

# Detection of Cell-Dissociated Non-Typeable *Haemophilus influenzae* in the Airways of Patients with Chronic Obstructive Pulmonary Disease

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**Background:** Non-typeable *Haemophilus influenzae* (NTHi) is the most commonly found pathogen in the lower respiratory airways of patients with COPD. NTHi is predominantly regarded as an intracellular pathogen; however, like most pathogens, it can exist and co-exist in two broad forms: cell-associated (intracellularly or adhered to cells) or cell-dissociated (biofilm dispersed or planktonic). We sought to investigate if cell-dissociated NTHi can be detected from the sputum of COPD patients and assess this relationship to disease severity and airway inflammation.

**Methods:** DNA was extracted from the sputum plug and cell-free supernatant to quantify absolute (cell-associated and cell-dissociated NTHi) and cell-dissociated NTHi, respectively, from 87 COPD subjects attending an observational longitudinal COPD exacerbation study. NTHi was quantified using TaqMan hydrolysis probes, targeting the OMP P6 gene using qPCR.

**Results:** At stable state cell-dissociated NTHi was detected 56% of subjects with a median (IQR) of  $9.95 \times 10^2$  gene copies ( $1.26 \times 10^2$  to  $1.90 \times 10^4$ ). Cell-dissociated NTHi correlated with absolute NTHi levels ( $r=0.34$ ,  $p<0.01$ ) but not airway inflammation or spirometry at stable state. At exacerbation, cell-dissociated NTHi correlated with lung function ( $FEV_1$   $r=0.629$ ,  $p=0.005$ ;  $FEV_1\%$  predicted  $r=0.564$ ,  $p=0.015$ ;  $FVC$   $r=0.476$   $p=0.046$ ) and sputum neutrophilic inflammation (% neutrophils  $r=0.688$ ,  $p=0.002$ ; total neutrophils  $r=0.518$ ,  $p=0.028$ ).

**Conclusion:** In patients with COPD, NTHi can exist in both cell-associated and cell-dissociated forms. Cell-dissociated NTHi is associated with neutrophilic airway inflammation during exacerbations of COPD and may be a driving factor in worsening lung function during these episodes.

**Keywords:** NTHi, infection, neutrophils, COPD

## Introduction

The lungs are the main interface between the host and the external environment, with constant exposure to pathogens.<sup>1</sup> Mucus production, cilia action and phagocytosis by inflammatory cells play an integral part in host-pulmonary defence. Patients with chronic obstructive pulmonary disease (COPD) have impaired pulmonary defences, as a consequence of chronic inflammation, epithelial cell damage and mucus hypersecretion.<sup>2</sup> This is likely to play a role for increasing the susceptibility of patients with COPD to respiratory tract infections.<sup>3</sup> Furthermore, in these patients, bacterial infection can be found in up to 30% at stable state, termed colonisation, akin to chronic infection; whilst acute bacterial infection can be found in up to 50% during an exacerbation.<sup>3</sup> The most frequently found bacteria irrespective of the disease state is non-typeable *Haemophilus influenzae* (NTHi).<sup>4</sup>

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Bacteria and the host can co-exist in two broad forms: unattached from the cell, termed cell-dissociated or related to the cell, termed cell-associated.<sup>5-7</sup> Cell-dissociated bacteria can be either planktonic or dispersed.<sup>7,8</sup> Planktonic bacteria have been shown to infiltrate otherwise sterile sites within the host.<sup>9</sup> Cell-associated bacteria can be attached to the host cell by bacterial adhesin and pili or can reside intracellularly.<sup>6</sup> Bacteria that exists within a biofilm can either be cell-associated or cell-dissociated (Figure 1). Moreover, bacteria within a biofilm have been shown to evade antibiotic therapy and reside within the host for a longer duration.<sup>10,11</sup> The ability of bacteria to adopt different forms could lead to improved bacterial survival rates leading to colonisation.<sup>12</sup> However, there is little data examining the different existential forms of bacteria in patients with COPD. NTHi has been shown to be particularly adept at invading host tissue,<sup>13</sup> suggesting it could be more prone to adopting a cell-associated form. Otherwise, how NTHi resides within the lungs is generally unstudied. In this study, we sought to investigate if cell-dissociated NTHi can be detected in patients with COPD and how this relates to clinical outcomes.

## Materials and Methods

### Subjects and Sampling

Sputum samples from COPD subjects entering a longitudinal study looking at biomarkers in COPD were analysed. Subject inclusion and exclusion criterion, study design and measurements are as previously described.<sup>14</sup> In brief, subjects attended a stable visit every 3 months over a 12-month period and also during an exacerbation. An exacerbation was

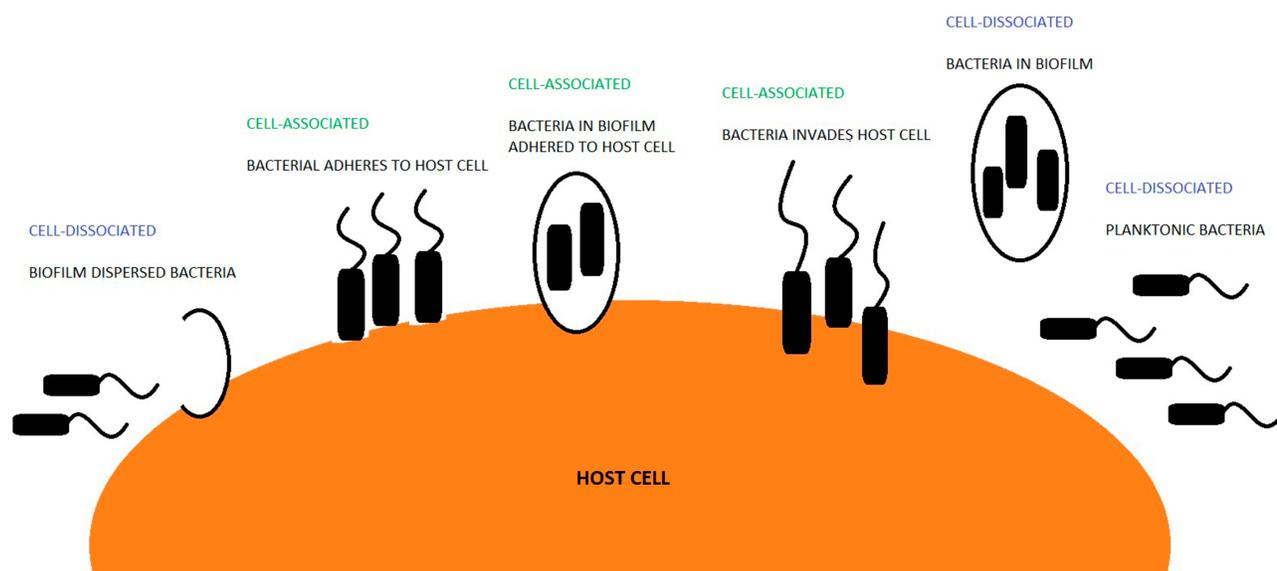
defined according to Anthonisen criteria<sup>15</sup> and/or health-care use.<sup>16</sup> At each visit, pre- and post-bronchodilator spirometry, health status (chronic respiratory questionnaire, CRQ),<sup>17</sup> symptoms (visual analogue scale, VAS)<sup>18</sup> and blood and sputum samples were obtained. All subjects gave written informed consent and the study was approved by the Leicestershire, Northamptonshire and Rutland ethics committee (reference number: 07/H0406/157). This study was conducted in accordance with the Declaration of Helsinki.

### Sputum Processing

Selected sputum plugs were dispersed with Dulbecco phosphate-buffered saline (PBS) and dithiothreitol (DTT). The PBS and DTT sputum supernatants were then stored at  $-80^{\circ}\text{C}$ . Cytospin preparation and quantification of cell differential count was performed. A separate sputum plug was sent to the routine microbiology laboratory for bacteria identification. An additional sputum plug was weighed and processed by SYBR green (Applied Biosystems®; Life Technologies Corp, Carlsbad, CA, USA) to determine total 16S and absolute NTHi (cell-associated and dissociated) bacterial DNA using real-time polymerase chain reaction (qPCR) as described previously.<sup>14</sup>

### Cell-Dissociated NTHi Quantification

Quantification of cell-dissociated NTHi was performed by extracting DNA from confirmed acellular sputum supernatant samples using the DNeasy blood and tissue kit (QIAGEN Ltd, Hilden Germany) according to the



**Figure 1** Schematic interpretation of how bacteria can reside within a host.

manufacturer's gram-negative bacteria protocol (see [Supplementary Data](#)). TaqMan qPCR was used to quantify cell-dissociated NTHi. Primers (Sigma, Poole, Dorset) previously determined to be sensitive and specific for the identification of NTHi in respiratory secretions<sup>19</sup> were used to target the OMP P6 gene (see [Supplementary Data](#)). A plasmid was used for standards for qPCR (see [Supplementary Data](#)). All samples were processed in duplicate with standards (serial dilutions of housekeeping gene OMP P6) and negative controls. The lower limit of detection (LLD) was 126 gene copies; all samples below this were considered negative and assigned a value of 0, and included in the analysis.

## Statistical Analysis

GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS Statistics version 22 (SPSS Inc. Chicago, IL, USA) were used for statistical analysis. The Kolmogorov–Smirnov test for normality was applied. Parametric and non-parametric data are presented as mean (SD) and median (IQR) unless stated. The Student's *T* test and Mann–Whitney test was performed for comparison between 2 groups in parametric and non-parametric data respectively. For paired analysis, the paired *T* test and Kruskal–Wallis test were used for parametric and non-parametric data, respectively. A  $p < 0.05$  was considered statistically significant.

## Results

Sputum was available from 87 COPD subjects (66 males) with a mean (range) age of 69 (43–88). There were 77 sputum samples at stable state and 30 sputum samples at exacerbation available for analysis. Of the 30 exacerbation samples, 20 of these had paired stable state data. The baseline characteristics for the COPD subjects are presented in [Table 1](#).

### Cell-Dissociated NTHi at Stable State

Cell-dissociated NTHi was detected in 56% of samples with a median (IQR) of  $9.95 \times 10^2$  gene copies ( $1.26 \times 10^2$ – $1.90 \times 10^4$ ) and was significantly lower than the levels of absolute NTHi detected in the sputum plug ( $p < 0.001$ , [Figure 2](#)). Absolute and cell-disassociated NTHi correlated ( $r = 0.34$ ,  $p < 0.01$ ). Levels of cell-dissociated NTHi were not associated with disease severity as defined by GOLD,<sup>20</sup> nor smoking status or inhaled corticosteroid use. Analysis of frequent exacerbators versus non-frequent exacerbators (a frequent exacerbator being more 2 or more treated exacerbations<sup>21</sup>) according to GOLD criteria showed no difference in levels

**Table 1** Baseline Characteristics of COPD Subjects Included in Analysis

Characteristics	
Subjects, n	87
Male, n (%)	66 (76)
Current smoker, n (%)	27 (31)
Ex-smoker, n (%)	60 (69)
Age, years <sup>a</sup>	69 (43–88)
Pack year history	50 (30)
Post-bronchodilator FEV <sub>1</sub> , L	1.37 (0.57)
Post-bronchodilator FEV <sub>1</sub> , % predicted	52.7 (20.5)
CRQ total, units	4.3 (1.2)
VAS total, mm	142 (73)
Total blood neutrophils, $\times 10^9/l^b$	5.28 (4.26–6.14)
Total blood eosinophils, $\times 10^9/l^b$	0.23 (0.13–0.36)
C-reactive protein, mg/L <sup>b</sup>	3 (3–11)
Sputum total cell count, $\times 10^3/mg^b$	4 (1.90–7.84)
Total sputum neutrophil count, $\times 10^3/mg^b$	2.30 (1.15–6.82)
Sputum neutrophil, %	67.5 (21.9)
Total sputum eosinophil count, $\times 10^3/mg^b$	0.05 (0.02–0.19)
Sputum eosinophil, % <sup>b</sup>	1.0 (0.3–3.8)
Colony forming units $\times 10^{6b}$	6.98 (1.80–55.56)
Total 16S bacterial load in sputum plug, $\times 10^8$ gene copies/mL <sup>b</sup>	2.04 (0.33–10.33)
Proportion of microbiology positive sputum samples, n (%)	17 (20)
Proportion of <i>Haemophilus influenzae</i> microbiology positive sputum samples, n (%)	10 (55)

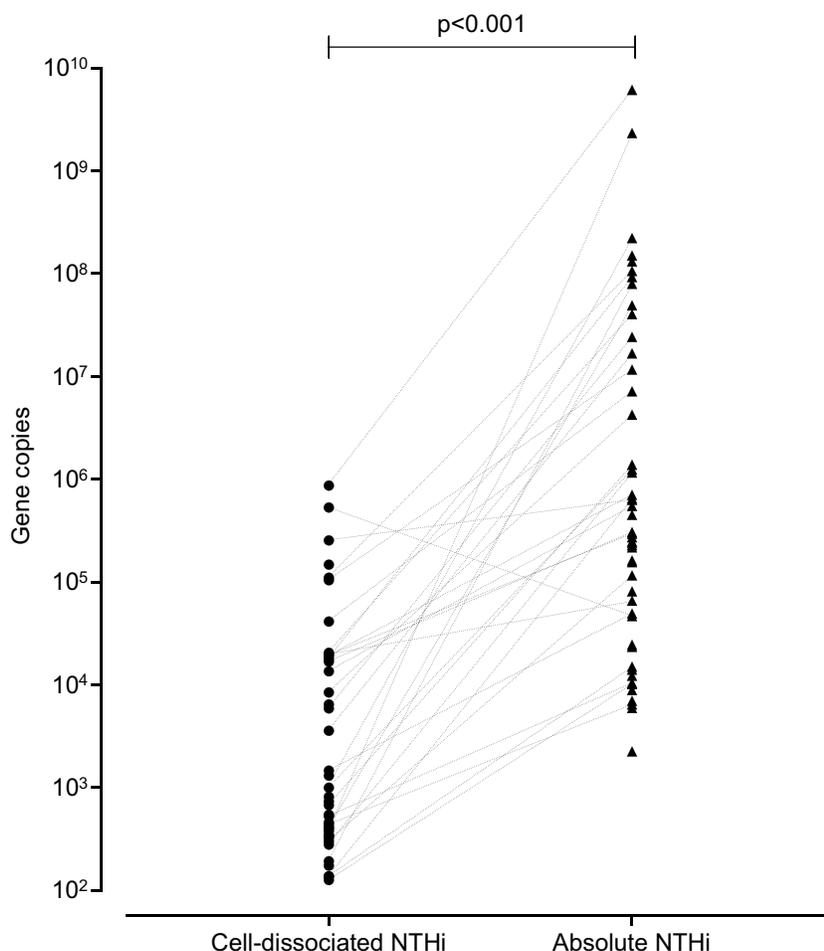
**Notes:** Chronic Respiratory Disease Questionnaire, scores range between 1 and 7 with higher score representing better health quality. Visual analogue scale, performed on 100 mm line from “no symptoms” to “worst symptoms”, higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data shown as mean (standard deviation) unless indicated otherwise. <sup>a</sup>Mean (range); <sup>b</sup>median (IQR).

**Abbreviations:** FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity.

of cell-dissociated NTHi,  $p = 0.820$ . Univariate analysis determined that only age and percentage predicted post-bronchodilator FEV<sub>1</sub> were statistically different between subjects that had detectable and non-detectable cell-dissociated NTHi ([Table 2](#)). Cell-dissociated NTHi levels were higher in sputum samples that were sputum culture positive compared to samples that were culture negative (median (IQR) in culture positive  $1.35 \times 10^3$  ( $0 \times 10^3$ – $110.00 \times 10^3$ ) gene copies vs 0 ( $0$ – $575.8$ ) gene copies in culture negative,  $p = 0.024$ ). There were no correlations of cell-dissociated NTHi with any other parameter at stable state ([Table 3](#)).

### Absolute NTHi at Stable State

Absolute NTHi was detected in 68% of samples with a median (IQR) of  $2.59 \times 10^5$  gene copies ( $2.0 \times 10^3$ – $1.1 \times 10^7$ ). Similar to cell-disassociated NTHi, absolute NTHi levels were not associated with disease severity as defined by GOLD.<sup>20</sup>



**Figure 2** Cell-dissociated NTHi levels compared to absolute NTHi levels at stable state.  
**Note:** Fifty-nine negative samples not plotted.

Absolute NTHi levels were significantly higher in ex-smokers compared to current smokers (median (IQR) in ex-smokers  $8.10 \times 10^4$  ( $0.41 \times 10^4$ – $131.00 \times 10^4$ ) gene copies vs 0 ( $0$ – $24.22 \times 10^4$ ) gene copies in current smokers,  $p=0.028$ ) and in subjects on ICS compared to those not on ICS (median (IQR) in those on ICS  $2.40 \times 10^4$  ( $0 \times 10^4$ – $60.79 \times 10^4$ ) gene copies vs  $24.26 \times 10^4$  ( $8.50 \times 10^4$ – $6425.85 \times 10^4$ ) gene copies in those not on ICS,  $p=0.034$ ). Bacterial load and CRQ correlated with absolute NTHi levels (Table 3).

Categorisation of absolute (<sub>a</sub>) and cell-dissociated (<sub>cd</sub>) non-detected (neg) and detected (pos) samples into 4 groups (Group 1: neg<sub>a</sub>/neg<sub>cd</sub>; Group 2: pos<sub>a</sub>/neg<sub>cd</sub>; group 3: neg<sub>a</sub>/pos<sub>cd</sub>; group 4: pos<sub>a</sub>/pos<sub>cd</sub>) no differences were apparent between these groups (Table 4).

### Cell-Dissociated NTHi at Exacerbation

At exacerbation cell-dissociated NTHi correlated with lung function (post-bronchodilator FEV<sub>1</sub>  $r=0.629$ ,  $p=0.005$ ;

percentage predicted post-bronchodilator FEV<sub>1</sub>  $r=0.564$ ,  $p=0.015$ ; FVC  $r=0.476$   $p=0.046$ ), blood eosinophils ( $r=-0.689$ ,  $p=0.002$ ) and sputum neutrophilic inflammation (sputum neutrophil percentage  $r=0.688$ ,  $p=0.002$ ; absolute sputum neutrophils  $r=0.518$ ,  $p=0.028$ ). A non-significant trend to increased levels of cell-dissociated NTHi was seen at exacerbation compared to stable state (median (IQR) at stable state  $1.91 \times 10^2$  ( $0 \times 10^2$ – $1.38 \times 10^2$ ) gene copies vs  $6.67 \times 10^2$  ( $0 \times 10^2$ – $944.75 \times 10^2$ ) gene copies at exacerbation,  $p=0.061$ ) (Figure 3). This was also seen when looking at the paired samples (median (IQR) at stable state  $62.94$  ( $0$ – $1.34 \times 10^3$ ) gene copies vs  $4.73 \times 10^2$  ( $6.36$ – $6.22 \times 10^4$ ) gene copies at exacerbation,  $p=0.081$ ).

### Absolute NTHi at Exacerbation

Absolute NTHi levels correlated with sputum total cell count ( $r=0.498$ ,  $p=0.050$ ), sputum neutrophils (total sputum neutrophil count  $r=0.612$ ,  $p=0.12$ ; %sputum neutrophil  $r=0.612$ ,

**Table 2** Differences Observed Between Subjects That Were Positive and Subjects That Were Negative for Cell-Dissociated NTHi at Stable State

Cell-Dissociated NTHi	Positive	Negative	P value
Subjects, n	43	34	
Male, n (%)	33 (77)	26 (76)	1.000
Current smoker, n (%)	10 (23)	14 (41)	0.137
Ex-smoker, n (%)	33 (77)	20 (59)	
Age, years <sup>a</sup>	72 (54–88)	67 (43–82)	0.017*
Exacerbation total <sup>b</sup>	2 (0–7)	3 (0–6)	0.111
Pack year history	48 (32)	50 (24)	0.781
Post-bronchodilator FEV <sub>1</sub> , % predicted	58 (21)	47 (19)	0.027*
CRQ total, units	16.65 (7.12)	16.87 (5.16)	0.889
VAS total, mm	119.70 (71.52)	136.70 (85.48)	0.383
Total blood neutrophils, ×10 <sup>9</sup> /l <sup>b</sup>	4.58 (3.73–6.00)	5.19 (4.27–5.64)	0.351
Total blood eosinophils, ×10 <sup>9</sup> /l <sup>b</sup>	0.23 (0.17–0.40)	0.19 (0.13–0.34)	0.109
C-reactive protein, mg/L <sup>b</sup>	2.50 (2.50–8.00)	2.50 (2.50–9.25)	0.800
Sputum total cell count, ×10 <sup>3</sup> /mg <sup>b</sup>	3.88 (1.32–6.38)	2.68 (1.28–5.06)	0.333
Total sputum neutrophil count, ×10 <sup>3</sup> /mg <sup>b</sup>	2.22 (0.89–4.10)	1.41 (0.94–3.04)	0.283
Sputum neutrophil, %	66 (20)	62 (21)	0.441
Total sputum eosinophil count, ×10 <sup>3</sup> /mg <sup>b</sup>	0.06 (0.01–0.20)	0.02 (0.01–0.12)	0.295
Sputum eosinophil, % <sup>b</sup>	1.25 (0.25–5.00)	1.00 (0.25–2.68)	0.385
Colony forming units × 10 <sup>6b</sup>	5.85 (0.80–54.75)	5.73 (3.04–23.85)	0.991
Total 16S bacterial load in sputum plug, × 10 <sup>8</sup> gene copies/mL <sup>b</sup>	2.71 (0.52–13.08)	3.42 (0.88–11.85)	0.741

**Notes:** Chronic Respiratory Disease Questionnaire, scores range between 1 and 7 with higher score representing better health quality. Visual analogue scale, performed on 100 mm line from “no symptoms” to “worst symptoms”, higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data shown as mean (standard deviation) unless indicated otherwise. <sup>a</sup>Mean (range); <sup>b</sup>median (IQR). \*Statistically significant (p<0.05).

**Abbreviations:** FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity.

p=0.012) and bacterial load measured by CFU (r=0.574, p=0.020). There was no significant increase in absolute NTHi at exacerbation compared to stable state (p=0.640).

## Discussion

Numerous studies have examined the role of bacteria, including NTHi in patients with COPD,<sup>22–25</sup> but these have all examined bacterial detection in sputum plugs, thereby quantifying absolute levels of bacteria which includes both cell-associated and cell-dissociated forms. To our knowledge, this is the first time cell-dissociated NTHi has been measured in

**Table 3** Correlations Between NTHi Levels and Lung Function, Patient Symptoms and Inflammatory Mediators in Blood and Sputum at Stable State

Variable	Cell-Dissociated NTHi		Cell-Associated and Dissociated NTHi	
	R value	P value	R value	P value
Pack year history, units	−0.056	0.723	−0.132	0.267
Total exacerbations	−0.063	0.588	−0.083	0.475
Percentage predicted post-bronchodilator FEV <sub>1</sub> , %	−0.106	0.500	0.149	0.209
CRQ, units	0.156	0.323	0.311	0.008*
VAS total, mm	−0.124	0.427	0.017	0.888
Total blood neutrophils, ×10 <sup>9</sup> /l	−0.029	0.803	−0.175	0.129
Total blood eosinophils, ×10 <sup>9</sup> /l	0.006	0.958	0.165	0.151
C-reactive protein, ×10 <sup>9</sup> /l	0.132	0.399	0.029	0.810
Sputum total cell count, ×10 <sup>3</sup> /mg	0.152	0.188	−0.194	0.091
Sputum neutrophil, %	0.131	0.404	0.023	0.849
Total sputum neutrophil count, ×10 <sup>3</sup> /mg	0.176	0.128	−0.140	0.229
Sputum eosinophil, %	−0.050	0.671	0.075	0.517
Total sputum eosinophil count, ×10 <sup>3</sup> /mg	−0.045	0.402	−0.043	0.714
Colony forming units/mL	0.019	0.879	0.362	0.002*

**Note:** \*Statistically significant (p<0.05).

sputum samples from patients with COPD. In this study, we have shown that cell-dissociated NTHi is detectable in the cell-free sputum supernatant samples from COPD subjects. Cell-dissociated NTHi was shown to be linked to lung function and airway inflammation, at exacerbation only. Associations were not seen between levels of cell-dissociated NTHi detected and disease severity. This study confirms the presence of cell-dissociated NTHi within sputum and that further investigation is warranted to investigate if this mechanism relates to exacerbations of COPD.

The microbiome in BAL and cell-free BAL supernatant have been shown to differ in their bacterial composition,<sup>7</sup> suggesting that pathogens can reside within the host in different forms (cell-associated and dissociated). Our study has examined NTHi, the most abundant pathogen within the lungs of patients with COPD.<sup>22</sup> NTHi is particularly adept at invading host tissue,<sup>13</sup> suggesting it could be more prone to adopting a cell-associated form. The ability of bacteria to adopt different forms within the host appears to greatly improve the survival rate and colonisation of the pathogen. The formation of a biofilm is associated with less response to antibiotic therapy and longer survival within the host.<sup>10</sup> Studies looking at *Streptococcus pneumoniae* biofilm formation have shown that within a biofilm the bacteria is

**Table 4** Looking at NTHi Levels at Stable State in the Sputum Plug and Supernatant

	Group 1: neg <sub>a</sub> /neg <sub>d</sub>	Group 2: pos <sub>a</sub> /neg <sub>d</sub>	Group 3: neg <sub>a</sub> /pos <sub>d</sub>	Group 4: pos <sub>a</sub> /pos <sub>d</sub>	P value
Subjects, n	12	22	13	30	
Age, year	63 (9)	69 (9)	71 (8)	71 (10)	0.134
Pack year history, units	46 (21)	52 (26)	50 (33)	54 (33)	0.883
Percentage predicted post- bronchodilator FEV <sub>1</sub> , %	45 (20)	48 (18)	62 (18)	57 (22)	0.076
CRQ, units	18.07 (5.23)	16.18 (5.11)	13.77 (7.20)	17.95 (6.10)	0.175
VAS, mm	144.90 (111.10)	132.20 (70.36)	149.20 (78.63)	116.70 (68.55)	0.560
Total blood neutrophils, x10 <sup>9</sup> /l <sup>b</sup>	5.16 (4.38–6.20)	5.19 (4.25–5.48)	4.63 (3.34–5.80)	5.12 (4.12–6.12)	0.744
Total blood eosinophils, x10 <sup>9</sup> /l <sup>b</sup>	0.21 (0.12–0.33)	0.17 (0.13–0.34)	0.30 (0.20–0.41)	0.20 (0.15–0.36)	0.378
C-reactive protein, x10 <sup>9</sup> /l <sup>a</sup>	2.5 (2.5–10.75)	2.5 (2.5–8.25)	3.0 (2.5–9.0)	2.5 (2.5–7.25)	0.997
Sputum total cell count, x10 <sup>3</sup> /mg <sup>a</sup>	2.88 (1.14–7.91)	2.68 (1.33–4.87)	3.48 (1.32–6.71)	3.55 (1.12–4.96)	0.946
Sputum neutrophil, %	63 (21)	62 (21)	60 (21)	69 (18)	0.455
Total sputum neutrophil count, x10 <sup>3</sup> /mg <sup>a</sup>	1.51 (0.53–4.06)	1.35 (1.02–2.94)	1.96 (0.88–4.62)	2.16 (0.77–3.81)	0.902
Sputum eosinophil, % <sup>a</sup>	1.63 (0.31–5.02)	1.00 (0.25–2.50)	4.50 (0.86–8.50)	0.75 (0.25–4.06)	0.164
Total sputum eosinophil count, x10 <sup>3</sup> /mg <sup>a</sup>	0.02 (0.01–0.17)	0.02 (0.01–0.11)	0.16 (0.02–0.31)	0.03 (0.01–0.12)	0.239
Colony forming units/mL (Chocolate blood agar), x10 <sup>6a</sup>	3.98 (1.13–8.42)	6.56 (4.65–50.44)	0.80 (0.53–233.00)	7.50 (3.08–61.50)	0.142

**Notes:** Absolute NTHi levels (a) or cell-dissociated (d) were positive (pos) or negative (neg). Chronic Respiratory Disease Questionnaire, scores range between 1 and 7 with higher score representing better health quality; Visual analogue scale, performed on 100 mm line from “no symptoms” to “worst symptoms”, higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data shown as mean (standard deviation) unless indicated otherwise. <sup>b</sup>Median (IQR).

**Abbreviations:** FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity.

non-invasive and non-toxic;<sup>7</sup> however, once dispersed from the biofilm there is adoption of a planktonic state. This was also associated with up-regulation of genes linked to virulence and may thus be related to the transition from asymptomatic colonisation to invasive bacterial *Streptococcus* infection.<sup>7</sup>

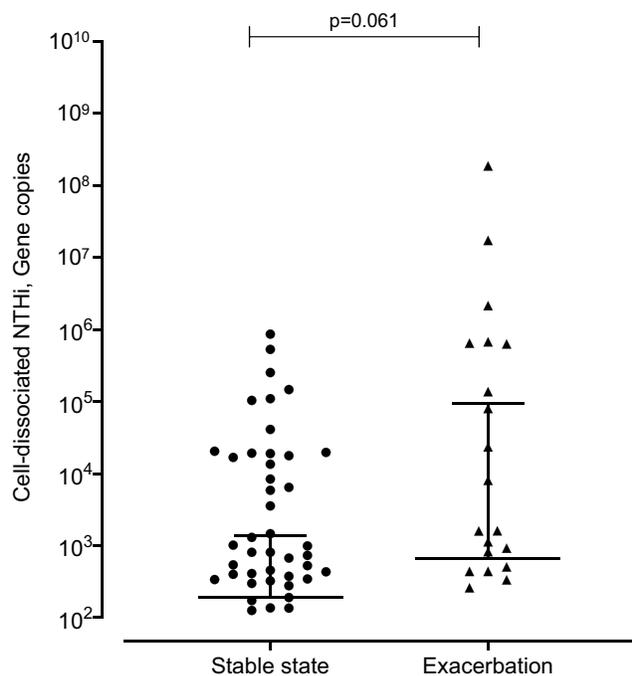
Exacerbations of COPD are associated with a pronounced decline in lung function,<sup>26</sup> an increased risk of further exacerbations<sup>27</sup> and increased bacterial colonisation of the lower respiratory tract.<sup>28</sup> NTHi has been shown to significantly increase at exacerbation state compared to stable state.<sup>14,28</sup> We showed that cell-dissociated NTHi was significantly associated with lung function and airway inflammation during an exacerbation and there was a trend to increased levels during the acute event. Our findings would suggest that cell-dissociated bacteria may play a vital role during exacerbations.

Neutrophils play an important role in innate immunity<sup>24,29,30</sup> and airway neutrophilic inflammation is dominant in COPD.<sup>14</sup> Observations demonstrate neutrophils are drawn to NTHi by secretory immunoglobulin A (IgA) and during phagocytosis release IL-8 further stimulating neutrophil recruitment.<sup>31</sup> The formation of neutrophil extracellular traps (NETs) aid in bacterial killing.<sup>30</sup> NTHi biofilms have been observed throughout NET structures and show resistance to both extracellular killing within NETs and phagocytic killing by recruited neutrophils.<sup>32</sup> Neutrophil

necrosis has been observed in the process of NTHi phagocytosis, encouraging NTHi persistence and further damage to lung epithelial cells.<sup>31</sup> In our study, we determined that the presence of cell-dissociated NTHi was associated with relative sputum neutrophil levels. Further work is needed to understand whether cell-dissociated NTHi in patients with COPD exhibits similar protective features in their interaction with neutrophils.

Studies have shown that biofilms are more resistant to immune clearance mechanisms<sup>33</sup> and to antibiotics compared to planktonic bacteria;<sup>34,35</sup> whilst other studies have shown bacteria biofilm dispersal has an up-regulation of genes linked to virulence.<sup>7</sup> The presence of cell-dissociated NTHi could lead to further understanding of bacterial differentiation states in the lungs of patients with COPD.

There are several limitations in this study. Firstly, the qPCR techniques used on the sputum plug and supernatant differ, in that the plug used a SYBR Green assay and the supernatant utilised a TaqMan assay, for which the sensitivity and expression levels between the two methods are likely to differ.<sup>36</sup> We ensured that we quantified this in a comparable way and in both instances the lower limits of detection were similar. Secondly, the detection of cell-dissociated NTHi in our study using these methods does not strictly inform whether this bacterial state has dispersed from biofilms, been released from lysed cells either prior to sampling or during processing or indeed if the



**Figure 3** Cell-dissociated NTHi levels from stable to exacerbation state.  
**Notes:** Data shown as median and interquartile range. Negative values are not displayed.

bacteria have always existed in a primary planktonic state; however, this is the first study to our knowledge that has attempted to detect cell-dissociated NTHi. Due to current widely evident qPCR limitations, we cannot comment if the cell-dissociated NTHi detected is alive or dead and this is a limitation in this current study. Finally, we are unable to make comment on the importance of cell-dissociated NTHi during exacerbations, due to the small number of samples studied, but suggest further investigation is required.

## Conclusion

We have shown that cell-dissociated NTHi can be detected in patients with COPD. Cell-dissociated NTHi is associated with lung function and neutrophilic airway inflammation at exacerbation state and is independent of disease severity and may play an important role during these episodes.

## Abbreviations

COPD, chronic obstructive pulmonary disease; CRQ, Chronic Respiratory Disease Questionnaire; DNA, deoxyribonucleic acid; DTT, dithiothreitol; FEV<sub>1</sub>, forced expiratory volume in 1 second; GOLD, Global Initiative for Chronic Obstructive Lung Disease; ICC, interclass co-efficient variation; IQR, inter-quartile range; LLD, lower limit of detection;

NETs, neutrophil extracellular traps; NTHi, non-typeable *Haemophilus influenzae*; OMP, outer membrane protein; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; VAS, Visual Analogue Scale.

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## Author Contributions

Mona Bafadhel is the guarantor of the content of the manuscript, including the data and analysis. MB, CEB, and MRB contributed to the design of the study. All authors contributed to data interpretation and analysis; took part in drafting or revising the article; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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