

RESEARCH ARTICLE



Lung mucosal immunity to NTHi vaccine antigens: Antibodies in sputum of chronic obstructive pulmonary disease patients

Federica Baffetta^{ID}^{a*}, Cecilia Buonsanti^{ID}^{a*}, Luca Moraschini^{ID}^a, Susanna Aprea^a, Martina Canè^{ID}^b, Stefano Lombardi^{ID}^a, Mario Contorni^{ID}^a, Simona Rondini^{ID}^c, Ashwani Kumar Arora^{ID}^c, Monia Bardelli^{ID}^a, Oretta Finco^{ID}^a, Davide Serruto^a, and Silvia Rossi Paccani^{ID}^a

^aGSK, Siena, Italy; ^bUniversity of Naples, Naples, Italy; ^cGSK Vaccines Institute for Global Health (GVGH), Siena, Italy

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a common chronic respiratory illness in older adults. A major cause of COPD-related morbidity and mortality is acute exacerbation of COPD (AECOPD). Bacteria in the lungs play a role in exacerbation development, and the most common pathogen is non-typeable *Haemophilus influenzae* (NTHi). A vaccine to prevent AECOPD containing NTHi surface antigens was tested in a clinical trial. This study measured IgG and IgA against NTHi vaccine antigens in sputum. Sputum samples from 40 COPD patients vaccinated with the NTHi vaccine were collected at baseline and 30 days after the second dose. IgG and IgA antibodies against the target antigens and albumin were analyzed in the sputum. We compared antibody signals before and after vaccination, analyzed correlation with disease severity and between sputum and serum samples, and assessed transudation. Antigen-specific IgG were absent before vaccination and present with high titers after vaccination. Antigen-specific IgA before and after vaccination were low but significantly different for two antigens. IgG correlated between sputum and serum, and between sputum and disease severity. Sputum albumin was higher in patients with severe COPD than in those with moderate COPD, suggesting changes in transudation played a role. We demonstrated that immunization with the NTHi vaccine induces antigen-specific antibodies in sputum. The correlation between IgG from sputum and serum and the presence of albumin in the sputum of severe COPD patients suggested transudation of antibodies from the serum to the lungs, although local IgG production could not be excluded.

Clinical Trial Registration: NCT02075541

PLAIN LANGUAGE SUMMARY

What is the context?

- Chronic obstructive pulmonary disease (COPD) is the most common chronic respiratory illness in older adults and the third leading cause of death worldwide.
- One bacterium in the lungs, non-typeable *Haemophilus influenzae* (NTHi), is responsible for acute exacerbation of the disease, characterized by an increase in airway wall inflammation and symptoms, leading to high morbidity and mortality.
- A vaccine targeting NTHi was previously developed but did not show efficacy in reducing exacerbations in COPD patients, probably because the vaccine did not elicit an immune response in the lung mucosae, where the bacteria are located.

What is the impact?

- Parenteral immunization with new vaccines targeting NTHi is able to elicit immune defense at the level of lung mucosae.
- Now that antibodies can be measured in sputum, new vaccines against COPD exacerbations or other lung infections can be tested for efficacy in the actual target tissue.
- Also, lung immunity against specific pathogens can now be tested.

What is new?

- We determined that antigen-specific antibodies were present in the lungs after vaccination; these were assessed in sputum after vaccination with NTHi surface antigens.
- NTHi-specific IgG were present in the lungs and appeared to have arrived there primarily by transudation, a type of leakage from the serum to the lung mucosae.
- Transudation appeared to be stronger in severe than in moderate COPD patients.

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CONTACT Silvia Rossi Paccani ✉ silvia.x.rossi-paccani@gsk.com 📍 GSK, Via Fiorentina 1, Siena, Italy.

*These authors contributed equally to this work.

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Introduction

Chronic obstructive pulmonary disease (COPD) is the most common chronic respiratory illness in older adults and the third leading cause of death worldwide, responsible for about 6% of all deaths in 2019.¹ It is characterized by progressive airflow-limitation and mucus production, and associated with an abnormal inflammatory response in the lungs. The abnormal response leads to airway obstruction, mucociliary dysfunction, structural changes to the airways, and systemic effects.^{2,3}

Acute Exacerbation of COPD (AECOPD) is characterized by an increase in airway wall inflammation and symptoms and AECOPD episodes are a major cause of COPD-related morbidity and mortality.^{4,5} AECOPD may, among others, be triggered by the acquisition of new bacterial strains in the lungs.⁶ According to an extensive review, in AECOPD sputum samples, *Haemophilus influenzae* was the main bacterial pathogen detected in 20% to 30% of samples, followed by *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Mcat) both detected in 10% to 15% of samples.⁷ In a more recent two-year follow-up of COPD patients, in the first year (with 306 exacerbations reported), 54% of AECOPD samples were PCR-positive for non-typeable *Haemophilus influenzae* (NTHi), and in the second year (with 177 exacerbations reported) 39% of AECOPD samples were PCR-positive for NTHi.^{8,9}

A multi-component adjuvanted vaccine targeting NTHi and Mcat surface antigens had been developed by GSK Vaccine and was tested in a phase 2 clinical trial, where it did not show overall efficacy in reducing exacerbations in COPD patients.¹⁰ Given that AECOPD-associated pathogens, such as NTHi and Mcat, are mainly localized in the respiratory mucosa, an efficacious vaccine is expected to elicit an immune response also in the lung mucosae. In addition, it would be convenient to be able to measure antibodies directly in sputum samples as a read-out of lung immunity after vaccination with an NTHi-Mcat vaccine.

The current study aimed to determine the feasibility of measuring antibodies against vaccine antigens in sputum samples. Hereto, we analyzed antigen-specific antibodies in sputum samples from adults with moderate or severe COPD that received a vaccine that contained only NTHi antigens – without Mcat antigens – as part of a placebo-controlled phase 2 trial (NCT02075541).¹¹ This NTHi vaccine contained three NTHi surface antigens, protein D (PD), protein E (PE), and type IV pilin subunit protein (PilA), and AS01_E as adjuvant.¹² AS01_E is a liposome-based adjuvant comprising 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) and QS-21, a saponin extracted from the bark of the *Quillaja saponaria* Molina tree.

The specific objectives of the study were to: 1) measure antigen-specific immunoglobulin G (IgG) and immunoglobulin A (IgA) in sputum before and after vaccination; 2) determine whether a correlation exists between the antibody levels in the sputum and previously determined antibody titers in serum; 3) determine whether a correlation exists between the antibody levels in the sputum and disease severity; and 4) evaluate transudation to determine the source of the antibodies.

Methods

Sputum samples

Sputum samples were obtained from the randomized, observer-blind, placebo-controlled phase 2 clinical trial (NCT02075541) conducted in Sweden and the United Kingdom (UK) between 8 July 2014 and 19 April 2017.¹¹ In that trial, sputum samples from patients (aged 40–80 years) with moderate to severe COPD were collected before the first dose (PRE) and 30 days (DAY90) after the second dose (which is given 60 days after dose 1) of vaccination with the NTHi vaccine. The DAY90 samples were chosen for analysis as the highest concentrations of antigen-specific antibodies were detected in serum in those samples.¹¹

Meso Scale U-plex assay plate preparation

An assay was set up to detect immunoglobulins specific for antibodies against the NTHi surface antigens PD, PE, and PilA, and Ovalbumin (OVA) as a negative control, in sputum. The U-plex assay plates are 96-well plates in which each well is coated at up to 10 separate microspots with unique linkers. Other unique complementary linkers attached to the antigens of interest self-assemble with the linkers on the plate to ensure each microspot binds only one specific antigen.

A 167 nM solution of each biotinylated protein was prepared in a diluent of phosphate-buffered saline (PBS) and 0.5% bovine serum albumin (BSA) to prepare the U-plex assay plates (Meso Scale Diagnostics, Rockville, MD). Each biotinylated protein was coupled to a unique linker, mixed by vortexing, and incubated for 30 minutes at room temperature. The reaction was stopped with a U-plex stop solution and incubated for another 30 minutes. Equivalent amounts of each linker-coupled protein solution and the appropriate amount of stop solution were added into a single tube, resulting in a 16.7 nM concentration of each protein. Then, 50 µl of this multiplex coating solution was added to each well of 5- or 7-assay U-plex plates. Plates were then incubated for 1 hour at room temperature while shaking and washed three times with 150 µl wash buffer (PBS, 0.05% Tween).

U-plex assay

Sputum samples were diluted with diluent buffer by serial dilution to 1:50, 1:500, and 1:5,000 for measuring antigen-specific IgA, and diluted to 1:10, 1:50, and 1:500 for measuring antigen-specific IgG. Then, 25 µl of diluted sample was added to each well of the assay plates and incubated for 1 hour at room temperature while shaking, followed by washing three times with 150 µl wash buffer (PBS, 0.05% Tween).

Sulfo-Tag detection antibodies (SULFO-TAG Anti-Hu/NHP IgG Antibody or SULFO-TAG Anti-Hu/NHP IgA Antibody, Meso Scale Diagnostics, Rockville, MD) were prepared in diluent buffer at 0.25 µg/ml, and 30 µl of the diluted detection antibodies were then added to all wells. Plates were incubated for 1 hour at room temperature while shaking at 650 rpm, then washed three times with 150 µl wash buffer. A 2× read buffer (Meso Scale Diagnostics, Rockville, MD)

solution was prepared in water, and 150 µl of the 2× read buffer was added per well. Plates were then read in the MESO QUICKPLEX SQ 120 plate reader (Meso Scale Diagnostics, Rockville, MD). The final response was computed by dividing the antigen-specific signal by the OVA signal, measured within the same well, to reduce the impact of the background signal and better discriminate the specific response.

To verify that the results from the U-plex assay correlated with results obtained with the traditional ELISA used to measure Ig in the blood¹¹ we compared the measurement of specific Ig in several sera. The correlation analysis results showed that there was a high correlation (*r* ranging from 0.914 to 0.980) between the results from the two assays (Figure S1).

Albumin measurement

Albumin was measured in the sputum samples as a marker of protein transudation from serum with a fluorometric Albumin Assay Kit (Abcam, Cambridge, UK), according to the manufacturer's protocol. The albumin was measured in duplicate sputum samples (at dilution 1:20) from 6 moderate and 6 severe patients at day 0 (PRE), 12 randomly selected moderate and all 12 severe patients (DAY90), and in three duplicate serum samples from normal controls (at dilution 1:200).

Data analysis

To reduce the background and determine the specific response for the Meso Scale data, the final response was computed by dividing the signal of the antigen-specific antibodies by the signal of the anti-OVA antibodies measured in the same well. The albumin data were quantified by interpolating the signal on the standard line as the average of two measurements. Random selection of samples for albumin analysis was performed with SAS 9.4. All geometric mean comparisons were performed with log10-transformed data by testing the equivalence of the mean, except for albumin which was analyzed in the original scale. The homogeneity of the variance was analyzed by a Bartlett test, and when the hypothesis of equal variance between the groups could not be rejected, a pooled t-test analyzed the equivalence of the means, otherwise the Welch Anova test was applied.

All correlation coefficients were computed with log10-transformed data. Correlation coefficients were calculated between antibody levels in sputum and serum at 30 days after the second vaccination (DAY90) for each antigen and between U-plex assay results and traditional ELISA results in several sera. Raw data were analyzed with JMP16 software (JMP, Cary, NC). Data from antibody titers in the serum of the same patients at the same time points (PRE and DAY90) were available from the phase 2 clinical trial.¹¹

Table 1. Patient characteristics.

	Participants N = 40
Age at enrollment in years, mean ± SD	66.3 ± 9.3
Female sex, n (%)	20 (50%)
COPD severity, n (%)	
moderate	28 (70%)
severe	12 (30%)
Number of exacerbations during the 12 study months, mean ± SD and median (IQR)	2.9 ± 2.3 and 2 (1–3.5)
Number of exacerbations 1 month post-dose 2 in moderate COPD patients (N = 28), mean ± SD	
moderate exacerbations	2.6 ± 2.1
severe exacerbations	2 ± NA
Number of exacerbations 1 month post-dose 2 in severe COPD patients (N = 12), mean ± SD	
moderate exacerbations	1.2 ± 0.4
severe exacerbations	0

IQR, interquartile range; N, number; SD, standard deviation.

Table 2. Respiratory function values.

	Moderate patients (N = 28)*		Severe patients (N = 12)	
	Range	Mean (SD)	Range	Mean (SD)
FEF25–75	0.37–1.41	0.67 (0.25)	0.20–0.68	0.38 (0.15)
FEV1	0.96–2.51	1.75 (0.44)	0.75–1.57	1.06 (0.26)
FEV1/FVC ratio	42.10–66.70	54.51 (7.24)	31.00–48.90	38.92 (5.88)
FEV1pp	52.30–79.80	64.37 (9.18)	32.30–45.70	37.53 (4.44)
FEV1REV	–208.00–163.00	33.89 (82.01)	–44.00–297.00	66.75 (93.72)
FEV1REV%	–9.06–10.04	2.35 (4.72)	–4.09–27.97	7.51 (9.35)
FVC	1.52–5.16	3.26 (0.88)	2.11–4.13	2.74 (0.60)
FVCpp	64.20–111.50	89.49 (12.60)	58.00–95.10	73.64 (12.27)
PEF	3.00–7.89	5.28 (1.49)	2.21–4.50	3.45 (0.71)
PEFpp	45.50–99.70	76.37 (15.93)	27.0–71.60	49.47 (10.80)

*data from 27 patients for FEV1REV and FEV1REVP.

FEF25–75, forced expiratory flow at 25–75% of forced vital capacity; FEV1, forced expiratory volume in one second; FEV1pp, percentage of predicted value of FEV1; FEV1REV, FEV1 after a reversibility test; FEV1REV%, percent change in FEV1 after a reversibility test; FVC, forced vital capacity; FVCpp, FVC percent predicted; N, number; PEF, peak expiratory flow; PEFpp, percent predicted.

Results

Patients and samples

Sputum samples from all COPD patients vaccinated with the NTHi vaccine for whom both PRE and DAY90 samples were available were obtained from participants in a phase 2 clinical trial (NCT02075541).¹¹ This resulted in 40 COPD patients (aged 40–70 years), of which 28 had moderate and 12 had severe COPD. Participants' characteristics are presented in Table 1 and their respiratory function values are presented in Table 2. Of these participants, PRE and DAY90 samples were available. In total, 80 sputum samples were analyzed, each in three dilutions per assay. Comparable conclusion can be drawn for all the dilutions for both IgG and IgA and none of the dilutions were at saturation, to illustrate this the dilution curves for the IgG levels are included in the Supplemental Material (Figure S2). All subsequent analyzes were performed with the data from dilutions 1:10 for IgG and 1:50 for IgA.

Vaccine antigen-specific IgG antibodies increased in sputum after vaccination

IgG antibodies were analyzed in the sputum samples at three different dilutions. Subsequent analyzes of the signals of the IgG antibodies were performed with the data from dilutions 1:10.

The PRE and DAY90 signals of the IgG antibodies against NTHi antigens were normalized for the signals of IgG antibodies against the negative control OVA signal and then plotted. Geometric mean IgG antibody signals specific for the three NTHi antigens PD (Figure 1a), PE (Figure 1b), and PiIA (Figure 1c) were 1.08, 0.94, and 1.01 respectively before vaccination, while after vaccination these were significantly higher at 4.89, 8.79, and 3.35, respectively ($p < .0001$ for each). The signal of the antibodies against OVA remained unchanged (data not shown), as expected for the negative control.

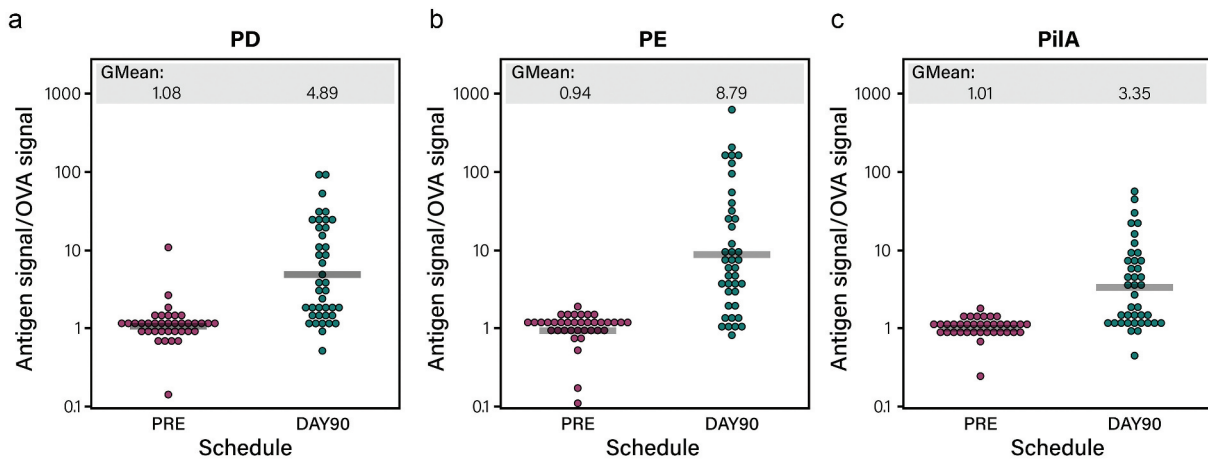


Figure 1. IgG response pre-vaccination vs post-vaccination. Signals (presented in log10) from IgG antibodies specific for a) PD, b) PE, and c) PiIA, normalized for the signals from IgG antibodies against OVA. Signals are from sputum samples collected before the first vaccination (PRE, $n = 37$) and 30 days after the second vaccination (DAY90, $n = 40$) with the NTHi vaccine. Each circle represents one sample, measured at dilution 1:10. The horizontal line represents the geometric mean. A value of ≤ 1 indicates background signal (as the specific signal is equal to the signal of the negative control). Gmean, geometric mean; IgG, immunoglobulin G; NTHi, non-typeable *Haemophilus influenzae*; OVA, ovalbumin; PD, Protein D; PE, Protein E; PiIA, type IV pilin subunit protein.

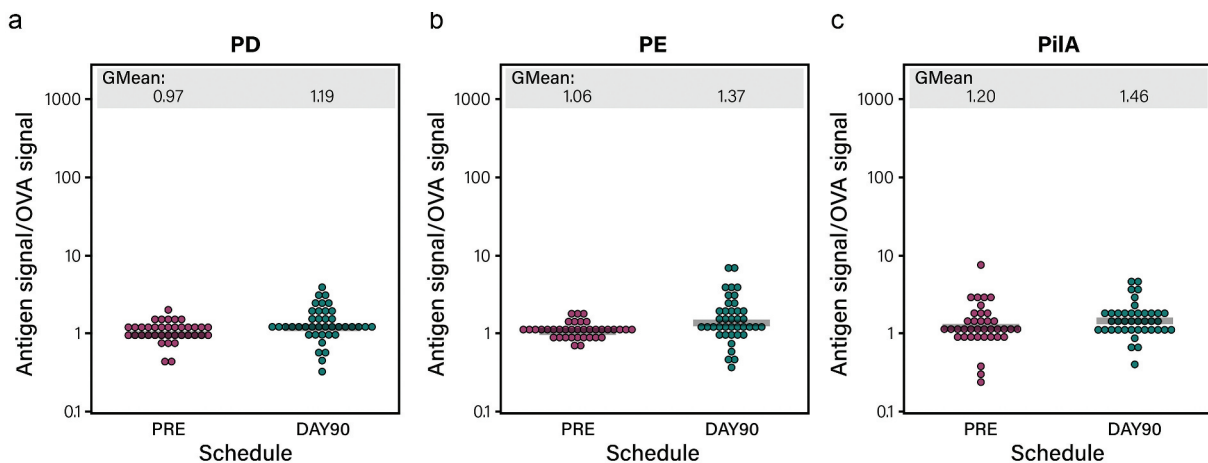


Figure 2. IgA response pre-vaccination vs post-vaccination. Signals (presented in log10) from IgA antibodies specific for a) PD, b) PE, and c) PiIA, normalized for the signals from IgA antibodies against OVA. Signals are from sputum samples collected before the first vaccination (PRE, $n = 37$) and 30 days after the second vaccination (DAY90, $n = 40$) with the NTHi vaccine. Each circle represents one sample, measured at dilution 1:50. The horizontal line represents the geometric mean. A value of ≤ 1 indicates background signal (as the specific signal is equal to the signal of the negative control). Gmean, geometric mean; IgA, immunoglobulin A; NTHi, non-typeable *Haemophilus influenzae*; OVA, ovalbumin; PD, Protein D; PE, Protein E; PiIA, type IV pilin subunit protein.

Vaccine antigen-specific IgA antibodies were low in sputum

IgA antibodies were analyzed in the sputum samples at three different dilutions. Subsequent analyzes of the signals of the IgA antibodies were performed with the data from dilutions 1:50 as the differences were more pronounced at that dilution.

Similar to the IgG antibodies, the PRE and DAY90 signals of the IgA antibodies against NTHi antigens were normalized for the signals of IgA antibodies against the negative control OVA signal and plotted. Geometric mean IgA antibody signals specific for the three NTHi antigens PD (Figure 2a), PE (Figure 2b), and PilA (Figure 2c) were 0.97, 1.06, and 1.20 respectively before vaccination, and these were only very slightly increased to 1.19, 1.37, and 1.46 respectively after vaccination. These increases were statistically significant for PD and PE (each $p < .05$) but not for PilA.

Correlation between IgG signals in serum and IgG titers in sputum samples

As NTHi antigen-specific IgG antibody signals were detected at high levels in sputum DAY90 samples, we next analyzed whether these sputum signals correlated with the IgG antibody titer in serum, as analyzed previously during the phase 2 trial. Indeed, positive correlations were found between log10 signals from sputum and IgG antibody titers from serum for PD ($r = 0.520$), PE ($r = 0.386$), and PilA ($r = 0.591$) (Figure 3(a,c)).

Correlation between IgG antibodies and disease severity

Because IgG in the lungs can be due to transudation of plasma through the lung tissue into the airways, and transudation is expected to increase with disease severity, we analyzed whether a correlation existed between concentrations of antigen-specific antibodies and severity. DAY90 antigen-specific IgG normalized for OVA were plotted of the 12 participants with severe COPD and 12 participants with moderate COPD

(randomly selected from 28 participants with moderate COPD). The geometric mean signal of antigen-specific IgG was significantly different against PD ($p < .05$) between moderate (2.18) and severe (9.78) COPD patients (Figure 4a), against PE ($p = .0005$) between moderate (3.21) and severe (37.57) COPD patients (Figure 4b), and against PilA ($p < .05$) between moderate (1.94) and severe (5.94) COPD patients (Figure 4c).

Another indication for transudation as a source for IgG in the lungs is the sputum to serum ratio of the antibodies. For the same 12 moderate and 12 severe COPD patients, the serum IgG signals were determined (presented in Figure S3), and the sputum to serum ratio of the IgG signals were plotted. While in the moderate patients these ratios were low, at 0.0019 for PD (Figure 4d), 0.0003 for PE (Figure 4e), and 0.0015 for PilA (Figure 4f), they were higher in severe patients at 0.0089 for PD (Figure 4d), 0.0031 for PE (Figure 4e), and 0.0031 for PilA (Figure 4f), the differences of over 4-fold for PD and over 10-fold for PE were statistically significant (both at $p < .001$).

Transudation of serum albumin into sputum

To determine whether protein transudation occurred from serum into the lungs, albumin was measured in the sputum samples. As albumin is not produced within the lungs, the albumin level in lung fluids (e.g., sputum) is considered the best transudation parameter. At day 0 (PRE), albumin concentrations in sputum samples were similar in moderate and severe patients. At DAY90, sputum albumin concentrations were undetectable in most patients with moderate COPD (mean 0.13 $\mu\text{g}/\mu\text{l}$), while albumin concentrations were significantly higher ($p < .005$) in severe COPD patients (mean 0.47 $\mu\text{g}/\mu\text{l}$) (Figure 5).

Discussion

In this study, we measured IgG and IgA against NTHi vaccine antigens in sputum samples from a placebo-controlled phase 2

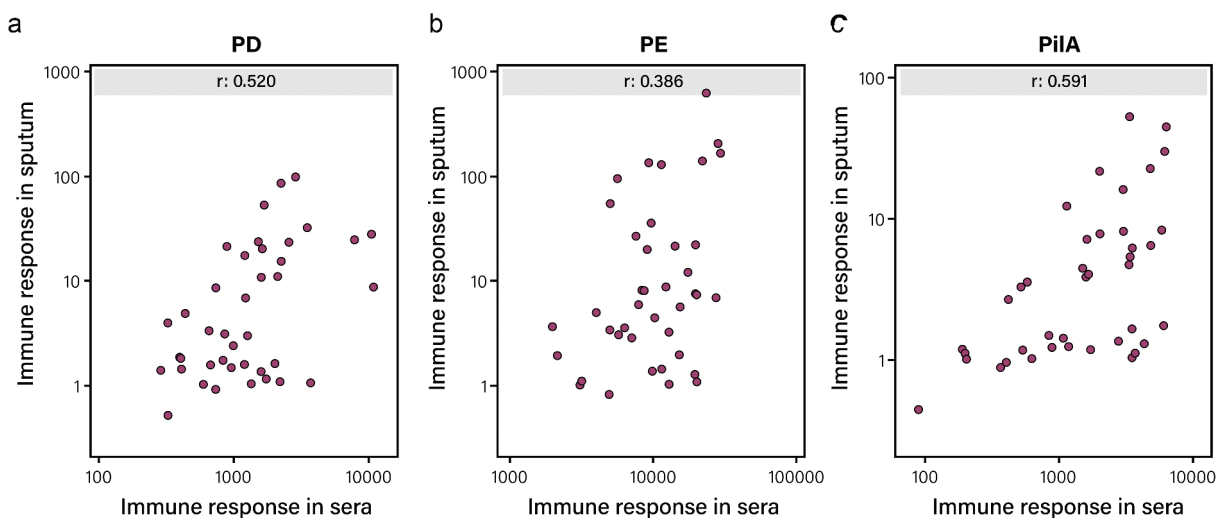


Figure 3. Correlation between IgG in serum and IgG in sputum. Signals (presented in log10) from DAY90 sputum normalized for the OVA signal (y-axis) and DAY90 serum (on x-axis) of IgG antibodies specific for a) PD, b) PE, and c) PilA. The r is the correlation coefficient between the signals in log10 scale. Each circle represents one individual. IgG, immunoglobulin G; OVA, ovalbumin; PD, Protein D; PE, Protein E; PilA, type IV pilin subunit protein; r , correlation coefficient.

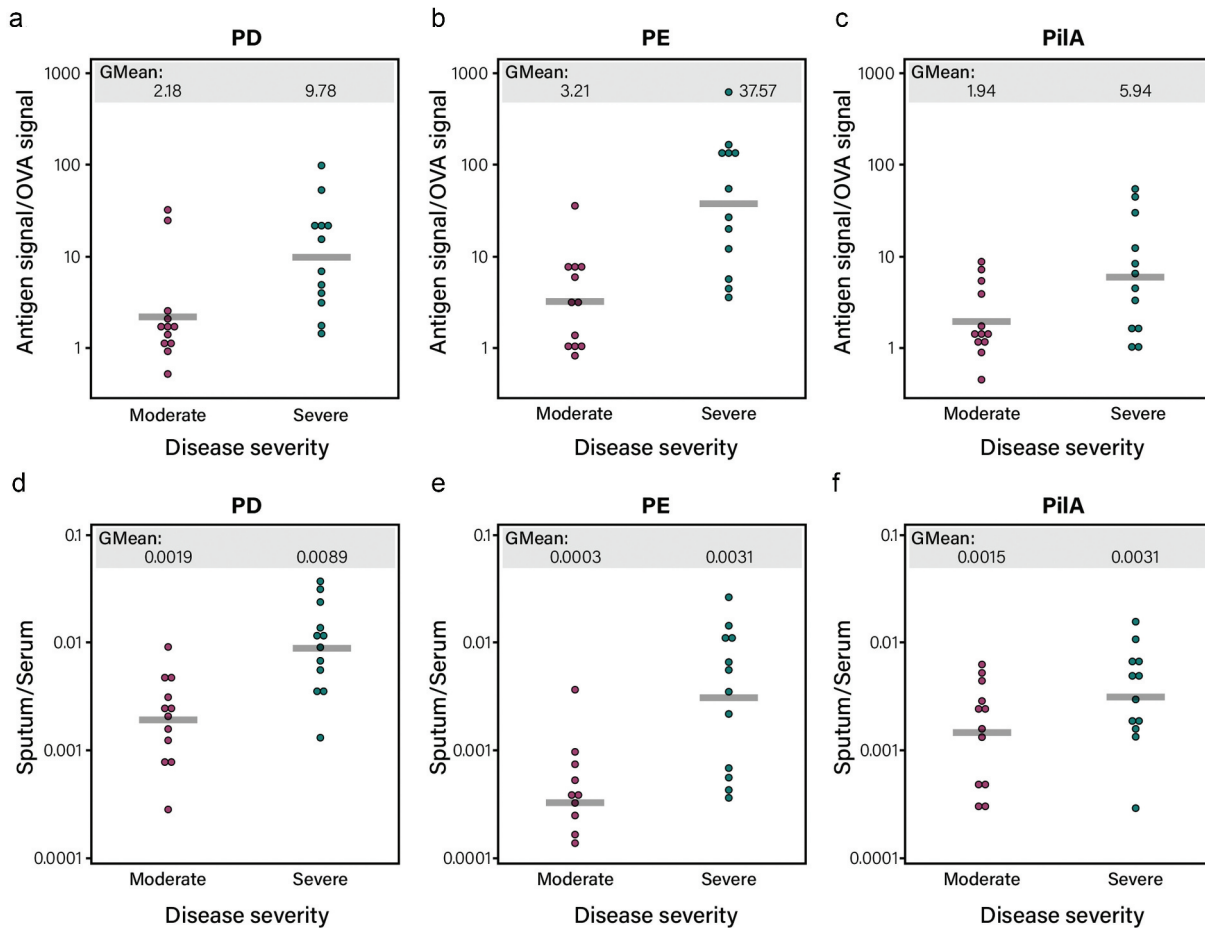


Figure 4. Correlation of disease severity with antigen-specific antibodies and sputum/serum ratio. a-c) Signals (presented in log10) from IgG antibodies specific for a) PD, b) PE, and c) PiIA, normalized for the signal from IgG antibodies against OVA. Signals are from sputum samples of 12 moderate and 12 severe COPD patients collected 30 days after the second vaccination (DAY90) with the NTHi vaccine. Each circle represents one sample measured at dilution 1:10. The horizontal line represents the geometric mean. d-f) Ratio of sputum/serum IgG antibodies specific for d) PD, e) PE, and f) PiIA. Ratios are from sputum samples of 12 moderate and 12 severe COPD patients collected 30 days after the second vaccination (DAY90) with the NTHi vaccine. Each circle represents the ratio of one sample, measured at dilution 1:10. The horizontal line represents the geometric mean. COPD, chronic obstructive pulmonary disease; IgG, immunoglobulin G; NTHi, non-typeable *Haemophilus influenzae*; OVA, ovalbumin; PD, Protein D; PE, Protein E; PiIA, type IV pilin subunit protein.

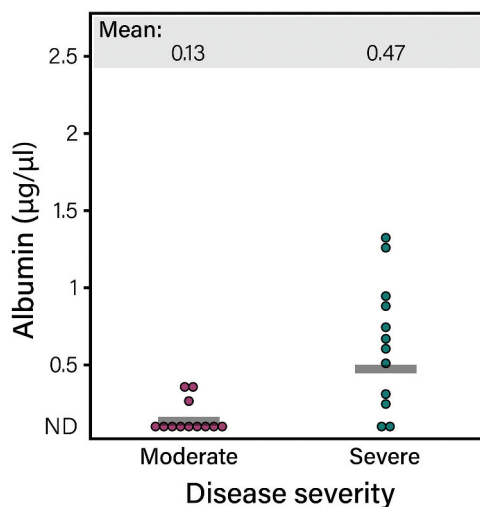


Figure 5. Transudation was detected in sputum from severe COPD patients. Albumin was measured in sputum of 12 moderate and 12 severe COPD patients as marker for transudation. Each circle represents one sample and is the average of two measurements. The horizontal line represents the mean. COPD, chronic obstructive pulmonary disease; ND, not detectable.

trial (NCT02075541), in which the NTHi vaccine was evaluated in adults with moderate and severe COPD.¹¹ Sputum samples from before vaccination and 30 days after the second vaccination dose were analyzed. We demonstrated that parenteral immunization with a protein-based vaccine induces antigen-specific IgG antibodies in the lung mucosae and that part of these antibodies may have entered the lung by transudation from plasma.

The mechanisms of protection against bacterial infections in patients with respiratory disease are elusive, and it was not known whether parenteral immunization is efficient in promoting an immune response at mucosal sites. We demonstrated that upon vaccination, IgG antibodies against three different NTHi antigens were present in sputum samples of vaccinated COPD patients and that there were clear and significant increases in IgG signals when compared to pre-vaccination. These results mirrored the data from both the NTHi phase 2 trial and the NTHi-MCat phase 2 trial that found that serum IgG against the NTHi antigens were strongly induced 30 days after the second vaccination.^{11,13}

Interestingly, although NTHi is the most commonly found pathogen in AECOPD sputum samples,⁷ preexisting immunity to NTHi antigens was not detectable in the patients. Each patient had a documented history of at least one moderate or severe AECOPD,¹¹ so exposure to NTHi would be expected in at least part of the patients. It is unknown why preexisting NTHi antibodies were not present in at least part of the samples. An explanation could be that NTHi is able to evade the host's immune system by hiding intracellularly, as has been documented transiently for airway epithelial cells and persistently for macrophages,^{14,15} or by hiding in biofilms.¹⁶

Vaccine antigen-specific IgA antibodies were only detected at a very low level in the sputum although their increase was significant after vaccination. Since IgA were not analyzed in the serum samples of the phase 2 trial,¹¹ we do not know at what level they were present in the serum. In a study of sputum samples collected >2 weeks after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination, also no antigen-specific IgA antibodies were detected in sputum from healthy controls and COPD patients, although antigen-specific IgA antibodies were increased in plasma.¹⁷ In an analysis of IgA kinetics in serum, throat swabs, and sputum samples from coronavirus disease 2019 patients, it was found that while antigen-specific IgA antibodies in serum were high 15 to 21 days from disease onset and remained stable, in the throat swabs and sputum IgA antibodies peaked at 8 to 14 days and waned after that.¹⁸ A similar, temporary increase in antigen-specific IgA was observed in nasal epithelial lining fluid at day 15 after SARS-CoV-2 vaccination of adults from the general population. The rapid waning of the antibodies was considered to correspond to the expected short half-life of IgA.¹⁹ In future studies of vaccine-specific antibodies, the sputum IgA should therefore be measured at earlier time points and be compared with serum IgA.

The source of the IgG antibodies in the sputum can be either local production in the mucosae or transudation from the serum. Immunoglobulins comprise the second largest class of proteins in bronchoalveolar lavage (BAL) fluids, and IgG is the major immunoglobulin in the lower respiratory tract.²⁰ In the past, IgG in nasal swabs after influenza vaccination were determined to be mainly derived from passive transudation from the serum.²¹ In patients with severe lung disease this is 1 to 6% depending on the subclass,²² while in healthy subjects, about 0.1% of serum IgG is known to transudate into the lungs. We found correlations of IgG between sputum and serum for all NTHi antigens, suggesting transudation from the serum to the lung contributed to the IgG in the sputum. In combination with the reported higher transudation in patients with severe lung disease compared to healthy people, this likely explains why in our patients the IgG levels were higher in sputum from patients with severe compared to moderate COPD.

However, the difference in ratio of sputum:serum between moderate and severe patients as compared to the difference measure in albumin between moderate and severe patients, suggested that a certain proportional degree of local IgG production cannot be excluded in the lungs of these patients. Some local IgG synthesis was therefore also suggested in the lung mucosae. In biopsies from patients with COPD, bronchiolar lymphoid follicles were found in the lungs and

in the patients with moderate to severe COPD these follicles often contained B-lymphocytes.²³ Another study also found higher numbers of B-cells in the subepithelial area of bronchial biopsies from patients with moderate and severe COPD than from controls.²⁴ B-cells were also found in lymphoid follicles in the parenchyma of COPD patients.²⁵ One hypothesis is that tertiary lymphoid follicles and the B-cells that they contain contribute to the immune response against bacterial (and viral) colonization and infection,^{26,27} and could be responsible for local IgG production after vaccination. This hypothesis still needs to be validated in clinical studies.

Another vaccine for the prevention of AECOPD, that is essentially the same as the NTHi vaccine except that it also contains the Mcat surface antigen UspA2 (the NTHi-Mcat vaccine), has also been developed. That vaccine showed good immunogenicity in adults with a smoking history of at least 10 pack-years.¹³ However, when this NTHi-Mcat vaccine was tested in COPD patients in a phase 2b clinical trial, it did not show efficacy in reducing the yearly rate of moderate and severe exacerbations in these patients,¹⁰ although a subsequent stratified post-hoc analysis suggested a signal of efficacy against severe exacerbations.²⁸ One explanation why the efficacy of that vaccine was not as expected is if the antigens used in the vaccine would not be expressed by the bacteria in the lungs. However, we recently determined that at least the NTHi antigens targeted by the vaccine are well expressed in the lungs during infection, thus, the targets of the antibodies are available.²⁹ Another explanation for the lack of efficacy of the NTHi-Mcat vaccine could be that the quantity of vaccine antigen-specific IgG in the lungs of COPD patients was not enough to control the infection. In a study of COPD patients, both the non-specific and NTHi-specific IgG1 were significantly lower in BAL fluids from COPD patients colonized with NTHi compared to those not colonized with NTHi.³⁰ Perhaps the quantity of NTHi-specific IgG1 antibodies in the lung was insufficient in certain COPD patients, resulting in them remaining NTHi colonized after infection, while those that did produce enough antibodies could solve or prevent the NTHi infection. We showed in the current study that vaccination can increase NTHi-specific IgG antibodies in the lungs, which we can speculate, if present in sufficient amounts, to contribute to help COPD patients fight NTHi infections. We also showed that the presence of specific IgG is more pronounced in severe patients. As mentioned, an efficacy signal was previously found against severe AECOPD in the NTHi-Mcat phase efficacy trial,²⁸ indicating that the vaccine can have a beneficial impact in those severe patients where, based on these data, we expect to have higher levels of vaccine-induced antibodies in the lungs.

The current study has several limitations. First, the sample size was small, with only 40 patients. Second, the participants were all Caucasians from Sweden and the UK, thus limiting the generalizability to other ethnic groups. Third, no healthy controls were included in the vaccination study, which prohibits the comparison of the antibodies present in the sputum with the normal situation. Insight into the situation in healthy lungs would improve the interpretation of the results.

In future studies, efforts should be made to avoid these limitations.

In conclusion, we demonstrated in this study that parenteral immunization with the protein-based NTHi vaccine induces antigen-specific IgG antibodies in the lung mucosae and that vaccination-induced IgG antibodies can be analyzed in sputum samples. The correlation between antigen-specific IgG levels from sputum and serum of COPD patients, as well as albumin present in the sputum, suggested transudation of antibodies from the serum to the lungs, although local IgG production in the mucosae could not be excluded. These results are relevant for the development of vaccines to prevent AECOPD as well as for vaccines against other lung infections.

Abbreviations

AECOPD	Acute exacerbation of COPD
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
COPD	Chronic obstructive pulmonary disease
IgA	Immunoglobulin A
IgG	Immunoglobulin G
Mcat	<i>Moraxella catarrhalis</i>
NTHi	Non-typeable <i>Haemophilus influenzae</i>
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PD	Protein D
PE	Protein E
PilA	type IV pilin subunit protein

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Disclosure statement

AKA, CB, DS, FB, LM, MB, MCo, OF, SA, SL, SR, and SRP are employed by GSK. AKA, LM, MB, MCo, SR, and SRP hold shares in GSK. The authors declare no other financial and non-financial relationships and activities. MCo declare no financial and non-financial relationships and activities and no conflicts of interest.

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ORCID

Federica Baffetta  <http://orcid.org/0009-0009-5508-4049>
 Cecilia Buonsanti  <http://orcid.org/0000-0001-5785-7695>
 Luca Moraschini  <http://orcid.org/0000-0002-3244-9043>
 Martina Canè  <http://orcid.org/0009-0007-5222-323X>
 Stefano Lombardi  <http://orcid.org/0009-0000-1395-1604>
 Mario Contorni  <http://orcid.org/0000-0001-9720-8972>
 Simona Rondini  <http://orcid.org/0000-0002-5114-1511>

Ashwani Kumar Arora  <http://orcid.org/0000-0003-3694-9878>
 Monia Bardelli  <http://orcid.org/0000-0002-9722-024X>
 Oretta Finco  <http://orcid.org/0000-0002-8652-6534>
 Silvia Rossi Paccani  <http://orcid.org/0000-0003-0136-9814>

Contributorship

FB, CB, SR, MB and SRP were involved in the study conception and design. CB, SA, and MCo were involved in acquisition and generation of data. FB, CB, SA, SL, LM, MB and SRP were involved in data analysis and FB, CB, MCo, SR, AKA, MB, LM, OF, DS, and SRP were involved in data interpretation.

All authors contributed substantially to the development of the manuscript and had full access to the data. All named authors meet the International Committee of Medical Journal Editors criteria for authorship for this article.

Data availability statement

GSK makes available anonymized individual participant data and associated documents from interventional clinical studies which evaluate medicines, upon approval of proposals submitted to www.clinicalstudydatarequest.com. To access data for other types of GSK sponsored research, for study documents without patient-level data and for clinical studies not listed, please submit an enquiry via the website. Clinical trial registration is NCT02075541.

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References

- World Health Organization. The top 10 causes of death; [accessed 2023 Feb 13] <https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death>.
- MacNee W. ABC of chronic obstructive pulmonary disease. Pathology, pathogenesis, and pathophysiology. *BMJ*. 2006;332(7551):1202–4. doi:10.1136/bmj.332.7551.1202.
- MacNee W, Tudor RM. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. *Proc Am Thorac Soc*. 2009;6(6):527–31. doi:10.1513/pats.200905-027DS.
- Donaldson GC, Seemungal TAR, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax*. 2002;57(10):847–52. doi:10.1136/thorax.57.10.847.
- European Respiratory Society. European Lung White Book. Respiratory Health and Disease in Europe. Sheffield, UK: European Respiratory Society; 2013.
- Sethi S, Evans N, Grant BJB, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *N Engl J Med*. 2002;347(7):465–71. doi:10.1056/NEJMoa012561.
- Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *N Engl J Med*. 2008;359(22):2355–65. doi:10.1056/NEJMra0800353.
- Wilkinson TMA, Aris E, Bourne S, Clarke SC, Peeters M, Pascal TG, Schoonbroodt S, Tuck AC, Kim V, Ostridge K, et al. A prospective, observational cohort study of the seasonal dynamics of airway pathogens in the aetiology of exacerbations in COPD. *Thorax*. 2017;72(10):919–27. doi:10.1136/thoraxjnl-2016-209023.
- Wilkinson TMA, Aris E, Bourne SC, Clarke SC, Peeters M, Pascal TG, Taddei L, Tuck AC, Kim VL, Ostridge KK, et al. Drivers of year-to-year variation in exacerbation frequency of COPD: analysis of the AERIS cohort. *ERJ Open Res*. 2019;5(1):00248–2018. doi:10.1183/23120541.00248-2018.
- Andreas S, Testa M, Boyer L, Brusselle G, Janssens W, Kerwin E, Papi A, Pek B, Puente-Maestu L, Saralaya D, et al. Non-typeable

- Haemophilus influenzae–Moraxella catarrhalis vaccine for the prevention of exacerbations in chronic obstructive pulmonary disease: a multicentre, randomised, placebo-controlled, observer-blinded, proof-of-concept, phase 2b trial. *Lancet Respir Med.* **2022**;10(5):435–46. doi:10.1016/S2213-2600(21)00502-6.
11. Wilkinson TMA, Schembri S, Brightling C, Bakerly ND, Lewis K, MacNee W, Rombo L, Hedner J, Allen M, Walker PP, et al. Non-typeable Haemophilus influenzae protein vaccine in adults with COPD: a phase 2 clinical trial. *Vaccine.* **2019**;37(41):6102–11. doi:10.1016/j.vaccine.2019.07.100.
 12. Leroux-Roels G, Van Damme P, Haazen W, Shakib S, Caubet M, Aris E, Devaster J-M, Peeters M. Phase I, randomized, observer-blind, placebo-controlled studies to evaluate the safety, reactogenicity and immunogenicity of an investigational non-typeable Haemophilus influenzae (NTHi) protein vaccine in adults. *Vaccine.* **2016**;34(27):3156–63. doi:10.1016/j.vaccine.2016.04.051.
 13. Van Damme P, Leroux-Roels G, Vandermeulen C, De Ryck I, Tasciotti A, Dozot M, Moraschini L, Testa M, Arora AK. Safety and immunogenicity of non-typeable Haemophilus influenzae–Moraxella catarrhalis vaccine. *Vaccine.* **2019**;37(23):3113–22. doi:10.1016/j.vaccine.2019.04.041.
 14. Brown MA, Morgan SB, Donachie GE, Horton KL, Pavord ID, Arancibia-Carcamo CV, Hinks TSC. Epithelial immune activation and intracellular invasion by non-typeable Haemophilus influenzae. *Front Cell Infect Microbiol.* **2023**;13:1141798. doi:10.3389/fcimb.2023.1141798.
 15. Ackland J, Heinson AI, Cleary DW, Christodoulides M, Wilkinson TMA, Staples KJ. Dual RNASeq Reveals NTHi-Macrophage Transcriptomic Changes During Intracellular Persistence. *Front Cell Infect Microbiol.* **2021**;11:723481. doi:10.3389/fcimb.2021.723481.
 16. Langereis JD, Hermans PW. Novel concepts in nontypeable Haemophilus influenzae biofilm formation. *FEMS Microbiol Lett.* **2013**;346(2):81–9. doi:10.1111/1574-6968.12203.
 17. Southworth T, Jackson N, Singh D. Airway immune responses to COVID-19 vaccination in COPD patients and healthy subjects. *Eur Respir J.* **2022**;60(2):2200497. doi:10.1183/13993003.00497-2022.
 18. Ren C, Gao Y, Zhang C, Zhou C, Hong Y, Qu M, Zhao Z, Du Y, Yang L, Liu B, et al. Respiratory mucosal immunity: kinetics of secretory immunoglobulin A in sputum and throat swabs from COVID-19 patients and vaccine recipients. *Front Microbiol.* **2022**;13:782421. doi:10.3389/fmicb.2022.782421.
 19. Aksyuk AA, Bansal H, Wilkins D, Stanley AM, Sproule S, Maaske J, Sanikommui S, Hartman WR, Sobieszczyk ME, Falsey AR, et al. AZD1222-induced nasal antibody responses are shaped by prior SARS-CoV-2 infection and correlate with virologic outcomes in breakthrough infection. *Cell Rep Med.* **2023**;4(1):100882. doi:10.1016/j.xcrm.2022.100882.
 20. Twigg HL 3rd. Humoral immune defense (antibodies): recent advances. *Proc Am Thorac Soc.* **2005**;2(5):417–21. doi:10.1513/pats.200508-089JS.
 21. Wagner DK, Clements ML, Reimer CB, Snyder M, Nelson DL, Murphy BR. Analysis of immunoglobulin G antibody responses after administration of live and inactivated influenza A vaccine indicates that nasal wash immunoglobulin G is a transudate from serum. *J Clin Microbiol.* **1987**;25(3):559–62. doi:10.1128/jcm.25.3.559-562.1987.
 22. Hill SL, Mitchell JL, Burnett D, Stockley RA. IgG subclasses in the serum and sputum from patients with bronchiectasis. *Thorax.* **1998**;53(6):463–8. doi:10.1136/thx.53.6.463.
 23. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med.* **2004**;350(26):2645–53. doi:10.1056/NEJMoa032158.
 24. Gosman MME, Willemse BWM, Jansen DF, Lapperre TS, van Schadewijk A, Hiemstra PS, Postma DS, Timens W, Kerstjens HAM. Increased number of B-cells in bronchial biopsies in COPD. *Eur Respir J.* **2006**;27(1):60–4. doi:10.1183/09031936.06.00007005.
 25. van der Strate BWA, Postma DS, Brandsma CA, Melgert BN, Luinge BB, Geerlings M, Hylkema MN, van den Berg A, Timens W, Kerstjens HAM, et al. Cigarette smoke-induced emphysema. A role for the B cell? *Am J Respir Crit Care Med.* **2006**;173(7):751–8. doi:10.1164/rccm.200504-594OC.
 26. Brusselle GG, Demoor T, Bracke KR, Brandsma CA, Timens W. Lymphoid follicles in (very) severe COPD: beneficial or harmful? *Eur Respir J.* **2009**;34(1):219–30. doi:10.1183/09031936.00150208.
 27. Yadava K, Bollyky P, Lawson MA. The formation and function of tertiary lymphoid follicles in chronic pulmonary inflammation. *Immunol.* **2016**;149(3):262–9. doi:10.1111/imm.12649.
 28. Arora AK, Chinsky K, Keller C, Mayers I, Pascual-Guardia S, Vera MP, Lambert C, Lombardi S, Rondini S, Tian S, et al. A detailed analysis of possible efficacy signals of NTHi-Mcat vaccine against severe COPD exacerbations in a previously reported randomised phase 2b trial. *Vaccine.* **2022**;40(41):5924–32. doi:10.1016/j.vaccine.2022.08.053.
 29. Brettoni C, Muzzi A, Rondini S, Weynants V, Rossi Paccani S. Ex-vivo RNA expression analysis of vaccine candidate genes in COPD sputum samples. *Respir Res.* **2023**;24(1):243. doi:10.1186/s12931-023-02525-z.
 30. Staples KJ, Taylor S, Thomas S, Leung S, Cox K, Pascal TG, Ostridge K, Welch L, Tuck AC, Clarke SC, et al. Relationships between Mucosal Antibodies, Non-Typeable Haemophilus influenzae (NTHi) infection and airway inflammation in COPD. *PLOS ONE.* **2016**;11(11):e0167250. doi:10.1371/journal.pone.0167250.