Predicting probability of tolerating discrete amounts of peanut protein in allergic children using epitope-specific IgE antibody profiling

Maria Suprun | Paul Kearney | Clive Hayward | Heather Butler | Robert Getts | Scott H. Sicherer | Paul J. Turner
Dianne E. Campbell | Hugh A. Sampson

ARTICLE SUMMARY

- Existing diagnostic testing is not predictive of severity or the threshold dose of clinical reactivity, and many patients still require an Oral Food Challenge (OFC). While OFCs are very useful for making an allergy diagnosis and determining clinical reactivity, they often cause anaphylaxis, which can increase patient anxiety. and are time and resource intensive.[1]
- An extensive validation was performed across 5 cohorts (all with confirmed oral food challenge results) across six different countries. Cohorts used: BOPI, OPIA, CAFETERIA, CoFAR6, and PEPITES with specimens from Australia, UK, US, Ireland, and Germany.
- This paper reports the first validated algorithm using two key peanut specific IgE epitopes to predict probabilities of reaction to different amounts of peanut in allergic subjects and may provide a useful clinical substitute for peanut oral food challenges.
- Using the algorithm, subjects were assigned into "high", "moderate", or "low" dose reactivity groups. On average, subjects in the "high" group were 4 times more likely to tolerate a specific dose, compared to the "low" group.[1] For example, 88% of patients in the high dose reactivity group were able to tolerate ≥ 144 mg of peanut protein whereas only 29% were able to tolerate the same amount in the low dose reactivity group.[1-2]

CLINICAL CONSIDERATIONS

- The new epitope test offers more granular information to help clinicians stratify treatment and peanut avoidance plans for their patients.
- See below for summary of clinical considerations based on threshold reactivity level.[1]

allergenis peanut diagnostic result	clinical considerations[1]
likely allergic – low dose reactor	• inform or avoid oral food challenge to reduce risk of anaphlyaxis • confirm strict avoidance of peanut • consider immunotherapy to reduce risk of reaction
likely allergic – moderate dose reactor	• consider a single oral food challenge (30 to 100 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider immunotherapy to reduce risk of reaction
likely allergic – high dose reactor	• consider a single oral food challenge (100 to 300 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider starting immunotherapy at higher doses to shorten time to maintenance dose
unlikely allergic	• oral food challenge to rule out the diagnosis of peanut allergy

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ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Nontypeable *Haemophilus influenzae* infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma

Jodie Ackland[1] | Clair Barber[1,2] | Ashley Heinson[1] | Adnan Azim[1,2] | David W. Cleary[1,2] | Myron Christodoulides[1] | Ramesh J. Kurukulaaratchy[1,2] | Peter Howarth[1,2] | Tom M. A. Wilkinson[1,2,3] | Karl J. Staples[1,2,3] | On behalf of the WATCH study investigators*

[1]Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, UK

[2]NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton, UK

[3]Wessex Investigational Sciences Hub, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, UK

Correspondence
Karl J. Staples, Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, UK.
Email: k.staples@soton.ac.uk

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Abstract

Background: Nontypeable *Haemophilus influenzae* (NTHi) is a respiratory tract pathobiont that chronically colonizes the airways of asthma patients and is associated with severe, neutrophilic disease phenotypes. The mechanism of NTHi airway persistence is not well understood, but accumulating evidence suggests NTHi can persist within host airway immune cells such as macrophages. We hypothesized that NTHi infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma.

Methods: Bronchoalveolar lavage (BAL) samples from 25 severe asthma patients were assessed by fluorescence in situ hybridisation to quantify NTHi presence. Weighted gene correlation network analysis (WGCNA) was performed on RNASeq data from NTHi-infected monocyte-derived macrophages to identify transcriptomic networks associated with NTHi infection.

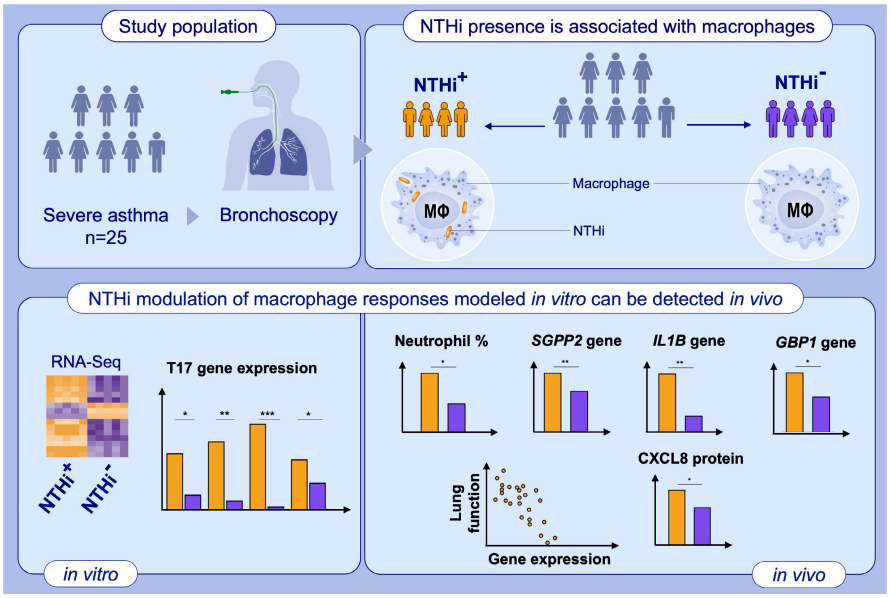
Results: NTHi was detected in 56% of BAL samples (NTHi+) and was associated with longer asthma duration (34 vs 22.5 years, $p = .0436$) and higher sputum neutrophil proportion (67% vs 25%, $p = .0462$). WGCNA identified a transcriptomic network of immune-related macrophage genes significantly associated with NTHi infection, including upregulation of T17 inflammatory mediators and neutrophil chemoattractants *IL1B*, *IL8*, *IL23* and *CCL20* (all $p < .05$). Macrophage network genes *SGPP2* ($p = .0221$), *IL1B* ($p = .0014$) and *GBP1* ($p = .0477$) were more highly expressed in NTHi+ BAL and moderately correlated with asthma duration (*IL1B*; rho = 0.41, $p = .041$) and lower prebronchodilator FEV1/FVC% (*GBP1*; rho = −0.43, $p = .046$ and *IL1B*; rho = −0.42, $p = .055$).

Abbreviations: BAL, bronchoalveolar lavage; CXCL8, C-X-C Motif Chemokine Ligand 8; *GBP1*, guanylate binding protein 1; *IL1B*, interleukin 1 beta; NTHi, nontypeable *Haemophilus influenzae*; RNASeq, RNA sequencing; *SGPP2*, sphingosine-1-phosphate phosphatase 2.

*See Acknowledgement section for all members of the WATCH study investigators.

Conclusions: NTHi persistence with pulmonary macrophages may contribute to chronic airway inflammation and T17 responses in severe asthma, which can lead to decreased lung function and reduced steroid responsiveness. Identifying therapeutic strategies to reduce the burden of NTHi in asthma could improve patient outcomes.

KEYWORDS
asthma, inflammation, macrophage, neutrophil, NTHi, T17 responses



GRAPHICAL ABSTRACT

Visualization of NTHi presence in the severe asthmatic airway demonstrates NTHi association with BAL macrophages, suggesting a mechanism of airway persistence. Modeling NTHi–macrophage interactions in vitro identifies upregulation of macrophage T17 responses. NTHi modulation of airway inflammation can be detected in vivo, with BAL macrophage gene expression correlated with decreased lung function in NTHi+ patients and increased neutrophilic inflammation detected in those with NTHi airway presence, suggesting that NTHi-infected macrophages may contribute to the complex chronic inflammatory environment in asthma.

Abbreviations: BAL, bronchoalveolar lavage; CXCL8, C-X-C Motif Chemokine Ligand 8; GBP1, guanylate binding protein 1; IL1B, interleukin 1 beta; NTHi, nontypeable Haemophilus influenzae; RNA-Seq, RNA sequencing; SGPP2, sphingosine-1-phosphate phosphatase 2

1 | INTRODUCTION

Asthma is a complex, heterogeneous disease of the airways characterized by episodic and reversible airway obstruction, hyperresponsiveness and inflammation.[1,2] Asthma has long been characterized by an excessive type 2 (T2) inflammatory response and eosinophilic infiltration, mediated by IL-4, IL-5 and IL-13.[3–5] As such, asthma is broadly classified into T2-high and T2-low endotypes based on the presence or absence of eosinophils and associated T2 markers.

Due to the heterogeneous nature of asthma, novel approaches to inflammatory endotyping based on sputum inflammatory profiles including eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic inflammatory phenotypes have been developed.[6,7] Advances in stratification methodologies utilizing biomarkers to develop sophisticated classification algorithms, and increased availability and analysis of longitudinal clinical data and patient history has demonstrated the predominance of eosinophilia in severe asthma.[8,9] As such, current asthma therapies including corticosteroids and monoclonal antibodies aim to reduce eosinophilic and T2 inflammation, with eosinophil levels determined to be a clinical marker of steroid-responsiveness.[10,11]

However, certain subsets of severe asthma patients do not respond well to conventional steroid treatment.[12] As individuals with severe asthma account for 50%–80% of the total health care burden of asthma, further studies aimed at uncovering the mechanisms underlying airway inflammation are needed to better understand the multifactorial nature of asthma.[13] The neutrophilic asthma phenotype accounts for up to 20% of adult asthma cases and is associated with increased steroid resistance, airflow obstruction, risk of hospitalization and lung microbial dysbiosis.[6,14–21] In particular, increased abundance of potentially pathogenic Proteobacteria, such as nontypeable *Haemophilus influenzae* (NTHi) is associated with more severe, neutrophilic phenotypes of asthma.[22–24] NTHi airway colonization in chronic respiratory disease is suggested to vary from days to years[25–28] and is detectable during both stable and exacerbated periods of asthma.[22,29,30]

Despite an increasing number of studies indicating associations between chronic NTHi airway colonization and asthma, it is not clear how NTHi persists in the airway and whether this persistence modulates the airway immune response. Although NTHi was previously regarded as an extracellular pathogen, accumulating evidence suggests that NTHi can invade and persist within host immune cells to facilitate airway survival.[31] Numerous in vitro studies have demonstrated the ability of NTHi to invade and persist within macrophages[32–35] with in vivo evidence of NTHi intracellular invasion within macrophage-like cells from adenoid tissue also apparent.[36,37] Despite this, the ability of NTHi to persist within macrophages in the asthmatic airway is less clear.

Although fundamental to the airway immune response, macrophages have an altered phenotype in asthma[38] and are functionally impaired, which worsens with disease severity.[39] A role for macrophages in shaping the T17 response in asthmatics and promoting neutrophilic inflammation has also been suggested, with the increased presence of IL-17+ macrophages detected in the bronchoalveolar lavage (BAL) of asthmatic patients.[40] Furthermore, we have recently shown that NTHi-infected macrophages upregulated genes involved in the IL-17 pathway and increased the release of neutrophil-related mediators including IL-8 and IL-17C.[41] It is unclear whether NTHi presence in the airway modulates macrophage activity and subsequently contributes to the complex chronic inflammatory environment characteristic of severe asthma.

We hypothesized that NTHi infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma. As such, the aim of our study was to investigate NTHi-macrophage interactions in severe asthma. We first assessed the presence of NTHi in severe asthma BAL samples. We then utilised RNASeq data (GSE180166) from an in vitro NTHi monocyte-derived macrophage (MDM) intracellular persistence model to investigate macrophage transcriptomic networks and identify the central NTHi-response genes. Finally, we determined whether NTHi modulation of these identified genes was detectable in severe asthma and correlated with patient clinical characteristics and airway inflammation.

2 | RESULTS

2.1 | NTHi association with airway macrophages can be detected in severe asthma

To first assess bacterial presence in severe asthma, fluorescence *in situ* hybridisation (FISH) was performed on BAL cytospins generated from 25 severe asthma patients undergoing bronchoscopy as part of the WATCH study.[42] BAL cytospin slides were stained with DAPI (Figure 1A), a universal bacterial (EUB338A) FITC probe (Figure 1B) and an NTHi-specific bacterial 16 s rRNA (HAIN16S1251) CY3 probe (Figure 1C), to visualize the presence of host cells, bacteria and NTHi, respectively. The NTHi-specific CY3 and pan-bacteria FITC signal overlapped, indicating NTHi presence only (Figure 1D). Furthermore, overlaying the individual host and NTHi fluorescent signals demonstrated that the NTHi signal closely associated with host BAL cell single-lobed nuclei (Figure 1E). Differential cell counting determined that macrophages were the predominant BAL cell type (median 91.6%, Figure S1), indicating NTHi association with pulmonary macrophages. In total, 56% of patient BAL samples (14/25) demonstrated a detectable NTHi-specific CY3 signal (herein referred to as NTHi+, Figure 1F).

To determine the impact of NTHi presence on airway inflammation and clinical characteristics, NTHi+ and NTHi- patient groups were compared (Table 1, S1). NTHi+ individuals exhibited increased sputum neutrophil proportion ($p = .0462$), which corresponded to a decrease in macrophage sputum proportion ($p = .0221$). Stratification of patients into neutrophilic or non-neutrophilic sputum inflammatory phenotypes found a higher number (12/14; 86%) of NTHi+ individuals were neutrophilic, compared with only 2/14 (14%) NTHi- individuals (Figure S2). However, this difference did not reach statistical significance ($p = .081$), which could be due to the overall neutrophilic nature of this cohort (17/25, 68%; $p = .0227$), with 5/11 NTHi- patients also categorized as neutrophilic (45.5%). NTHi presence was also associated with a longer asthma duration (34 years), compared with NTHi- individuals (22.5 years, $p = .0436$).

2.2 | WGCNA identifies modules significantly correlated to the macrophage response to NTHi

As NTHi presence in BAL was visualized to be associated with pulmonary macrophages, we next sought to determine the impact of NTHi presence on macrophage gene expression. We have previously modeled intracellular NTHi within human MDM and identified numerous macrophage differentially expressed genes (DEGs) using RNASeq.[41] However, this previous work did not consider the importance of gene co-expression or identify which hub genes are the crucial components within a transcriptomic network. In this current study, we further interrogated the RNASeq data (GSE180166) using WGCNA[43] to identify the key biological networks and macrophage hub genes, which could then be compared between the NTHI+ and NTHi- groups within our severe asthma cohort by qPCR.

Network topology analysis assigned 15,048 MDM genes to 15 modules (Figure 2A, B), with 10 modules significantly correlated with NTHi infection (Figure 2C, all $p < .05$). To determine the significance between the genes assigned to each module and NTHi infection, correlation of module membership (MM) and gene significance (GS) was performed. High correlation between GS and MM indicates that genes that are significantly correlated with the trait of interest (GS) are also important components of the module (MM). The blue module showed the strongest correlation, indicating that genes within this module are both significantly associated with the infection trait and are important components of the blue module (Figure S3A; 0.93, $p < 0.001$).

Clustering of modules with the infection trait further emphasized the significant relationship of the blue module with NTHi infection (Figure 2D). Four distinct clusters of modules were visible and gene list enrichment analysis identified enrichment of divergent processes for each cluster (Figure S4). Cluster I, which included the blue module and infection trait, was significantly enriched in immune processes, with a significant overlap of terms shared between the blue module

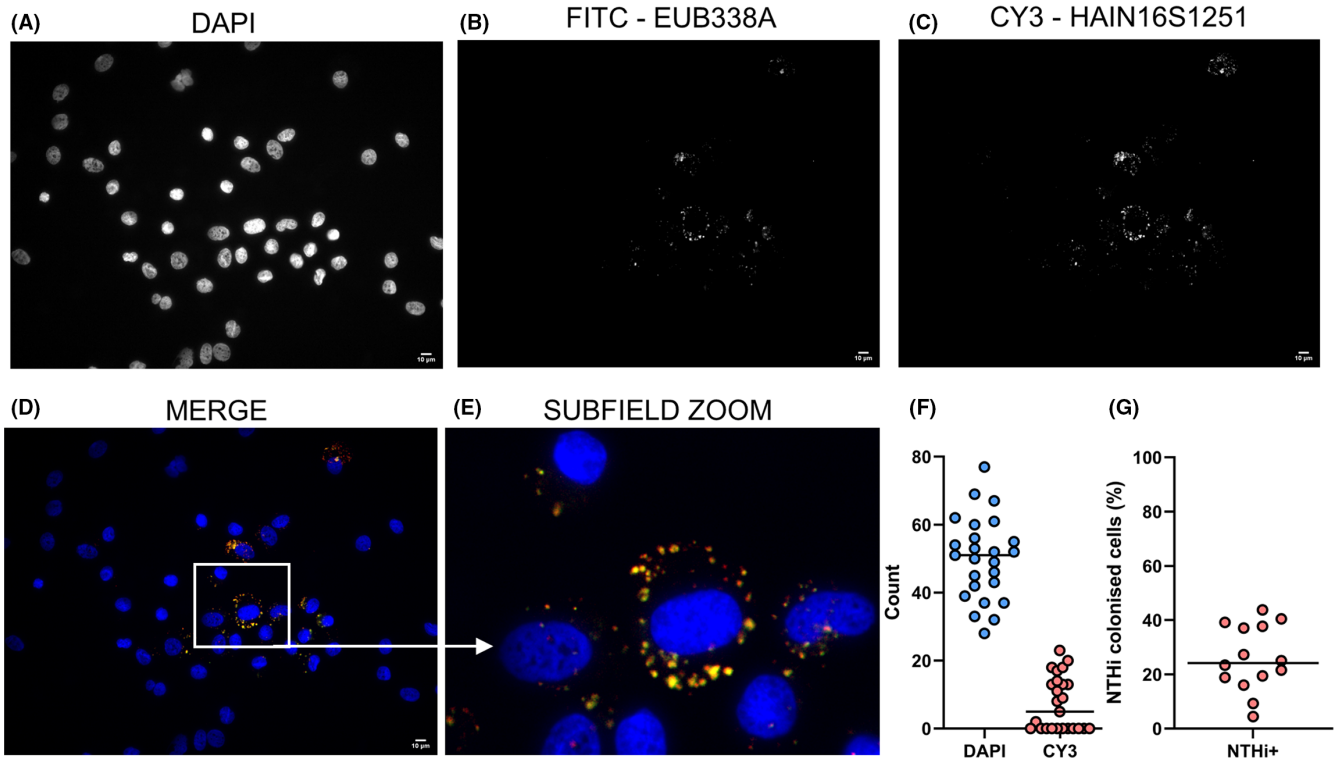


FIGURE 1 Detection of NTHi colonization of asthmatic BAL cells by FISH. Images show representative cytospin slide of severe asthma BAL cells colonized with NTHi. Slides were stained with (A) DAPI, (B) a pan-bacteria FITC probe (EUB338A) and (C) an NTHi-specific CY3 probe (HAIN16S1251). (D) Merged color composite of all three fluorescent channels. (E) Enlarged subfield view of (D), indicated by the white arrow. Images were acquired using a ×40 magnification immersion oil objective. Scale bar shows 10 μm. (F) Quantification of NTHi colonization of asthmatic BAL cells for all 25 patients within the study cohort. The total DAPI (host cell nucleus) and CY3 (NTHi) signal detected in 25 asthma BAL cytospins were quantified in Image J. Each individual dot indicates the total DAPI count and CY3 count for each patient cytospin. (G) The percentage of host cells colonized with NTHi per field of view for each cytospin slide was determined by the number of CY3 counts over the DAPI count for each individual patient. The median percentage of colonized cells per field of view was 24.2%. Only samples that returned a CY3 count >1, indicating NTHi presence, were included. Line on graphs indicates median

individually and Cluster I overall, suggesting the genes assigned to the blue module were driving the enrichment of terms in cluster I (Figure S5A,B), which were significantly enriched in immune processes (Figure 3A, B). The expression profile of the 2333 MDM genes assigned to the blue module clustered depending on infection status (Figure S5C), indicating these genes were associated with NTHi infection. As a result, the blue module was chosen to explore further.

2.3 | Identification of the most highly connected genes in the blue module gene network

To further explore the importance of the macrophage genes assigned to the blue module, a network of the 62 most highly connected macrophage genes was constructed in Cytoscape (Figure 3C). All 62 genes were more highly expressed in response to NTHi compared with uninfected controls (Figure S6A). To determine the key genes in this network, genes were ranked by the Maximal Clique Centrality (MCC) scoring method using the Cytoscape cytohubba plugin. *CASP4*, *PNRC1* and *SGPP2* were ranked as the top three genes in the network (Table S2).

Enrichment analysis of the 62 genes present in this network identified significant enrichment of Biological Processes involved in immune responses (Figure S6B). KEGG pathway analysis further confirmed enrichment of macrophage immune responses, including asthma-related inflammatory pathways 'IL-17 signaling pathway' and 'Th17 cell differentiation' (Figure S6C). To assess macrophage release of T17 mediators, levels of IL-1β, CXCL8, IL-23 and CCL20 were measured in cell culture supernatants. All T17 mediators were significantly upregulated at both time points during NTHi infection compared with uninfected controls (Figure S7A-D, all $p < .05$).

We also validated the expression of the 3 top-ranked in vitro NTHi response genes (*CASP4*, *SGPP2* and *PNRC1)* in our NTHi-MDM model by qPCR. We selected 3 other immune-related genes from the network (*IL1B*, *GBP1* and *LAMP3*), which have previously shown to be important for macrophage immune responses to intracellular pathogens.[44–46] Increased expression of all 6 genes was measured in response to NTHi compared with uninfected controls (Figure S8A–F, all $p < .05$).

2.4 | Expression of macrophage genes in severe asthma samples

Next, it was important to determine whether this transcriptomic signal of NTHi modulation of macrophage immune responses could

TABLE 1 Comparison of demographics and clinical characteristics between NTHi+ and NTHi- samples

	Total ($n = 25$)	NTHi + ($n = 14$)	NTHi – ($n = 11$)	p-value
General demographics				
Age (yr)	56 [47–66.5]	59 [54.25–67.25]	50 [41–68]	.2167
Gender (M/F)	15/10	10/4	5/6	.240
Smoking status (Ex/Never)	11/14	7/7	4/7	.6887
Pack years	0 [0–4.9]	0 [0–6.625]	0 [0–2]	.6805
Asthma-related characteristics				
Asthma duration	21 [9.5–42.5]	34 [18.5–47.75]	22.5 [8–27]	**.0436**
ACQ Score	2.3 [1.5–2.9]	2.25 [1.3–3.1]	2.3 [1.5–2.8]	.6959
Exacerbation (last 12 months)	1 [1–2.5]	1 [1–1.5]	2[0–4]	.4918
Pulmonary function				
BD FEV1%#	83.5% [71%–92.25%]	81.5 [47–90.75]	83.5 [90–93.5]	.3207
Pre BD FEV1/FVC %#	69.5% [64.42%–76.01%]	67.2 [54.9–75.3]	73.68 [67.41–76.88]	.1802
Asthma management				
Inhaled corticosteroids	18/25 (72%)	10/14 (71.4%)	8/11 (72.7%)	>.99
BDP equivalent dose(μg)	2000 [2000–2000]	2000 [1000–2000]	2000 [2000–2000]	.2279
Maintenance oral steroids	6/25 (24%)	4/14 (29%)	2/11 (18%)	.6609
Long-term antibiotics	4/25 (13%)	2/14 (14%)	2/11 (18%)	>.99
Sputum cell count				
Macrophage (%)	35.6 [16.6–53.5]	19.1 [12.9–46.4]	51.3 [51.3–63.6]	**.022**
Neutrophil (%)	57 [24.8–73.7]	67 [49.2–80.7]	24.9 [16.3–62]	**.046**
Eosinophil (%)	0.8 [0.2–5.4]	0.9 [0.3–3.2]	0.8 [0.1–9.6]	.43
Lymphocyte (%)	0 [0–0]	0 [0–0.1]	0 [0–0]	.08

Note: The 25 severe asthma patients were split based on the outcome of the FISH analysis into NTHi+14 and NTHi-11 groups. Values reported are medians [IQR] or (%). # indicates missing patient data; prebronchodilator spirometry measures and asthma duration history were not available for 2 patients. Statistical analysis was performed between NTHi+ and NTHi- groups. Continuous data were analyzed by the Mann–Whitney U test; categorical data were analyzed by the Fisher's exact test. Bold indicates p-values determined as statistically significant ($p < 0.05$).

Abbreviations: ACQ, asthma control questionnaire; BD, bronchodilator; BDP, beclometasone dipropionate; BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity.

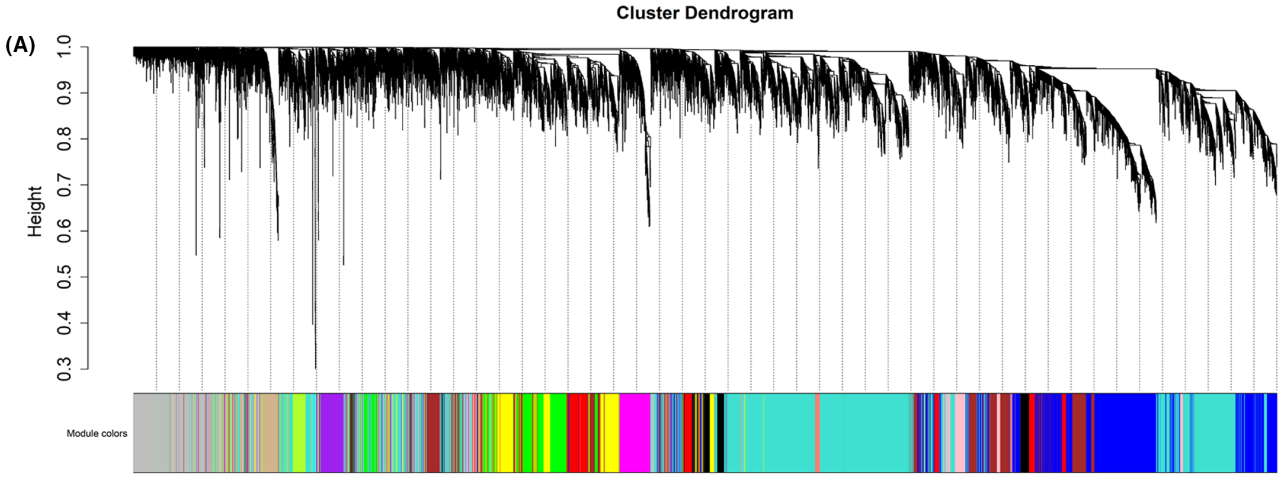
be detected in vivo. To do this, 25 BAL cell pellets matched to the 25 BAL cell cytospins were used to investigate the expression of the selected 6 macrophage blue module network genes using qPCR (Figure 4). While expression of *CASP4*, *PNRC1* and *LAMP3* did not significantly differ between groups ($p > .05$), *SGPP2* ($p = .0221$), *IL1B* ($p = .0014$) and *GBP1* ($p = .0477$) expression was higher in NTHi+ compared with NTHi- samples (Figure 4C–E respectively). We also assessed BAL supernatants for CXCL8 and found significantly higher levels of CXCL8 in NTHi+ samples (Figure S7E, $p = .0217$).

2.5 | Correlation of NTHi-related macrophage gene expression with clinical characteristics

Correlation analysis for all 25 patients found levels of co-expression between the 6 chosen genes in BAL (Figure 5), in line with the in vitro WGCNA MDM data. In particular, the two genes both more highly expressed in NTHi+ patients, *IL1B* and *GBP1*, were the most strongly positively correlated genes in BAL samples (rho = 0.67, $p = .00031$). Furthermore, these two genes correlated with clinical characteristics. A weak to moderate, but significant, positive correlation between *IL1B* expression and asthma duration was identified (rho = 0.41, $p = .041$), with both *IL1B* (rho = −0.42; $p = .055$) and *GBP1* (rho = −0.43, $p = .046$) negatively correlated with pre-BD FEV_1/FVC%. Therefore, NTHi modulation of macrophage gene expression could be detected in the severe asthmatic airway and importantly, was significantly associated with decreased lung function.

3 | DISCUSSION

Our study suggests that NTHi airway presence is facilitated by interactions with airway macrophages and is associated with sputum neutrophilic inflammation in severe asthma. We demonstrate NTHi association with BAL macrophages obtained from individuals with severe asthma during a stable period of disease. We investigated these interactions by performing WGCNA on RNASeq data from an NTHi-MDM intracellular persistence model,[41] which identified a transcriptomic gene network enriched in immune responses, including T17 responses. Confirmation of MDM release of T17 mediators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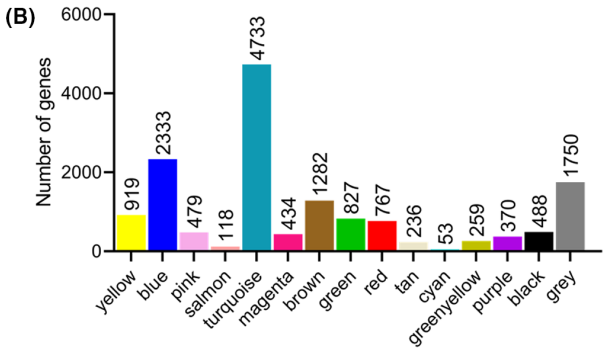
(B)
Number of genes
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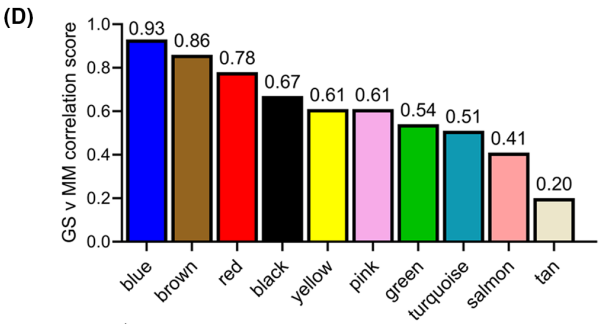
(D)
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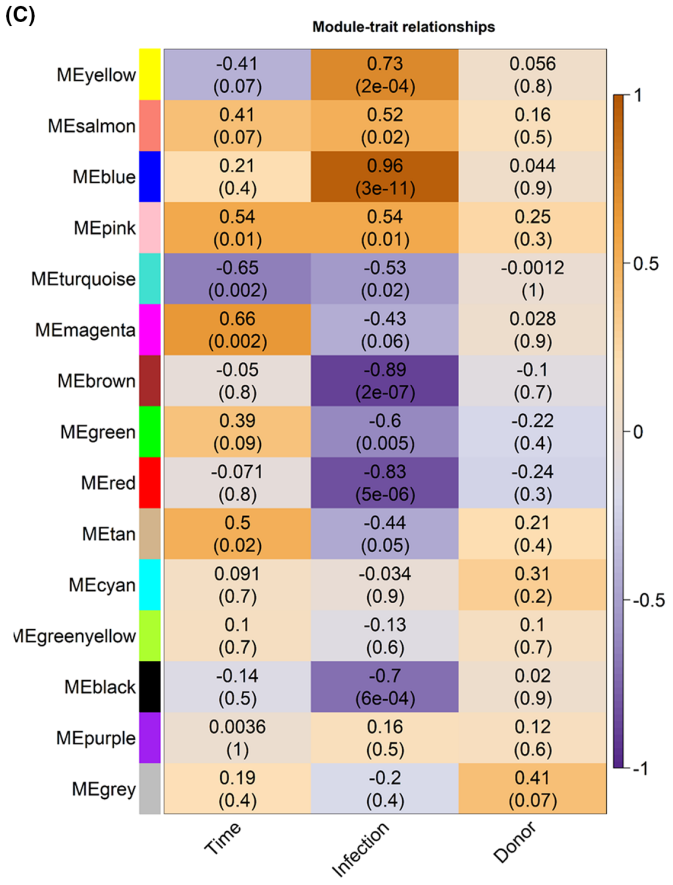
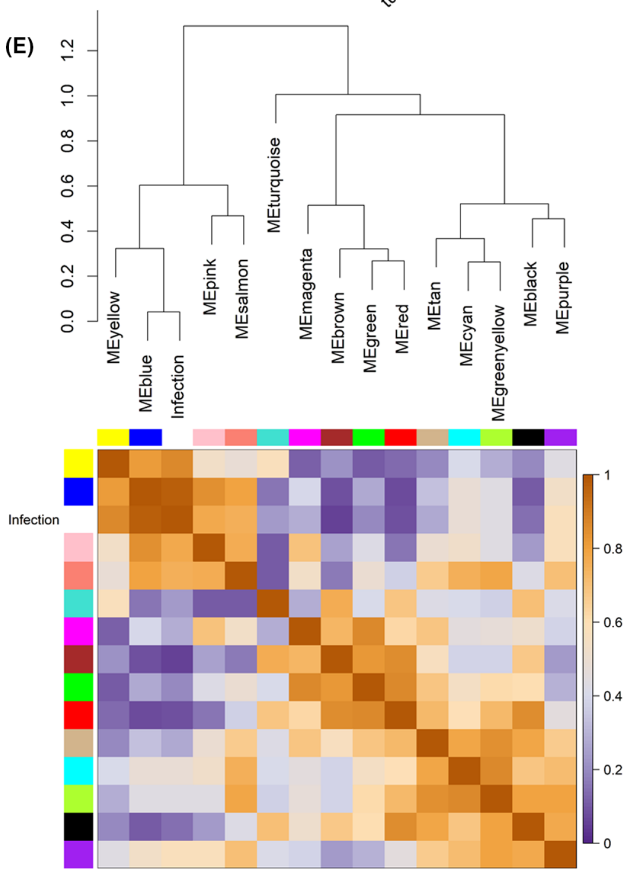

(C)
Module-trait relationships
MEyellow -0.41 (0.07) 0.73 (2e-04) 0.056 (0.8)
MEsalmon 0.41 (0.07) 0.52 (0.02) 0.16 (0.5)
MEblue 0.21 (0.4) 0.96 (3e-11) 0.044 (0.9)
MEpink 0.54 (0.01) 0.54 (0.01) 0.25 (0.3)
MEturquoise -0.65 (0.002) -0.53 (0.02) -0.0012 (1)
MEmagenta 0.66 (0.002) -0.43 (0.06) 0.028 (0.9)
MEbrown -0.05 (0.8) -0.89 (2e-07) -0.1 (0.7)
MEgreen 0.39 (0.09) -0.6 (0.005) -0.22 (0.4)
MEred -0.071 (0.8) -0.83 (5e-06) -0.24 (0.3)
MEtan 0.5 (0.02) -0.44 (0.05) 0.21 (0.4)
MEcyan 0.091 (0.7) -0.034 (0.9) 0.31 (0.2)
MEgreenyellow 0.1 (0.7) -0.13 (0.6) 0.1 (0.7)
MEblack -0.14 (0.5) -0.7 (6e-04) 0.02 (0.9)
MEpurple 0.0036 (1) 0.16 (0.5) 0.12 (0.6)
MEgrey 0.19 (0.4) -0.2 (0.4) 0.41 (0.07)
Time
Infection
Donor
1
0.5
0
-0.5
-1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MEred
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FIGURE 2 Construction of macrophage transcriptomic gene network using WGCNA identifies significant modules associated with NTHi infection. (A) Clustering dendrogram of all 15,048 genes with dissimilarity based on topological overlap with assigned module colors below. The gray module was categorized as the 'bin' module containing genes not assigned to any module. (B) Bar plot depicting the number of genes assigned to each module. (C) Association between modules and sample trait data. Each row corresponds to a module eigengene and each column to a trait. Each cell contains the correlation score (top) and p-value (bottom) assigned to each relationship. (D) Module membership (MM) and gene significance (GS) correlation scores for infection. The correlation score for each module is present above each bar, and modules are ordered based on significance (most significant to least significant, left to right) for the correlation (all $p < .05$). (E) Visualization of the eigengene network as a dendrogram and heatmap showing the relationships between individual modules and the NTHi infection. Four distinct clusters of modules were visible in the dendrogram; cluster I (yellow, blue, pink and salmon), cluster II (turquoise), cluster III (magenta, brown, green and red) and cluster IV (tan, cyan, greenyellow, black and purple). Each row and column in the heatmap corresponds to one module eigengene (labeled by color) or NTHi infection. In the heatmap, purple color represents low adjacency, while orange represents high adjacency

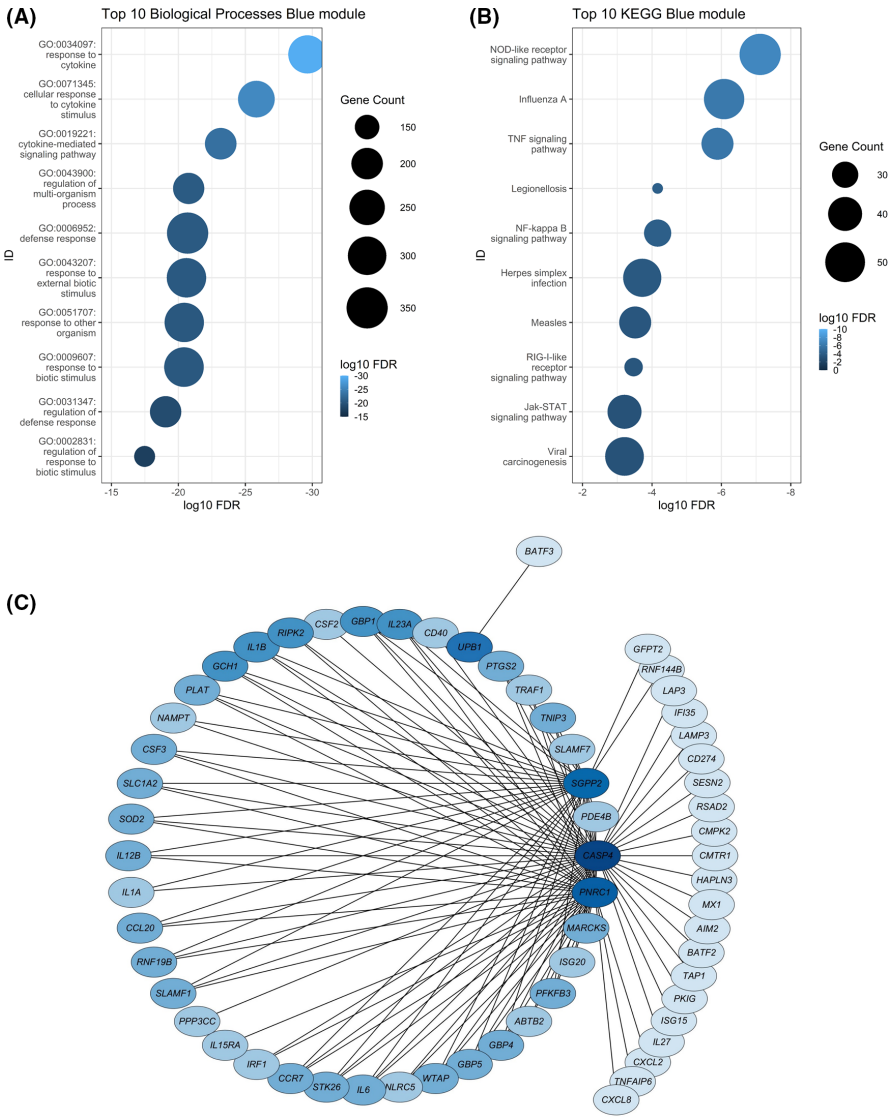

FIGURE 3 Blue module gene expression profile and gene ontology analysis indicates the enrichment of macrophage immune processes in response to intracellular NTHi. (A) Enrichment of biological processes indicates activation of MDM immune responses in response to NTHi, with (B) KEGG pathway analysis indicating enrichment of immune responses involved in the response to an intracellular pathogen. Pathway/category IDs are ordered by enrichment significance (FDR), which is indicated by the color of each dot, with the size of the dot representing the number of genes assigned to each pathway/category. (C) To identify the top connected genes within this blue module, visualization of the blue module network was generated in Cytoscape using a topological overlap threshold of 0.34. Gene ranking performed by Maximal Clique Centrality (MCC) using the cytoHubba plugin in Cytoscape. Nodes are colored blue, with darker shades indicating high network importance, lighter shades indicate decreasing network importance and solid lines show connecting edges/interactions between nodes

in cell culture supernatants further demonstrated the contribution of NTHi-infected macrophages to T17 inflammation. NTHi presence and modulation of *IL1B* and *GBP1* gene expression in vivo correlated with clinical characteristics including increased sputum neutrophilic inflammation and lower lung function measures. These findings have important implications for understanding the drivers of chronic airway inflammation and disease progression in severe asthma.

The mechanisms underlying NTHi airway persistence remain unclear. Although NTHi has the capacity to form biofilms in vitro, NTHi biofilm formation in the human lung is less understood.[47] We did not observe any evidence of NTHi biofilm formation in our samples but rather saw the presence of cell-associated NTHi in 56% of individuals during a stable period of disease. A limitation of our fluorescence microscopy is that it did not allow for ascertainment of whether the

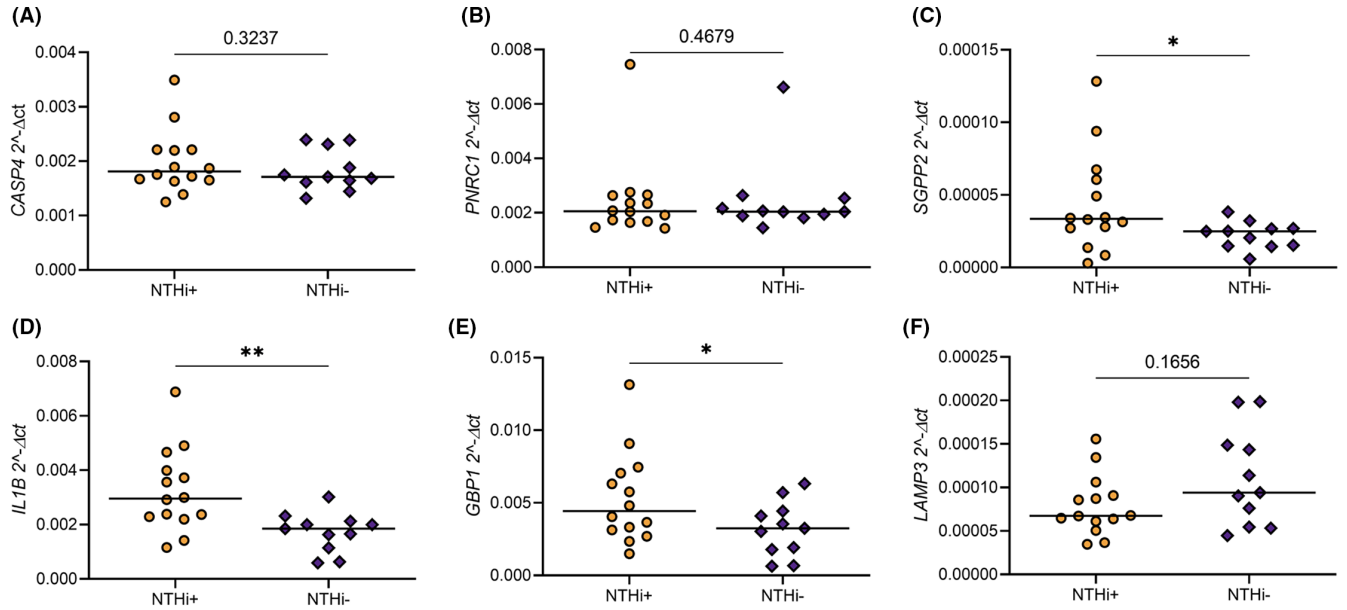

FIGURE 4 Expression of macrophage genes was detectable in BAL from severe asthma. The expression of six MDM genes identified in the blue module network deemed to be significantly associated with NTHi infection were investigated in BAL samples by qPCR. Patients were split into NTHi+ or NTHi- groups based on the outcome of the FISH analysis identifying NTHi presence in matched BAL cell cytospins. The top three ranked genes, (A) *CASP4*, (B) *PNRC1* and (C) *SGPP2*, and three other genes present in the blue module network and associated with an intracellular immune response, (D) *IL1B*, (E) *GBP1* and (F) *LAMP3*, were measured. Gene expression was normalized to *B2M*. Graphs show medians, $n = 25$. Data were analyzed by the Mann–Whitney U test, $^{*}p < .05$, $^{**}p < .01$

BAL macrophage-associated NTHi was intracellularly or extracellularly localized within our samples. Nonetheless, our findings are corroborated by Dickson and colleagues, who found that significant components of the lung microbiota are in fact cell-associated.[48] Indeed, our recent work reported the ability of multiple clinical NTHi strains to invade macrophages and persist intracellularly.[41]

In this current study, we performed WGCNA on macrophage RNASeq data to identify biologically significant transcriptomic networks and the hub genes that were important during NTHi-macrophage interactions. *CASP4* was the top-ranked gene in the blue module gene network, suggesting a role of the noncanonical inflammasome pathway in the macrophage response to NTHi. Elevated expression of inflammasome components in severe asthma including NLRP3, caspase-1 and caspase-4 have previously suggested a role for the inflammasome pathway in asthma.[49] However, we found no difference in *CASP4* expression between NTHi+ and NTHi- patients. The lack of *CASP4* expression between patients stratified by NTHi presence may be due to the underlying inflammatory phenotype-dependent differences. Simpson and colleagues detected higher *CASP4* gene expression in neutrophilic asthmatics compared with eosinophilic and paucigranulocytic asthmatics.[49] As such, *CASP4* expression may already be elevated compared with healthy individuals or other asthma inflammatory phenotypes, independently of NTHi presence.

Our results indicate an important role for NTHi modulation of IL-1β pathways; we found significant upregulation of *IL1B* gene expression and protein release by NTHi-infected MDM and elevated *IL1B* gene expression in NTHi+ BAL samples, with this signal absent in uninfected MDM or NTHi− BAL. Elevated BAL *IL1B* gene expression is in agreement with previously published studies identifying elevated airway IL-1β gene and protein expression in asthma.[49–54] Moreover, a recent substudy of the AMAZES trial demonstrated a strong correlation between *H. influenzae* load and IL-1β in those with noneosinophilic asthma following add-on azithromycin, further emphasizing the role of NTHi in driving airway inflammation in asthma.[55]

Macrophages have recently been shown to display an altered transcriptomic profile in neutrophilic asthma,[56] thus could be an alternative cell type, other than the neutrophil, to therapeutically target in neutrophilic asthma. Here, we show co-expression of *IL1B* and *GBP1* in BAL, suggesting modulation of *IL1B* by macrophage intracellular immune pathways involving GBPs, which could offer insights into how to target IL-1-mediated inflammatory pathways. As our work suggests that NTHi-infected macrophages may be the source of IL-1β-induced inflammation in the asthmatic airway, stratifying patients based on NTHi presence may result in better therapeutic outcomes. IL-1 pathway interventions have yet to be shown to be successful in asthma, and may only be effective in certain patient cohorts, such as those colonized with NTHi.[57]

NTHi presence in our severe asthma sub-cohort was associated with increased sputum neutrophil proportion, supporting previous work demonstrating that individuals possessing a *Haemophilius*-dominant microbiome were more likely to be neutrophilic.[23,24] It is not clear why NTHi is associated with neutrophilic asthma; however, we found NTHi presence was associated with increased asthma duration. Long-term exposure of mice to *H. influenzae* resulted in

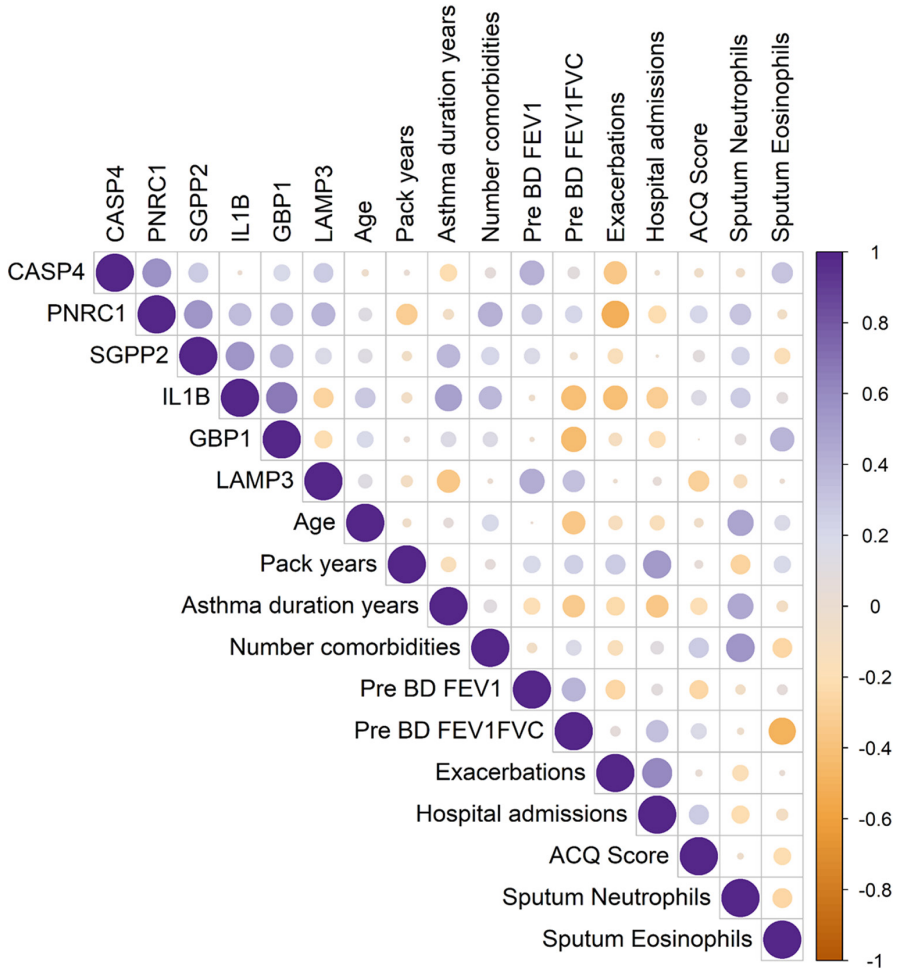

FIGURE 5 Correlation of expression of genes from the blue module network with asthma clinical characteristics in severe asthma BAL samples Spearman's correlation was used to determine correlations between the level of gene expression and patient characteristics or demographics. Larger sized dots indicate more significant correlations, with the correlation coefficient (rho) represented by the colored bar (right), with blue representing positive correlations and red representing negative correlations

airway remodeling following a bacteria-induced switch from a T2, eosinophilic inflammatory phenotype to T17, neutrophilic inflammation.[58] Furthermore, murine studies have demonstrated that the combination of NTHi infection and allergic airways disease promotes a steroid-resistant, neutrophilic inflammatory phenotype.[59,60]

We have previously shown NTHi-infected macrophages upregulate IL-17C release, providing further evidence of NTHi-macrophage interactions contributing to T17 responses.[41] In this current work, we demonstrate enrichment of immune pathways involved in T17 inflammation and confirmed MDM release of T17 mediators in response to NTHi intracellular persistence, and elevated CXCL8 levels in NTHi+ BAL samples. Given previous studies reporting independent associations of NTHi and macrophage contribution to T17 inflammation,[40,60,61] our work links these observations and proposes that NTHi association with pulmonary macrophages may contribute to and propagate the development of the T17-driven neutrophilic phenotype in asthma.

Therefore, investigating the role of NTHi persistence in the airway of asthmatics prior to severe disease development could elucidate the exact role of NTHi interactions with macrophages on asthma progression. Although the main aim of our study was to specifically investigate NTHi modulation of the severe asthmatic airway, we acknowledge the small cohort size and severe nature of asthma experienced by our cohort is a limitation. Furthermore, WGCNA identified clusters of genes that were significantly associated with NTHi infection using an in vitro MDM NTHi persistence model, which demonstrated significant enrichment of innate immune responses. However, the gene signature from this analysis may be too acute to be recapitulated in the chronically colonized airway, with NTHi known to persist from days to years.[25–28] Thus, future transcriptomic work using a larger cohort of BAL samples longitudinally obtained from both health and asthma will better allow for determining global transcriptomic alterations associated with NTHi persistence in vivo. Such studies will also allow for the determination of whether potential confounding factors, such as steroid usage or antibiotic treatment, impact the expression of the genes identified in this current analysis. A further potential confounding factor is the heterogeneous nature of our BAL cell population; although 91.6% of cells were macrophages, other cell types are present and may influence gene expression results. Although we only observed NTHi association with BAL macrophages within our samples, both in vitro and in vivo work has demonstrated the ability of NTHi to infect epithelial cells and induce inflammatory responses,[62] so these cells could also be an important source of airway inflammation. The use of single-cell methodologies would provide further resolution of cell-specific gene expression within the asthmatic airway.

Finally, although GM-CSF differentiated MDM are commonly used and are phenotypically comparable to alveolar macrophages,

including similar phagocytic capacity,[63] cytokine production[64] and expression of cell surface markers,[65] they may not fully recapitulate in vivo NTHi-macrophage interactions, especially given the known functional impairments of macrophages in asthma.[38,39] Future modeling work would benefit from using macrophages isolated from both healthy and asthmatic airways, and using a culture system that attempts to retain the 3D lung architecture and complex cellular milieu, to allow for a better understanding of NTHi-macrophage interactions in vivo. Due to the complex, multifactorial nature of asthma, it is likely that additional factors contribute to disease progression, rather than a single transitional event. Unpicking the complex relationship between NTHi persistence, the host immune response and severe asthma pathogenesis over an extended period using a longitudinal cohort could reveal the opportune moment to therapeutically intervene to prevent disease progression.

In conclusion, NTHi infection of pulmonary macrophages may drive components of complex chronic airway inflammation, including inflammasome activation and T17 responses, leading to the development of a neutrophilic, severe asthma phenotype. Our work highlights a potential benefit of stratifying patients based on NTHi presence to improve treatment efficacy. Although antibiotic therapy has shown to be successful in reducing *H. influenzae* load, not all patients experienced depletion of *H. influenzae* and the treatment was associated with increased antibiotic resistance gene carriage.[66] Therefore, uncovering novel, nonantibiotic therapeutic targets to reduce the burden of NTHi in asthma may alleviate chronic airway inflammation, prevent the progression to more severe disease and improve patient outcomes.

4 | MATERIALS AND METHODS

4.1 | WATCH patient recruitment

Bronchoalveolar lavage (BAL) samples were obtained from a subcohort of patients undergoing bronchoscopy as part of the Wessex Asthma Cohort of Difficult Asthma (WATCH) study in accordance with the protocol approved by the West Midlands—Solihull Research Ethics Committee (REC:14/WM/1226), as per Azim et al.[42] All patients included did not have a clinical diagnosis of COPD. All subjects who provided samples gave informed consent. Further information about lung sample processing for the generation of cytospin slides and RNA extraction is available in the supplementary material.

4.2 | Fluorescence in situ hybridisation (FISH)

FISH was performed on stored BAL cytospin slides as previously described,[67] using an NTHi-specific Cy3-labeled probe (HAIN16S1251) and a pan-bacteria FITC-labeled probe (EUB338A). Slides were visualized using a Zeiss Axioskop2 Mot fluorescence microscope using x 40 magnification immersion oil objective. Images were acquired using Micro-Manager and analyzed in ImageJ/FIJI (National Institutes of Health, USA, version 1.53c). Further details are supplied in the Appendix S1.

4.3 | MDM NTHi intracellular infection model

MDM culture and infection were performed as previously described.[41] Briefly, monocytes were isolated from the blood of healthy volunteers (in accordance with Hampshire A Research Ethics Committee, 13/SC/0416) by magnetic associated cell sorting (MACS) and differentiated into monocyte-derived macrophages (MDM) in the presence of 2 ng/ml GM-CSF over 12 days as previously described.[68] NTHi ST14 was grown and stored in aliquots at −80°C until required in accordance with the protocol described by Kirkham and colleagues.[69] NTHi infection of MDM was performed at a multiplicity of infection (MOI) 100 for 6 h in antibiotic-free RPMI media. After 6 h, MDM was incubated in RPMI media supplemented with 500 μg/mL gentamicin for 90 min to remove and kill extracellular NTHi. Gentamicin-containing media was removed, and cells were incubated in antibiotic-free media until 24 h.

4.4 | Weighted gene correlation network analysis (WGCNA)

We utilised RNA Sequencing data from an NTHi-MDM intracellular persistence model previously reported by Ackland et al[41] and deposited at http://www.ncbi.nlm.nih.gov/geo/ under GEO accession number GSE180166. Normalized and transformed data were analyzed using the WGCNA R package.[43] Further information is available in the Appendix S1.

4.5 | RNA isolation and qPCR

RNA was extracted from BAL cell pellets or MDM and reverse transcribed to cDNA as previously described.[41] Expression of macrophage genes was assessed by quantitative PCR (qPCR) using TaqMan universal PCR master mix (Applied Biosystems), with all primers obtained from Applied Biosystems (Table S3). The qPCR reactions were performed at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min using a 7900HT Fast Real-Time PCR System. The expression of target genes was normalized to *B2M* expression and expressed as deltaC.

4.6 | Mediator release assays (ELISA and Luminex)

IL-1β and CXCL8 release into cell culture or BAL supernatants were assessed by DuoSet ELISA kits, which were carried out according to the manufacturer's instructions (R&D Systems). IL-23 and CCL20 release into cell culture supernatants was assessed by a customized Luminex Human Magnetic Assay according to the manufacturer's

instructions (R&D Systems). Further information is supplied in the Appendix S1.

4.7 | Statistics

Statistical analysis was performed using GraphPad Prism (version 8 GraphPad Software, San Diego, USA), and statistical significance was determined as $p < .05$. The Wilcoxon signed-rank test between paired data or the Mann–Whitney U test between unpaired data were performed. Correlation analyses were performed using the Spearman rank correlation coefficient. The Fisher's exact test was used for the analysis of categorical data.

AUTHOR CONTRIBUTIONS

JA contributed to the study design, data collection and analysis and drafted the initial manuscript. CB, AA and RJK contributed to study design, undertook longitudinal data collection and contributed to manuscript preparation. AH contributed to data analysis and manuscript preparation. DWC, MC, PH and TMAW contributed to the study design and manuscript preparation. KJS contributed to the study design, drafted the initial manuscript and acted as a guarantor for the paper.

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CONFLICT OF INTEREST

DC reports that he was a postdoctoral researcher on projects funded by Pfizer and GSK between April 2014 and October 2017. RJK received a nonpromotional grant (£35,000) from Novartis to assist in funding initial database support for the WATCH study. PH reports that he is an employee of GSK. TW reports grants and personal fees from AstraZeneca, personal fees and others from MMH, grants and personal fees from GSK, personal fees from BI, and grants and personal fees from Synairgen, outside the submitted work. KS reports grants from AstraZeneca, outside the submitted work. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORCID

Jodie Ackland https://orcid.org/0000-0003-3120-3620
Clair Barber https://orcid.org/0000-0001-5335-5129
Adnan Azim https://orcid.org/0000-0001-5960-1159
Karl J. Staples https://orcid.org/0000-0003-3844-6457

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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