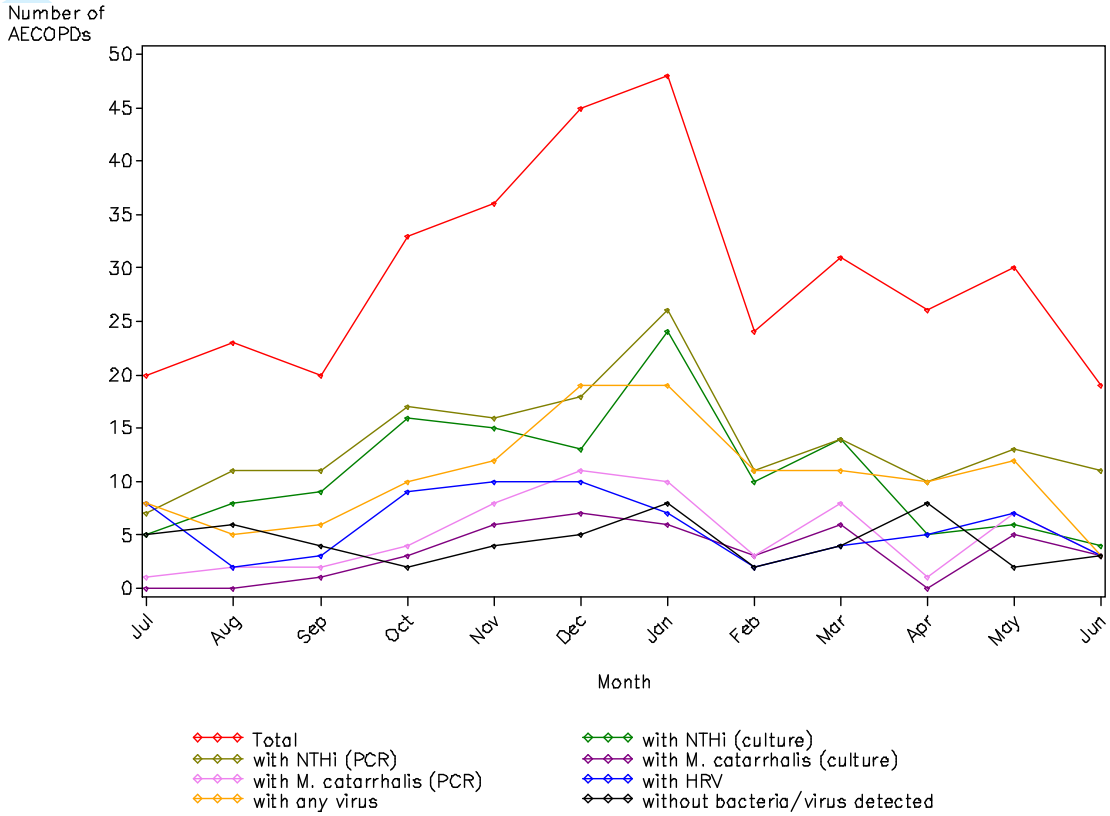
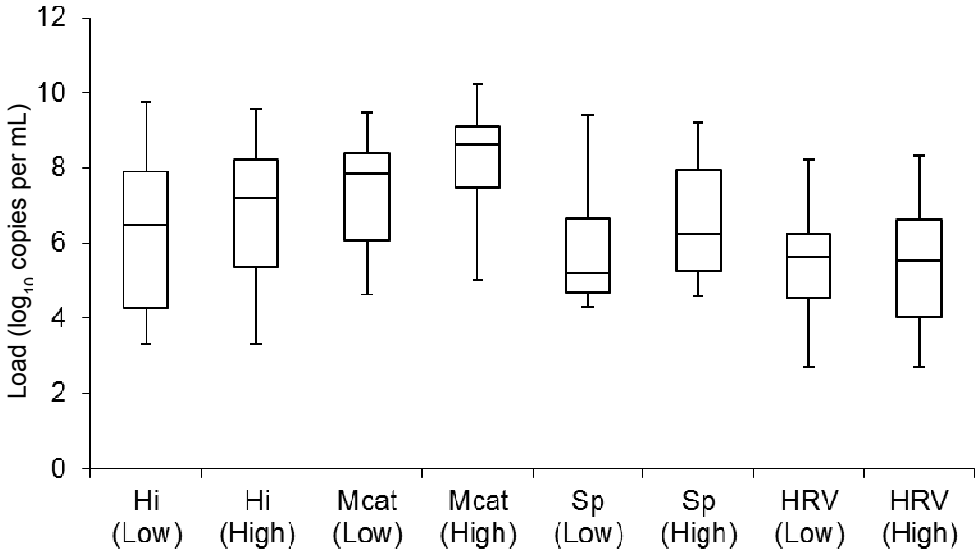
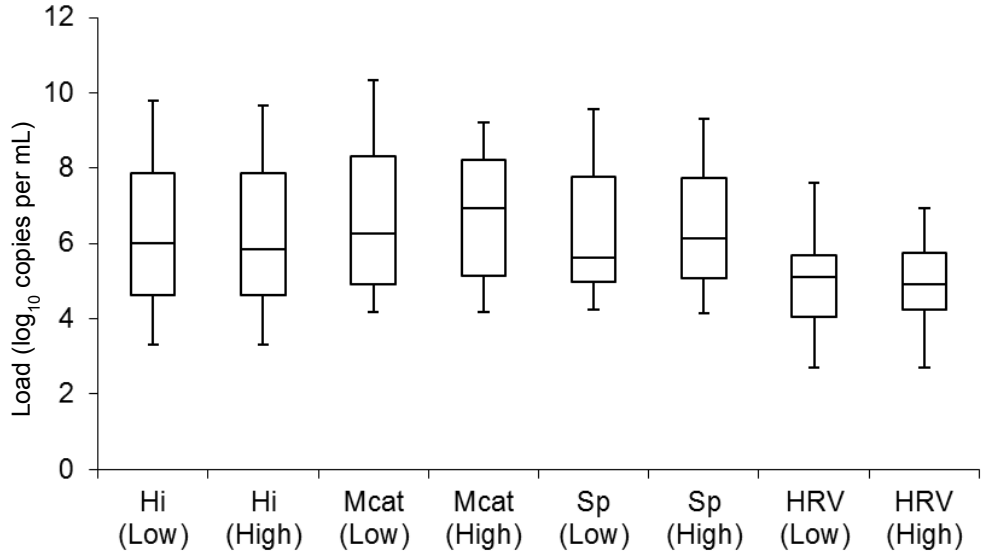


Figure S2. Bacterial and viral load by PCR in sputum at low and high season at exacerbation and stable visits (full cohort year 1). Box and whisker plots show median, interquartile range, and minimum and maximum values. Mann-Whitney test was used to test for significant differences between low and high season.

A. Exacerbation visits. Difference between low and high season was statistically significant for NTHi (p=0·015) and *M. catarrhalis* (p=0·048) only.



B. Stable visits. No statistically significant differences were detected between low and high season.



Hi = non-typeable *Haemophilus influenzae*. Mcat = *Moraxella catarrhalis.* Sp = *Streptococcus pneumoniae.* HRV = human rhinovirus. Low = low season (April to September). High = high season (October to March).