Neisseria species as pathobionts in bronchiectasis

Li, Liang; Mac Aogáin, Micheál; Xu, Tengfei; Jaggi, Tavleen Kaur; Chan, Louisa L. Y.; Qu, Jing

Published in: Cell Host & Microbe

DOI: 10.1016/j.chom.2022.08.005

Publication date: 2022

Licence: CC BY

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Discovery Research Portal

Citation for published version (APA):
Neisseria species as pathobionts in bronchiectasis

Graphical abstract

Highlights

- Some bronchiectasis patients exhibit increased airway abundance of Neisseria spp.
- The culturable species N. subflava weakens barrier integrity and induces inflammation
- N. subflava elicits distinct transcriptomic/metabolipidomic signatures in the mouse lung
- Neisseria-associated pathogenic signatures are observed in bronchiectasis patients

Authors
Liang Li, Micheál Mac Aogain, Tengfei Xu, ..., Mingliang Fang, James D. Chalmers, Sanjay H. Chotirmall

Correspondence
schotirmall@ntu.edu.sg

In brief
Bronchiectasis exhibits geographically variable airway microbiomes, including an increased abundance of Neisseriaceae, which is linked to exacerbations. Systems-level analysis of clinical N. subflava identified distinct transcriptomic and metabolipidomic profiles in a murine model of airway exposure. These signatures concurred with those observed in human Neisseria-dominant patients with bronchiectasis, supporting a “pathobiont” role for Neisseria.
Clinical and Translational Report

**Neisseria** species as pathobionts in bronchiectasis


1Department of Pharmacology, School of Medicine, Southern University of Science and Technology, Shenzhen, China
2Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China
3Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore
4Biochemical Genetics Laboratory, Department of Biochemistry, St. James’s Hospital, Dublin, Ireland
5Clinical Biochemistry Unit, School of Medicine, Trinity College Dublin, Dublin, Ireland
6School of Civil and Environmental Engineering, Nanyang Technological University, Singapore, Singapore
7College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, PRC
8University of Dundee, Ninewells Hospital, Medical School, Dundee, Scotland
9Department of Otolaryngology, Infectious Disease Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
10Department of Respiratory and Critical Care Medicine, Singapore General Hospital, Singapore, Singapore
11Department of Respiratory and Critical Care Medicine, Tan Tock Seng Hospital, Singapore, Singapore
12Department of Respiratory and Critical Care Medicine, Changi General Hospital, Singapore, Singapore
13Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia
14Department of Respiratory Medicine and Critical Care, Peking University Shenzhen Hospital, Shenzhen, China
15Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute, School of Medicine and Public Health, University of Newcastle, Newcastle, NSW, Australia
16Department of Respiratory and Sleep Medicine, John Hunter Hospital, New Lambton Heights, NSW, Australia
17Woolcock Institute of Medical Research, University of Sydney, Sydney, NSW, Australia
18School of Life Sciences, University of Technology Sydney, Sydney, NSW, Australia
19Singapore Centre for Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, Singapore, Singapore
20School of Biological Sciences, Nanyang Technological University, Singapore, Singapore
21Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, China
22These authors contributed equally
23Lead contact

*Correspondence: schotirmall@ntu.edu.sg
https://doi.org/10.1016/j.chom.2022.08.005

**SUMMARY**

**Neisseria** species are frequently identified in the bronchiectasis microbiome, but they are regarded as respiratory commensals. Using a combination of human cohorts, next-generation sequencing, systems biology, and animal models, we show that bronchiectasis bacteriomes defined by the presence of **Neisseria** spp. associate with poor clinical outcomes, including exacerbations. **Neisseria subflava** cultivated from bronchiectasis patients promotes the loss of epithelial integrity and inflammation in primary epithelial cells. In vivo animal models of **Neisseria subflava** infection and metabolipidome analysis highlight immunoinflammatory functional gene clusters and provide evidence for pulmonary inflammation. The murine metabolipidomic data were validated with human **Neisseria**-dominant bronchiectasis samples and compared with disease in which **Pseudomonas**-, an established bronchiectasis pathogen, is dominant. Metagenomic surveillance of **Neisseria** across various respiratory disorders reveals broader importance, and the assessment of the home environment in bronchiectasis implies potential environmental sources of exposure. Thus, we identify **Neisseria** species as pathobionts in bronchiectasis, allowing for improved risk stratification in this high-risk group.

**INTRODUCTION**

The study of microbes in chronic respiratory disease is evolving. Departing from traditional single pathogen-centric models to more holistic and integrative approaches will facilitate an appreciation of the airway microbiome composition and its associated host response (Budden et al., 2019; O’Dwyer et al., 2016). Microbial networks, composed of pathogenic and commensal “pathobiont” constituents, and understanding the microbiome better defines airway ecology and the associated clinical
endophenotypes of respiratory disease (Gao et al., 2018; Layeghifard et al., 2019; Mac Aogain et al., 2021). Recent work suggests that commensal airway bacteria play dynamic roles in shaping the host immune response, including the maintenance of mucosal homeostasis where micro-aspiration of upper airway taxa engenders robust immune tone and protection from pathogenic microbes (Wu et al., 2021). These observations align with human studies that define subclinical microbiome-related clusters that exhibit distinct metabolic profiles, enhanced expression of inflammatory cytokines, and elevated Th17 lymphocytes (Segal et al., 2016). As such, the microbiome potentially exerts influence across a spectrum of clinical states and disease endophenotypes, from daily homeostatic interaction with host immunity that dictates subclinical immunophenotypes to a more extreme dysregulated immune-inflammatory milieu observed in the chronically diseased airway (Richardson et al., 2019).

Bronchiectasis, a disease characterized by permanent, progressive, and irreversible dilatation of the airways, is increasing in prevalence globally (Chalmers et al., 2018; Dhar et al., 2019). Renewed focus on its molecular underpinning has led to improved understanding of this complex and heterogeneous condition, including the association of microbiome composition with clinical phenotype (Chalmers and Chotirmall, 2018; Flume et al., 2018; Mac Aogain et al., 2021; Metersky and Chalmers, 2019). Recurrent cycles of infection and inflammation characterize its pathogenesis and result in structural abnormalities; however, the inherent clinical heterogeneity is exemplified by failed clinical trials due to marked differences between patients and their response to therapy (Chotirmall, 2018; Chotirmall and Chalmers, 2018; Flume et al., 2018). Chronic infection is a hallmark of bronchiectasis, and infection represents a cause and consequence of disease; however, the identification of potential culprit pathogens exhibits considerable geographic variation. In 1 of 3 patients, sputum cultures are negative, despite a detectable inflammatory response and clinical phenotype suggestive of chronic infection (Dickson et al., 2013; Fuschillo et al., 2008; Richardson et al., 2019). Recent work highlights the importance of geographic variation to microbiomes in bronchiectasis, including a failure to replicate key findings from partner clinical trials such as RESPIRE (Aksamit et al., 2018; Chotirmall and Chalmers, 2018; Chotirmall et al., 2017; De Soya et al., 2018). Taken together, these data underscore the complex and heterogeneous microbial consortia associated with bronchiectasis; however, they provide potential scope for their use in improving patient stratification (Mac Aogain et al., 2021; Rogers et al., 2014b). Although microbiomes associate with clinical phenotypes in bronchiectasis, studies accounting for geographic variation and which advance testable hypotheses or provide mechanisms for such observations remain limited. Furthermore, “classical” bronchiectasis pathogens, including S. pneumoniae, H. influenzae, and gram-negative organisms such as Pseudomonas aeruginosa, all identified for their ability to cause invasive pneumonia, do not account for the possibility that upper airway commensals, which cause local inflammation but not necessarily bloodstream infection, may have pathogenic roles.

Here, we leverage on the cohort of Asian and matched European bronchiectasis (CAMEB) and employ a bedside-to-bench approach using a combination of next-generation sequencing and systems biology to identify and characterize Neisseria spp. as pathobionts in bronchiectasis. We demonstrate (in geographically matched individuals) that Neisseria spp. is associated with poorer clinical outcomes in bronchiectasis and that Neisseria subflava demonstrates immunopathologic potential in primary human cell and mouse infection models. The multimics response to Neisseria subflava infection was characterized and integrated using transcriptomic, metabolomic, and lipidomic approaches, with validation in patients with bronchiectasis corroborating its “pathobiont” role. Finally, we demonstrate that Neisseria spp. can be identified in other respiratory diseases and indoor environments. Identifying Neisseria species as pathobionts in bronchiectasis allows improved patient risk stratification and potential intervention.

RESULTS

Bacteriomes demonstrate geographic variation characterized by Neisseria species

We performed 16S targeted amplicon sequencing of the airway microbiome in the CAMEB cohort which “matched” individuals of Asian (Singapore-Kuala Lumpur, Malaysia [SG-KL]) and European (Dundee, Scotland [DD]) origin by age, sex, and disease severity (n = 225; Table S1) (Mac Aogain et al., 2018). This revealed geographic differences characterized by established bronchiectasis pathogens, including Pseudomonas, Haemophilus, Streptococcus, and Moraxella and included Neisseria spp., considered commensal, but represent the predominant discriminant bacterial taxa in Asians (Figures 1A–1F and S1; Rogers et al., 2014b). Neisseria spp. were overrepresented in Asians (Figures 1A–1D), corroborated by linear discriminate analysis (LDA) effect size (LEfSe) (Figures 1E and 1F). Having identified Neisseria spp. by a growth-independent methodology, we next evaluated n = 10 Asian airway specimens (with the highest relative abundance of Neisseria spp. by 16S sequencing) for the presence of Neisseria isolates. Growth-based assessment identified multiple specimens yielding significant growth of gray unpigmented colonies on chocolate agar, consistent with Neisseria morphology. Commercial biochemical and MALDI TOF bacterial identification assays were consistent with the identification of these isolates as members of the genus Neisseria (data not shown). This was further confirmed by whole-genome sequencing and phylogenetic analyses identifying isolates to the species level as N. subflava based on comparison of high-quality genome assemblies with publicly available reference genomes (Figure 1G). To prove this organism was present in the lower airway and not simply transiently detected due to aspiration from the upper respiratory tract, fluorescent in situ hybridization (FISH) probes specific for N. subflava were designed (see key resources table) and used to assess formalin-fixed-paraffin-embedded lung tissue biopsies obtained from control individuals (n = 12, lung cancer without bronchiectasis) and individuals with bronchiectasis and Neisseria-dominant 16S microbiome profiles (n = 9) (Table S2). FISH imaging confirmed N. subflava in the lower airways of individuals with bronchiectasis (Figure 1H). Sub-analysis of Asians exhibiting Neisseria-dominant profiles revealed a significant correlation between Neisseria abundance and exacerbation frequency independent of disease severity, lung function, or sputum bacterial 16S ribosomal RNA (rRNA) amplicon concentration and/or read counts (Figures 1I and S2).
and S2). There was no observed effect of either antibiotics or inhaled corticosteroids on Neisseria abundance (Mann-Whitney U test, p > 0.05) or dominance (chi-squared, p > 0.05) in Asian patients. Taken together, these findings potentially implicate Neisseria spp. as airway pathobionts in bronchiectasis where it associates with increased exacerbations.

Airway N. subflava infection promotes loss of epithelial barrier integrity and induces inflammation

To assess the ability of the Neisseria subflava isolates to infect the human airway, we employed primary differentiated human nasopharyngeal epithelial cells (HNECs) and human bronchial epithelial cells (HBECs) at air-liquid interface (ALI), representative of the upper and lower airways, respectively. Primary airway epithelial cells were susceptible to infection and N. subflava rRNA was detectable as early as 2 h post infection (hpi). N. subflava was internalized by 24 hpi with intracellular bacterial load peaking at 72 hpi, as quantified by qRT-PCR of cell lysates (Figure 2A). Significant disruption of the epithelial cell-cell barrier was demonstrable through reduction in trans-epithelial electrical resistance (TEER) (Figures 2B and 2C). Neisseria subflava infection triggered airway inflammatory responses characterized by increased gene expression of interleukin (IL)-6, IL-8, CCL2, and CXCL10 (Figures 2D–2H).

Intratracheal administration of N. subflava induces bronchoalveolar infection and immunoinflammatory responses in vivo

Intratracheal delivery of clinical N. subflava isolates to C57BL/6 mice resulted in decreased body weight and clinical score indicative of successful infection (Figures 3A and 3B). For both N. subflava and the P. aeruginosa comparator group, mouse body weight dropped at day 2 post infection and increased thereafter (Figure 3A). P. aeruginosa infected mice show poorer outcomes at day 2, demonstrating a rapid evolution of infection when compared with N. subflava (Figure 3B). The bacterial load
of *P. aeruginosa* in mouse lungs dropped continuously and were cleared from mouse lungs by day 7 post infection, whereas *N. subflava* continue to demonstrate increased bacterial loads at day 7 post infection which remain high at day 14 post infection. These findings demonstrate *N. subflava*’s ability to persist for a prolonged time period, whereas *P. aeruginosa* inhalation, following an acute response, is cleared more rapidly (Figure 3C).

*N. subflava* exhibits distinct bronchiolar epithelial damage in comparison with *P. aeruginosa* suggestive of distinct pathology (Figure S4). Assessment of *N. subflava* load, measured by culture of lung homogenates, reveal the establishment of infection in the mouse lung by day 4 and up to day 14 (Figure 3C). To further confirm successful lower airway infection, FISH analysis detected *N. subflava* within infected mouse lung parenchyma up to day 14 post infection (Figure 3D). *N. subflava* infection induced macroscopic lung tissue damage, including bleeding and lobar inflammation on day 4 (Figure 3E). Acute inflammation was confirmed by Giemsa staining of bronchoalveolar lavage fluid (BALF), demonstrating predominantly alveolar macrophages initially (day 0) followed by neutrophilic infiltration (day 2) (Figure 3F). Bronchoalveolar infection and tissue damage induced by *N. subflava* were evident from H&E staining of mouse lungs: bronchiolar damage and pro-inflammatory immune cell infiltration into alveolar spaces were observed between days 2 and 7 post infection, peaking at day 4, followed by recovery at day 14 (Figures 3G and 3H). The immune-inflammatory response *in vivo* was further confirmed by flow cytometry, revealing an acute (early) inflammatory phase marked by neutrophilic (Ly6G+ F4/80− CD45+) and NK cell (NK1.1+ CD19− NK1.1− CD45+) infiltration followed by an adaptive (late phase) response characterized by T helper cells (CD4+ CD3+), cytotoxic T cells (CD8+ CD3+), macrophages (F4/80+ Ly6G− CD45+), and B cells (CD19+ NK1.1− CD45+) consistent with acute respiratory infection (Figure 3I).

Lung transcriptomics demonstrates immunoinflammatory functional gene clusters following *N. subflava* infection *in vivo*

Having observed *N. subflava* infection and its immunoinflammatory consequence *in vivo*, we next sought to characterize the post infection lung transcriptomic response. RNA sequencing of infected mouse lung was performed over a 14-day infection course, revealing dynamic change (Figure 4A). Principal component analysis (PCA) identified major shifts in gene expression...
between day 0 and day 2 post infection, followed by return to baseline and relative quiescence by day 7 (Figure 4B). This was followed by a delayed but marked shift in gene expression during recovery at day 14 (Figure 4B). To better define functional changes, we clustered the observed differential gene expression patterns according to enriched functional pathways, revealing seven “functional” clusters (Figure 4C). Early phase changes were characterized by immune cell function, infiltration, and antimicrobial responses, which peaked at day 2, followed by an inflammatory/oxidative stress response at day 4 (Figure 4C). These changes corroborate our macroscopic, microscopic, and flow cytometry data, where at day 2, an antibacterial response ensues, marked by infiltration of neutrophils (Figures 3F, 3H, and 3I), confirmed by gene expression patterns of hypercytokinemia, phagosome formation, and pattern recognition receptor activity (Figure 4C) reflected in an upper right shift in the PCA compared with day 0 (Figure 4B). At days 4 and 7, the acute host response to bacterial infection was reduced, marked by lower neutrophil infiltration (Figures 3F and 3I), and a curtailed immune response, most notably at day 7 (Figure 4C). Interestingly, day 7 gene expression was comparable with baseline (day 0), in the number of differentially expressed genes (DEGs) (Figure 4A) and reflected by a shift toward baseline in PCA and functional pathway analysis, respectively (Figures 4B and 4C) potentially indicative of immune tolerance toward N. subflava at this later time point. Importantly, increased N. subflava load in mouse lung was observed, but in the absence of excessive host immune responses at this later time point (Figure 3C). In contrast, host responses at day 14 demonstrate a highly altered profile relative to both baseline (day 0) and day 7 (Figure 4A), suggestive of an airway remodeling response (Figure 4C), concomitant with sustained levels of N. subflava in the airway (Figure 3C). Therefore, taken together, intratracheal exposure in vivo leads to a sustained presence of N. subflava in the airway and an altered host immune response characterized by an initial pro-inflammatory profile, oxidative stress response, and macroscopic tissue damage (days 2 and 4), followed by immune quiescence (day 7), and subsequent airway remodeling (day 14). Late-stage gene expression was marked by significant shifts in immune function to airway remodeling involving gap junction and endothelin signaling, suggesting a potentially prolonged immunopathology linked to airway damage following initial N. subflava infection (Figure 4C). Interestingly, patterns observed from N. subflava infection were distinct from those observed following P. aeruginosa infection when transcriptomes are directly compared (Figure S4 G). PCA reveals diverse lung transcriptomes across the 14-day infection course for both organisms. Lung transcriptomes following P. aeruginosa infection formed a separate cluster to those following N. subflava infection and demonstrate a major shift between days 0 and 2 with marginal change from day 4 onward (Figure S4 H). Temporal expression of key inflammatory genes indicates acute inflammatory changes identified in our ALI models such as IL-6, TNF, CCL2, and CXCL10 (Figure 2D) were elevated following infection by both organisms; however, changes associated with N. subflava were two to three orders of magnitude larger than the baseline, compared with moderate upregulation observed with P. aeruginosa (Figure S4I). Taken together, although both microbes induce vastly diverse pulmonary responses, N. subflava appears more immunogenic, triggering a more intense inflammatory response following acute infection, followed by a prolonged airway remodeling effect (Figures S4G–S4I).

### N. subflava induces a pro-inflammatory pulmonary metabololipidome

The overall lung sampling strategy for metabololipidome analyses is illustrated and includes sampling from a control (uninfected) group (H) (Figure 5A). Paired tissue sampling from the ipsilateral lobe of the infected lung was performed that included areas demonstrating macroscopic infection (ID, infected (diseased)) paired to uninfected areas (IH, infected [healthy]) (Figure 5A). Analysis of the mouse metabololipidome reveals that N. subflava-infected mice—even in apparently (macroscopically) uninfected regions, demonstrate distinct immunoinflammatory profiles characterized by upregulated coenzyme Q10 biosynthesis, pyrimidine ribonucleotide biosynthesis, and nitric oxide signaling, mirroring innate immune signatures of the transcriptome and suggesting an inflammatory response at the infection site and surrounding regions (Figures 5 and S5A–S5E). In comparing uninfected (H) and infected (ID) mice, 113 significantly dysregulated metabolites and lipids were identified, at fold changes ≥1.5, including several involved in immune regulation, immunometabolism, second messaging, and plasma membrane-mediated cell signaling (Figures 5B, 5C, and 5E; Table S3). Through connectivity network analyses, we assessed for interrelationships between the transcriptomic gene clusters (Figure 4C) and the dysregulated metabololipidome (Figures 5B–5D). This revealed activation of lipid-associated metabolic pathways related to itaconic acid, oleic acid, and ceramide (d34:2 and d34:0) biosynthesis, following N. subflava infection. In addition, perturbations in cell signal transduction linked to nitric oxide signaling and...
Figure 4. Mouse lung transcriptomics demonstrate functional gene clusters in vivo in response to *N. subflava* infection

(A) RNA sequencing of infected mouse lung at the indicated time points following *N. subflava* infection reveals change to differentially expressed genes (DEGs) across the 14-day course of infection.

(B) Principal component analysis (PCA) of mouse lung transcriptomes across the 14-day course of infection demonstrates shifts in host response. Colored arrows indicate directionality of the relative transcriptomic change observed.

(C) Heatmap indicating seven functional gene clusters from mouse lung transcriptomes illustrating significantly DEGs and respective enriched pathways in response to *N. subflava* infection. All illustrated changes are presented relative to control (uninfected) mice at day 0 with red coloration (including boxes) indicative of gene-pathway upregulation and blue coloration downregulation. All experiments were performed using n = 5 mice for each respective time point.
inflammation were observed, whereas metabolic pathways related to redox homeostasis and metabolism of several macronutrients including amino and nucleic acids were altered. Taken together, metabolipidome analyses confirmed significant shifts in metabolic and immune responses toward a pro-inflammatory state in *Neisseria*-infected lung tissue independent of macroscopic appearance (Figure 5A: IH and ID). Consistent with RNA sequencing data, the pro-inflammatory profile of the metabolipidome further underscores the immunopathogenic potential of *N. subflava* implicating it in lung damage seen in our murine infection model.

**Systemic change to the metabolipidome is detectable in *Neisseria*-dominant bronchiectasis**

Having characterized transcriptomic and metabolipidomic lung tissue responses to *N. subflava*, we next assessed the systemic metabolipidome (using mouse serum) revealing infection-related profile shifts, marked by an altered lipidome as early as day 2 post infection, consistent with lung transcriptomes (Figures 4, 6, and S5F–S5J). Mice exhibited two distinct metabolipid clusters defining dynamic change over the course of *N. subflava* infection (Figure 6A). To link lung alteration to the observed systemic change, we next performed weighted gene co-expression...
network analysis (WGCNA) on mouse lung transcriptomes that identified seven distinct gene modules, three of which significantly correlated with our derived serum clusters (Figure 6B).

Functional enrichment of these modules (incorporating the systemic metabolipidome profiles) identified key pathways linking the transcriptome with the metabolipidome. *N. subflava* lung exposure leads to metabolic shifts associated with activation of pro-inflammatory signaling pathways including leukotriene biosynthesis, nitric oxide, and eicosanoid signaling (Figure 6C). Pathways related to airway remodeling, such as endothelin-1,
epithelial adherens, and actin cytoskeleton signaling were also associated with serum metabolomic clusters, as were Phosphatase and Tenasin homolog (PTEN), gap junction, and relaxin signaling, further implicating an airway remodeling response (Figure 6C).

To translate mouse findings to Neisseria-dominant bronchiectasis (Figure 1), we performed systemic metabololipidome analyses using human serum obtained from non-diseased (healthy) controls and individuals from the Asian arm of the CAMEB cohort with Neisseria- and/or Pseudomonas-dominant bronchiectasis, the latter an established bronchiectasis pathogen (Finch et al., 2015). Interestingly, overlapping profiles were observed but distinct from healthy individuals (controls) (Figures S6A–S6D). Eighty-one metabololipids were identified in Neisseria-dominant bronchiectasis (Figure S6E), of which 8 were selected for further validation based on correlations with mouse serum (i.e., taurine, L-lactate, and fatty acids FA[20:4] and FA[18:2]) and mouse lung (L-ornithine, oleic acid, L-aspartic acid, and hypoxanthine) profiles, respectively (Figure 6D). The strong validation between human serum profiles in Neisseria-dominant bronchiectasis and those obtained in the murine infection model suggest shared mechanisms of Neisseria-associated infection (Figure 6D).

Further confirmation of the clinical value to our findings is the comparable results between Neisseria- and Pseudomonas-dominant microbiomes, the latter an established bronchiectasis pathogen associated with poor clinical outcome (Aliberti et al., 2016; Finch et al., 2015; Figure 6D). The potential influence of the dual presence of Neisseria and Pseudomonas (to differing extents) was assessed (Figure S7) and revealed important subtle influences on their respective systemic metabololipidic profiles suggestive of differing pathogenic mechanisms in individual patients related to a combination of colonization and respective relative abundance. Therefore, employing a systems biology approach combining human and mouse sampling, we demonstrate that Neisseria spp. induces inflammatory and airway remodeling responses with strong measurable clinical correlation that is comparable with the established bronchiectasis pathogen P. aeruginosa.

Metagenomic surveillance of Neisseria in chronic respiratory disease and the home environment in bronchiectasis

Our identification and characterization of N. subflava as an airway pathobiont in bronchiectasis leads to the consideration of Neisseria spp. as pathobionts in other chronic respiratory diseases. To build on our targeted 16S rRNA sequencing approach (Figure 1), we employed shotgun metagenomics to evaluate Neisseria spp. in bronchiectasis, severe asthma (SA), and chronic obstructive pulmonary disease (COPD) including healthy (non-diseased) individuals recruited from Asia (SK-KL) and the United Kingdom (DD), respectively (Figure 7A). We confirmed the presence of Neisseria species (including N. subflava) in all diseased groups with increased abundance noted in individuals recruited from our SG-KL cohort. In contrast, non-Asians, though exhibiting Neisseria spp. in all groups, had comparatively less abundance, a feature consistent with prior 16S rRNA findings in bronchiectasis (Figures 1 and 7A). Although Neisseria spp. are considered upper airway commensals, except for established pathogenicity in the setting of meningitis and gonorrhea, our findings point toward potential clinical association upon airway exposure in respiratory disease. Prior metagenomic analysis from our group highlights the potential of the home environment as a source of microbial exposure (Mac Aogain et al., 2020; Tiew et al., 2020). We therefore prospectively investigated ten homes, selected based on individuals demonstrating Neisseria-dominant bronchiectasis, and performed metagenomic evaluation of microbial content in the air, in airway, and on environmental surfaces in their residence. A significant Neisseria spp. presence was confirmed in host airways and on associated inhaler devices. Interestingly, Neisseria spp. (including N. subflava) was identified on air conditioning filters, fans, and indoor air samples, in contrast to outdoor air (sampled on the balcony or outside home windows) concurrently. Outdoor air metagenomes revealed minimal Neisseria detection, suggesting the indoor home environment as a potential exposure source of this pathobiont (Figure 7B).

DISCUSSION

Here, employing a bedside-to-bench translational approach leveraging in-vitro, in vivo, and ex-vivo approaches in human and animal models, the integration of next-generation sequencing and systems biology demonstrates that Neisseria species and N. subflava, in particular, represent a pathobiont in bronchiectasis. Neisseria-dominant microbiome profiles were detected at variable frequency based on geographic origin and relate to poor clinical outcomes in bronchiectasis. N. subflava, cultured from respiratory specimens, were subjected to primary human epithelial and mouse infection models to reveal its pathogenicity. Systems biology including transcriptomics, metabolomics, and lipidomics characterized the immune-inflammatory response in vivo which was validated in Neisseria-dominant bronchiectasis. These data reveal comparable features with those of Pseudomonas-dominant disease, an established bronchiectasis pathogen. Metagenomics applied to specimens from other respiratory diseases, including SA and COPD, demonstrates the broader significance of detecting Neisseria species in vivo, and the home environment potentially represents a key source of exposure.

Applying microbiome-based analysis to stratify disease subphenotypes is gaining traction across translational studies and, in bronchiectasis, demonstrates that the poor prognosis associated with Pseudomonas and Veillonella dominance (Rogers et al., 2014a, 2014b). Our current work builds significantly on these early observations, by identifying geography as an important microbial determinant, utilizing carefully curated and “matched” patient cohorts. We demonstrate a bronchiectasis microbial phenotype characterized by Neisseriaceae of strong clinical relevance. Culture followed by molecular characterization leads to the identification of N. subflava by whole-genome sequence analysis, followed by confirmation of its presence in the lower airways in bronchiectasis by FISH. Neisseria spp. represent a significant component of the human microbiome, constituting part of the “core microbiome” in the oral cavity (Donati et al., 2016). Traditionally, “non-pathogenic” genera remain poorly characterized with regard to their pathogenicity, with the notable exception of limited reports in bronchiectasis (Liu et al., 2015). N. subflava impacted tight junctions and cell barrier integrity in
Figure 7. Metagenomic surveillance of Neisseria spp. in respiratory disease and the home environment of Neisseria-dominant bronchiectasis

(A) Metagenomic species level analysis indicating the relative abundance of the top 40 microbes in non-diseased (healthy) controls (n = 8) and individuals with respiratory disease, including bronchiectasis (BE) (n = 12), severe asthma (SA) (n = 8), and chronic obstructive pulmonary disease (COPD) (n = 8). Recruited individuals are illustrated based on the country of origin where SG-KL reflects recruits from Singapore-Kuala Lumpur, Malaysia (left), and DD individuals from Dundee, Scotland (right). The various Neisseria species are indicated at the top of each respective barplot to highlight their variable abundance across disease states and by geographic region. All taxa are colored according to the legend.

(B) Metagenomic analysis of the home environment in n = 10 Neisseria-dominant individuals with bronchiectasis reveals Neisseria spp. in indoor air and home surfaces. Metagenomic sequencing of outdoor and indoor air, individual sputum, and swabs of an accompanying inhaler device and surfaces in the home environment including aircon filter and fan were performed, and top 40 detected Neisseria species (and relative abundance) are illustrated. Bubble size corresponds to the number of reads assigned to each respective taxon.
our primary human nasopharyngeal and bronchial cellular models, features previously observed only for overt pathogenic members of the Neisseriaceae, suggestive of invasive potential (Sutherland et al., 2010). Immunological assessment reveals increased expression of IL-6 and IL-8 with markedly enhanced CCL2 (MCP-1), all features of immune dysregulation in bronchiectasis (King, 2018; McShane, 2019). Induction of CXCL10 (IP-10) is noteworthy, given its increased expression in response to pathogenic N. meningitidis (and in fulminant meningitis) possibly due to conserved antigenic moieties between members of the Neisseriaceae (Ovstebø et al., 2008). Critically, microbiome-based signatures in bronchiectasis exhibit demonstrable association with host immune responses: H. influenzae-dominance exhibits increased matrix metalloprotease-2 and -8 (MMP2 and MMP8) relative to Pseudomonas-dominance, whereas overall taxonomic diversity relates to IL-1β and IL-8 expressions. Increased microbiome richness further predicts improved clinical outcomes and reduced inflammatory signaling (Rogers et al., 2014a; Taylor et al., 2015). Our work further supports such association between individual microbiome profiles and airway immunological signatures, here in relation to airway Neisseria, lending increased credence to the emerging role of upper airway bacterial migration in shaping lower airway immunology (Dickson et al., 2017; Segal et al., 2016; Wu et al., 2021).

Animal models of infection are described for Neisseria; however, these largely focus on N. meningitidis (Weyand, 2017). Dedicated studies of “non-pathogenic” Neisseria in the mouse remain lacking. Further, no animal model representative of bronchiectasis is currently available, representing a challenge to establishing causality in this field. The inherent disease heterogeneity and chronic progressive nature of bronchiectasis (varying between individuals) makes modeling this disease ex-vivo challenging and a key knowledge gap in the field. Current approaches remain inadequate in reproducing the in vivo state in bronchiectasis, limiting our experimental options, despite infection remaining central to its pathogenesis (Flume et al., 2018). Here, we demonstrate, in an acute intratracheal mouse model, that N. subflava promotes broncho-alveolar infection, significant pulmonary inflammation and tissue destruction. Although Neisseria-associated alveolar infection clears within days, bronchiolar infection persists in an inimitable pattern distinct from that seen in S. pneumoniae or P. aeruginosa pulmonary infection (Kadioglu et al., 2000; Kukavica-Ibrulj et al., 2014). Our murine model identifies an early innate immune response characterized by neutrophilic and NK cell infiltration into lung parenchyma, followed by a late adaptive phase involving T helper cells, cytotoxic T cells, and B cells accompanied by persistence of N. subflava. Taken together, these results implicate N. subflava in triggering innate and adaptive immunity in line with other pathogens: a classical phagocytic phase followed by lymphocytic response. Recent work assessing the episodic aspiration of upper airway commensals similarly investigated their ability to trigger airway immune responses (Wu et al., 2021). Given strong associations between microbiology and bronchiectasis phenotypes, more refined chronic disease models may require incorporation of specific microbial exposures—as is the case for other model systems such as the use of H. influenzae in COPD models (Gaschler et al., 2009) or respiratory syncytial virus (RSV) in asthma (You et al., 2006). Although our murine model is unlikely to be completely reflective of the clinical situation in all patients, we note that the bronchiolar inflammation observed points to Neisseria-specific mechanisms of inflammation and remodeling (defined by our transcriptomics/metabolomics) and is distinct to that of a P. aeruginosa challenge. This may direct the future development of murine bronchiectasis models, at least for a subset of patients. The relatively sustained infective potential and extensive macro- and microscopic pulmonary damage observed in our model likely reflect our direct inoculation strategy, mimicking deeper and prolonged lower airway exposure to N. subflava compared with the upper airway aspiration of commensal Neisseria spp.

To characterize the host response, transcriptomics, performed longitudinally over the course of N. subflava infection, closely reflected the immune response observed in our mouse model. Enrichment of antibacterial and phagocytic response signals was observed early, which progressed to an inflammatory and oxidative stress response, before emergence of an airway remodeling signature. Although bronchiectasis is primarily a neutrophilic disease, emerging work implicates allergic sensitization in disease pathogenesis in subgroups (Mac Aogain et al., 2019). Enrichment of the endothelin pathway, as part of a broader functional airway remodeling, potentially suggests a role for hypersensitization associated with N. subflava (Gregory et al., 2013).

Metabolipodinic approaches, accompanied by validation, provide scope for biomarker discovery and refined stratification of subphenotypes in respiratory diseases (Wheelock et al., 2013). Having established N. subflava pathogenicity in vivo, we next identified a dysregulated metabolipidome, validated in human disease, and characterized by significant immunometabolic reprogramming implicating arginine and tryptophan metabolism and increased ceramide (Bronte and Zanovello, 2005). Host defense against respiratory pathogens such as P. aeruginosa is characterized by ceramide release, due to lipid raft remodeling, a common site for pathogen-host signaling (Grassmé et al., 2003). Importantly, this represents a potential mechanism of mucosal invasion, employed by N. gonorrhoeae, which may serve as an important signaling platform for interaction with commensal Neisseriaceae (Grassmé et al., 1997). Systemic murine metabolomes, sampled serially following N. subflava infection, reveal metabolic clusters that upon functional integration with transcriptomes identify key analytes of interest that were validated with human serum from Neisseria- and Pseudomonas-dominant bronchiectasis. Major markers include arachidonic, linoleic, and oleic acids, whose alteration is consistent with an infection-driven fatty acid profile and indicates a leukotriene-associated response. These were accompanied by taurine, lactate, ornithine, aspartic acid, and hypoxanthine, all linked to oxidative stress (Schmidt et al., 2001; Wenzel, 1997).

Given the clinical relevance of Neisseria-dominant microorganisms in bronchiectasis, we explored other chronic respiratory diseases including SA and COPD. We confirmed detection and high relative abundance of Neisseria spp. across these diseases, particularly in individuals recruited in Asia, suggesting clinical relevance beyond bronchiectasis. Geographic variability in bronchiectasis airway microbiology is not limited to Neisseria, as significant differences in the prevalence of pathogens are observed...
including *H. influenzae*, the driving factors of which are multifactorial including host, environmental, exposure, and climatic influences (Chalmers and Chotirmall, 2018; Chotirmall et al., 2017; Rogers et al., 2014b). Our group has previously applied metagenomic profiling to establish associations between microbial exposure in the indoor environment and respiratory disease outcomes (MacAogain et al., 2020; Tiew et al., 2020). Using similar approaches, we sought to identify potential sources of *Neisseria* spp. in the home environment of bronchiectasis patients. The presence of *N. subflava*, among other members of the *Neisseriaceae*, was detectable in the homes of patients with bronchiectasis highlighting the organism’s ability to persist in the home and represent an environmental source for repeated exposure. These observations suggest that microbial signatures in the home may influence human microbiome profiles and potentially clinical course and outcomes. Importantly, the nature and directionality of such environmental microbial exposures (i.e., environment influencing host or vice versa) and its direct impact on bronchiectasis and other respiratory diseases requires further exploration in controlled studies.

Our study has several strengths: well-matched human cohorts across geographic locations, use of a broad range of primary human cellular and animal infection models, and application of a variety of next-generation sequencing and systems biology approaches to characterize *Neisseria* as pathobionts in bronchiectasis.

**Limitations of the study**

Although we successfully isolated *N. subflava* from respiratory specimens in *Neisseria*-dominant microbiomes using established culture methodologies, our approach may have biased detection toward non-fastidious species and selectively favored the culture of *N. subflava*. Broader metagenomic sequencing of airway samples or the application of advanced “culture-omics” may better serve in determining if other *Neisseria* spp. are implicated in bronchiectasis (Whealan et al., 2020). Thus, although our work highlights the potential pathobiont role of airway *Neisseriaceae*, we acknowledge that they likely represent one of several commensal airway taxa implicated by a growing body literature to be involved in microbial host interaction as part of complex airway microbial consortia (Dickson et al., 2017; Rigouts et al., 2022; Segal et al., 2016; Wu et al., 2021). In identifying other geographically distinct taxa, we applied LEfSe analysis that corroborated our main finding with respect to *Neisseria* abundance. However, it is clear that the identification of discriminant taxa may give differing results based on analytical methods chosen for assessment of compositional data (Nearing et al., 2022). Novel approaches for mitigating these challenges such as analysis of compositions of microbiomes with bias correction (ANCOM-BC) continue to be developed for more robust microbiome comparisons (Lin and Peddada, 2020). We did not directly assess bacterial load using qPCR, which would have provided insight to bacterial burden, a factor that could have an important role in *Neisseria*-mediated airway pathology. Our work did not include any therapeutic intervention targeting *Neisseria*-dominant microbiomes, and hence, clinical benefit of targeting *Neisseria* in bronchiectasis remains to be determined. Future clinical validation through well-designed intervention studies will be necessary. Although we took great effort to match Asian and European patients, geographic confounders potentially remain. These include exacerbations, use of corticosteroids and antibiotics, and microbiobiological culture results. This was partly due to a clinical matching criterion based on the bronchiectasis severity index (BSI) score which allows for variable phenotypes within patients of similar scores and the known geographic heterogeneity of this disease (Chandrasekaran et al., 2018; Dhar et al., 2019). This issue was mitigated to some extent as *Neisseria*-associated clinical phenotypes were largely assessed in Asian patients in this work. Related to this, our analysis of *Neisseria*-associated clinical relationships among Europeans was limited by the low numbers of *Neisseria*-dominant patients in the Dundee cohort. This, however, does not exclude the possibility of such phenomena occurring among patients from this region that may require broader sampling to detect. Finally, our use of human bacterial strains in a murine model likely triggered heightened immune responses, which should be considered in interpreting our data.

In summary, we present clinical and experimental data indicative of *Neisseria* spp. as unrecognized lung “pathobionts” in bronchiectasis and other respiratory diseases representing a “targetable” microbial trait of the lung microbiome in at-risk individuals.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Human subjects
  - Human airway epithelial cells
  - Husbandry and housing conditions of experimental animals
  - Murine intratracheal infection model
  - Ethics approval
- **METHOD DETAILS**
  - Sputum, serum, and environmental sample collection
  - DNA extraction from clinical and environmental samples
  - Bacterial microbiome sequencing and analysis
  - Microbiological culture of *Neisseria* spp. isolates from the bronchiectasis airway
  - Infection of Air-liquid interface (ALI) cultures with *Neisseria subflava*
  - Trans-epithelial electrical resistance (TEER)
  - Quantification of gene expression by qRT-PCR
  - Immune profiling of mouse bronchoalveolar lavage fluid (BALF)
  - Staining of lung tissue
  - Mouse transcriptome sequencing
  - Metabolite and lipid extraction
  - Instrumental analysis and metabolite-lipid identification
QUANTIFICATION AND STATISTICAL ANALYSIS
- Statistical analysis and data visualization
- Transcriptomic analysis, functional enrichment analysis and weighted correlation network analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2022.08.005.

ACKNOWLEDGMENTS

This research is supported by the Singapore Ministry of Health’s National Medical Research Council under its Clinician-Scientist Individual Research Grant (CS-IRG) (MOH-000141) (S.H.C.) and Clinician Scientist Award (CSA) (MOH-000710) (S.H.C.). It is also supported by the National Natural Science Foundation of China (81900007) and the Natural Science Foundation of Guangdong Province of China (2021A1515010004). LL is supported by the Research Initiation Fund for Introduction of Talents from the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (Y9G037). J.D.C. is supported by a Senior Research Fellowship from the Chief Scientist Office, Scotland (SCAF/17/03) and the British Lung Foundation Chair of Respiratory Research. The authors would like to thank The Academic Respiratory Initiative for Pulmonary Health (TARIPH), Lee Kong Chian School of Medicine, NTU Singapore, for collaboration support.

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

J.D.C. has received research grants from GSK, BI, AZ, Gilead Sciences, Gelifols, and Insmed and has received personal fees from GSK, BI, AZ. Chiesi, Gelifols, Napp, Novartis, Insmed, and Zambon, all outside the submitted work. S.H.C. is on advisory boards for CSL Behring, Pneumagen, and BI, serves on Data and Safety Monitoring Boards for Inovio Pharmaceuticals and Imam Abdulrahman Bin Faisal University, and has received personal fees from AZ, Chiesi, Gelifols, Napp, Novartis, Insmed, and Zambon, all outside the submitted work. J.D.C. has received research grants from GSK, BI, AZ, Gilead Sciences, Gelifols, and Insmed and has received personal fees from GSK, BI, AZ, Chiesi, Gelifols, Napp, Novartis, Insmed, and Zambon, all outside the submitted work. Chalmers, J.D., Chang, A.B., Choitirmall, S.H., Dhar, R., and McShane, P.J. (2018). Bronchiectasis. Nat. Rev. Dis. Primers 4, 45. https://doi.org/10.1038/s41572-018-0042-3.


REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE anti-mouse CD45 Antibody Clone 30-F11</td>
<td>Biolegend</td>
<td>RRID:AB_312971</td>
</tr>
<tr>
<td>APC/Cyanine7 anti-mouse CD45 Antibody clone 30-F11</td>
<td>Biolegend</td>
<td>RRID:AB_312981</td>
</tr>
<tr>
<td>APC anti-mouse Ly-6G Antibody Clone 1A8</td>
<td>Biolegend</td>
<td>RRID:AB_2227348</td>
</tr>
<tr>
<td>Anti-F4/80 antibody [BM8] (FITC)</td>
<td>Abcam</td>
<td>RRID:AB_941505</td>
</tr>
<tr>
<td>APC anti-mouse NK-1.1 Antibody clone PK136</td>
<td>Biolegend</td>
<td>RRID:AB_313397</td>
</tr>
<tr>
<td>FITC anti-mouse CD19 antibody clone 6D5</td>
<td>Biolegend</td>
<td>RRID:AB_313641</td>
</tr>
<tr>
<td>Ms CD3 Molecular Complex FITC 17A2</td>
<td>BD Biosciences</td>
<td>RRID:AB_2920870</td>
</tr>
<tr>
<td>PE anti-mouse CD4 Antibody Clone H129.19</td>
<td>Biolegend</td>
<td>RRID:AB_2075573</td>
</tr>
<tr>
<td>APC anti-mouse CD8a Antibody Clone 53-6.7</td>
<td>Biolegend</td>
<td>RRID:AB_312751</td>
</tr>
<tr>
<td><strong>Bacterial and virus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical respiratory isolates of <em>Neisseria subflava</em></td>
<td>Singapore General Hospital, Tan Tock Seng Hospital, Chang General Hospital, Singapore</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Biological samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum and serum samples from bronchiectasis patients (Singapore)</td>
<td>Singapore General Hospital, Tan Tock Seng Hospital, Chang General Hospital, Singapore</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum samples from bronchiectasis patients (Malaysia)</td>
<td>UKM Medical Centre, Kuala Lumpur, Malaysia</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum samples from bronchiectasis patients (Dundee)</td>
<td>Ninewells Hospital and Medical School, Dundee, Scotland, United Kingdom</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum samples from COPD patients (Singapore)</td>
<td>Singapore General Hospital, Singapore</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum samples from Severe Asthma patients (Singapore)</td>
<td>Singapore General Hospital, Singapore</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum samples from COPD patients (Dundee)</td>
<td>Ninewells Hospital and Medical School, Dundee, Scotland, United Kingdom</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum samples from Severe Asthma patients (Dundee)</td>
<td>Ninewells Hospital and Medical School, Dundee, Scotland, United Kingdom</td>
<td>N/A</td>
</tr>
<tr>
<td>Sputum and serum samples from healthy controls (Singapore)</td>
<td>Nanyang Technological University, Singapore</td>
<td>N/A</td>
</tr>
<tr>
<td>Lung biopsy samples from bronchiectasis patients and controls</td>
<td>Department of Respiratory Medicine and Critical Care, Peking University Shenzhen Hospital</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Chemicals, peptides, and recombinant proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputasol</td>
<td>Thermo Fisher</td>
<td>SR0233A</td>
</tr>
<tr>
<td>RNAlater</td>
<td>Thermo Fisher</td>
<td>AM7021</td>
</tr>
<tr>
<td>Giemsa Staining Solution</td>
<td>Beyotime</td>
<td>C0133</td>
</tr>
<tr>
<td>DAPI</td>
<td>Beyotime</td>
<td>C1006</td>
</tr>
<tr>
<td>TRIzol</td>
<td>Thermo Fisher</td>
<td>15596026</td>
</tr>
<tr>
<td><strong>Critical commercial assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematoxylin and Eosin Staining Kit</td>
<td>Beyotime</td>
<td>C0105M</td>
</tr>
</tbody>
</table>

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sanjay H. Chotirmall (schotirmall@ntu.edu.sg).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All raw sequencing and metabolipidomic data have been deposited in NCBI-SRA and MetaboLights database (EMBL-EBI) respectively. Public accession numbers associated with 16S rRNA gene amplicon sequencing data, mouse transcriptomic RNA sequencing data, metabolomics and lipidomics data, sputum and environmental metagenomic whole genome shotgun (WGS) data and Neisseria subflava genome assemblies are described in the key resources table.

---

Continued

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Pure PCR Template Preparation Kit</td>
<td>Roche diagnostics</td>
<td>11796828001</td>
</tr>
<tr>
<td>Deposited data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S amplicon sequencing data</td>
<td>NCBI</td>
<td>BioProject: PRJNA590225</td>
</tr>
<tr>
<td>Whole genome sequence assemblies of <em>Neisseria subflava</em> clinical respiratory isolates</td>
<td>NCBI</td>
<td>BioProject: PRJNA714914</td>
</tr>
<tr>
<td>RNA sequencing data of the <em>Neisseria subflava</em> infection model</td>
<td>NCBI</td>
<td>BioProject: PRJNA706545</td>
</tr>
<tr>
<td>Metabolomics and lipidomics datasets from the <em>Neisseria subflava</em> infection model</td>
<td>EMBL-EBI</td>
<td>MetaboLights: MTBLS3009</td>
</tr>
<tr>
<td>Metagenomic sequencing data</td>
<td>NCBI</td>
<td>BioProject: PRJNA595703</td>
</tr>
<tr>
<td>Experimental models: Cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Human nasopharyngeal epithelial cells</em></td>
<td>Singapore General Hospital and National University Hospital, Singapore, John Hunter Hospital, NSW, Australia</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Human bronchial epithelial cells</em></td>
<td>John Hunter Hospital, NSW, Australia</td>
<td>N/A</td>
</tr>
<tr>
<td>Experimental models: Organisms/strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse: C57BL/6J</td>
<td>Jackson Laboratory</td>
<td>000664</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>See Table S7 in supplemental information.</td>
<td></td>
</tr>
<tr>
<td>Software and algorithms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All original code</td>
<td>This manuscript</td>
<td><a href="https://github.com/RespiratoryMicrobiome/Neisseria">https://github.com/RespiratoryMicrobiome/Neisseria</a>; <a href="https://doi.org/10.5281/zenodo.6969817">https://doi.org/10.5281/zenodo.6969817</a></td>
</tr>
<tr>
<td>CLC Genomics Workbench 9.0</td>
<td>CLC genomics.</td>
<td>N/A</td>
</tr>
<tr>
<td>DESeq2</td>
<td>Love et al., 2014</td>
<td>N/A</td>
</tr>
<tr>
<td>Ingenuity Pathway Analysis (IPA) software</td>
<td>Qiagen Bioinformatics.</td>
<td>N/A</td>
</tr>
<tr>
<td>XCMS</td>
<td>Smith et al., 2006</td>
<td>N/A</td>
</tr>
<tr>
<td>R package “Hmisc”</td>
<td>Harrell, 2019</td>
<td>N/A</td>
</tr>
<tr>
<td>R package “ggplot2”</td>
<td>Wickham, 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>R package “vegan”</td>
<td>Oksanen et al., 2013</td>
<td>N/A</td>
</tr>
<tr>
<td>R Package “Weighted correlation network analysis (WGCNA)”</td>
<td>Langfelder and Horvath, 2008</td>
<td>N/A</td>
</tr>
<tr>
<td>LEfSe</td>
<td>Segata et al., 2011</td>
<td>N/A</td>
</tr>
<tr>
<td>Cytoscape</td>
<td>Reimand et al., 2019</td>
<td>N/A</td>
</tr>
<tr>
<td>MEGAN</td>
<td>Huson et al., 2016</td>
<td>N/A</td>
</tr>
</tbody>
</table>
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects
Four human cohorts were recruited in this study each receiving appropriate ethical approvals (detailed below) from all respective participating centers. All recruited individuals provided written informed consent for participation in this study. The four cohorts are summarized as follows:

1. A cross-sectional Cohort of Asian and Matched European Bronchiectasis (CAMEB), consisting of individuals with high-resolution computed tomography (HRCT) confirmed bronchiectasis and recruited during outpatient attendance when clinically stable, defined as the absence of new symptoms or change to bronchiectasis therapy in the preceding four-weeks. Patients were recruited from three sites in Singapore (Singapore General Hospital, Changi General Hospital and Tan Tock Seng Hospital), one Malaysian site (UKM Medical Centre, Kuala Lumpur) and an age-, sex- and disease-severity matched group (based on Bronchiectasis Severity Index - BSI) from a single European site (Ninewells Hospital, Dundee, UK) to control for confounding geographic factors (Chalmers et al., 2014; Mac Aogáin et al., 2018). Patients with another concurrent major respiratory disease as their primary diagnosis (i.e., asthma or COPD) (Bateman et al., 2008; Celli et al., 2004), those pregnant or breast-feeding, active mycobacterial disease or on chemotherapy were excluded along with patients with active infection (necessitating acute use of antibiotics) and/or received systemic corticosteroids in the four weeks preceding recruitment. Central to the CAMEB cohort strategy was the ‘matching’ of individuals recruited from Asia (Singapore and Kuala Lumpur, Malaysia) to an individual from Europe (Dundee, Scotland) to permit geographic comparisons between the cohorts (Chalmers et al., 2014; Mac Aogáin et al., 2018). This current study focused on the assessment of geographic variation in the bacterial microbiome between Asian and European patients from the CAMEB cohort. A total of n=225 patients from the cohort had their sputum assessed, generating 16S rRNA bacteriome profiles. This included n=95 ‘matched’ Asian-European pairs. Further details about the CAMEB cohort, including demographics, recruitment, patient matching, and inclusion and exclusion criteria are described previously (Mac Aogáin et al., 2019; Mac Aogáin et al., 2018, 2021) and demographics for the individuals included in this particular study are summarized in Table S1.

2. For the assessment of Neisseria spp. in the lower airway, a cohort of n=21 individuals from a single center in Peking University Shenzhen Hospital, China was recruited. Lung biopsies were obtained from n=9 individuals with clinically and radiologically confirmed bronchiectasis and n=12 non-bronchiectasis (control) subjects. Patients in the bronchiectasis group had a prior history of bronchiectasis confirmed by clinical and radiological assessment, including computed tomography (CT) of the chest. Control subjects represented individuals undergoing bronchoscopy with a radiological suspicion of lung cancer. All control subjects had no demonstrable radiological evidence of bronchiectasis as defined by established criteria including increased or abnormal bronchiolar diameter or appearance, signet ring sign or thickening of bronchiolar walls (Pasteur et al., 2010). Formalin-fixed paraffin-embedded specimens of lung tissue were obtained in all patients, which for all subjects were taken approximately 2 cm from the carina from within a main bronchi in macroscopically ‘normal’ appearing regions. Demographics for the individuals included in this cohort are summarized in Table S2.

3. For whole-genome shotgun (WGS) metagenomics analysis of the airway microbiome in non-diseased (healthy) individuals (n=8) and those with chronic respiratory disease (n=28), a spontaneously expectorated sputum sample was obtained from a deep cough in healthy subjects (n=8, Singapore) and patients with bronchiectasis (n=12; 6 Singapore and 6 Dundee); severe asthma (n=8; 4 Singapore and 4 Dundee) and chronic obstructive pulmonary disease (COPD) (n=8; 4 Singapore and 4 Dundee). Non-diseased (healthy) subjects had no active or past history of any respiratory or other medical disease and had normal spirometry measured in accordance with ERS/ATS criteria (Pellegrino et al., 2005). Severe asthma was defined as an individual attending a dedicated severe asthma service at a tertiary referral center for at least 2 years. Patients were free from exacerbation over the preceding four-week period prior to sampling and were at least at step four of the Global Initiative for Asthma (GINA) treatment ladder (Global Initiative for Asthma, 2019; Chung et al., 2014), while COPD was defined according to the Global initiative for chronic obstructive lung disease (GOLD), where FEV1/FVC<0.7, sampled during periods of disease stability defined as the absence of exacerbation over the preceding 6 weeks before study recruitment (GOLD, 2018 [http://goldcopd.org]); Tiew et al., 2020). The demographics and clinical details of the individuals included in this cohort are summarized in Table S5.

4. Stable, Neisseria-dominant bronchiectasis patients (n=10) were recruited in Singapore for home and environmental sampling. As outlined above, patients had HRCT-confirmed bronchiectasis and were recruited during outpatient attendance during a period of clinical stability, consistent with the CAMEB study protocol. Sampling in this study arm involved collection of 1) an indoor (bedroom) air sample, 2) a concurrently obtained outdoor (balcony) air sample, 3) a surface swab obtained from...
either an air-conditioning filter or fan within the individual bedroom in addition to 4) a surface swab of the patient inhaler device and 5) an individual sputum sample. Demographics for the individuals included in the home sampling part of the study are summarized in Table S6.

For validation of metabolomic experiments, serum samples from six Neisseria-dominant and six Pseudomonas-dominant patients from Asia and the CAMEB cohort, in addition to six healthy controls were compared (Table S4).

For demographic reporting and the presented analysis, bronchiectasis disease severity was assigned according to the multimodal Bronchiectasis Severity Index (BSI) and further divided into ‘mild’ (BSI; 0-4), ‘moderate’ (BSI; 5-8) or ‘severe’ (BSI; 9 and above) categories. Clinical data comprising the BSI including age, body mass index (BMI), Medical Research Council (MRC) dyspnea score, FEV1 percentage predicted values, radiological severity, number of exacerbations (defined by BTS consensus criteria) in the preceding year, hospitalizations in the preceding year, microbial colonization with other organisms and colonization by P. aeruginosa were recorded for each patient, including data on sex, disease etiology and smoking status (Chalmers et al., 2014; Hill et al., 2017).

Individuals recruited into this study were either healthy (with normal immune status) or diagnosed with a chronic respiratory disease (as described above). In this latter group, relative functional immunodeficiencies related to underlying disease pathogenesis may be present however unless otherwise stated, none had any formal diagnosis of a primary or secondary immunodeficiency syndrome.

Human airway epithelial cells

Nasopharyngeal and bronchial sampling respectively were performed according to established protocols by trained personnel using flocked nasopharyngeal swabs (FNPS) with the BBL Universal Viral Transport Standard Kit or obtained using a single sheathed nylon cytology brush applied under direct vision during bronchoscopy. For nasopharyngeal sampling, a FNPS was inserted into the nostril to an appropriate depth and rotated several times before removal. For bronchial sampling, approximately 4-8 brushings were taken from second to third generation bronchi, and cells were washed from brushes with DMEM. Samples were immediately transferred on ice to the laboratory for further processing as described. Human nasopharyngeal epithelial cells (HNECs): Flocked nasopharyngeal swabs (FNPS) were flushed with transport medium at least 20 times to release HNECs. Cells were plated on human collagen IV precoated 6-well plates and cultured in B/D expansion medium (1:1 advanced DMEM/F12 and Bronchial epithelial cell medium supplemented with 1x BEpiCGS, 1% of HEPES, 1% of Glutamax, 1x B27, penicillin/streptomycin (100 U/mL) coated 6-well plates and cultured in B/D expansion medium (1:1 advanced DMEM/F12 and Bronchial epithelial cell medium). Swabs (FNPS) were flushed with transport medium at least 20 times to release HNECs. Cells were plated on human collagen IV precoated 6-well plates and cultured in B/D expansion medium (1:1 advanced DMEM/F12 and Bronchial epithelial cell medium (BEPICM) supplemented with 1x BEpiCGS, 1% of HEPES, 1% of Glutamax, 1x B27, penicillin/streptomycin (100 μg/ml), primocin (50 μg/ml), R-spondin 1 (500 ng/ml), nicotinamide (5 mM), Y-27632 (5 μM), DMH-1 (1 μM), 3',5-triido-L-thyronine (100 nM), SB202190 (500 nM), A83-01 (1 μM), FGF-7 (25 ng/ml), FGF-10 (100 ng/ml) and N-[3-(3,5-difluorophenacyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (5 μM)). Medium was refreshed every two days until confluent. Human bronchial epithelial cells (HBECs): Cells were washed with HBSS and plated in a tissue-culture flask in bronchial epithelial growth medium (BEGM) (Lonza, USA) with growth supplements. Medium was refreshed every two days until confluent.

Husbandry and housing conditions of experimental animals

For intratracheal mouse infection experiments, 6- to 10-week old C57BL/6 mice were obtained (Jackson laboratories) and maintained in specific Pathogen Free (SPF) facilities on a 12-hour light-dark cycle with ad libitum access to chow and water, in biosafety level 2 (BSL-2) conditions, until experimental inoculation.

Murine intratracheal infection model

At the time of inoculation, mice were anesthetized using 80 mg/kg body weight ketamine and 10 mg/kg body weight xylazine and infected via intratracheal delivery with the cultured Neisseria subflava (isolate SG-KL01) at 2 x 10^7 CFU or Pseudomonas aeruginosa (strain PAO1) at 1 x 10^8 CFU in 40 μL of sterile phosphate-buffered saline (PBS) once. Mice were monitored for body weight and clinical score (Burkholder et al., 2012). Phenotypic scoring was performed based on indicators of general well-being such as coat condition, normal posture, eyes, and body stance. Mice were scored as follows: ‘100’ - normal posture and shining fur; spontaneous and normal behavior and social contact, clear and clean eyes; normal breathing; ‘80’ - minor symptoms of ruffled fur, spontaneous but reduced behavior, altered breathing; ‘60’ - loss of body weight, moderately reduced activity, ruffled fur, hunched posture, accelerated breathing; ‘40’ - loss of body weight, ruffled fur, motility only after stimulation, massively hunched posture, unclean and sticky, closed or squinted eyes, strongly accelerated breathing; ‘20’ - moribund, ‘0’ - death. Mice were euthanized at specified time points, and lung tissue was harvested. Lung lobes were either fixed in 4% paraformaldehyde for histological analysis or snap-frozen in liquid nitrogen and kept at -80°C for subsequent assays.

Ethics approval

All human cohorts recruited for this study are described as relevant. Participants were recruited from several hospitals and/or academic sites from 5 countries (Singapore, Malaysia, Scotland, China, and Australia) as described in the methods and recruitment approved by their respective institutional review boards as follows. All included participants provided written informed consent: CIRB 2016/2073, UKMMC FF-2016-440, NHD 12/ES/0059, BDSYLSY-053A (bronchiectasis) CIRB 2016/2628, 16/NW/0101 (severe asthma), CIRB 2016/2715, CIRB 2016/2549, 16/NW/0101 (COPD), CIRB 2017/2109 (Home sampling) (all mutually recognized by DSRB). NTU IRB-2017-12-010 (Healthy individuals). HBECS were obtained from the John Hunter Hospital, Australia (H-163-1205, The Hunter New England LHD ethics committee, Australia), and HNECs from CIRB 2020/2338 (Singapore), IRB-2020-05-004 (Nanyang Technological University, Singapore) and NUS under IRB L13-509 and DSRB 2011/00228. All animal experiments were conducted under standard conditions, and ethical clearance was obtained according to the guidelines set by national regulatory authorities.
approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University (A18089) and Xiamen University, China (XMULAC20190029).

METHOD DETAILS

Sputum, serum, and environmental sample collection
Spontaneously expectorated ‘representative’ sputum from a deep cough with the assistance of a chest physiotherapist or induction protocol (where appropriate) was collected into sterile containers and transported (on ice) for evaluation (Chotirmall et al., 2010). All specimens from clinical sites were transported promptly, appropriately and processed centrally at a single site to ensure consistency and standardization of all experimental work and downstream analysis. Samples from the Singaporean hospitals were transported on ice by courier to Nanyang Technological University (within 4 hours of collection). To ensure quality control of materials transported from sites outside Singapore, specimens were shipped on dry ice in temperature-controlled containers and their integrity checked on arrival before experimental use. Equal volumes of Sputasol (Thermo Fisher Scientific) and sputum samples were combined and shaken for 15 minutes at 37°C. Sputasol-homogenized samples were mixed with two volumes of RNAlater (Sigma Aldrich) (Coughlan et al., 2012; Mac Aogáin et al., 2018). Treated sputum samples were sorted at −80°C prior to processing. DNA extraction experiments were performed at Nanyang Technological University, Singapore using a single standardized protocol (outlined in detail below) (Mac Aogáin et al., 2018). Serum was collected from bronchiectasis patients whose sputum microbiome profile was either Neisseria- or Pseudomonas-dominant in composition, as well as from healthy control subjects (Table S4). To obtain serum, blood specimens were collected in vacutainer serum tubes (BD Biosciences) and centrifuged at 1300 g for 10 minutes at 18°C to separate serum which was subsequently used for the described studies. In addition to sputum and serum, environmental air samples were collected from patient homes using filter-based air samplers SASS3100 (Research International) with a lowered flow rate of 100 L·min−1 (to avoid sleep disruption) for eight consecutive hours. Samplers were placed in the patient’s bedroom and near an outdoor air source (i.e., balcony) and programmed to run concurrently overnight at the same time each evening (8pm – 4am). In addition, surface swabs (either air-conditioner or fan where applicable) were taken from the patients’ homes: the surfaces of air-conditioning filters or fans in the bedroom and inhaler devices were swabbed using 4N6Floq (Copan) swabs pre-moistened with 0.1% PBS-Triton-X100 and snapped into DNeasy PowerWater kit (Qiagen) bead tubes directly after use. Air filters from SASS samplers were transferred to filter pouches and transported with swabs in bead tubes to the lab for immediate processing or stored at −20°C prior to processing (Gusareva et al., 2019; Mac Aogáin et al., 2020).

DNA extraction from clinical and environmental samples
Sputum DNA was extracted from a 250 mg sample using methods previously described (Coughlan et al., 2012; Mac Aogáin et al., 2018). Stored sputum samples were thawed on ice and transferred to sterile bead mill tubes (VWR) containing 1 mm sterile glass beads (Sigma-Aldrich). Samples were homogenised using a bead mill homogeniser (VWR) and DNA was purified using the Roche High-pure PCR Template Preparation Kit (Roche) according to manufacturers’ instructions. The integrity of extracted sputum DNA was confirmed using the Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen). Swab samples in DNeasy PowerWater kit (Qiagen, Germany) bead tubes were processed according to the manufacturer’s protocol with the addition of proteinase K (Sigma-Aldrich, USA) and sonication at 65°C (Mac Aogáin et al., 2020). For processing, the SASS filter was transferred to a sterile tube and washed with PBS-Triton X-100 in triplicate. Washed solutions from SASS air filters were further processed by filtering through 0.02 μm Anodisc filters (Whatman) using a vacuum manifold (DHI). DNA was then extracted from the Anodisc with the DNeasy PowerWater kit (Qiagen) according to the manufacturer’s protocol with modifications to increase DNA yield (Luhung et al., 2021). Sterile swabs, filters and sputum DNA extraction reagents were processed simultaneously as extraction controls to assess the levels of experimental background contamination (Figure S3).

Bacterial microbiome sequencing and analysis
Targeted amplicon sequencing of the 16S rRNA gene (bacteriome) was carried out on DNA extracted from individuals with bronchiectasis recruited from the CAMEB cohort using a previously validated amplicon shotgun sequencing protocol with paired-end analysis (2 x 101 bp reads) on an Illumina HiSeq2500 platform (Mac Aogáin et al., 2021; Ong et al., 2013). Blank samples were subjected to 16S rRNA analysis capturing and allowing adjustment for background levels of experimental contamination, as shown in Figure S3. Low DNA yield from blanks necessitated pooling (n=4) to ensure sufficient material for amplification and sequencing. The concentration of 16S rRNA amplicons was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). The amplicon concentrations and read counts observed (as a proxy for bacterial contamination/burden) in test samples and extraction blanks were assessed. Blank samples exhibited considerably lower amplicon concentrations and read counts than those of test samples and therefore background contamination is unlikely to have had a substantial influence on the observed microbiome profiles. Read counts in blanks as well as their taxonomic assignments are illustrated in Figure S3. All 16S rRNA gene short-read sequence data from this study have been uploaded to the National Center for Biotechnology Information (NCBI) sequence read archives under project accession number PRJNA590225. Metagenomic sequencing of sputum and environmental samples was sequenced on a HiSeq 2500 platform (Illumina, USA) at the NTU core sequencing facility according to library preparation and DNA sequencing methods as described (Mac Aogáin et al., 2020, 2021). Read counts in sequencing blank controls for metagenomic analysis as well as their taxonomic assignments are illustrated in Figure S3. The metagenomics sequence data were processed for adaptor removal and quality trimming with a Phred
quality score threshold of 20 (Q>20) using Cutadapt v. 1.8.1. The trimmed reads were then aligned against the NCBI non-redundant protein database using the taxonomic classifier Kaiju (v.1.7.2) (Menzel et al., 2016). Kaiju outputs were imported into MEGAN (v. 5.11.3) (Huson et al., 2016), which uses lowest common ancestry (LCA) algorithm to assign taxon IDs to metagenomic reads based on NCBI taxonomy. The minimum number of reads required for taxonomic assignment was set to 25. Short-read metagenomic sequencing data from this study have been uploaded to the National Center for Biotechnology Information (NCBI) sequence read archives under project accession number PRJNA595703.

**Microbiological culture of Neisseria spp. isolates from the bronchiectasis airway**
The presence of viable *Neisseria* spp. in the sputum of Asian bronchiectasis patients was assessed by inoculation of chocolate agar (BD-BBL) with a loopful of sputum incubated overnight at 37 °C under 5% CO₂. Colourless colonies isolated on chocolate agar were presumptively identified by Gram staining, oxidase test and BD BBL™ Crystal™ microbial ID strips (N/H), and further assessed using a commercial MALDI TOF bacterial identification system (Bruker MALDI Biotyper Identification system). Using pure cultures of presumptively identified *Neisseria* spp., high quality genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit for use in more definite confirmation by whole-genome sequence analysis. DNA was further purified by the phenol/chloroform method. DNA was quantified by both nanodrop and qubit quantification. For 10-20kb SMRTbell libraries, the g-DNA concentration was maintained at least at 200ng/ul. Libraries for long-read sequencing were constructed using the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences, USA) and subjected to SMRT sequencing (Pacific Biosciences RSII). Short reads (2x250 bp paired end) were also generated on a MiSeq platform (Illumina) using the NexteraXT library preparation kit (Illumina). De novo assembly of PacBio sequence reads was performed using the Hierarchical Genome Assembly Process (HGAP) version 3 included in the PacBio SMRT Analysis 2.3.0 package (Chin et al., 2013). The assembly was then error corrected and polished with paired end reads from an Illumina MiSeq run using Quiver and Pilon version 1.16, respectively (Walker et al., 2014). Phylogenetic comparison of polished genome assemblies with publicly available reference genomes for several *Neisseria* species, based on core genome alignment, was performed using the Roary pan-genome analysis pipeline with tree visualisation in iTol (Letunic and Bork, 2019; Page et al., 2015). Genome assemblies have been deposited at the National Center for Biotechnology Information (NCBI) under whole genome shotgun (WGS) project accession PRJNA714914.

**Infection of Air-liquid interface (ALI) cultures with Neisseria subflava**
HNECs and HBECs cells were grown to confluence as a monolayer from which 2x10⁵ cells seeded on the apical chamber of a 24-well transwell (Coming transwell 3470) pre-coated with human collagen I (Advanced Biomatrix, USA). The transwell with HNECs or HBECs was first cultured in submerged phase. Once the cell layer became intact, the medium on the apical chamber was removed and the cells were cultured at air-liquid interface (ALI) thereafter. Medium was refreshed twice a week and the HNECs and HBECs were differentiated for 21 days using ALI-Diff medium (Advanced DMEM/F12 supplemented with Penicillin/Streptomycin (100 μg/ml), hydrocortisone (0.5 μg/ml), Epinephrine (0.5 μg/ml), 3,3',5-Triido-L-thyronine (100 nM), human EGF (0.5 ng/ml), TTNPB (100 nM) and A83-01 (50 nM)) before infection experiments. ALI-HNECs or HBECs were cultured in antibiotic-free medium one-week before and throughout infection. The *Neisseria subflava* clinical isolate SG-KL01 was cultured on chocolate agar plate at 37 °C under 5% CO₂ for 24h. Colonies were picked and dispersed in PBS and gently bead-beaten to yield a homogenous solution. Twenty microlitres of homogenous bacterial suspension at a concentration of at least ~8.7x 10⁷ CFU/ml was added to the apical chamber for infection and incubated at 37 °C, 5% CO₂ for 2h. For gene expression experiments, cells were lysed with buffer RLT Plus (Qiagen, USA) with β-mercaptoethanol at 2, 24, 48 and 72 hours post infection (hpi). To quantify internalized bacteria, infected cells were washed twice with PBS both apically and basolaterally to remove external bacteria at 24, 48 and 72 hpi. The cells were refreshed with ALI-Diff medium with 1% penicillin/streptomycin and incubated at 37 °C, 5% CO₂ for 1h to kill all the adhered and external bacteria. 0.5% of Triton X-100 was added to the cells for 15 mins at room temperature to detach cells and release the internalized bacteria. Serial dilution of bacterial suspensions was performed and spread-plated on LB agar plates. Plates were incubated at 37 °C, 5% CO₂ for 24h to determine CFU/ml.

**Trans-epithelial electrical resistance (TEER)**
Cellular junction integrity of ALI cultures was assessed before and during infection by measuring the TEER using EVOM2 with STX2 chopstick electrode (World Precision Instruments, USA). TEER values were indicated as Ω•cm² and calculated as TEER = (measured value – background value) *surface area of transwell insert in cm².

**Quantification of gene expression by qRT-PCR**
Total RNA was extracted from cell lysates using a Qiagen RNeasy Plus mini kit following manufacturer’s instructions. Extracted RNA was then reverse transcribed into cDNA with a PrimeScript RT reagent kit (Takara, Japan). mRNA expression was measured by real-time PCR amplification with GoTaq® qPCR Master Mix (Promega, USA) with specific primers (see Table S7) using an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA).

**Immune profiling of mouse bronchoalveolar lavage fluid (BALF)**
Immune cells were harvested from infected mice by bronchoalveolar lavage (BAL). One millilitre of sterile PBS was injected into the mouse lung through the trachea and retrieved into a syringe. BALF contains immune cells and debris from damaged lung tissue. Cells
were pelleted by centrifugation at 500 \times g for 10 min and stained by Giemsa (Beyotime) or subjected to flow cytometry. Flow cytometry was performed using a BD FACSAria. The following surface markers were used: macrophages (F4/80-fluorescein isothiocyanate [FITC] and CD45-phycocerythrin [PE]), neutrophils (Ly6G-FITC), natural killer cells (NK1.1-PE), T cytotoxic cells (CD3-allophycocyanin [APC] and CD8-FITC), T-helper cells (CD3-APC and CD4-PE), and B cells (CD19-APC). Unstained cells served as negative controls. Antibodies for cell staining are listed and further detailed in the key resources table.

Staining of lung tissue
Lung tissue samples from either mouse experiments or human biopsies were dehydrated and embedded in paraffin. Five-micrometre sections were next mounted on slides coated with Superfrost Plus (Thermal Scientific). Sections were dewaxed and rehydrated in water. Hematoxylin and eosin (H&E) staining (Beyotime) was performed for histopathological analysis of lung tissues. The presence of *Neisseria subflava* in lung sections was assessed by employing fluorescent in situ hybridization (FISH) (Fanger et al., 1997). Briefly, following dehydration of fixed lung sections in an ethanol series (3 min each in 50, 80, and 98% ethanol), overnight hybridization to 16S rRNA of *N. subflava* was performed with a probe tagged with Cy5 in a hybridization buffer containing 30% formamide. After washing steps, sections were mounted with an antifade reagent containing DAPI (4',6-diamidino-2-phenylindole, Beyotime), and images captured using an Observer Z1 fluorescence microscope (Carl Zeiss).

Mouse transcriptome sequencing
Murine lung tissue specimens were harvested employing a double-blinded experimental design and involving three independent operators. Samples at each timepoint were harvested in parallel across the experimental conditions and processed promptly (within 2 hours), thus limiting both potential operator-specific and temporal processing effects. Total RNA was extracted from frozen mouse lung tissues using TRIzol (Thermo Fisher). The integrity of the total RNA and level of DNA contamination was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies) and Qubit 2.0 fluorometer (Invitrogen). Library preparation was performed according to the mRNA-seq sample preparation kit (Illumina) and RNA sequencing performed on an Illumina MiSeq platform with single-end 75-bp sequencing. Sequencing reads were mapped to the mouse genome using the CLC Genomics Workbench 9.0. Differential gene expression was determined using the R statistics package DESeq2. The following criteria were adopted to filter the unique sequence reads: maximum number of hits for a read of 1, minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of two mismatches. A constant of 1 was added to the raw transcript count value to avoid any issues with zero values. Transcript counts were normalized to the effective library size. The differentially expressed genes were identified by performing a negative binomial test and transcripts determined as differentially expressed when they showed a >=2-fold change with an adjusted P value of at least 0.05. Pathway analysis and data presentation was performed using Ingenuity Pathway Analysis (IPA) software (Qiagen Bioinformatics). RNA sequencing data of the *Neisseria subflava* infection model has been deposited in NCBI’s Sequence Read Archive (SRA) database under the Accession Number PRJNA706545.

Metabolite and lipid extraction
Lung tissue or serum metabolite (Ivanisevic et al., 2013) and lipid (Chen et al., 2013; Xu et al., 2020) extraction methods were obtained from prior studies. For the lung tissue samples, weighted (~10 mg) freeze-tissue samples were homogenized using steel beads with thaw twice. The suspension was then further split into two portions of 1,000 \mu L and 200 \mu L, to be used for metabolomics and lipidomics analysis, respectively. The metabolomic portion was placed at -20°C for 1 hour, followed by cryopreservation at -20°C for 20 mins, followed by drying of the supernatant by speed-vacuum evaporator at 4°C. Next, this was dissolved with a weight-normalized volume (10 mg lung tissue equals 100 \mu L) of ice-cold acetonitrile:H2O (1:1, v/v) for further mass spectrometry analysis. The 200 \mu L lipidomic portion was added to methyl tert-butyl ether (MTBE) and water and kept at a 20:6:7 (MTBE: H2O v/v/v) ratio in a glass vial, followed by sonicating (40 kHz and 100 W) for 30 mins in an ice bath, with centrifugation at 3,000 rpm/min at 4°C for 15 mins to facilitate phase separation. The upper organic phase (with enriched lipids) was collected and dried under nitrogen blow, and reconstituted with weight-normalized volume (10 mg lung tissue equals 50 \mu L) of isopropanol:chloroform:methanol (1:1:1, v/v/v) which was further diluted with the same volume of the initial LC-phases for further mass spectrometry analysis. For serum metabolite extraction, both mouse and human samples (Table S4) were used. Here, 400 \mu L ice-cold methanol:acetonitrile (1:1, v/v) was added to 100 \mu L serum, vortexed for 30s, and placed at -20°C for 1 h, followed by centrifugation at 14,000 rpm/min at 4°C for 20 mins to obtain supernatant. The supernatant was then dried by speed-vacuum evaporator at 4°C followed by dissolution in 100 \mu L ice-cold acetonitrile:H2O (1:1, v/v) for further mass spectrometry analysis. For serum lipid extraction, and comparable to lung samples, 100 \mu L of serum was added to MTBE and methanol (final ratio of MTBE:methanol:serum maintained at 20:6:7 (v/v/v)), sonicated and centrifuged to obtain the upper organic phase. This was followed by drying under nitrogen blow and reconstitution with 50 \mu L isopropanol:chloroform:methanol (1:1:1, v/v/v) with further dilution with 50 \mu L initial LC-phases for mass spectrometry analysis. The metabolomics and lipidomics quality control (QC) samples were prepared by mixing identical volumes (as described in the respective figure legends) obtained from all metabolome and/or lipidome samples, respectively.

Instrumental analysis and metabolite-lipid identification
Metabolite and lipid fractions were separated and acquired using an Agilent ultra-high-performance liquid chromatography (1290 UHPLC series, Agilent Technologies, USA) system coupled with quadrupole time-of-flight (Q-TOF) mass spectrometry (6550 iFunnel Q-TOF, Agilent Technologies, USA). Metabolite separation was performed on an Amide column (UPLC BEH Amide,
1.7 μm, 100×2.1 mm, Waters Corporation, Milford, USA) and a hydrophilic interaction chromatography (HILIC) aminopropyl column (3 μm, 150×10 mm, Phenomenex, Torrance, USA) in positive and negative ion mode, respectively. In positive ion mode, the mobile phases comprised of phase A (25 mM NH₄OH and 25 mM NH₄OAc in water) and phase B (ACN), gradient eluted by 95% B (0~0.5 min); 95% B to 65% B (0.5~7 min); 65% B to 40% B (7~8 min); 40% B (8~9 min); 40% B to 95% (9~9.1 min) and 95% (9.1~12 min), with a consistent flow rate of 0.5 mL min⁻¹. Injection volume and column temperature was set at 5 μL and 25 °C, respectively. In the negative ion mode, the mobile phases comprised of phase A (ACN: water (5:95, v/v, with 40 mM ammonium hydroxide, pH 9.8)) and phase B (ACN: water (95:5, v/v, with 20 mM ammonium acetate)), gradient eluted by 100% B (0~2 min); 100% B to 10% B (2~15 min); 10% B to 0% B (15~17 min); and 0% B (17~33 min), with a consistent flow rate of 0.25 mL min⁻¹. Injection volume and column temperature was set at 6μL and 37 °C, respectively. Lipid separation was conducted on a C18 Column (UPLC BEH C18, 1.7 μm, 50×2.1 mm, Waters Corporation, Milford, USA) in dual ion modes. The gradient elution comprised of phase A (ACN: water (6:4, v/v, with 10 mM ammonium formate)) and phase B (IPA: ACN (9:1, v/v, with 10 mM ammonium formate)), gradient eluted by 40% to 100% B (0~10min), 100% B (10~12min), 100% B to 0% B (12~12.2 min) and 40% B (12.2~15 min) at a consistent flow rate of 0.4mL min⁻¹. Injection volume and column temperature was set at 5μL and 55 °C, respectively. Before running samples for metabolomic and lipidomic analysis, eight QC samples were first run to stabilize the system and then repeated after every six samples to monitor system stability. The acquired raw data was imported into XCMS (Smith et al., 2006) to obtain the ion correction and alignment and significant ions (with p-value<=0.05 and |fold change|>=1.5) were selected for further processing. Metabolite and lipid identification was confirmed with reference to the online databases METLIN (Domingo-Almenara et al., 2019), MS-DIAL (Tsugawa et al., 2015) and LipidMaps (Sud et al., 2007).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis and data visualization
The Shapiro-Wilk normality test was used to examine data distributions. For continuous variables, statistical significance was determined using Mann-Whitney and/or Kruskal-Wallis test with Dunn’s test for post-hoc analysis and Benjimini-Hochberg correction where more than two groups were present. Correlations were assessed using the “rcorr” function within the R package “Hmisc” (Harrell, 2019). For categorical variables, Pearson’s Chi-squared test or Fisher’s Exact test (as appropriate) was implemented with Bonferroni correction for multiple comparisons when contingency extended beyond 2x2 (MacDonald and Gardner, 2000). In all cases, two-tailed analysis was considered, and differences were deemed significant at p<0.05. Analysis was performed using R Statistical Software (v3.5.1) using the “ggplot2” package for visualisation (Wickham, 2016). For microbiome analysis, alpha and beta diversity were assessed using the “vegan” package (Oksanen et al., 2013) while discriminant taxa were identified using Linear Discriminant Analysis Effect Size (LEfSe, http://huttenhower.sph.harvard.edu/galaxy) (Segata et al., 2011). Metagenomic data were visualised using MEGAN (Huson et al., 2016).

Transcriptomic analysis, functional enrichment analysis and weighted correlation network analysis
Differential gene expression between treatment groups was analyzed with DESeq2 (Love et al., 2014). Genes were considered differentially expressed when a log₂ Fold Change > ± 1 and false discovery rate < 0.05 was obtained. The functional enrichment analysis of the differentially expressed genes (DEGs) and differential metabolites was performed using Ingenuity Pathway Analysis (IPA) (Qiagen Inc., Germany). The functional network of DEGs and differential metabolites was constructed using Cytoscape (Reimand et al., 2019). Weighted correlation network analysis (WGCNA) was performed to identify gene modules that strongly correlate to temporal changes of serum metabolite markers (Langfelder and Horvath, 2008).