The Role of Non-typeable Haemophilus Influenzae Biofilms in Chronic Obstructive Pulmonary Disease

Jake R. Weeks[1*], Karl J. Staples[1], C. Mirella Spalluto[1], Alastair Watson[2], Tom M. Wilkinson[1]

[1]Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, United Kingdom, [2]University of Southampton, United Kingdom

Submitted to Journal:
Frontiers in Cellular and Infection Microbiology

Specialty Section:
Biofilms

Article type:
Review Article

Manuscript ID:
720742

Received on:
04 Jun 2021

Revised on:
07 Jul 2021

Journal website link:
www.frontiersin.org

Conflict of interest statement

The authors declare a potential conflict of interest and state it below

Karl Staples reports grants from AstraZeneca, outside the conduct of the study; Tom Wilkinson reports grants and personal fees from AstraZeneca, outside the conduct of the study; personal fees and other from MMH, grants and personal fees from GSK, grants and personal fees from AZ, personal fees from BI, grants and personal fees from Synairgen, outside the submitted work; Jake Weeks, C. Mirella Spalluto and Alastair Watson report no conflicts of interest.

Author contribution statement

Jake Weeks: conceptualization, investigation, literature searching, analysis, project administration, writing original draft, reviewing and editing, approval of final draft. Karl Staples: supervision, conceptualization, reviewing & editing, approval of final draft. C. Mirella Spalluto: supervision, conceptualization, reviewing & editing, approval of final draft. Alastair Watson: supervision, reviewing & editing, approval of final draft. Tom Wilkinson: supervision, conceptualization, reviewing & editing, approval of final draft.

Keywords

NTHI, Biofilms, Chronic Obstructive Pulmonary Disease (COPD), Airways diseases, Lung Microbiome, Host-Pathogen Interactions, Antimicrobial tolerance

Abstract

Word count: 339

Non-typeable Haemophilus influenzae (NTHi) is an ubiquitous commensal-turned-pathogen that colonises the respiratory mucosa in airways diseases including Chronic Obstructive Pulmonary Disease (COPD). COPD is a progressive inflammatory syndrome of the lungs encompassing chronic bronchitis that is characterised by mucus hypersecretion and impaired mucociliary clearance and creates a static, protective, humid, and nutrient-rich environment, with dysregulated mucosal immunity; a favourable environment for NTHi colonisation

Several recent large COPD cohort studies have reported NTHi as a significant and recurrent aetiological pathogen in acute exacerbations of COPD. NTHi proliferation has been associated with increased hospitalisation, disease severity, morbidity and significant lung microbiome shifts. However, some cohorts with patients at different severities of COPD do not report that NTHi is a significant aetiological pathogen in their COPD patients, indicating other obligate pathogens including Moraxella catarrhalis, Streptococcus pneumoniae and Pseudomonas aeruginosa as the cause. NTHi is a ubiquitous organism across healthy non-smokers, healthy smokers and COPD patients from childhood to adulthood, but it currently remains unclear why NTHi becomes pathogenic in only some cohorts of COPD patients, and what behaviours, interactions and adaptations are driving this susceptibility.

There is emerging evidence that biofilm-phase NTHi may play a significant role in COPD. NTHi displays many hallmarks of the biofilm lifestyle and expresses key biofilm formation-promoting genes. These include the autoinducer-mediated quorum sensing system, epithelial- and mucus-binding adhesins and being embedded in a protective, self-produced polymeric substance matrix. These NTHi biofilms exhibit extreme tolerance to antimicrobial treatments and the immune system as well as expressing synergistic interspecific interactions with other lung pathogens including S. pneumoniae and M. catarrhalis.

Whilst the majority of our understanding surrounding NTHi as a biofilm arises from otitis media or in-vitro bacterial monoculture models, the role of NTHi biofilms in the COPD lung is now being studied. This review explores the evidence for the existence of NTHi biofilms and their impact in the COPD lung. Understanding the nature of chronic and recurrent NTHi infections in acute exacerbations of COPD could have important implications for clinical treatment and identification of novel bactericidal targets.

Contribution to the field

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death worldwide. Whilst treatments help manage symptoms, there is currently no known cure. Non-typeable Haemophilus influenzae (NTHi) is the predominant cause of COPD exacerbations, periodic episodes of worsening symptoms which often do not return to baseline. Furthermore, there is now emerging evidence of NTHi biofilms which provide numerous advantages and could play a significant role in COPD disease. NTHi displays many hallmarks of biofilms and expresses key biofilm-formation genes including the autoinducer-mediated quorum sensors and epithelial and mucus-binding adhesins. Furthermore, they can produce and embed themselves in a protective matrix. NTHi biofilms exhibit extreme tolerance to antimicrobial treatments and the immune system as well as expressing synergistic inter-specific interactions with other lung pathogens. Whilst the majority of our understanding surrounding NTHi as a biofilm historically arose from otitis media or in-vitro bacterial monoculture models, the role of NTHi biofilms in the COPD lung is now being studied. This review explores the evidence around NTHi biofilms and their impact in the COPD lung. Understanding the

nature of chronic and recurrent NTHi infections and acute exacerbations of COPD could be important for understanding this disease and identifying future therapeutic targets.

Funding statement

This work was undertaken using a BBSRC iCASE PhD studentship awarded for Jake Weeks' doctoral studies.

The Role of Non-typeable Haemophilus Influenzae Biofilms in Chronic Obstructive Pulmonary Disease

Weeks, J. R[1*], Staples, K. J[1,2], Spalluto, C. M[1,2], Watson, A[1,2,3], & Wilkinson, T. M. A [1,2*]

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

[2] NIHR Southampton Biomedical Research Centre, Southampton Centre for Biomedical Research, Southampton General Hospital, Southampton, UK

[3] Birmingham Medical School, University of Birmingham, Birmingham, UK

*** Correspondence: Jake Weeks J.R.Weeks@soton.ac.uk**

Keywords: Non-typeable Haemophilus influenzae (NTHi)[1], biofilms[2], chronic obstructive pulmonary disease (COPD)[3], airways diseases[4], lung microbiome[5], host-pathogen interactions[6], antimicrobial tolerance[7]

Abstract

Non-typeable *Haemophilus influenzae* (NTHi) is an ubiquitous commensal-turned-pathogen that colonises the respiratory mucosa in airways diseases including Chronic Obstructive Pulmonary Disease (COPD). COPD is a progressive inflammatory syndrome of the lungs encompassing chronic bronchitis that is characterised by mucus hypersecretion and impaired mucociliary clearance and creates a static, protective, humid, and nutrient-rich environment, with dysregulated mucosal immunity; a favourable environment for NTHi colonisation

Several recent large COPD cohort studies have reported NTHi as a significant and recurrent aetiological pathogen in acute exacerbations of COPD. NTHi proliferation has been associated with increased hospitalisation, disease severity, morbidity and significant lung microbiome shifts. However, some cohorts with patients at different severities of COPD do not report that NTHi is a significant aetiological pathogen in their COPD patients, indicating other obligate pathogens including *Moraxella catarrhalis, Streptococcus pneumoniae* and *Pseudomonas aeruginosa* as the cause. NTHi is a ubiquitous organism across healthy non-smokers, healthy smokers and COPD patients from childhood to adulthood, but it currently remains unclear why NTHi becomes pathogenic in only some cohorts of COPD patients, and what behaviours, interactions and adaptations are driving this susceptibility.

There is emerging evidence that biofilm-phase NTHi may play a significant role in COPD. NTHi displays many hallmarks of the biofilm lifestyle and expresses key biofilm formation-promoting genes. These include the autoinducer-mediated quorum sensing system, epithelial- and mucus-binding adhesins and being embedded in a protective, self-produced polymeric substance matrix. These NTHi biofilms exhibit extreme tolerance to antimicrobial treatments and the immune system as well as expressing synergistic interspecific interactions with other lung pathogens including *S. pneumoniae* and *M. catarrhalis*.

Whilst the majority of our understanding surrounding NTHi as a biofilm arises from otitis media or *in-vitro* bacterial monoculture models, the role of NTHi biofilms in the COPD lung is now being studied. This review explores the evidence for the existence of NTHi biofilms and their impact in the COPD lung. Understanding the nature of chronic and recurrent NTHi infections in acute exacerbations of COPD could have important implications for clinical treatment and identification of novel bactericidal targets.

1 Introduction

1.1 Chronic Obstructive Pulmonary Disease

COPD is currently the third leading cause of global mortality affecting an estimated 174 – 384 million people worldwide and is responsible for 3.2 million deaths annually (1). COPD is caused by the inhalation of noxious particles and gases and at least 90% of COPD patients live in developing countries where the disease is mainly associated with biomass fuel burning and pollution. The remaining 10% live in developed countries where the predominant cause is cigarette smoking (1). COPD is a heterogenous progressive, inflammatory syndrome of the lungs, characterised by the co-existence of predominantly two distinct syndromes; chronic bronchitis and emphysema (2). Together these conditions result in a chronic productive cough, poorly reversible airflow obstruction and alveolar parenchymal destruction (3), punctuated by episodic acute COPD exacerbations (AECOPD), that lead to hospitalisation (4).

Chronic bronchitis is particularly important with respect to the role of bacteria and chronic infections. An immunological cascade in response to inhaled particles and gases drives airway structural remodelling that leads to hypertrophy and hyperplasia of sub-mucosal bronchial glands and mucus-producing goblet cells (3), forming a viscous and alkaline mucus layer to trap particles and bacteria. Excess mucus production and damage to the airway epithelium by inflammation and oxidative stress results in ciliary dysfunction, which is also caused by the direct effects of smoking. This impairs mucociliary clearance and leads to the formation of obstructive mucus plugs, changing the biotic and abiotic conditions of the lung (5, 6)**.** Static mucus provides a nutrient-rich substrate for the colonisation of bacteria and mucus plugs trapped in bronchioles may form local anaerobic regions (6). Further immunological responses to bacterial infection include the formation of neutrophil extracellular traps (NETs) in severe COPD patients that has been associated with more frequent exacerbations and a reduction in lung microbiome diversity (7), as well as modulation of the complement system that enhances phagocytotic clearance of bacteria (8).

Together these conditions may lead to significant microbiome shifts and favour the proliferation of fastidious, facultatively anaerobic pathogens, and drive a biofilm lifestyle.

1.2 The Lung Microbiota in Airways Diseases & COPD

Contrary to historical views, the lung is not a sterile environment but has a rich and diverse microflora. The role of the lung microbiome and microbial dysbiosis in respiratory diseases is becoming increasingly understood through the advent of 16S rRNA sequencing of sputum and deep lung brushes and biopsy samples (9), where the microbial composition of sputum samples closely resembles that of the low biomass bronchial and peripheral lung samples (10). This has led to the characterisation of microbial patterns of respiratory disease and of COPD sub-types (11), supporting a patient-specific approach to treatment. However, there is currently no formal curated database of the Human Lung

Microbiota, which has not formed part of the Human Microbiome Project (12). Therefore, there is disparity across lung microbiome investigations (Table. 1.) into airways diseases partly driven by the lack of standardisation of methods of sample acquisition and processing (10).

The general consensus reports that the healthy lung microbiota is largely comprised of by *Pseudomonas, Streptococcus, Prevotella, Fusobacterium, Veillonella Porphyromonas and Haemophilus spp.* (12, 13) that together represent >85% of the core lung microbiome. These genera have an important commensal role in modulating the immune response mechanisms of the lung and priming of the immune system (14). These genera are ubiquitous inhabitants of the lung microenvironment in both health and disease such as COPD, which is associated with an overall reduction in this microbial diversity. However, this microbial community composition remains diverse between healthy, non-smoker individuals, and microbial diversity further varies in healthy-smokers and COPD patients. Microbiota composition changes also occur in COPD patients as a consequence of the chronic infection process, host immune system responses and long-term use of bronchodilators, antibiotics and corticosteroid therapy (15). Previously, significantly different clusters of microbial communities have been reported between severe (GOLD 3/4) and less severe (GOLD 1/2) COPD patients (16), with correlations also being drawn between the abundance of Moraxellaceae and Streptococcaceae with the expression of immune system factors including IL-10 and TNF-α (16). Lung microbiome comparisons also detected significant increases in Proteobacteria and *Haemophilus sp.* and reductions in *Bacteroidetes, Prevotella and Veillonella* (11) in severe and very severe COPD patients compared to moderate COPD patients (11). Coupled with a decrease in overall diversity, these studies highlight how the lung microbiota increasingly destabilises throughout COPD progression.

Whilst this core, heterogeneous microbial diversity exists without necessarily causing disease, individually, all of these genera are also associated with microbial diversity shifts, or dysbiosis, that contribute to oral and airways disease states. These include periodontitis, cystic fibrosis, pneumonia, and inflammatory lung diseases (9). The normal lung microbiome is extremely diverse, and some studies report *Neisseria* (17), *Acinetobacter* (18), *Sphingomonas, Megasphaera* and *Staphylococcus* (19) as predominant genera in both health and disease. Additionally, fungi, bacteriophages and viruses including human rhinovirus (HRV) (20) constitute part of this microbiome and have complex interactions with the bacteria, driving dysbiosis such as significantly increasing the proportion of Proteobacteria including *Haemophilus spp.* in the sputum microbiome of COPD patients (20). Therefore, the existence of these bacteria in individuals in both health and disease states suggests that the aetiological factor for disease is not solely due to the presence of pathogens but a change in how those pathogens interact with the host and other bacteria – their behaviour.

Of these genera, there is a substantial amount of evidence that suggests Non-typeable *Haemophilus influenzae* (NTHi) is responsible for a large subset of AECOPD hospitalisations (4, 21, 22), alongside *Streptococcus pneumoniae* and *Moraxella catarrhalis* in several recent studies (Table. 1.). NTHi is an ubiquitous, non-encapsulated Gram-negative coccobacillus and opportunistic pathogen (23) despite having commensal properties (24). This commensal-turned-pathogen is the most common cause of *Haemophilus spp.* infection across all ages (25), is present in 70% COPD patients (4) and is associated with 24% AECOPD hospitalisations (21). Furthermore, higher bacterial loads of NTHi are correlated more severe airway inflammation, more severe AECOPD and increased disease burden on the patient (26). However, NTHi presence does not explain every incidence of AECOPD across different patient cohorts, raising the question of how the behaviour of NTHi has changed in these cases to cause a disease state. One current hypothesis is that in developed countries, the routine immunisation of infants with the conjugated *H. influenzae* type B (Hib) vaccine and prescription of COPD maintenance drugs (27) (11) has contributed to lung microbiome dysbiosis that favours NTHi colonisation and provides

immunological pressures for the selection of biofilm-forming NTHi strains (28). For example, selection for NTHi strains expressing different outer-membrane-proteins (OMPs) has led to a change in behaviour, favouring attachment and colonisation of the airways epithelia (28, 29), implicated in COPD infections (30). Similarly, antibiotic and maintenance treatments have been shown to be largely ineffective in combating the microbial causes of COPD in the long term. Systematic reviews have revealed that regular, long-term prophylactic antibiotic prescription did not result in reduced hospitalisation or improvement in lung function nor mortality (31). In addition, long-term maintenance drugs including inhalation of corticosteroids has been found to increase sputum bacterial load (32). Moreover, short-term 3-month treatments with either moxifloxacin, doxycycline or azithromycin antibiotics were all found to not significantly decrease airway bacterial load but instead promoted antibiotic resistance in all treatment groups (33). These observations demonstrate how changes in the behaviour of NTHi, driven by disease progression and treatment strategies, may promote the biofilm lifestyle, contributing to disease.

Many severe COPD patients are receiving 'long-term' treatment with macrolide antibiotics that exhibit secondary anti-inflammatory properties and immune-modulating effects (34, 35). Meta-analysis of several COPD cohorts has shown that macrolide antibiotic treatment successfully decreases the frequency of AECOPD when prescribed over 6 – 12 months (35) by suppressing bacterial load and reducing inflammation (36). However, macrolides exert no benefit in the short term over 3 months (35). These findings have been corroborated using low-dose, long-term macrolide therapy up to 12 months (37) and current guidelines indicate optimal usage over 6 – 12 months in patients who experience 3 or more AECOPD per year, are prescribed steroids and are hospitalised due to AECOPD at least once per year (38). However, these guidelines do not describe long-term treatment that exceeds 12-months. This presents an issue for patients living with a progressive and currently incurable respiratory disease. Long term macrolide therapy also presents several risks, with studies reporting non-fatal but adverse effects including gastrointestinal reactions, liver injury and ototoxicity (35). In addition, due to the increased risk of comorbidities, macrolide therapy has been reported as being unsuitable for the elderly (37, 39). Further systematic reviews have reported the increasing risk of these adverse events as well as macrolide antibiotic resistance (40) achieved by multiple mechanisms including modification of macrolide target sequences and upregulation of efflux pumps that increase with prolonged use of macrolides over 12-months (41). Despite the benefit of macrolides in reducing AECOPD, much of the supporting evidence is attributed to azithromycin and not wider macrolides (36), and 'long-term' treatment is limited to only 12 months. Accompanied by the reported risks of serious adverse events and acquisition of antibiotic resistance, macrolide therapy does not represent a substantial solution to controlling the bacterial and inflammatory components of COPD.

It is also important to recognise the limitations of these previous lung microbiome studies. There was previously no standardisation of microbiome analysis, such as the use of different 16S rRNA hypervariable regions (12). Many studies contained only small, cross-sectional cohorts (42). Furthermore, unprotected specimen brushings or sputum samples have also been widely used, which may introduce oropharyngeal contamination (10). Together, these factors may limit the accuracy with which the healthy lung microbiome is reported and how it differs in disease states over time. However, results highlighting the importance of NTHi are reciprocated across several COPD cohorts by PCR, culture (4, 21) and 16S rRNA techniques (11), warranting further investigation of NTHi and this pathogen's life cycle within the COPD lung.

Different microbiome identification techniques should also be taken into consideration to allow the fair appraisal of results between studies. A major advantage of culture techniques is that media is often

selective and enriched with supplements that promote the growth of fastidious organisms, allowing low-abundance organisms to be detected and cultured (43), whilst also eliminating contaminants. Furthermore, culture methods can lead to useful downstream experimental models and assays including antimicrobial susceptibility testing. However, whilst culture methods are improving, it is low-throughput and a central dogma is that only around 1% bacteria are culturable (44), and those that can be cultured are likely altered under the nutrient-rich growth conditions. In addition, culture techniques may also introduce bias, depending on which selective growth media are used, based upon the microorganisms that are expected to be present in the sample. Together, these limitations mean the composition of bacteria detected using culture may not be a valid representation of the lung microbiome. Whilst 16S rRNA sequencing does not detect fungi and viral constituents, its major advantage is its sensitivity to the detection of phenotypically aberrant, rarely isolated or unculturable bacteria as well as novel pathogens (45). Advances in Illumina sequencing and bioinformatic approaches also allows high-throughput detection, analysis and quantification of entire microbiome communities from multiple samples. However, high 16S rRNA sequence similarities may make it difficult to distinguish beyond the genus level in some taxa. The analysis is also dependent on the choice of one of nine bacterial 16S hypervariable regions (V1 – V9) with no single hypervariable region exhibiting a different enough degree of sequence diversity to distinguish amongst all bacteria at the species level (46). Whilst not a sterile environment, the lung microbiome does have extremely low biomass which increases its susceptibility to contaminant sequences during sample acquisition, processing and 16S rRNA sequencing (47). This may also explain high variances between studies.

It is clear however, that NTHi presents a significant problem for a large number of COPD patients and their clinicians alike, being detected across culture and 16S rRNA techniques. These patient-cohort focused studies show that, despite widespread use of antibiotics, NTHi persist in the COPD lung and lead to chronic and recurrent infections, exhibiting extreme tolerance to antimicrobial immune system defences and pharmacological treatments as well as displaying synergistic or co-infection interactions with other pathogens. Whilst NTHi intracellular infection is a well-documented mode of persistence in COPD, by becoming internalised and surviving within host cells as a protected reservoir that facilitates recurrent infection (23), NTHi exhibits distinct adaptations that are typical hallmarks of the biofilm lifestyle (48). These include biotic surface adherence, extreme tolerance and resistance to antibiotics and antimicrobials, persistence of sub-populations of bacteria following treatment leading to recurrent infection, extreme capacity for evading host antimicrobial immunological defences and complex interspecific or polymicrobial interactions (48).

1.3 Non-typeable *Haemophilus Influenzae* is Adapted for the Biofilm Lifestyle

NTHi is well adapted for colonisation of the human airways. This fastidious organism survives in a mucus-rich environment and has a complex nutritional requirement for haemin and nicotinamide adenine dinucleotide, that are both available in the human lungs (49). NTHi expresses an arsenal of proteins that allow it to adhere to and invade respiratory epithelial cells. These proteins include type IV pilin protein and the adhesin OMP P1 that bind to the host cell-surface protein ICAM-1 and glycoprotein CEACAM1 (50, 51) as well as mucin proteins and lactoferrins in human mucus (52). These mucus secretions and plugs observed in COPD patients that coat the airways epithelium may be especially important for the initial attachment stage in the development of bacterial biofilms (53) (Figure 1), which are sessile, three-dimensional multicellular communities of bacteria embedded within a self-produced extracellular polymeric substance (EPS) matrix (54). Biofilms exhibit complex strategies that confer persistent or chronic infection and dissemination to secondary sites of infection, characterised by tolerance and resistance to the immune system and antibiotic drugs.

2 NTHi Biofilm Strategies

The biofilm lifestyle confers several advantages to survival compared to the planktonic or free-living state (Figure 2). Typically, biofilm bacteria exhibit pleiomorphic behaviour, altering their morphology, coordinating gene expression and differentiating metabolomic functions in a heterogeneous manner. These changes occur in response to many factors, such as bacteria population density and quorum sensing that regulates gene expression cascades, stressful environmental conditions such as oxygen and nutrient gradients that develop as the biofilm matures, as well as immune system or pharmacological antimicrobial effectors. Additionally, biofilms exhibit physical and chemical strategies against the immune system through the formation of a protective barrier, the EPS matrix, as well as virulence factor modulation, interspecific synergistic interactions with other bacteria and the sharing of antimicrobial-sequestering compounds. The differentiation of metabolically inactive persister cells and dissemination of biofilm to secondary sites are a major cause of chronic, recurrent infection following antibiotic treatment of the initial infection, which may be implicated in COPD.

2.1 Quorum Sensing

Quorum sensing (QS) is the density-dependent coordination of gene expression (53, 54) across bacteria within a community that is integral for biofilm formation, development and dispersal. Most biofilms achieve QS through autoinducer-2 (AI-2) chemical signalling (53). Autoinducer expression is encoded by the *LuxS* homologue (55) and these signals are detected by the ABC transporter RbsB, (56) leading to further downstream transcription cascades that drive biofilm formation (Fig. 2). This signalling mechanism highlights a target for interrupting biofilm development.

Like most other biofilm-forming bacteria, AI expression has been shown to promote NTHi biofilm formation and persistence, although much of this research is in the context of NTHi otitis media (OM) strains grown *in-vitro* (55) or the chinchilla middle ear model of NTHi-induced OM (57). Scanning confocal laser microscopy (SCLM) of NTHi *LuxS* mutants grown in continuous flow chambers show significantly reduced biomass, thickness and persistence (55). Disruption of the NTHi RbsB transporter reciprocated these results (56) showing that detection of QS signals is just as important as production. Downstream QS cascades result in increased transcription of biofilm-promoting genes including *gstA* – a glycosyltransferase that increases sialyation of the biofilm matrix (58), with *gstA* mutants producing sialyation deficient and low biomass biofilms.

However, NTHi also express a second, AI-2 independent QS system; the QSeB/C two component system that drives biofilm formation (59). However, QSeB/C disruption does not affect the biofilm in the same way as LuxS mutants. Instead, QSeB/C mutants show significant biofilm biomass and surface coverage in continuous flow models over 24 – 48 hours (59). Whilst this system is less well understood, the expression of a secondary QS system may explain why NTHi with a disrupted AI-2-RbsB system are still capable of forming reduced biofilms rather than no biofilms at all.

2.2 Defence Against Immune System Effectors

Biofilms exhibit defence strategies against the immune system including survival and persistence within neutrophil extracellular traps (NETs) (Fig 2.) NETs are extruding DNA-rich (57) webs of

decondensed chromatin containing histones, neutrophil elastase and granules that trap bacteria (7). One of the biofilm's tolerance strategies is the expression of peroxiredoxin-glutaredoxin (pdgx) and catalase that protect bacteria against NET-derived oxidative stress effectors (60) and expression is upregulated in biofilm compared to planktonic strains (61). This NET-survival strategy has been demonstrated in NTHi biofilm OM models that form greater biomass over 14 days (57). Immunofluorescence and viability staining techniques demonstrated NTHi biofilms surviving and persisting within the NET lattices themselves. It is hypothesised that NETs can be incorporated into and benefit the structure of the biofilm (57). By increasing biomass, biofilms drive metabolism-limiting gradients that induce metabolically inactive persister cells at the centre of the biofilm and provide a physical and chemical barrier to immune system effectors and antimicrobial agents (53).

One of the lung's primary defences against bacteria is activation of complement that opsonises invading pathogens for phagocytosis by neutrophils and macrophages (8). However, NTHi have adapted to evade or even 'hijack' the complement system, driving collateral damage and host tissue destruction (62, 63). Several studies report up- and down-regulation of virulence and complement activating factors including lipoligosaccharide (endotoxin) (64), sialic acid, outer membrane proteins (OMP P2 and P5) and inflammatory mediators (26) that trigger a local neutrophil response (Fig 2.). However, because some NTHi have adapted to hijack complement and are inherently tolerant to NETosis, this increased neutrophil response instead promotes airways inflammation, facilitating intracellular invasion and persistent infection by NTHi.

2.3 Extracellular Polymeric Substance: Key Components and Functions

The EPS matrix is a hallmark of biofilms which confers a protective barrier against the immune system and antimicrobials and is comprised of by polysaccharides, proteins, lipids and eDNA (Fig 2.), that may constitute 90% of the dry mass of most biofilms (65). Until recently there was little evidence for the production of an EPS matrix (53) as part of the NTHi biomass. Unlike the polysaccharide-rich and alginate producing biofilms of *P. aeruginosa,* the aetiological pathogen in chronic cystic fibrosis (CF) infections, NTHi biofilms have a limited carbohydrate or exopolysaccharide component (66), containing only lipooligosaccharide (LOS) endotoxin. The composition of NTHi EPS is largely DNA and protein-rich which may contribute to its success in the hostile lung environment and tolerance to pharmaceuticals through a cascade of host-pathogen interactions, (67) despite not being embedded in a thick alginate.

Analysis of 18 biofilm-specific multifunctional proteins has demonstrated that the protein constituent of the EPS is essential for NTHi biofilm formation, maintenance, structural integrity, survival and tolerance to immune-system and antimicrobial effectors (66, 68). The EPS is structurally stabilised by Nuclear Associated Proteins DNABII that binds bent double stranded extracellular DNA (ds-eDNA) forming a strong nucleoprotein mesh (65). Bent ds-eDNA are curved rather than straight architectures of extracellular DNA for which DNABII family proteins have a higher affinity (65). This eDNA is independent of DNA derived from cell lysis or outer membrane vesicle biogenesis and instead NTHi employs an active mechanism of eDNA release via Tra-dependent inner membrane transit and release through the comE pore (69). Bent ds-eDNA-DNABII complexes confer amoxicillin tolerance and increased biomass that is relinquished through antibody degradation of DNABII (65). Immunofluorescent techniques have shown that eDNA provides structural stabilisation, maintenance and expansion of the biofilm to secondary sites (66) and it is arranged in a meshwork of fine strands and thicker, rope-like ds-eDNA structures (70). Additionally, the negative charge of eDNA sequesters positively charged antimicrobials through a cation chelating interaction, conferring biocide resistance, requiring1000-fold increases in antibiotic concentration to kill biofilms compared to planktonic

phenotypes (71). In the chinchilla OM model, eDNA was also found to reduce the activity of the antimicrobial defence peptide (AMP) Beta Defensin-3 (72). DNase I mediated degradation of eDNA resulted in reduced biofilm formation and increased susceptibility to ampicillin and ciprofloxacin *in-vitro* (73). Degradation of eDNA also destabilised the strong biofilms formed by clinical NTHi isolates and decreased surface adhesion and biofilm formation in planktonic cultures (72).

However, the NTHi biofilm itself has its own eDNA control, mediated by the endogenous nuclease Nuc (74). Nuc is thought to be under the control of the QS system, leading to structural remodelling of the biofilm such as the formation of water and nutrient channels (74). Low-level expression of endogenous Nuc in NTHi biofilms exerts a similar destructive effect on these discrete areas of the biofilm in a similar way to treatment with DNAse I, but with a 1,400 fold greater activity (74). The high expression present in planktonic NTHi likely contributes to maintenance of the planktonic lifestyle. A second mechanism of structural remodelling is expression of the transcription inhibitor ModA2 methyltransferase that decreases eDNA and DNABII expression (75), destabilising the biofilm. Therefore, both Nuc and ModA2 may be considered therapeutic targets of biofilm disruption, by increasing expression to drive NTHi biofilm instability and cause a biofilm to planktonic transition, making the bacteria more susceptible to treatment.

2.4 Surface Component Modification & Adhesion Molecules

The NTHi outer membrane is particularly protein-rich and the expression of key proteins including type IV Pilus, OMP P1 and P5 and HMW1/2 adhesins together facilitate initial attachment directly to the bronchial epithelium and mucin-rich mucus (50, 52, 69, 70, 76), as well as trafficking of the aforementioned eDNA and DNABII components (Fig. 2). Whilst NTHi are rich in only one polysaccharide, lipooligosaccharide (LOS), there are a diverse number of LOS and cell-surface modifications expressed in NTHi that are consistent with biofilm-forming bacteria (77). These LOS modifications include sialyation and phosphorylcholination that together increase biofilm formation and reduce LOS endotoxin bioactivity, reducing the host's innate immune response to these substances. (64, 77, 78). These modifications summarised below (Table. 2.) may also serve as therapeutic targets as interfering with key biofilm proteins and adhesins shows great potential in disruption of initial biofilm formation and maturation, and thus increases the biofilm's susceptibility to treatments.

2.5 Antibiotic Resistance & Tolerance Mechanisms

NTHi biofilms exhibit complex chemical multi-drug resistant strategies (Table. 3.) against a variety of widely used antibiotics (79). Clinically important antibiotics including Ciprofloxacin, Azithromycin, and Amoxicillin were 100% effective in eliminating 28 NTHi OM planktonic isolates whilst only killing 68%, 57% and 4% of NTHi biofilms, respectively (79). Furthermore, 7% of biofilms even survived combined rifampicin - ciprofloxacin treatment. NTHi biofilms are inherently tolerant to high concentrations of gentamycin (80) and erythromycin (81). This tolerance is achieved partly through upregulated carbohydrate metabolism which drives transformation into the metabolically inactive biofilm phenotype dependent on stored glycogen (82). This is a protective, epigenetic response to sub-minimum inhibitory concentrations (sub-MIC) of ß-lactam antibiotics (82) that target and inhibit the growth of metabolically active bacteria. Other proteomic changes occur acting against reactive oxygen species and promoting a semi-dormant lifestyle, rendering immune system factors and β-lactam antibiotics ineffective (29). The aforementioned eDNA-rich EPS matrix protects against a range of antimicrobials including chlorhexidine glucoronate (73) and antibiotics including ampicillin and

ciprofloxacin (72). However, NTHi biofilms are, counter-intuitively, susceptible to sub-inhibitory concentrations of the macrolide antibiotic, azithromycin (81), which exhibits effective antibiofilm properties not demonstrated by erythromycin or gentamycin (81), even against clinical NTHi strains resistant to a wide range of antibiotics. However, the efficacy of azithromycin may not be due to only its antibacterial properties in this context. Recently azithromycin has proved a popular and effective for treatment of lung infections possibly due to its secondary anti-inflammatory properties (83). The COPD lung is characterised by chronic inflammation, increasing the risk of recurrent infection following treatment. The use of an antibiotic such as azithromycin with secondary anti-inflammatory properties may help decrease the lung's innate immune system that causes collateral tissue destruction. Additionally, azithromycin has been reported to antagonise the quorum sensing system in *P. aeruginosa,* leading to decreased biofilm forming capacity, diminished virulence and an impaired oxidative stress response (84) offering one explanation as to why β-lactam – azithromycin combination therapy in a cohort of critical care community-acquired pneumonia (CAP) patients reduced mortality by ~20% (85).

2.6 Multispecies Interactions with *S. pneumonia* and *M. catarrhalis*

Many studies investigate the role of biofilm-phase bacteria in the context of mono-species biofilms. However, because the lung microbiome is polymicrobial (4, 12, 13) this diversity may facilitate complex but poorly understood interspecific interactions between bacteria as well as viruses (20). These interspecific interactions (Fig 2.) may be indirect, through changing of both biotic and abiotic conditions that favour the proliferation of secondary colonisers in airways diseases, analogous to ecological succession (86). For example, NTHi infection has been found to upregulate the pro-inflammatory responses that may drive wider microbial shifts and exacerbate COPD (87, 88) as well as upregulate MUC2 production, increasing airway obstruction and providing a viscous substrate for secondary bacterial colonisers (89) over time. Alternatively these interactions may be more direct through the growth of multi-species biofilms (90) that result in co-operative adhesion and stability of the biofilm structure. NTHi and *S. pneumoniae* multi-species biofilms, for example, together produce and share a single EPS that is constituted by type IV pili, eDNA, LOS, QS signals and other proteins, carbohydrates, adhesins and transcription factors (90) from both species. Whilst further research is required to provide direct evidence as to whether NTHi is a primary coloniser of the human respiratory tract in COPD, NTHi does express an arsenal of adhesins and invasins that facilitate initial attachment directly to bronchial epithelia and mucin-rich mucus (50, 52, 69, 70, 76). Additionally, NTHi can express factors that change the environment making subsequent colonisation by other bacteria more favourable. NTHi and *S. pneumoniae* often co-colonise the respiratory tracts of COPD patients (91) and interact synergistically, promoting initial attachment, biofilm formation and survival. These interactions include increased expression of virulence genes such as the *pilA* of type IV Pilus (TfP), a strong and flexible transmembrane filament that has diverse functional roles in pathogenicity. Other interactions include the sharing of β-lactamases and eDNA (91), and the release of biocidal H_2O_2 (92) that provides a nutrient and DNA reservoir at the expense of killed bacterial cells and senescent host cells, supporting biofilm survival. Co-culture of NTHi and *Streptococcus pneumoniae* strains was found to significantly increase biofilm formation on cultured respiratory epithelial cells (93) compared to mono-species biofilms. Whilst these bacteria also interact competitively, competition may not be a negative for NTHi survival in the long term. One such example is that *S. pneumoniae* expresses bactericidal H_2O_2 (94) and stimulates neutrophils that together provide a selection pressure for ROS-tolerant and persistent strains of NTHi (92).

M. catarrhalis is a major respiratory pathogen that colonises 5-32% of COPD patients and accounts for 10% acute exacerbations (95). *M. catarrhalis* infections usually clear after 40 days but can result

in long term biotic changes that favour secondary colonisation of the airways by NTHi (95) following initial *M. catarrhalis* infection. *M. catarrhalis* binds to the tips of healthy ciliated epithelia forming aggregates, significantly reducing cilia beat frequency in bronchial epithelial cultures (96) resulting in impairment of the mucociliary clearance pathway and formation of mucus plugs then colonised by NTHi. In OM models, *M. catarrhalis* has been demonstrated to stabilise NTHi - *S. pneumoniae* - *M. catarrhalis* polymicrobial biofilms by protecting NTHi from the bactericidal properties of *S. pneumoniae*, further promoting NTHi viability and persistence (94).

3 Evidence for NTHi Biofilms in the COPD Lung

A plethora of studies have identified single key genes and transcriptional products associated with biofilm formation and persistence *in-vitro* and *in-vivo* in bronchial epithelial and OM models, using clinical NTHi isolates (Table. 4.) Whilst these studies provide good evidence that NTHi has the *capacity* to form biofilms under nutrient-rich conditions, it does not confirm whether they actually form stable, persistent biofilms within the human COPD lung. Recently however, the ferret COPD model involving chronic long-term exposure to cigarette smoke has provided immunofluorescent and gene expression evidence for NTHi biofilm formation and persistence (97). Here, smoke-exposed ferret lungs presenting characteristic histological and immunological hallmarks of COPD showed increased NTHi aggregation and the expression of four key biofilm-associated genes, *pdgX*, *luxS*, *dps* and *hktE*, that are together involved in growth, quorum sensing, environmental stress and oxidative stress tolerance (97).

Whilst recent reviews have highlighted several mechanisms of NTHi that favor epithelial cell adherence and biofilm formation in the lower airways, (98) there remains a gap in the research pertaining to NTHi biofilm development in the context of the COPD lung of which there is limited direct evidence (8). Some of the major limits to our understanding can be attributed to current methods of biofilm detection, which include isolation of NTHi from sputum and BAL samples and culture under nutrient-rich conditions *in-vitro* or non-lung *in-vivo* models.

Recently, PCR-based direct gene expression studies have revealed associations between adhesin expression and *in-vitro* biofilm formation, providing the first set of NTHi biofilm gene biomarkers (99). However, this sub-set of genes is limited as thus far a gene expression profile using a comprehensive array of biofilm genes in NTHi clinical lung-derived samples has not been completed. Moreover, current imaging methods of *ex-vivo* biofilms are limited to transmission electron microscopy (80). Some studies report poor evidence of biofilm formation and in one cohort of COPD patients only 10% of NTHi isolates formed biofilm whilst 80% of these were classified as weak biofilms (100). Similarly, in a cohort of pulmonary sarcoidosis patients, another airways disease characterised partly by inflammation, there was a reduction in biofilm-forming *Haemophilus spp.* isolates in disease compared to health (101) using culture-methods.

Another study provided evidence for the existence of NTHi biofilm in the human respiratory tract as well as host responses to the biofilm, in a cohort of COPD patients (61). NTHi strains isolated from the sputum of six COPD patients grew biofilms expressing significantly more abundant pdgx compared to planktonic cultures and were shown to elicit an immune response in 44.4% of a further 18 COPD patients through detection of pdgx antibodies by ELISA (61). This increased association of pdgx expression with biofilm formation is likely due to the protective properties against reactive oxygen species and survival within NETs (60) which may serve as a biomarker for NTHi biofilm infection.

Overall, this evidence points to the existence of NTHi biofilms in infections across airways diseases. However, at the current time explicit evidence is limited in COPD. Further understanding the role of NTHi biofilms in COPD may elucidate novel, specific, therapeutic targets that may improve the disease management of patients where otherwise COPD maintenance drugs and antibiotics are ineffective in the long term.

4 Discussion – Clinical Implications of NTHi Biofilm Research

The current literature provides strong genetic and phenotypic evidence that NTHi are well-adapted to have the capacity to colonise the human lung and form biofilms on bronchial epithelia with impaired mucociliary clearance, characteristic of COPD amongst others. NTHi isolates are highly heterogeneous and further express an arsenal of strategies that confer immune system evasion, antimicrobial tolerance, persistence and chronic infection, that make it a particularly challenging pathogen to clinically treat. Considering the role of NTHi infections in the context of biofilms and their physical and chemical properties may explain the differences between patient cohorts, and why airways diseases are characterised by chronic, recurring lung infections. However, the presence of NTHi is not synonymous with AECOPD, so perhaps it is not necessarily the presence of a particular pathogen but the phase in which bacteria are present, biofilm or planktonic. Conversely, whilst some studies report NTHi as a non-significant bacteria in AECOPD or hospitalisation, this does not rule out the potential historical role of NTHi colonisation in the airways and host-pathogen interactions leading up to the present time of study. It is also important to consider that NTHi may have commensal benefits for the host's immune system. For example intracellular NTHi infection of airway epithelia protects against respiratory syncytial virus (RSV) (24) through stimulation of immune effectors. Conversely, NTHi can take advantage of compromised immune systems following viral infection (20).

Understanding COPD and NTHi infections in the context of biofilms has several clinical implications. Firstly, the potential to improve diagnosis; currently, physical symptoms of chronic bronchitis and emphysema are used in the clinical diagnosis of COPD, despite recent microbiome associations. Whilst bacteria are understood to have a significant impact on the progression of this disease, dysbiosis of the lung microbiome is not currently used but could be a biomarker for the prediction of early onset COPD. Secondly, improving AECOPD treatment; NTHi isolates are highly heterogeneous in terms of biofilm-forming, antimicrobial tolerance and immune system stimulation or evasion mechanisms so a 'one-treatment-fits-all approach' may not be appropriate. Understanding the underlying biofilm mechanisms may reveal several targets to disrupt biofilm integrity, harmful host-pathogen interactions and increase antimicrobial susceptibility. Recently, nitric oxide donors have been successful in triggering biofilm dispersal from β-lactam resistant cystic fibrosis *P. aeruginosa* isolates and improving the efficacy of antibiotics (102, 103). Mucolytic drugs are currently widely prescribed by clinicians as an adjunctive therapy for COPD (104) and include erdosteine that has been shown to increase the efficacy of antibiotics against chronic respiratory infections (105). The adhesins OMP P5 and TfP that facilitate initial attachment have been targeted for vaccine development to prevent biofilm formation, which was successful in evoking a protective immune response to NTHi in the OM model (50). The literature demonstrates that targeting biofilm components are an effective way to reduce biofilm formation and increase antimicrobial susceptibility *in-vitro*, including antibody-mediated DNABII degradation (65) and DNase I mediated eDNA degradation (73) with a plethora of surface modifications and tolerance mechanisms identified as future targets for development. Being able to profile the genotype or transcriptome of clinical isolates for biofilm-genes may reveal strain-specific susceptibilities or drug-targets where airways infections could be treated with greater efficacy using a precision medicine approach.

Despite recent advancements, there is a lack of evidence that NTHi forms biofilms within the human COPD lung. The majority of the research investigating this biofilm concept involves isolated strains cultured under nutrient-rich conditions *in-vitro* or in OM or bronchial epithelial cell culture models – not within the hostile lung environment with a cascade of immunological responses and potentially nutrient deprived and anaerobic microenvironments. To our knowledge, there are no current studies that have attempted to detect NTHi biofilm within sputum, BALF samples or tissue biopsies from COPD patients *ex-vivo,* using PCR detection of biofilm-associated genes or immunofluorescence imaging techniques. This would provide direct evidence of biofilms existing within the COPD lung.

Additionally, microbiome studies have a great deal of disparity reporting the core lung microbiome. Different studies take sputum and BAL samples which are different lung niches and may account for some of this disparity in microbiota shifts and diversity changes during AECOPD. Disparity can also be attributed to having no standard methodology, including sampling and sequencing inconsistencies, for example using different 16S hypervariable regions or using Illumina or Pyrosequencing platforms, that may yield different results when quantifying the abundance of bacteria taxa.

Future research should be directed towards clinical COPD NTHi isolates and model development that better represent the lung microenvironment and polymicrobial communities, to understand the behaviour of NTHi during chronic airways infections. Developing a comprehensive list of 'biotypes' for NTHi strains by profiling biofilm-gene expression could reveal patient-strain specific biofilm mechanisms, susceptibilities and potential therapeutic targets. Understanding the complex interspecific and host-pathogen interactions and microbiota shifts over time would allow us to model disease progression and design effective, precision-medicine treatment strategies to improve the prognosis of COPD patients and slow down the progression of COPD by targeting the microbial cause.

Understanding the behaviour of NTHi as a biofilm-producing pathogen reveals a growing number of mechanisms that could be potentially targeted by therapeutics. Research in model systems has already shown that disruption of key biofilm systems including LuxS quorum sensing, the eDNA – DNABII binding complex and expression of key adhesins and cell surface modifications alters the NTHi biofilm phenotype and increases antimicrobial susceptibility (55, 71, 72, 76, 81). However, the challenge now is to alter these processes within the human lung. Increasing our understanding of how NTHi coordinates the expression of genes that control these biofilm- and survival-promoting processes is essential. Recently, the role of bacterial exosomes or outer membrane vesicles (OMVs) have been implicated in NTHi biofilms in the air-liquid-interface epithelium model (106). Gram negative bacteria including NTHi produce spherically bilayered OMVs of approximately 100-300nm in size by a process of outward blebbing from the bacteria outer membrane (107) and have multifunctional roles including supporting the biofilm lifestyle (67). Such proposed functions include the shuttling of quorum-sensing molecules (106) as well as enrichment with key outer membrane proteins (OMPs) and LOS that have previously been associated with biofilm development (108) as well as NET recruitment (109). NTHi OMVs also traffic cargo destined for the EPS matrix (67), a hallmark feature of biofilms that confers physical and chemical survival against the immune system and antimicrobial drugs. As analogous structures of eukaryotic extracellular vesicles that deliver a cargo of DNA, RNA and miRNA (110, 111), it is possible that NTHi biofilm-derived OMVs represent a novel target for the disruption of gene expression pathways controlling biofilm development and contributing to disease.

This review has highlighted several key questions that need exploring in the field. Firstly, how does the behaviour of biofilm-phase NTHi affect progression of COPD? Specifically, there is a need to conduct research into lung derived NTHi biofilms and develop valid airways models. Furthering our understanding of host-biofilm interactions, such as the proinflammatory cascade, could lead to slowing

the progression of COPD. Next, the NTHi biofilm itself. Are the biofilm-specific strategies of NTHi the reason why disease management drugs and antimicrobials are largely ineffective in the long term? Whilst current treatment strategies do help to alleviate AECOPD and slow the progression of COPD, infections remain recurrent throughout the chronic disease leading to increased hospitalisation and morbidity (112-114). This leads us to hypothesise that NTHi biofilms present potential, novel therapeutic targets (115). For example, there is potential for biofilm development to be heavily impaired by targeting the aforementioned NTHi biofilm strategies including the eDNA-DNABII binding complex, adhesins, outer membrane proteins, high molecular weight proteins, LOS modifications and biofilm-promoting gene expression pathways.

5 Conclusion

NTHi is a major aetiological commensal-turned-pathogen implicated in COPD progression and AECOPD across several recent large-cohort patient COPD studies. However, reports differ on the prevalence of this ubiquitous organism across healthy and non-healthy individuals, the reported significance of this pathogen in the onset or exacerbation of disease, as well as treatment efficacy. NTHi are well-adapted to colonisation of the respiratory epithelium and express many genes that promote the biofilm lifestyle, as well as proteins that confer immune system evasion, antibiotic tolerance, and metabolomic changes that drive survival, persistence and chronic, recurrent infections. Furthering our understanding of the mechanisms of NTHi colonisation in chronic lung infections in COPD patients in the context of biofilms is now essential and may help to explain these vast differences. Whilst several studies report that clinical NTHi isolates can express the key hallmarks of a biofilm, these are often in the context of *in-vivo* OM or *in-vitro* BEC models under nutrient-rich conditions that poorly represent the human lung. Further research is required to characterise the biofilm-forming capacity of NTHi and identify the COPD lung host-pathogen interactions and anti-biofilm therapeutic targets, which could have a significant impact on the diagnosis and treatment of patients living with chronic airways diseases.

6 Tables

Table 1. Summary of Key Studies Investigating AECOPD Microbiology

Finding	Main Result(s)	Citation
NTHi proliferations cause AECOPD leading to hospitalisation.	NTHi was detected in the sputum of 70% patients in a large cohort study of 105 moderate (45%) and severe (40%) COPD patients (GOLD stage 2 - 3). During AECOPD, culture and PCR analysis revealed NTHi populations significantly proliferate compared to baseline.	(4)
Streptococcus spp. and M. catarrhalis contribute to AECOPD.	Culture and PCR analysis of sputum revealed *Streptococcus pneumoniae* remained a dominant lung taxon during AECOPD (but proportions did decrease). *M. catarrhalis* proliferated during AECOPD.	(4)
Viral interactions contribute to AECOPD and drive NTHi proliferation.	PCR showed increased co-infection of NTHi and HRV between exacerbation-state (29.2%) and stable state (9.1%) patients, with 46.5% patients having at least one HRV-positive exacerbation.	(4)
	Inoculation of 14 mild COPD (GOLD stage 2) patients with human rhinovirus (HRV) resulted in a 21% *Haemophilus spp.* proliferation driving a persistent infection. NTHi was detected in sputum by 16S rRNA pyrosequencing using the V3-V5 16S hypervariable regions.	(20)
NTHi proliferation is associated with AECOPD severity.	Microbiome analysis of 584 sputum samples from a cohort of 101 COPD patients with moderate (44.6%), severe (39.6) to very severe (15.8%) COPD (GOLD stage 2 – 4) showed that there was a significant increase in NTHi in patients with very severe COPD compared to moderate COPD. Microbiome sequencing was conducted using 16S rRNA Illumina sequencing, V4 hypervariable region,	(11)
	Overall, there were significant microbiome shifts during AECOPD over one year, across moderate, severe and very severe COPD patients, between stable and exacerbation	(11)

	states. NTHi proliferation was associated with exacerbation events.	
Pseudomonas aeruginosa causes AECOPD and prolongs hospitalisation.	Sputum culture and PCR analysis identified NTHi as most common bacteria (~24%) across 92 hospitalised AECOPD patients with mild (4.3%), moderate (19.5%), severe (10.8%) and very severe (65.2%) COPD (GOLD stage 1 – 4). However, NTHi was not significantly associated with hospitalisation duration.	(21)
	P. aeruginosa was detected by culture as the second most common species across 92 patients hospitalised (~14%) and was significantly associated with increased hospitalisation duration.	(21)
NTHi infections do not contribute to the COPD lung microbiome.	NTHi was not detected as part of the core microbiome in a small cohort of stable-state COPD patients. The microbiome was assessed using a terminal restriction fragment (TRF) length polymorphism and clone library analysis technique on bronchoalveolar lavage (BAL) samples from 9 stable state COPD patients with moderate (6/9) or severe (3/9) COPD (GOLD stage 2 - 3) compared to 9 healthy controls.	(19)
NTHi infections do not contribute to AECOPD.	*Haemophilus spp*. were not identified as clinically important genera during AECOPD (0.7%). *Pseudomonas spp*. were also not clinically important (1.8%). In this study the sputum microbiota from a cohort of 9 patients with mild (1/9), moderate (3/9), and severe (5/9) COPD (GOLD stage 1 – 3) was analysed using 16S rRNA pyrosequencing and qPCR techniques.	(22)
Moraxella spp. and *Streptococcus spp*. contribute to severe AECOPD.	During exacerbation episodes, sputum samples from the 5 severe COPD patients (GOLD stage 3) showed increases of *M. spp*. and *S. spp*. in 90% and 88% patients, respectively.	(22)

AECOPD is associated with sputum microbiome shifts in *Proteobacteria, Firmicutes or Bacteroidetes*.	Between stable- and exacerbation-states, alpha-diversity analysis showed shifts in the proportion of Proteobacteria, Firmicutes and Bacteroidetes phyla. However, this dysbiosis was heterogeneous across patients.	(22)
M. catarrhalis infection is responsible for a subset of AECOPD but infection is cleared following immune response.	*M. catarrhalis* is estimated to cause 10% AECOPD. *M. catarrhalis* was detected by sputum culture in a prospective cohort study involving 104 COPD patients with 3009 clinic visits over 81 months. 560 visits occurred during exacerbation episodes and 2449 visits occurred during clinically stable periods. *M. catarrhalis* was detected in the sputum of 50 patients with 47.5% presenting with AECOPD. Immunoassays showed that patients cleared *M. catarrhalis* infections efficiently and molecular typing techniques showed that reacquisition of the same strain was rare, demonstrating development of strain-specific protection.	(95)
Interspecific co-colonisation interactions exist in COPD patients.	A prospective cohort study involving monthly sputum cultures from 181 COPD patients exhibiting chronic bronchitis with 8843 clinic visits over 4.5 years revealed NTHi was the most common bacteria isolated (14.4%) and colonisation was positively correlated with *S. pneumoniae*. Co-colonisation correlation was consistent between stable-state and exacerbation states.	(91)
Lung tissue microbiota shifts in very severe COPD patients compared to smoker and non-smoker controls.	There is a reduction in diversity in severe COPD patients which also correlates with alveolar destruction. Bacterial DNA was isolated from lung tissue from 8 very severe COPD (GOLD stage 4) patients undergoing lung transplantation. Bacterial communities were analysed using qPCR amplification of 16S rRNA hypervariable region V2 and terminal restriction fragment length polymorphism analysis and pyrotag sequencing.	(116)

Bronchial wash microbiota shifts in COPD patients compared to smoker and non-smoker controls.	COPD is associated with a reduction in microbial diversity compared to smoking and non-smoking healthy controls, highlighting a microbial cause for COPD. The microbiome of bronchial wash samples in 18 clinically stable COPD patients with mild to severe (GOLD 1 – 3) airflow obstruction was significantly different to 8 healthy smokers and 3 non-smoker controls, detected by culture and Illumina MiSeq sequencing.	(117)
Lung microbiota does not shift during AECOPD and there does not account for exacerbation events.	Whilst dominant bacteria cultured from COPD patient sputum have included *P. aeruginosa* and *H. influenzae,* there were no significant microbiota changes before and after exacerbations. Overall microbial load and community composition remained stable following antibiotic treatment for AECOPD. Microbiota was analysed using anaerobic culture and 16S rDNA pyrosequencing of sputum from 40 patients with mild (17/40), moderate (17/40) or severe (6/40) COPD (GOLD stage 1 – 3).	(118)
	In a longitudinal study analysing the microbiome of 476 sputum samples from 87 patients with mild (1/87), moderate (35/87), severe (32/87) and very severe (19/87) COPD (GOLD stage 1 – 4) patients, *Streptococcus, Haemophilus, Moraxella and Pseudomonas* accounted for 41.1%, 18.9%, 5.6% and 4.4%, respectively, of the total 366 genera and showed no statistically significant differences in composition before and during exacerbation. Sputum microbiota was analysed 16S rRNA pyrosequencing using the V3-V5 hypervariable region.	(42)
Dominant COPD lung microbiota are shared with healthy lung microbiota.	PCR amplification and 16S rRNA pyrosequencing of stable state sputum, bronchial aspirate, bronchoalveolar lavage and bronchial mucosa samples from 8 patients with moderate COPD (GOLD stage 3) showed 60% of the microbiota was dominated by genera shared with the healthy lung microbiota, including Streptococcus,	(18)

Prevotella, Moraxella, Haemophilus, Acinetobacter, Fusobacterium, and Neisseria.

GOLD, Global Initiative for Chronic Obstructive Lung Disease;

544

545

Table 2. Summary of Key NTHi Surface Modifications

Surface Component Modification	Function	Experimental Effects	Citation
Sialyation	Sialytransferases modify the LOS with environmentally available sialic acid.	Sialyated LOS glycoforms have increased biofilm formation and persistence in the rat lung model system *in-vivo*. NTHi grown in sialic acid deficient conditions and *siaB* (CMO-sialic acid synthetase) have reduced biofilm formation.	(77)
		NTHi increase LOS sialyation during planktonic to biofilm transition which promotes aggregation and further sialyation.	(78)
Phosphorycholine (ChoP)	A hydrophilic fatty-acid that promotes initial attachment and reduces immune response to infection.	Increased ChoP glycoforms reduces LOS endotoxin bioactivity, reducing host innate response stimulation and evasion of the immune system.	(77) (64)
Type IV Pilus (TfP)	Filamentous structure, 6-7nm in diameter common across many bacteria cell surfaces.	Immunofluorescent studies have revealed that TfP binds to human bronchial epithelia ICAM-1 receptor facilitating non-reversible attachment.	(50)
	In NTHi, encoded by PilA and transported to the cell membrane by ComE secretin in NTHi, contributing to biofilm formation.	In otitis media models, TfP was visualised to be part of the integral structure of the ds-eDNA component of the EPS matrix, constituting a function in further stability.	(70)
	Initial attachment by breaking through the substratum's repulsive forces and forming weak but	TfP machinery facilitates eDNA and DNABII trafficking and release into EPS matrix, via ComE pore where TfP is expressed.	(69)

	attractive van der Waals forces.		
OMP P5/P1	A fimbrial structure that binds to mucin, a constituent of mucus. Chronic bronchitis mucus hypersecretion provides ideal nutrient-rich substrate to bind.	OMP p5 facilitates NTHi adherence to human tracheobronchial mucin and lactoferrins, promoting biofilm formation in the human lung.	(52) (50)
		16S rRNA sequencing revealed *Streptococcus pneumoniae* was a dominant lung taxa during AECOPD.	(70)
		A recent study has reported OMP P1 as the genuine e CEACAM-binding invasin of *H. influenzae* leading to attachment and internalization in the absence of OMP P5 expression.	(51)
HMW1/2 adhesins	Support direct adhesion to the upper respiratory tract	Present in 80% of clinical NTHi isolates however the expression varies.	(76)

546

547

Table 3. Summary of NTHi Biofilm Antibiotic Resistance & Tolerance Mechanisms

Mechanism & Drug	Model / Experiment	Experimental Effects	Citation
Gentamycin tolerance	Clinical CF NTHi isolates adhered to human airway epithelia	Biofilms survived treatment with high concentration gentamycin (10 – 25µl).	(80)
Sub-MIC ß-lactam antibiotics & carbohydrate metabolism	NTHi biofilms grown on airway epithelia	mRNA transcriptional changes showed an increase in carbohydrate metabolism gene expression in response to sub-MIC ampicillin and amoxicillin.	(82)
		A subset of five genes functioned in glycogen biosynthesis, a component of biofilm biomass, as well as in the secretion of type IV pilin involved in adhesion. Furthermore, this exposure was found to 'prime' biofilms against stronger antibiotics and make them less sensitive to cefuroxime.	(82)
eDNA-rich EPS matrix protects against antimicrobials and antibiotics	8 clinical NTHi isolate static biofilms *in-vitro*	Biofilms were largely not susceptible to a range of detergents, antiseptics and disinfectants including sodium dodecyl sulfate, ceptpyridinium chloride, povidone and widely used clinical and commercially available chlorhexidine glucoronate.	(73)
		Treatment with DNase I degrades the eDNA component of the protective EPS matrix, drove dispersal and led to significantly greater killing of the bacteria by these agents.	(72)
		Findings were reciprocated with ampicillin and ciprofloxacin treatment.	(72)
Low metabolic activity protects	Metabolomic and proteomic	Generally, proteins involved in protein synthesis and energy metabolism,	(29)

biofilms against β-lactam antibiotics	analysis of 814 proteins across biofilm and planktonic strains revealed that 127 products were differentially expressed.	including cysteinyl-tRNA synthetase and aerobic respiration control protein ArcA respectively, were largely downregulated. DNA metabolism proteins and co-factor binding proteins including NAD nucleosidase (involved in oxidative stress) and heme-binding protein A were upregulated. Downregulation of metabolomic proteins suggests that NTHi biofilms survive in a dormant state with decreased energy metabolism and protein synthesis (Post et al., 2014) which may make β-lactam antibiotics, that act by inhibiting bacterial cell wall peptidoglycan, ineffective.

548

Table 4. Summary of Clinical Evidence for NTHi Biofilms (*ex-vivo*).

Evidence	Model / Experiment	Experimental Effects	References
Biofilm promoting adhesin genes	PCR analysis of 108 clinical NTHi strains for adhesin genes correlated with biofilm formatting *in-vitro*	Variability in the presence of key adhesin genes including hifA (22%), hmw (48%), hia (57%), hap (22%) and siaB (38%), of which hemagglutinating pili and Hia were significantly associated with increased biofilm forming capacity. Biofilm forming strains of NTHi were significantly more likely to be identified in patients with chronic (90%) rather than acute (63%) respiratory infections.	(99)
TEM showing biofilm adhesion to cell surfaces	*Ex-vivo* BALF imaged using TEM	Analysis of BAL from CF patients has provided good evidence of NTHi biofilm attached to cell surfaces, using transition election microscopy techniques These clinical NTHi isolates also formed mature biofilms on cultured airway epithelial cells, showing microcolony formation and EPS production.	(80)
Biofilm NTHi isolates	61 patients hospitalised with lower respiratory tract infection patients. NTHi isolated and grown *in-vitro*	Claimed to have an association with NTHi biofilm formation and increased hospitalisation duration. However, the evidence of biofilm production was poor, with only 10% actually forming biofilms, the majority of which (80%) were weak biofilm producers with no strong biofilms produced. Additionally, biofilms were no more significantly resistant to antimicrobials than non-biofilms so did not display a major hallmark of biofilm formation.	(100)
Biofilm NTHi isolates	Sarcoidosis patient sputum samples	*Haemophilus spp*. was identified in both 31/37 healthy and 30/31 sarcoidosis patients, 67% of *H. influenzae* isolates formed biofilm, all of which were weak using the standard crystal violet assay and classification.	(101)
Immune response to	6 COPD patient derived NTHi isolates	COPD patient derived NTHi strains express greater pdgx under biofilm than planktonic growth conditions. ELISA detects pdgx antibodies in 44.4%	(61)

biofilm strategies	and 18 COPD patients for ELISA analysis	COPD patients' respiratory tract by ELISA showing host immune response to biofilm-survival strategies.

7 Conflict of Interest

Karl Staples reports grants from AstraZeneca, outside the conduct of the study; Tom Wilkinson reports grants and personal fees from AstraZeneca, outside the conduct of the study; personal fees and other from MMH, grants and personal fees from GSK, grants and personal fees from AZ, personal fees from BI, grants and personal fees from Synairgen, outside the submitted work; Jake Weeks, C. Mirella Spalluto and Alastair Watson report no conflicts of interest.

8 Author Contributions

Jake Weeks: conceptualization, investigation, literature searching, analysis, project administration, writing original draft, reviewing and editing, approval of final draft. Karl Staples: supervision, conceptualization, reviewing & editing, approval of final draft. C. Mirella Spalluto: supervision, conceptualization, reviewing & editing, approval of final draft. Alastair Watson: supervision, reviewing & editing, approval of final draft. Tom Wilkinson: supervision, conceptualization, reviewing & editing, approval of final draft.

9 Funding

This work was undertaken using a BBSRC iCASE PhD studentship awarded for Jake Weeks' doctoral studies.

10 References

1. Rabe KF, Watz H. Chronic obstructive pulmonary disease. The Lancet. 2017;389(10082):1931-40.
2. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med. 2007;176(6):532-55.
3. MacNee W. ABC of chronic obstructive pulmonary disease Pathology, pathogenesis, and pathophysiology. BMJ. 2006;332:3.
4. Wilkinson TMA, Aris E, Bourne S, Clarke SC, Peeters M, Pascal TG, 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.
5. Panchabhai TS, Mukhopadhyay S, Sehgal S, Bandyopadhyay D, Erzurum SC, Mehta AC. Plugs of the Air Passages: A Clinicopathologic Review. Chest. 2016;150(5):1141-57.
6. McDaniel CT, Panmanee W, Hassett DJ. An Overview of Infections in Cystic Fibrosis Airways and the Role of Environmental Conditions on Pseudomonas aeruginosa Biofilm Formation and Viability. Cystic Fibrosis in the Light of New Research2015.
7. Dicker AJ, Crichton ML, Pumphrey EG, Cassidy AJ, Suarez-Cuartin G, Sibila O, et al. Neutrophil extracellular traps are associated with disease severity and microbiota diversity in patients with chronic obstructive pulmonary disease. J Allergy Clin Immunol. 2018;141(1):117-27.
8. Ahearn CP, Gallo MC, Murphy TF. Insights on persistent airway infection by non-typeable Haemophilus influenzae in chronic obstructive pulmonary disease. Pathog Dis. 2017;75(4).
9. Pragman AA, Berger JP, Williams BJ. Understanding persistent bacterial lung infections: clinical implications informed by the biology of the microbiota and biofilms. Clin Pulm Med. 2016;23(2):57-66.
10. Pragman AA, Lyu T, Baller JA, Gould TJ, Kelly RF, Reilly CS, et al. The lung tissue microbiota of mild and moderate chronic obstructive pulmonary disease. Microbiome. 2018;6(1):7.
11. Mayhew D, Devos N, Lambert C, Brown JR, Clarke SC, Kim VL, et al. Longitudinal profiling of the lung microbiome in the AERIS study demonstrates repeatability of bacterial and eosinophilic COPD exacerbations. Thorax. 2018;73(5):422-30.
12. Beck JM, Young VB, Huffnagle GB. The microbiome of the lung. Transl Res. 2012;160(4):258-66.
13. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, et al. Analysis of the lung microbiome in the "healthy" smoker and in COPD. PLoS One. 2011;6(2):e16384.
14. Segal LN, Rom WN, Weiden MD. Lung microbiome for clinicians. New discoveries about bugs in healthy and diseased lungs. Ann Am Thorac Soc. 2014;11(1):108-16.
15. Toraldo DM, Conte L. Influence of the Lung Microbiota Dysbiosis in Chronic Obstructive Pulmonary Disease Exacerbations: The Controversial Use of Corticosteroid and Antibiotic Treatments and the Role of Eosinophils as a Disease Marker. J Clin Med Res. 2019;11(10):667-75.
16. Mika M, Nita I, Morf L, Qi W, Beyeler S, Bernasconi E, et al. Microbial and host immune factors as drivers of COPD. ERJ Open Res. 2018;4(3).
17. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. PLoS One. 2010;5(1):e8578.
18. Cabrera-Rubio R, Garcia-Nunez M, Seto L, Anto JM, Moya A, Monso E, et al. Microbiome diversity in the bronchial tracts of patients with chronic obstructive pulmonary disease. J Clin Microbiol. 2012;50(11):3562-8.

19. Zakharkina T, Heinzel E, Koczulla RA, Greulich T, Rentz K, Pauling JK, et al. Analysis of the airway microbiota of healthy individuals and patients with chronic obstructive pulmonary disease by T-RFLP and clone sequencing. PLoS One. 2013;8(7):e68302.
20. Molyneaux PL, Mallia P, Cox MJ, Footitt J, Willis-Owen SA, Homola D, et al. Outgrowth of the bacterial airway microbiome after rhinovirus exacerbation of chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2013;188(10):1224-31.
21. Nakou A, Papaparaskevas J, Diamantea F, Skarmoutsou N, Polychronopoulos V, Tsakris A. A prospective study on bacterial and atypical etiology of acute exacerbation in chronic obstructive pulmonary disease. Future Microbiol 2014;9(11):10.
22. Jubinville E, Veillette M, Milot J, Maltais F, Comeau AM, Levesque RC, et al. Exacerbation induces a microbiota shift in sputa of COPD patients. PLoS One. 2018;13(3):e0194355.
23. Clementi CF, Murphy TF. Non-typeable Haemophilus influenzae invasion and persistence in the human respiratory tract. Front Cell Infect Microbiol. 2011;1:1.
24. Hartwig SM, Ketterer M, Apicella MA, Varga SM. Non-typeable Haemophilus influenzae protects human airway epithelial cells from a subsequent respiratory syncytial virus challenge. Virology. 2016;498:128-35.
25. Slack MPE. The evidence for non-typeable Haemophilus influenzae as a causative agent of childhood pneumonia. Pneumonia (Nathan). 2017;9:9.
26. King PT, Sharma R. The Lung Immune Response to Nontypeable Haemophilus influenzae (Lung Immunity to NTHi). J Immunol Res. 2015;2015:706376.
27. Van Eldere J, Slack MPE, Ladhani S, Cripps AW. Non-typeable Haemophilus influenzae, an under-recognised pathogen. The Lancet Infectious Diseases. 2014;14(12):1281-92.
28. Foxwell AR, Kyd J, Cripps AW. Nontypeable Haemophilus Influenzae: Pathogenesis and Prevention. Microbiology and Molecular Biology Reviews. 1998;62(2):15.
29. Post DM, Held JM, Ketterer MR, Phillips NJ, Sahu A, Apicella MA, et al. Comparative analyses of proteins from Haemophilus influenzae biofilm and planktonic populations using metabolic labeling and mass spectrometry. BMC Microbiol. 2014;14:329.
30. Cerquetti M, Giufre M. Why we need a vaccine for non-typeable Haemophilus influenzae. Hum Vaccin Immunother. 2016;12(9):2357-61.
31. Herath SC, Normansell R, Maisey S, Poole P. Prophylactic antibiotic therapy for chronic obstructive pulmonary disease (COPD). Cochrane Database Syst Rev. 2018;10:CD009764.
32. Contoli M, Pauletti A, Rossi MR, Spanevello A, Casolari P, Marcellini A, et al. Long-term effects of inhaled corticosteroids on sputum bacterial and viral loads in COPD. Eur Respir J. 2017;50(4).
33. Brill SE, Law M, El-Emir E, Allinson JP, James P, Maddox V, et al. Effects of different antibiotic classes on airway bacteria in stable COPD using culture and molecular techniques: a randomised controlled trial. Thorax. 2015;70(10):930-8.
34. Qiu S, Zhong X. Macrolides: a promising pharmacologic therapy for chronic obstructive pulmonary disease. Ther Adv Respir Dis. 2017;11(3):147-55.
35. Yao G-Y, Ma Y-L, Zhang M-Q, Gao Z-C. Macrolide Therapy Decreases Chronic Obstructive Pulmonary Disease Exacerbation: A Meta-Analysis. Respiration. 2013;86(3):254-60.
36. Kelly C, Chalmers JD, Crossingham I, Relph N, Felix LM, Evans DJ, et al. Macrolide antibiotics for bronchiectasis. Cochrane Database Syst Rev. 2018;3:CD012406.
37. Cao Y, Xuan S, Wu Y, Yao X. Effects of long-term macrolide therapy at low doses in stable COPD. Int J Chron Obstruct Pulmon Dis. 2019;14:1289-98.
38. Smith D, Du Rand I, Addy CL, Collyns T, Hart SP, Mitchelmore PJ, et al. British Thoracic Society guideline for the use of long-term macrolides in adults with respiratory disease. Thorax. 2020;75(5):370-404.

39. Watson A, Wilkinson TMA. Respiratory viral infections in the elderly. Ther Adv Respir Dis. 2021;15:17534666621995050.
40. Cui Y, Luo L, Li C, Chen P, Chen Y. Long-term macrolide treatment for the prevention of acute exacerbations in COPD: a systematic review and meta-analysis. Int J Chron Obstruct Pulmon Dis. 2018;13:3813-29.
41. Djamin RS, Talman S, Schrauwen EJA, von Wintersdorff CJH, Wolffs PF, Savelkoul PHM, et al. Prevalence and abundance of selected genes conferring macrolide resistance genes in COPD patients during maintenance treatment with azithromycin. Antimicrob Resist Infect Control. 2020;9(1):116.
42. Wang Z, Bafadhel M, Haldar K, Spivak K, Mayhew D, Miller B, et al. Lung microbiome dynamics in COPD exacerbations. Eur Respir J. 2016;47(4):1034-6.
43. Hiergeist A, Glasner J, Reischl U, Gessner A. Analyses of Intestinal Microbiota: Culture versus Sequencing. ILAR J. 2015;56(2):228-40.
44. Martiny AC. High proportions of bacteria are culturable across major biomes. ISME J. 2019;13(8):2125-8.
45. Clarridge JE, 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev. 2004;17(4):840-62, table of contents.
46. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods. 2007;69(2):330-9.
47. Zubiria-Barrera C, Stock M, Neubert R, Vester A, Kulle A, Schneegans A, et al. A simple sequence-based filtering method for the removal of contaminants in low-biomass 16S rRNA amplicon sequencing approaches. J Microbiol Methods. 2020;178:106060.
48. Bjarnsholt T. The Role of Bacterial Biofi lms in Chronic Infections. APMIS. 2013;121:51.
49. Sriram KB, Cox AJ, Clancy RL, Slack MPE, Cripps AW. Nontypeable Haemophilus influenzae and chronic obstructive pulmonary disease: a review for clinicians. Crit Rev Microbiol. 2018;44(2):125-42.
50. Novotny LA, Bakaletz LO. Peptide Vaccines for Otitis Media. Handbook of Biologically Active Peptides2013. p. 603-11.
51. Tchoupa AK, Lichtenegger S, Reidl J, Hauck CR. Outer membrane protein P1 is the CEACAM-binding adhesin of Haemophilus influenzae. Mol Microbiol. 2015;98(3):440-55.
52. Kubiet M, Ramphal R. Adhesion of Nontypeable *Haemophilus influenzae* biofilms from Blood and Sputum to Human Tracheobronchial Mucins and Lactoferrin American Society for Microbiology. 1995;63(3):4.
53. Langereis JD, Hermans PW. Novel concepts in nontypeable Haemophilus influenzae biofilm formation. FEMS Microbiol Lett. 2013;346(2):81-9.
54. Costerton JW, Lewandowski Z. MICROBIAL BIOFILMS Annu Rev Microbiol 1995;49:34.
55. Armbruster CE, Hong W, Pang B, Dew KE, Juneau RA, Byrd MS, et al. LuxS promotes biofilm maturation and persistence of nontypeable haemophilus influenzae in vivo via modulation of lipooligosaccharides on the bacterial surface. Infect Immun. 2009;77(9):4081-91.
56. Armbruster CE, Pang B, Murrah K, Juneau RA, Perez AC, Weimer KE, et al. RbsB (NTHI_0632) mediates quorum signal uptake in nontypeable Haemophilus influenzae strain 86-028NP. Mol Microbiol. 2011;82(4):836-50.
57. Hong W, Juneau RA, Pang B, Swords WE. Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable Haemophilus influenzae persistence in the chinchilla model for otitis media. J Innate Immun. 2009;1(3):215-24.
58. Pang B, Armbruster, C., Foster, G., Learman, B., Gandi, U., Swords, E. . Autoinducer 2 (AI-2) Production by Nontypeable *Haemophilus influenzae* 86-082NP Promotes Expression of a Predicted

708 Glycosyltransferase That Is a Determinant of Biofilm Maturation, Prevention of Dispersal, and
709 Persistence *In Vivo*. Infection and Immunity. 2018;86(12).
710 59. Unal CM, Singh B, Fleury C, Singh K, Chavez de Paz L, Svensater G, et al. QseC controls
711 biofilm formation of non-typeable Haemophilus influenzae in addition to an AI-2-dependent
712 mechanism. Int J Med Microbiol. 2012;302(6):261-9.
713 60. Juneau RA, Pang B, Armbruster CE, Murrah KA, Perez AC, Swords WE. Peroxiredoxin-
714 glutaredoxin and catalase promote resistance of nontypeable Haemophilus influenzae 86-028NP to
715 oxidants and survival within neutrophil extracellular traps. Infect Immun. 2015;83(1):239-46.
716 61. Murphy TF, Kirkham C, Sethi S, Lesse AJ. Expression of a peroxiredoxin-glutaredoxin by
717 Haemophilus influenzae in biofilms and during human respiratory tract infection. FEMS Immunol Med
718 Microbiol. 2005;44(1):81-9.
719 62. Santocki M, Kolaczkowska E. On Neutrophil Extracellular Trap (NET) Removal: What We
720 Know Thus Far and Why So Little. Cells. 2020;9(9).
721 63. Su YC, Jalalvand F, Thegerstrom J, Riesbeck K. The Interplay Between Immune Response and
722 Bacterial Infection in COPD: Focus Upon Non-typeable Haemophilus influenzae. Front Immunol.
723 2018;9:2530.
724 64. West-Barnette S, Rockel A, Swords WE. Biofilm growth increases phosphorylcholine content
725 and decreases potency of nontypeable Haemophilus influenzae endotoxins. Infect Immun.
726 2006;74(3):1828-36.
727 65. Goodman SD, Obergfell KP, Jurcisek JA, Novotny LA, Downey JS, Ayala EA, et al. Biofilms
728 can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated
729 proteins. Mucosal Immunol. 2011;4(6):625-37.
730 66. Domenech M, Pedrero-Vega E, Prieto A, Garcia E. Evidence of the presence of nucleic acids
731 and beta-glucan in the matrix of non-typeable Haemophilus influenzae in vitro biofilms. Sci Rep.
732 2016;6:36424.
733 67. Gunn JS, Bakaletz LO, Wozniak DJ. What's on the Outside Matters: The Role of the
734 Extracellular Polymeric Substance of Gram-negative Biofilms in Evading Host Immunity and as a
735 Target for Therapeutic Intervention. J Biol Chem. 2016;291(24):12538-46.
736 68. Wu S, Baum MM, Kerwin J, Guerrero D, Webster S, Schaudinn C, et al. Biofilm-specific
737 extracellular matrix proteins of nontypeable Haemophilus influenzae. Pathog Dis. 2014;72(3):143-60.
738 69. Jurcisek JA, Brockman KL, Novotny LA, Goodman SD, Bakaletz LO. Nontypeable
739 Haemophilus influenzae releases DNA and DNABII proteins via a T4SS-like complex and ComE of
740 the type IV pilus machinery. Proc Natl Acad Sci U S A. 2017;114(32):E6632-E41.
741 70. Jurcisek JA, Bakaletz LO. Biofilms formed by nontypeable Haemophilus influenzae in vivo
742 contain both double-stranded DNA and type IV pilin protein. J Bacteriol. 2007;189(10):3868-75.
743 71. Jones EA, McGillivary G, Bakaletz LO. Extracellular DNA within a nontypeable Haemophilus
744 influenzae-induced biofilm binds human beta defensin-3 and reduces its antimicrobial activity. J Innate
745 Immun. 2013;5(1):24-38.
746 72. Cavaliere R, Ball JL, Turnbull L, Whitchurch CB. The biofilm matrix destabilizers, EDTA and
747 DNaseI, enhance the susceptibility of nontypeable Hemophilus influenzae biofilms to treatment with
748 ampicillin and ciprofloxacin. Microbiologyopen. 2014;3(4):557-67.
749 73. Izano EA, Shah SM, Kaplan JB. Intercellular adhesion and biocide resistance in nontypeable
750 Haemophilus influenzae biofilms. Microb Pathog. 2009;46(4):207-13.
751 74. Cho C, Chande A, Gakhar L, Bakaletz LO, Jurcisek JA, Ketterer M, et al. Role of the nuclease
752 of nontypeable Haemophilus influenzae in dispersal of organisms from biofilms. Infect Immun.
753 2015;83(3):950-7.

75. Brockman KL, Azzari PN, Branstool MT, Atack JM, Schulz BL, Jen FE, et al. Epigenetic Regulation Alters Biofilm Architecture and Composition in Multiple Clinical Isolates of Nontypeable Haemophilus influenzae. mBio. 2018;9(5).
76. Giufre M, Carattoli A, Cardines R, Mastrantonio P, Cerquetti M. Variation in expression of HMW1 and HMW2 adhesins in invasive nontypeable Haemophilus influenzae isolates. BMC Microbiol. 2008;8:83.
77. Swords WE, Moore ML, Godzicki L, Bukofzer G, Mitten MJ, VonCannon J. Sialylation of lipooligosaccharides promotes biofilm formation by nontypeable Haemophilus influenzae. Infect Immun. 2004;72(1):106-13.
78. Greiner LL, Watanabe H, Phillips NJ, Shao J, Morgan A, Zaleski A, et al. Nontypeable Haemophilus influenzae strain 2019 produces a biofilm containing N-acetylneuraminic acid that may mimic sialylated O-linked glycans. Infect Immun. 2004;72(7):4249-60.
79. Slinger R, Chan F, Ferris W, Yeung SW, St Denis M, Gaboury I, et al. Multiple combination antibiotic susceptibility testing of nontypeable Haemophilus influenzae biofilms. Diagn Microbiol Infect Dis. 2006;56(3):247-53.
80. Starner TD, Zhang N, Kim G, Apicella MA, McCray PB, Jr. Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis. Am J Respir Crit Care Med. 2006;174(2):213-20.
81. Starner TD, Shrout JD, Parsek MR, Appelbaum PC, Kim G. Subinhibitory concentrations of azithromycin decrease nontypeable Haemophilus influenzae biofilm formation and Diminish established biofilms. Antimicrob Agents Chemother. 2008;52(1):137-45.
82. Wu S, Li X, Gunawardana M, Maguire K, Guerrero-Given D, Schaudinn C, et al. Beta- lactam antibiotics stimulate biofilm formation in non-typeable haemophilus influenzae by up-regulating carbohydrate metabolism. PLoS One. 2014;9(7):e99204.
83. Southern KW, Barker PM. Azithromycin for cystic fibrosis. Eur Respir J. 2004;24(5):834-8.
84. Nalca Y, Jansch L, Bredenbruch F, Geffers R, Buer J, Haussler S. Quorum-sensing antagonistic activities of azithromycin in Pseudomonas aeruginosa PAO1: a global approach. Antimicrob Agents Chemother. 2006;50(5):1680-8.
85. Wise MP, Williams DW, Lewis MA, Frost PJ. Macrolides and community-acquired pneumonia: is quorum sensing the key? Critical Care. 2010;14(181):3.
86. Khanolkar RA, Clark ST, Wang PW, Hwang DM, Yau YCW, Waters VJ, et al. Ecological Succession of Polymicrobial Communities in the Cystic Fibrosis Airways. mSystems. 2020;5(6):16.
87. Rotta Detto Loria J, Rohmann K, Droemann D, Kujath P, Rupp J, Goldmann T, et al. Nontypeable Haemophilus Influenzae Infection Upregulates the NLRP3 Inflammasome and Leads to Caspase-1-Dependent Secretion of Interleukin-1beta - A Possible Pathway of Exacerbations in COPD. PLoS One. 2013;8(6):e66818.
88. Staples KJ, Taylor S, Thomas S, Leung S, Cox K, Pascal TG, et al. Relationships between Mucosal Antibodies, Non-Typeable Haemophilus influenzae (NTHi) Infection and Airway Inflammation in COPD. PLoS One. 2016;11(11):e0167250.
89. Zhang J, Zhu Z, Zuo X, Pan H, Gu Y, Yuan Y, et al. The role of NTHi colonization and infection in the pathogenesis of neutrophilic asthma. Respir Res. 2020;21(1):170.
90. Kyd JM, Krishnamurthy A, Kidd S. Interactions and Mechanisms of Respiratory Tract Biofilms Involving Streptococcus Pneumoniae and Nontypeable Haemophilus Influenzae. Microbial Biofilms - Importance and Applications2016.
91. Jacobs DM, Ochs-Balcom HM, Zhao J, Murphy TF, Sethi S. Lower Airway Bacterial Colonization Patterns and Species-Specific Interactions in Chronic Obstructive Pulmonary Disease. J Clin Microbiol. 2018;56(10).
92. Tikhomirova A, Kidd SP. Haemophilus influenzae and Streptococcus pneumoniae: living together in a biofilm. Pathog Dis. 2013;69(2):114-26.

803 93. Krishnamurthy A, Kyd J. The roles of epithelial cell contact, respiratory bacterial interactions
804 and phosphorylcholine in promoting biofilm formation by Streptococcus pneumoniae and nontypeable
805 Haemophilus influenzae. Microbes Infect. 2014;16(8):640-7.
806 94. Bair KL, Campagnari AA. Moraxella catarrhalis Promotes Stable Polymicrobial Biofilms With
807 the Major Otopathogens. Front Microbiol. 2019;10:3006.
808 95. Murphy TF, Brauer AL, Grant BJ, Sethi S. Moraxella catarrhalis in chronic obstructive
809 pulmonary disease: burden of disease and immune response. Am J Respir Crit Care Med.
810 2005;172(2):195-9.
811 96. Velkova S, Petris A, Lee D, Freestone P, Williamson R, Beinke S, et al. Moraxella catarrhalis
812 infection of healthy and COPD ciliated epithelial cultures. European Respiratory Journal. 2018;52.
813 97. Hunt BC, Stanford D, Xu X, Li J, Gaggar A, Rowe SM, et al. Haemophilus influenzae persists
814 in biofilm communities in a smoke-exposed ferret model of COPD. ERJ Open Res. 2020;6(3).
815 98. Short B, Carson S, Devlin AC, Reihill JA, Crilly A, MacKay W, et al. Non-typeable
816 Haemophilus influenzae chronic colonization in chronic obstructive pulmonary disease (COPD). Crit
817 Rev Microbiol. 2021;47(2):192-205.
818 99. Nishi J, Imuta N, Kamenosono A, Tokuda K, Tanaka S, Manago K. Prevalence of adhesin
819 genes and biofilm formation among clinical isolates of nontypable *Haemophilus influenzae* J-STAGE.
820 2006;17(2):8.
821 100. Martinez-Resendez MF, Gonzalez-Chavez JM, Garza-Gonzalez E, Castro-Fuentes LN,
822 Gutierrez-Ferman JL, Echaniz-Aviles G, et al. Non-typeable Haemophilus influenzae biofilm
823 production and severity in lower respiratory tract infections in a tertiary hospital in Mexico. J Med
824 Microbiol. 2016;65(12):1385-91.
825 101. Kosikowska U, Rybojad P, Stepien-Pysniak D, Zbikowska A, Malm A. Changes in the
826 prevalence and biofilm formation of Haemophilus influenzae and Haemophilus parainfluenzae from
827 the respiratory microbiota of patients with sarcoidosis. BMC Infect Dis. 2016;16(1):449.
828 102. Soren O, Rineh A, Silva DG, Cai Y, Howlin RP, Allan RN, et al. Cephalosporin nitric oxide-
829 donor prodrug DEA-C3D disperses biofilms formed by clinical cystic fibrosis isolates of Pseudomonas
830 aeruginosa. J Antimicrob Chemother. 2020;75(1):117-25.
831 103. Cai YM, Webb JS. Optimization of nitric oxide donors for investigating biofilm dispersal
832 response in Pseudomonas aeruginosa clinical isolates. Appl Microbiol Biotechnol. 2020;104(20):8859-
833 69.
834 104. Papi A, Avdeev S, Calverley PMA, Cordeiro CR, Jesenak M, Koblizek V, et al. Use of
835 mucolytics in COPD: A Delphi consensus study. Respir Med. 2020;175:106190.
836 105. Dal Negro R, Pozzi E, Cella SG. Erdosteine: Drug exhibiting polypharmacy for the treatment
837 of respiratory diseases. Pulm Pharmacol Ther. 2018;53:80-5.
838 106. Ren D, Nelson KL, Uchakin PN, Smith AL, Gu XX, Daines DA. Characterization of extended
839 co-culture of non-typeable Haemophilus influenzae with primary human respiratory tissues. Exp Biol
840 Med (Maywood). 2012;237(5):540-7.
841 107. Jan AT. Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update.
842 Frontiers in Microbiology. 2017;8.
843 108. Winter LE, Barenkamp SJ. Immunogenicity of Nontypeable Haemophilus influenzae Outer
844 Membrane Vesicles and Protective Ability in the Chinchilla Model of Otitis Media. Clin Vaccine
845 Immunol. 2017;24(10).
846 109. Sharpe SW, Kuehn MJ, Mason KM. Elicitation of epithelial cell-derived immune effectors by
847 outer membrane vesicles of nontypeable Haemophilus influenzae. Infect Immun. 2011;79(11):4361-9.
848 110. Guiot J, Struman I, Louis E, Louis R, Malaise M, Njock MS. Exosomal miRNAs in Lung
849 Diseases: From Biologic Function to Therapeutic Targets. J Clin Med. 2019;8(9).

111. Rodrigues M, Fan J, Lyon C, Wan M, Hu Y. Role of Extracellular Vesicles in Viral and Bacterial Infections: Pathogenesis, Diagnostics, and Therapeutics. Theranostics. 2018;8(10):2709-21.
112. Wedzicha JA, Wilkinson T. Impact of chronic obstructive pulmonary disease exacerbations on patients and payers. Proc Am Thorac Soc. 2006;3(3):218-21.
113. Wilkinson T WJ. Strategies for improving outcomes of COPD exacerbations. Int J Chron Obstruct Pulmon Dis. 2006;1:7.
114. Kong CW, Wilkinson TMA. Predicting and preventing hospital readmission for exacerbations of COPD. ERJ Open Res. 2020;6(2).
115. Wilkinson TMA, Schembri S, Brightling C, Bakerly ND, Lewis K, MacNee W, et al. Non-typeable Haemophilus influenzae protein vaccine in adults with COPD: A phase 2 clinical trial. Vaccine. 2019;37(41):6102-11.
116. Sze MA, Dimitriu PA, Hayashi S, Elliott WM, McDonough JE, Gosselink JV, et al. The lung tissue microbiome in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2012;185(10):1073-80.
117. Einarsson GG, Comer DM, McIlreavey L, Parkhill J, Ennis M, Tunney MM, et al. Community dynamics and the lower airway microbiota in stable chronic obstructive pulmonary disease, smokers and healthy non-smokers. Thorax. 2016;71(9):795-803.
118. Tunney MM, Einarsson GG, Wei L, Drain M, Klem ER, Cardwell C, et al. Lung microbiota and bacterial abundance in patients with bronchiectasis when clinically stable and during exacerbation. Am J Respir Crit Care Med. 2013;187(10):1118-26.

11 Figure legends

Figure 1. Model of NTHi Biofilm Formation and Lifecycle

(a) The impaired mucociliary clearance system coupled with inflammatory mucus secretion hyperplasia creates a suitable substrate for the initial colonisation and reversible attachment of NTHi to the air-liquid interface of the tracheobronchial respiratory mucosa, a pseudostratified, ciliated, columnar epithelium predominated by ciliated epithelial cells and interspersed by secretory cells (mucus-secreting goblet cells and secretoglobin secreting club cells) and basal cells. In COPD disease states, the mucus layer extends into the periciliary layer. (b) NTHi irreversibly attach to respiratory epithelial cells by adhesins including Type IV Pilus and OMP P1 that bind ICAM-1, CEACAM-1 and CLEC7A. NTHi also binds to the mucus layer itself by having adhesins that bind tracheobronchial mucins and lactoferrins. (c) Irreversibly attached NTHi begin to aggregate and produce an EPS matrix, favouring secondary colonisers. (d) Biofilms exhibit complex intra-specific and interspecific interactions, signalling, metabolism changes, gene expression changes that drive nutrient and oxygen gradients. This leading to differentiation of bacteria and maturation of the biofilm with structurally large microcolonies with nutrient and water channels. (e) Quorum sensing changes drive dispersal of the biofilm as planktonic bacteria and aggregates of biofilm leading to (f) dissemination and colonisation of secondary sites, facilitating persistence. Created with BioRender.com

Figure 2. Model of NTHi Biofilm Strategies and Host-Pathogen Interactions Facilitating Chronic Infection in the COPD Airways. (a) The LuxS-RbsB AI-2 quorum sensing system facilitates coordination of intra-specific and inter-specific gene expression that promotes a transition from the planktonic to biofilm lifestyle in response to the local environmental conditions, leading to the downstream transcription of biofilm-associate genes. NTHi also express a QSeB two-component secondary system that may support biofilm gene expression coordination where the LuxS-RbsB system has been impaired. The quorum sensing system drives dissemination of biofilm bacteria to secondary sites, and formation of oxygen, nutrient and water channels towards the centre of the biofilm. (b) NTHi biofilms produce a thick protein- and eDNA-rich EPS matrix that provides a physical and chemical barrier, slowing penetration against pharmacological and immune system antimicrobial agents by blocking phagocytes and macrophages and chelating peptides and antibiotics such as β-defensin and β-lactams. The EPS matrix also provides structural stability and provides a site for mature biofilm structures to form, whilst also limiting metabolic activity, altering microenvironments, and driving persister cells through the development of gradients towards the centre of the biofilm. (c) NTHi express chemical defence strategies that allow for the evasion of the host's NETosis response through recruitment of NETs into the EPS and expression of catalase that provides oxidative stress tolerance. Recognition by the immune system is also modulated through the differential expression of LOS and OMP P5 and OMP P2 that recruit neutrophils to form NETs but by evading the phagocytosing and cytotoxic mechanisms. NTHi biofilms further undergo a series of surface modifications that promote irreversible attachment, recruit secondary colonisers and impair recognition and/or clearance by immune system factors. These include sialyation, phosphorycholination, and the expression of TfP, OMPs and HMWs. (d) NTHi biofilms exhibit multidrug resistance due to upregulated carbohydrate metabolism and a preference for glycogen metabolism, as well as expressing antibiotic resistance genes including penicillin-binding protein 3 (PBP3) and β-lactamases. Negatively charged eDNA in the EPS matrix also sequesters positively charged antimicrobials. (e) Biofilms are seldom mono-species and exhibit complex inter-specific interactions with other inhabitants of the microbiome. In particular NTHi interacts with other COPD-associated pathogens including *S. pneumoniae* and *M. catarrhalis* that selects for hypoxia tolerant NTHi and damages cilia and mucociliary clearance, respectively,

favouring NTHi biofilm formation. Tissue shown is the tracheobronchial respiratory mucosa, a pseudostratified, ciliated, columnar epithelium predominated by ciliated epithelial cells and interspersed by secretory cells (mucus-secreting goblet cells and secretoglobin secreting club cells) and basal cells. Created with BioRender.com

Figure 1.JPEG

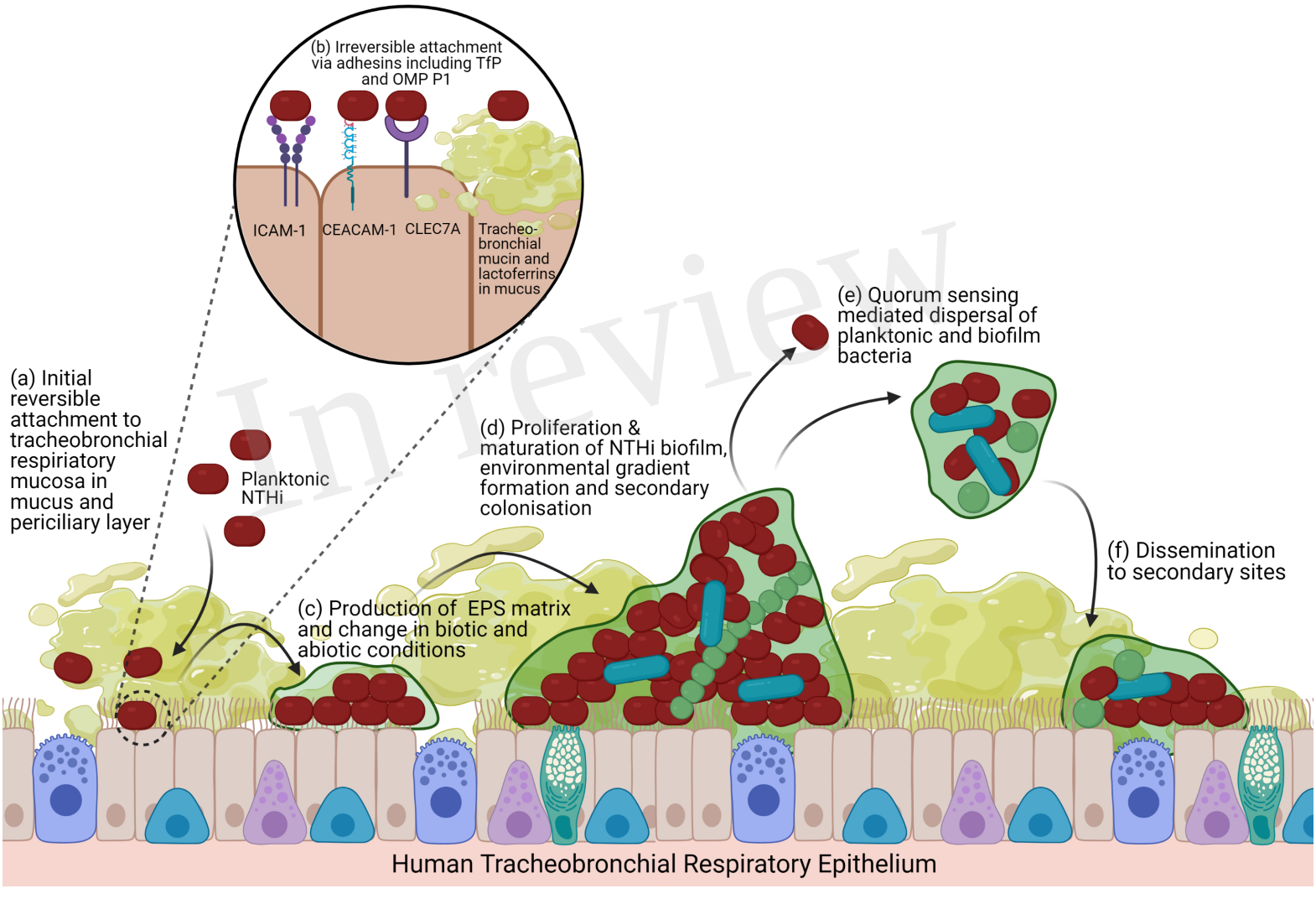

Figure 2.JPEG