PATHOGENESIS OF NON-TYPEABLE HAEMOPHILUS INFLUENZAE INFECTIONS IN CHRONIC SUPPURATIVE LUNG DISEASE

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Abstract

The respiratory tract antimicrobial defense system is a multilayered defense mechanism that relies upon mucociliary clearance and components of both the innate and adaptive immune systems to protect the lungs from inhaled or aspirated microorganisms. One of these potential pathogens, non-typeable *Haemophilus influenzae* (*NTHi*), adopts several, multifaceted redundant strategies to successfully colonize the lower airways and establish a persistent infection. *NTHi* can impair mucociliary clearance, express multiple multi-functional adhesins for various cell types within the respiratory tract and evade host defenses by surviving within and between cells, forming biofilms, increasing antigenic drift, secreting proteases and antioxidants, and by host-pathogen cross-talk, impair macrophage and neutrophil function. *NTHi* is recognized as an important pathogen in several chronic lower respiratory disorders, such as protracted bacterial bronchitis, bronchiectasis, cystic fibrosis and primary ciliary dyskinesia. The persistence of *NTHi* in human airways, including its capacity to form biofilms, results in chronic infection and inflammation, which can ultimately injure airway wall structures. The complex nature of the molecular pathogenetic mechanisms employed by *NTHi* is incompletely understood but improved understanding of its pathobiology will be important for developing effective therapies and vaccines, especially given the marked genetic heterogeneity of *NTHi* and its possession of phase-variable genes. Currently, no vaccine candidates are ready for large phase III clinical trials.

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Running head: *H. influenzae* and chronic suppurative lung disease

ABBREVIATIONS

AECs ciliated airway epithelial cells

AI-2 autoinducer-2

AMPs antimicrobial peptides

ASL airway surface liquid

BAL bronchoalveolar lavage

C4bP C4b binding protein

CEACAM-1 carcinoembryonic antigen-related cell adhesion molecule

CF cystic fibrosis

CFTR cystic fibrosis transmembrane conductance regulator

COPD chronic obstructive pulmonary disorder

CSLD chronic suppurative lung disease

ECM extracellular matrix

eDNA extracellular deoxyribonucleic acid

FISH fluorescent in-situ hybridization

Hap *Haemophilus influenzae* adhesion protein

Hia *Haemophilus influenzae* adhesion

HMW high-molecular weight

ICAM-1 intracellular adhesion molecule-1

Ig immunoglobulin

IL interleukin

LOS lipo-oligosaccharide

MAC membrane attack complex

MCC mucociliary clearance

METs macrophage extracellular traps

NETs neutrophil extracellular traps

NLRP3 NLR family pyrin domain containing 3

nNO nasal nitric oxide

NTHi nontypeable *Haemophilus influenzae*

OMP outer membrane protein

PAMPs pathogen associated molecular patterns

PBB protracted bacterial bronchitis

PCD primary ciliary dyskinesia

PHiD-CV *Haemophilus influenzae* protein D conjugate vaccine

QSS quorum sensing system

ROS reactive oxygen species

SOD superoxide dismutase

TLR toll-like receptor

ABSTRACT

The respiratory tract antimicrobial defense system is a multilayered defense mechanism that relies upon mucociliary clearance and components of both the innate and adaptive immune systems to protect the lungs from inhaled or aspirated microorganisms. One of these potential pathogens, non-typeable *Haemophilus influenzae* (*NTHi*), adopts several, multifaceted redundant strategies to successfully colonize the lower airways and establish a persistent infection. *NTHi* can impair mucociliary clearance, express multiple multifunctional adhesins for various cell types within the respiratory tract and evade host defenses by surviving within and between cells, forming biofilms, increasing antigenic drift, secreting proteases and antioxidants, and by host-pathogen cross-talk, impair macrophage and neutrophil function.*NTHi* is recognized as an important pathogen in several chronic lower respiratory disorders, such as protracted bacterial bronchitis, bronchiectasis, cystic fibrosis and primary ciliary dyskinesia. The persistence of *NTHi* in human airways, including its capacity to form biofilms, results in chronic infection and inflammation, which can ultimately injure airway wall structures. The complex nature of the molecular pathogenetic mechanisms employed by *NTHi* is incompletely understood but improved understanding of its pathobiology will be important for developing effective therapies and vaccines, especially given the marked genetic heterogeneity of *NTHi* and its possession of phase-variable genes. Currently, no vaccine candidates are ready for large phase III clinical trials.

INTRODUCTION

Haemophilus influenzae are facultative, anaerobic, Gram-negative coccobacilli requiring two accessory growth factors, X (hemin) and V (nicotinamide adenine dinucleotide, NAD). They are restricted human pathogens comprising both encapsulated (a–f) and unencapsulated or non-typeable (non-typeable *H. influenzae* [*NTHi*]) strains. Encapsulated serotypes cause invasive disease, while*NTHi* strains are genetically diverse and have adapted to colonize the nasopharynx and cause local mucosal infections. *NTHi* are acquired by either person-to-person transmission from respiratory droplets or from direct contact with respiratory secretions. Nasopharyngeal colonization by *NTHi* begins during infancy, where one-third are colonized by 1-year of age and nearly one-half by age 2-years.Colonization rates are higher in Indigenous children, those with older siblings, amongst childcare attendees, and during viral respiratory infection*NTHi* carriage is dynamic, with frequent strain turnover; the same child can acquire a new strain with loss of the old ones or carry multiple strains simultaneously, some of which are shared with primary caregivers, suggesting that transmission is occurring between them.

In children, *NTHi* colonizing the nasopharynx can cause otitis media, sinusitis, and conjunctivitis, while in preterm and young infants, and those with serious underlying comorbidities, they may occasionally invade leading to sepsis and bacteremic pneumonia. As an upper airway colonizer, *NTHi* can also access the lungs by inhalation or via micro-aspiration episodes and direct mucosal dispersion. In healthy children,

these incursions are transient, as microbes are removed by mucociliary clearance (MCC) and innate immune defenses. However, *NTHi* are able to colonize the lower airways of children with chronic suppurative lung disease (CSLD) where impaired MCC, airway wall injury, and regional microenvironments favor microbial growth. This review describes the lower airway defenses, and how*NTHi* evades these defenses to establish infection. In so doing,*NTHi* contributes to the pathogenesis of CSLD, an umbrella term used here to encompass protracted bacterial bronchitis (PBB), bronchiectasis, cystic fibrosis (CF) and primary ciliary dyskinesia (PCD). The role of *NTHi* in asthma is beyond the scope of this review and is discussed elsewhere.[17]

LOWER AIRWAY DEFENSES

The lung has layered defenses involving innate and adaptive immune protection against infection. Airway epithelia, through their barrier and MCC functions, production of antimicrobial peptides (AMPs), inflammatory mediators, and ability to transport immunoglobulin (Ig) A and IgM antibodies into the airway lumen, play a central role.

MCC is the main innate defense mechanism and involves mucus production and its proximal transport by ciliary beating. The apical surfaces of ciliated airway epithelial cells (AECs) are bathed by airway surface liquid (ASL) comprised of an upper gel-like mucus layer composed principally of MUC5AC and MUC5B mucins that entrap inhaled microorganisms, and beneath it the periciliary fluid allowing rapid ciliary beating. The mucus layer also secretes potent antimicrobial molecules, such as lysozyme, defensins, IgA, and IgG. Respiratory cilia are hair-like projections from the apical membranes of AECs that, by beating synchronously, propel airway mucus and entrapped microorganisms towards the oropharynx, where they are either expectorated or swallowed.[21] Respiratory cilia may also have chemosensory, signal transduction, and cellular growth regulatory functions. They express members of the bitter taste family of receptors to direct innate immune defenses responding to foreign antigens.

Microorganisms penetrating the mucus layer reach a second line of defense that includes AMPs secreted by AECs that are activated after sensing microbes by pattern recognition receptors, such as Toll-like receptors (TLR). AMPs, like lysozyme and lactoferrin, are also expressed constitutively into the ASL and are now supplemented locally by defensins and other AMPs, cytokines, and chemokines from recruited phagocytes and activated AECs. AMPs selectively target vital microbial structures, taking advantage of structural and biochemical differences between the host and the microbes. Microorganisms resistant to AMPs are killed by reactive oxygen species (ROS) produced by neutrophils and alveolar macrophages.

The third line of defense is adaptive immunity mediated by B-lymphocytes (humoral immunity) and T-lymphocytes (cellular immunity) where clonal rearrangement of antigen receptor genes generates long-term antigen-specific memory. The importance of adaptive immunity is underlined by congenital and acquired disorders of adaptive immunity, such as agammaglobulinemia, common variable immunodeficiency, and human immunodeficiency virus infections, which are all risk factors for recurrent pneumonia and bronchiectasis. Establishment of a new pathogen in the lower airways requires them to evade these defenses, to compete with other resident microbes, and to adapt to the nutrient availability and physicochemical properties of the local microenvironment. Here we describe how *NTHi* adapt to survive in the lungs of children with CSLD, where they exploit impaired local defenses and contribute to airway wall injury.

HOW *NTHi* ESTABLISHES LOWER AIRWAY INFECTION

Following either inhalation of respiratory droplets or micro-aspiration and mucosal dispersion from the upper to lower airways, *NTHi*employs several strategies to establish infection: perturbation of MCC, adherence to AECs, evading immune defenses, forming biofilms, and scavenging iron and other essential nutrients, allowing them to persist and survive in the lungs (Figure-1).

Perturbation of MCC *NTHi* colonization is facilitated by impaired MCC. Such impairment may be congenital, as in CF or PCD, or acquired following exposure to respiratory viruses, chronic inflammation, cigarette smoke, indoor air pollution or *NTHi* itself. In CF, mutations in the CF transmembrane conductance regulator (CFTR) chloride channel compromise MCC by decreasing chloride and bicarbonate secretion into

the ASL, thereby reducing its volume and pH, increasing mucus viscosity, and impeding ciliary function. While in PCD, mutations in motile ciliopathy-associated genes result in AECs with dyskinetic or static cilia.

Respiratory viruses, notably rhinoviruses, respiratory syncytial virus and influenza, downregulate genes critically involved in cilia formation through an ill-defined mechanism.Moreover, viruses can lead to shedding of ciliated AECs or dysregulated cilial function, further reducing MCC and increasing the risk of secondary bacterial infection. Rhinovirus challenge studies in adults with chronic obstructive pulmonary disease (COPD) induced bacterial co-infection in 60% of subjects compared with 10% of healthy, non-smoking controls. *NTHi* was the principal secondary bacterial pathogen, with bacterial load peaking at 2-weeks and persisting for at least 6-weeks after the rhinovirus infection. Possible mechanisms for secondary *NTHi* infections are that rhinoviruses disrupt AEC barrier function by damaging tight inter-epithelial cell junctions, permitting *NTHi* to enter paracellular sites.Rhinoviruses and*NTHi* also share a common cellular receptor and rhinoviruses inhibit macrophage interleukin (IL)-1 responses to *NTHi* and diminish IL-8 responses via TLR-2 dependent degradation of IRAK-1.

CSLD in children is characterized by chronic airway inflammation, which may also adversely impact upon MCC. Chronic inflammation in asthma is associated with decreased cilial motility, disorientated beating direction, and ultrastructural damage. In COPD, the intraflagellar transport of structural proteins in the respiratory cilia is dysregulated; consequently, cilia length is shortened and less capable of propelling the overlying mucus.Furthermore, in adults with COPD, squamous cells replace pseudostratified epithelia, resulting in a significant reduction in ciliated cuboidal cell numbers. Cigarette smoke exposure also has detrimental effects upon ciliogenesis and cilia function,decreasing CFTR activity, and it is associated with decreased ciliary beat frequency. Nevertheless, it is difficult to disentangle the relationships between inflammation, infection, resident lung microbiota, and aerotoxicants, and their individual impact upon MCC.

Experimental models indicate *NTHi* may also interfere with MCC directly. *NTHi* adheres initially to the mucus layer via its outer membrane proteins (OMP), such as P2 and P5, following which isolates expressing the highly conserved surface lipoprotein, protein-D, and lipo-oligosaccharide (LOS) inhibit cilial function. Activation of the host protein, protein kinase C epsilon, mediates these effects, but this is an inconsistent finding, and penetrating paracellular foci is also thought to be an important strategy for escaping the mucociliary apparatus.

Adherence to AECs

Having bypassed MCC defenses, *NTHi* employs multiple surface-expressed multifunctional adhesion proteins, such as proteins E and F, that bind to nonciliated AECs and extracellular matrix (ECM) proteins, in particular laminin and vitronectin. Adherence is enhanced when there is structural damage to the airway mucosa from viral infections or chronic inflammation exposing ECM proteins. The trimeric autotransporters, Hi adhesin (Hia), Hi adhesion protein (Hap), and high–molecular weight (HMW) proteins 1 and 2, play an important role in colonization, even though only Hap is highly conserved in *NTHi*strains. Moreover, the type IV pili on *NTHi* cell surfaces mediate adherence to AECs by interacting with the intracellular adhesion molecule-1 (ICAM-1), which also serves as a cellular receptor for rhinoviruses that may help explain their frequent co-infections. The ICAM-1 receptors on the AEC surface increase in numbers in COPD patients, in smokers, and exhibit a positive feedback loop following*NTHi* exposure. Additionally, P1 fimbriae bind to the carcinoembryonic antigen-related cell adhesion molecule (CEACAM-1), while P5 fimbriae adhere to both CEACAM-1 and ICAM-1 on AEC surfaces, respectively.[59,60] As well as binding to mucins, other OMPs adhere to exposed ECM proteins.[61] Indeed, the interaction between the ECM exposed within the COPD airway environment and Hap facilitates *NTHi* attachment to AECs and this interaction may also operate in the lungs of children with CSLD.

Evasion of immune defenses

Having overcome the first layer of host lung defenses, *NTHi* must also evade innate and adaptive immune responses. Pulmonary phagocytic cells (alveolar macrophages and neutrophils) constantly sense the local microenvironment for pathogen associated molecular patterns (PAMPs), which are molecules with highly

conserved motifs associated with human pathogens. PAMPs bind to pattern recognition receptors, such as TLRs, which activate intracellular pathways to secrete chemotaxins, including IL-8 to recruit and upregulate neutrophils to the site of infection, and to initiate intracellular killing of phagocytosed pathogens.[64] When phagocytes encounter *NTHi*there is a sustained release of ROS, both within intracellular phagolysosomes and into the extracellular environment. The generation of ROS is also associated with neutrophil and macrophage extracellular traps (NETs and METs, respectively), which are composed of a mesh of DNA, histones and bactericidal proteins to neutralize invading pathogens. The production of ROS represents the primary mechanism adopted by these cells to eliminate phagocytosed pathogens. Nevertheless, *NTHi* can neutralize the effects of oxidative stress by producing antioxidants, including catalase, peroxiredoxin-glutaredoxin and superoxide dismutase (SOD), which in addition to forming biofilms is one of the mechanisms it uses to resist NET.

Furthermore, submucosal plasma cells produce dimeric IgA molecules, which bind to an epithelial cell membrane protein, the secretory component, and are transported through AECs to be released as secretory IgA into mucosal secretions.[67] IgA aids lung defenses by preventing microbial adherence, neutralizing toxins and boosting adaptive immunity. There are two types of IgA (IgA1 and IgA2) of which IgA1 accounts for more than 90% of the total IgA. However, *NTHi*produces an IgA1 protease, IgaA, encoded by the *IgaA* gene as an extracellular endopeptidase that cleaves IgA1 at the hinge region, disabling its antibacterial functions and aiding adherence to and invasion of AECs. Approximately half the *NTHi* strains also possess a second IgA1 protease gene, *IgaB,* that encodes IgaB, which seems necessary for intracellular survival.

The human complement system is also part of the innate defenses against pathogenic bacteria. It has multiple functions and when activated complement mediates the inflammatory response and elimination of pathogens and enhances adaptive immunity. The complement system leads to C3b deposition on the bacterial cell surface with formation of the membrane attack complex (MAC) and bacterial lysis. There are three complement activation pathways, which can all be inhibited by*NTHi* . The classical and lectin pathways are blocked by the C4b binding protein (C4bP), whereas factor H is believed to be main inhibitor of the alternative pathway. C4bP and factor H are captured by OMP P5 and protect*NTHi* against opsonization and phagocytosis, while also preventing MAC formation. Vitronectin, a potent negative regulator of the terminal pathway can also be bound by *NTHI* proteins E and F, further inhibiting the formation of the MAC by *NTHi* . Moreover, LOS covering the outer cell wall of *NTHi* can block bactericidal antibodies accessing cell surface structures and activating the classical complement pathway.[73] Finally, *NTHi*may benefit from other co-pathogens, such as *Moraxella catarrhalis,* and their inhibition of the complement system.[74]

Persistence and survival of *NTHi* in the lungs

After adhering successfully to AECs, *NTHi* must still obtain nutrients and continue evading host defenses.' *NTHi* have absolute growth requirements for iron, but are unable to synthesize heme, which is essential for their survival. Consequently,*NTHi* has multiple core and accessory genes producing proteins expressed on the cell surface to acquire iron from free heme and hemoglobin sequestrators, such as hemopexin and haptoglobin.[77] However, the exact mechanisms remain poorly understood because of the number, diversity and functional redundancy of the encoding genes. Recent studies identified that some of these genes are phase-variably expressed and rapidly acquire point mutations during persistent infection, resulting in accumulated amino acid changes in surface exposed regions of iron acquisition proteins, suggesting these are survival strategies following immune selection pressures.[79] Other OMPs, such as P2, also undergo antigenic drift with key amino acid sequence changes occurring in key epitopes, which enable *NTHi* to persist despite the presence of potentially protective antibodies.

NTHi utilize phase variation to facilitate adaptation and survival within multiple niches throughout the respiratory tract. Phase variation is the random and reversible switching of gene expression, which permits reversible loss or gain of surface structures, such as adhesins (HMW1 and HMW2), LOS and iron-acquisition proteins (heme receptors and hemagglutinating pili).[81] Phase-variable genes have changes in the length of simple sequence repeats and when located in the open reading frame of a gene this variation in length determines if the encoded protein is either expressed (ON) or not (OFF). Phase-variable DNA methyltransferases

also control the expression of multiple genes involved in colonization and survival within different ecological niches and such systems are termed phasevarions to describe phase-variable regulons.[81] Taken together, these random changes in gene expression enable *NTHi* to generate phenotypic variants, which are better suited to the local lung environment or more able to evade host defenses.

Widely regarded as an extra-cellular pathogen, *NTHi* can access and survive in the paracellular spaces between AECs due to the adherence and invasion protein, HMW1,[85] and following downregulation of cadherin and claudin proteins responsible for epithelial cell-to-cell interactions and epithelial tight junction integrity, respectively.[86] As a further survival strategy, *NTHi* can also invade mononuclear cells and AECs by utilizing multiple virulence factors, including IgaA protease, LOS via the platelet-activating factor receptor leading to cytoskeletal remodeling, several adhesins, including protein D, Hap, or P1 fimbrae binding to the CEACAM-1 receptor and the phase-variable adhesin HMW1 and protein E binding to vitronectin.[69,85,87] Under iron restricted conditions within the lungs, *NTHi* enter AECs by macropinocytosis to form bacterial communities within the cytoplasm.[88] Once inside the cell, *NTHi* must adapt to the low pH and increased oxidative state within the phagosome of macrophages or the limited nutrient availability within the AEC cytoplasm. Relatively little is known of how *NTHi* survives and traffics within this environment. Transcriptional factors, such as the ferric uptake regulator cassette which regulates the expression of multiple genes controlling the uptake and utilization of iron, and IgaB that cleaves lysosomal-associated membrane protein-1, appear to be important, and *NTHi* can survive for at least 72-hours inside AECs.

Furthermore, lung explant cultures from COPD and CF patients undergoing transplantation for end-stage lung disease revealed that despite negative sputum and lung tissue cultures, *NTHi* was detected widely throughout the lung parenchyma and lower airway tissue sections by immunoperoxidase staining and polymerase chain reaction assays. While most NTHi were detected in clusters in extracellular and paracellular locations, in CF patients they were found within macrophages too. The discrepancy between culture and non-culture results was attributed to pre-operative antibiotics and the inhibitory effects of*Pseudomonas* aeruginosa upon *NTHi*growth.[91] However, paracellular and intracellular locations of *NTHi* and biofilms may also be important.

Biofilm aggregates

NTHi can form biofilms (Figure-2), which are aggregated bacterial communities encased in an ECM of polysaccharides, proteins and extracellular DNA (eDNA) that adhere to a surface.[92]As biofilms mature, the resident bacteria undergo a series of behavioral changes, including differential gene expression and increased ECM production, in response to increasing cell density and environmental stresses from decreasing oxygen tension and nutrient availability.[93] Crucial bacterial factors for biofilm formation include LOS, pili, eDNA, and a functioning quorum sensing system (QSS). Bacteria utilize QSS for inter-cellular communication where cells collectively regulate their gene expression in response to cell density as sensed by the concentration of small soluble autoinducer signal molecules produced and secreted by bacteria.[94]In *NTHi* , biofilm development and dispersal are mediated by autoinducer-2 (AI-2), which is controlled by the LuxS/RbsB system regulating LOS and pili expression, including PilA, P2 and P6. DNA-NET-like structures captured by DNA-binding proteins from the DNABII family form a mesh network within biofilms where they help make up important structural components.[95]

The ECM of biofilms helps protect *NTHi* from environmental stresses, including acting as a physiochemical barrier to cellular and innate host defenses and to antimicrobial agents. For example, in addition to stimulating biofilm formation, eDNA binds the AMP, human beta-defensin-3, while peroxiredoxin-glutaredoxin and catalase protect against neutrophil-induced oxidative stress, and IgA proteases cleaving IgA are found on the ECM.[96] There are several potential mechanisms within biofilms capable of mediating antimicrobial resistance. These include the (i) ECM limiting antibiotic access to bacteria within the biofilm, (ii) negatively charged eDNA sequestering positively charged antibiotics, (iii) hypoxic and nutrient restricted environment deep within biofilms transforming bacteria into a semi-dormant, metabolically inert state rendering antibiotics relying upon active cell growth and division ineffective, and (iv) horizontal gene transfer of antibiotic resistance genes that result in deactivating enzymes, altered membrane permeability and cellular targets,

and upregulated multidrug efflux pumps.[97] Nevertheless, despite much in-vitro and experimental model work on biofilms, evidence for their presence in patients with CSLD is relatively limited. They have however, been detected in the sputum of adults with CF and COPD,[98,99] and in bronchoalveolar lavage (BAL) fluid from children with PBB and bronchiectasis.[100]Importantly, by using fluorescent in-situ hybridization (FISH) staining in a small subset of BAL samples, researchers were able to demonstrate the polymicrobial nature of the biofilms, which included in addition to*NTHi* other recognised respiratory pathogens (*Streptococcus pneumoniae, M. catarrhalis*) and upper airway commensals, such as*Prevotella* species.[100]

NTHi-induced host damage

Incursion by *NTHi* into the lower airways induces both innate and adaptive immune responses. *NTHi* infection stimulate TLRs and NLR family pyrin domain containing 3 (NLRP3) inflammasomes stimulate AECs to release AMPs and proinflammatory mediators, including tumor necrosis factor-alpha, IL-6 and IL-8, that recruit and activate macrophages and neutrophils leading to increased ROS production, and NETs formation, the latter designed to immobilize and destroy pathogens. In this hyperinflammatory environment, high concentrations of proteolytic enzymes, such as neutrophil elastase, and ROS are released by activated and dying neutrophils, with the latter leading to local increases in free radicals. Despite these host defensive measures, *NTHi* are resistant to NETs and survive oxidative stress by forming biofilms and possessing antioxidants, while also capitalizing on the ROS-induced tissue damage as a nutrient source to establish and sustain their presence in the airways. The tissue damage results from excessive oxidative stress secondary to *NTHi*persistence and an imbalance between oxidants and antioxidants, leading to oxidation-induced damage of proteins, lipids, and DNA in the airways. These changes, labelled by some as NETosis, contribute to a pathway leading to impaired lung function and destruction of local airway wall architecture as demonstrated by airway hyperresponsiveness, mucus hypersecretion, AEC shedding, vascular exudation, and, finally, airway remodeling. Moreover, the damaged mucosa facilitates *NTHi*persistence because of further disruption to MCC and the higher affinity of *NHTi* for damaged tissues, which sustains the airway inflammatory response and promotes a vicious cycle of infection, inflammation and impaired bacterial clearance. (Figure-3).

NTHi and chronic suppurative lung diseases

NTHi is a normal upper airway commensal, but when it becomes established in the lower airways it elicits a strong, but ineffective inflammatory response.[26] As it is an important pathogen in patients with COPD, much of our understanding of the clinical implications of chronic *NTHi* infection is from this patient group. Indeed, in COPD it accounts for nearly half of all isolates and is responsible for over 30% of disease exacerbations. Moreover, the severity of COPD exacerbations is associated with*NTHi* airway colonization. However, large knowledge gaps remain to explain why *NTHi* only affects a subset of COPD patients and what host-pathogen and microbial (eg. bacteria-bacteria and virus-bacteria) interactions cause this susceptibility.[112] There is accumulating evidence that the biofilm-phase of *NTHi* may be important and knowing more about the mechanisms involved may promote targeted treatment of this chronic disease. Recent studies question the ability of antibiotics to eradicate lower airway *NTHi* infection in COPD patients since antibiotics do not prevent reinfection, nor do they alter the disease course[76]. Consequently, there has been substantial interest in creating an effective *NTHi*vaccine.

NTHi is the major pathogen in bronchiectasis and is accompanied by airway neutrophilic infiltration and inflammatory markers, such as IL-8 and neutrophil elastase. Alveolar macrophages from PBB and bronchiectasis patients have reduced capacity to phagocytose *NTHi*and apoptotic neutrophils, allowing the latter to become necrotic and release ROS and proteolytic enzymes into the airways.[115] NETs, which in bronchiectasis are a key marker of disease severity,[116] may induce IL-17 secreting lymphocytes (Th17 and NK cells) in bronchial submucosa to further drive neutrophil recruitment and mucus hypersecretion perpetuating the cycle of inflammation.[117] Compared to healthy controls, peripheral blood mononuclear cells from bronchiectasis patients also have an attenuated γ-interferon response to *NTHi* , but not to mitogens, suggesting a specific impairment in cell mediated immunity,[118] which improves after receiving the highly conserved *NTHi* surface protein, protein D, incorporated into the pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV).

In CF patients *NTHi* are most often detected in children and adolescents and can be the predominant pathogen cultured in BAL fluid from young children.[14] Nevertheless, their role as pathogens has been questioned.[119] However, studies from the 1990s revealed that high *NTHi* loads ($> 10^5$ colony-forming units) in BAL cultures were associated with significantly higher neutrophil and IL-8 levels than when no pathogens were present.[120,121] A North American CF registry-based study also found that isolating*NTHi* from respiratory secretions was associated with accelerated decline in lung function in the transition from adolescence to adulthood.[122] Recently, *NTHi* isolates from CF patients inoculated into a murine lung model induced chronic neutrophilic inflammation driven by Th17 cells and IL-17 cytokines. Taken together, these studies suggest a pathogenic role for *NTHi*in CF patients.

In PCD, nasal nitric oxide (nNO) levels are dramatically reduced for uncertain reasons. Recent in-vitro data suggest low levels of nNO make it easier for *NTHi* to adhere to primary AECs from PCD patients and form biofilms, while exogenous nitric oxide with azithromycin enhanced bacterial killing in biofilms compared to azithromycin alone.

PBB and bronchiectasis are on a continuum, and in children they have similar lower airway pathogen profiles, core microbiota, neutrophilic airway inflammation and impaired cellular immune responses to*NTHi* .[40,128-130] A recent 5-year follow-up study of 194 children with PBB reported recurrent PBB and *NTHi* in BAL cultures predicted a future diagnosis of bronchiectasis, emphasizing the importance of *NTHi* in disease progression and outcome.

Summary

NTHi is a highly adapted human commensal and opportunistic lower airway pathogen possessing multifunction virulence factors exhibiting efficient adherence, immune evasion and nutrient scavenging properties. These allow it to invade cells and form biofilms in order to survive within the lungs of children with CSLD. The molecular pathogenesis of*NTHi* is not fully understood and large knowledge gaps exist over its mechanistic role in progressive lung disease. Identifying critical cellular pathways and, including host-pathogen cross-talk and interactions with other lung microbes, are crucial for developing novel therapeutic agents and vaccine candidates (Box).[132]NTHi vaccines are an active area of research, but *NTHi* strains have both marked genetic heterogeneity from recombination with other*NTHi* and phase-variability, and no vaccine candidate is ready for large field trials. Two recent, relatively small vaccine trials, one in children with CSLD targeting *NTHi* protein D in the PHiD-CV vaccine and another in adults with COPD targeting three*NTHi* surface proteins, failed to meet their primary outcome of reducing exacerbations, despite both demonstrating good immunogenicity highlighting that further mechanistic studies are needed involving*NTHi* and patients with CSLD.[133,134]

FIGURE LEGENDS

Figure-1 . The main strategies used by nontypeable*Haemophilus influenzae* to establish a persistent infection in the lower airways of patients with chronic suppurative lung disease.

Figure-2. *NTHi* biofilm life cycle. (A) *NTHi* in planktonic form escapes local airway defenses and irreversibly attaches to receptors on airway cells. (B) After evading local niches,*NTHi* aggregates and starts the production of extracellular polymeric substance matrix which favors *NTHi* survival in the form of biofilm. (C) Biofilm properties enhance growth and survival of*NTHi* and other bacteria. (D) Maturation of biofilm is followed by*NTHi* dispersal in planktonic forms to subsequently develop biofilms in secondary niches. *NTHi* : nontypeable *Haemophilus influenzae* . *Created by biorender.com*

Figure-3 . Schematic representation of *NTHi* -induced host damage. AMs: alveolar macrophages, AMPs: antimicrobial peptides, Aps: antioxidant peptides, IL: interleukin, NE: neutrophil elastase, NKs: natural killer cells, NPs: neutrophils, *NTHi* : nontyeable*Haemophilus* influenzae, ROS: reactive oxygen species, Th: T helper cells. *Created by biorender.com*

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Box: Future research questions on the pathogenesis and role of *NTHi* infections in pediatric chronic suppurative lung disease

Can animal models replicate the complexity of *NTHi* infection in CSLD?

What are the important interactions between *NTHi* and the host microbiome?

What role do cilia play in *NTHi* host-pathogen interactions?

How long do *NHTi* survive within cells and in intercellular spaces?

How long do *NTHi* strains survive in the lungs? Are they replaced or constantly changing as a result of intraspecies transformation and recombination of genes from the supragenome?

What are the critical functions of host-pathogen cross-talk that allow *NTHi* to evade or manipulate host defences?

What part does NETosis have in the pathogenesis of CSLD?

What is the role of *NTHi* biofilms in progressing CSLD and how might this be overcome by increasing susceptibility to host defences and antimicrobial agents?

What are the regulatory mechanisms controlling a relatively small number of multi-functional proteins that allow *NTHi* to adapt to environmental changes?

What is the nature, timing and frequency of antigenic, epigenetic and phase variation in lung *NTHi* isolates?

What are the critical phase-variable genes for establishing *NTHi* lung infection and how they might be important targets for vaccine candidates?

Can *NTHi* virulence be manipulated by small molecules targeting the quorum sensing system?

What are the mechanisms of antimicrobial resistance in biofilms and where is the reservoir of antibiotic resistant genes?

What impacts do CFTR modulators have upon *NTHi* infection in CF patients?

What are the host mechanisms of protective immunity?

Will multi-valent vaccines containing highly conserved surface antigens, including antigens under phase-variable control expressed during critical periods of establishing infection overcome the challenges of *NTHi* genetic heterogeneity and protect against *NTHi* infection?

CF: cystic fibrosis, CFTR: cystic fibrosis transmembrane conductance regulator, CSLD: chronic suppurative lung disease, *NTHi* , nontypeable *Haemophilus influenzae.*

Figure-1 . The main strategies used by nontypeable *Haemophilus influenzae* to establish a persistent infection in the lower airways of patients with chronic suppurative lung disease.

Figure-2. *NTHi* biofilm life cycle. (A) *NTHi* in planktonic form escapes local airway defenses and irreversibly attaches to receptors on airway cells. (B) After evading local niches, *NTHi* aggregates and starts the production of extracellular polymeric substance matrix which favors *NTHi* survival in the form of biofilm. (C) Biofilm properties enhance growth and survival of *NTHi* and other bacteria. (D) Maturation of biofilm is followed by *NTHi* dispersal in planktonic forms to subsequently develop biofilms in secondary niches. *NTHi* : nontypeable *Haemophilus influenzae* . *Created by biorender.com*

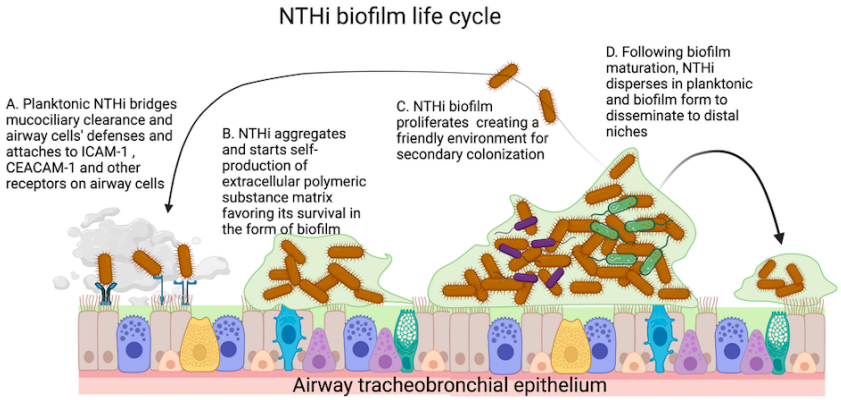

Figure-3 . Schematic representation of *NTHi* -induced host damage. AMs: alveolar macrophages, AMPs: antimicrobial peptides, Aps: antioxidant peptides, IL: interleukin, NE: neutrophil elastase, NKs: natural killer cells, NPs: neutrophils, *NTHi* : non-typable*Haemophilus* influenzae, ROS: reactive oxygen species, Th: T helper cells. *Created by biorender.com*