The Lung Microbiome in Virus-induced Asthma Exacerbations

Dr Ernie Hoi Cheung Wong

Airways Disease Infection Section
National Heart and Lung Institute
Imperial College London
Norfolk Place
London, United Kingdom
W2 1PG

A thesis submitted for the degree of Doctor of Philosophy:

February 2017
Abstract

Acute asthma exacerbations cause significant morbidity and healthcare burden. Up to 60% of acute exacerbations are associated respiratory viruses, particularly human rhinoviruses. The role of bacteria in acute exacerbations is unclear yet antibiotics are frequently prescribed. Recent studies revealed a greater abundance of potentially pathogenic bacteria (e.g. *Haemophilus* spp.) within the airway microbiota in asthma, whilst a greater abundance of commensals (e.g. *Prevotella* spp.) were observed in health. The current project examined the dynamics of the airway microbiota in the context of a virus-induced asthma exacerbation.

The airway microbiota was assessed in a cohort of mild/ moderate asthmatic subjects. Sputum samples were obtained at baseline and following naturally-occurring cold and underwent 16S rRNA gene sequencing. During acute cold, increased relative abundance of *Neisseria* sp. (*Neisseria_2974*) significantly correlated with greater peak flow (PEF) decline and IL-1β level. In contrast, *Prevotella* and *Veillonella* sp. (*Veillonella_10839*) relative abundances correlated with reduced PEF decline and lower IL-1β and IL-8 levels respectively.

To validate these findings and evaluate the impact of human rhinovirus on the airway microbiota, a cohort of moderate asthmatic and healthy subjects were experimentally infected with rhinovirus-16. Bronchoalveolar lavage was obtained at baseline and at two time-points post infection. The microbiota community between asthmatic and healthy subjects did not differ significantly at baseline or post rhinovirus-16 infection. Following rhinovirus-16 infection, increased *Neisseria_2074* relative abundance again correlated with greater PEF decline whilst increased *Prevotella* relative abundance correlated with reduced clinical symptoms. Furthermore, rhinovirus-16 viral load exhibited a significant linear
relationship with the extent of microbiota community change, suggesting that severity of rhinovirus-16 infection may directly impact on the microbiota.

In conclusion, an imbalanced airway microbiota was associated with greater PEF decline and pro-inflammatory cytokine levels during a virus-induced asthma exacerbation, though the precise role of the microbiota remains to be determined.
# Table of contents

Abstract ........................................................................................................................................... 2

Table of contents ........................................................................................................................... 4

Figures and tables ........................................................................................................................... 14

Acknowledgements ......................................................................................................................... 25

Statement of personal contribution to this study ............................................................................. 27

Copyright declaration ...................................................................................................................... 28

Commonly used abbreviations ....................................................................................................... 29

1 Introduction .................................................................................................................................. 30

1.1 Asthma ...................................................................................................................................... 30

1.1.1 Definition ............................................................................................................................ 30

1.1.2 Epidemiology and socio-economic burden ......................................................................... 30

1.1.3 Aetiology ........................................................................................................................... 31

1.1.4 Pathophysiology, phenotypes and endotypes ..................................................................... 34

1.1.5 Diagnosis ........................................................................................................................ 35

1.1.6 Management ..................................................................................................................... 36

1.2 Acute asthma exacerbations ..................................................................................................... 39

1.2.1 Definition and classification ............................................................................................. 39

1.2.2 Epidemiology and socio-economic impact ....................................................................... 39

1.2.3 Predisposing factors ......................................................................................................... 40

1.2.4 Triggers of asthma exacerbations ...................................................................................... 40

1.2.5 Management ..................................................................................................................... 41

1.3 The role of viruses in asthma exacerbations ........................................................................... 42
1.3.1 Human rhinoviruses................................................................. 43
1.3.2 Mechanism of human rhinovirus-induced asthma exacerbations ......................................................... 46
1.4 The role of bacteria in asthma.............................................................................................................................. 48
  1.4.1 Development of asthma ................................................................................................................................. 48
  1.4.2 Persistence of chronic asthma ......................................................................................................................... 49
  1.4.3 Acute asthma exacerbations ......................................................................................................................... 51
  1.4.4 Summary .......................................................................................................................................................... 53
1.5 The concept of microbiota ..................................................................................................................................... 54
1.6 16S rRNA gene sequencing...................................................................................................................................... 55
  1.6.1 A brief history of sequencing technology ........................................................................................................ 55
  1.6.2 Operational taxonomic units (OTUs) ............................................................................................................. 56
  1.6.3 Technological variations and limitations ....................................................................................................... 57
1.7 The airway microbiome in health and disease ........................................................................................................ 58
  1.7.1 Complex microbial community within healthy airways .................................................................................... 58
  1.7.2 The airway microbiota in chronic lung disease during stable disease and exacerbations 60
  1.7.3 Current understanding on the airway microbiota ............................................................................................ 64
1.8 The asthmatic airway microbiome ......................................................................................................................... 65
  1.8.1 Imbalanced microbiota composition ............................................................................................................ 65
  1.8.2 Microbiota composition/structure associations with disease features ......................................................... 65
1.9 The concept of dysbiosis in asthma .......................................................................................................................... 68
1.10 Viral-bacterial interactions .................................................................................................................................... 70
  1.10.1 Viral infections perturb the airway microbiota ............................................................................................. 71
  1.10.2 Potential mechanisms by which viral infections may enhance bacterial colonisation and infection ................................................................................................................................. 73
  1.10.3 Microbiota may modulate response to viral infection ............................................................................... 75
1.11  Research rationale ................................................................. 75

1.12  Project aims ............................................................................. 76
    1.12.1  Naturally occurring cold ..................................................... 76
    1.12.2  Experimental RV-16 infection ............................................. 76

1.13  Hypothesis ............................................................................. 77

2  Methods ..................................................................................... 78
    2.1  Outline ................................................................................. 78
    2.2  Materials .............................................................................. 78

2.3  The asthmatic airway microbiota in naturally occurring colds ........ 81
    2.3.1  Study aim .......................................................................... 81
    2.3.2  Study cohort ....................................................................... 81
    2.3.3  Ethics and consent ............................................................... 83
    2.3.4  Study design ....................................................................... 83
    2.3.5  Visit breakdown .................................................................. 84
    2.3.6  Spirometry ......................................................................... 87
    2.3.7  Histamine challenge test ...................................................... 87
    2.3.8  The Wisconsin Upper Respiratory Symptom Survey (WURRS-21) ......................................................... 87
    2.3.9  Nasosorption ....................................................................... 89
    2.3.10 Nasal lavage ....................................................................... 89
    2.3.11 Throat swab ....................................................................... 90
    2.3.12 Sputum induction ............................................................... 90

2.4  The airway microbiota in experimental rhinovirus infection ........... 91
    2.4.1  Study aim .......................................................................... 91
    2.4.2  Study cohort ....................................................................... 91
    2.4.3  Ethics and consent ............................................................... 94
    2.4.4  Study design ....................................................................... 94
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.5</td>
<td>Histamine challenge</td>
</tr>
<tr>
<td>2.4.6</td>
<td>Portable spirometry</td>
</tr>
<tr>
<td>2.4.7</td>
<td>Symptom scores</td>
</tr>
<tr>
<td>2.4.8</td>
<td>Bronchoscopic sampling</td>
</tr>
<tr>
<td>2.4.9</td>
<td>Nasosorption and nasal lavage</td>
</tr>
<tr>
<td>2.4.10</td>
<td>RV-16 inoculation</td>
</tr>
<tr>
<td>2.4.11</td>
<td>Diagnosis of clinical cold</td>
</tr>
<tr>
<td>2.4.12</td>
<td>Confirmation of RV-16 infection</td>
</tr>
<tr>
<td>2.5</td>
<td>Virology testing</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Neutralising antibodies to RV-16</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Quantitative PCR for RV-16</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Qualitative PCR for respiratory viruses</td>
</tr>
<tr>
<td>2.6</td>
<td>Quantitative bacterial culture</td>
</tr>
<tr>
<td>2.7</td>
<td>Pro-inflammatory cytokine measurement</td>
</tr>
<tr>
<td>2.8</td>
<td>IP-10 measurement</td>
</tr>
<tr>
<td>2.9</td>
<td>16S rRNA gene sequencing</td>
</tr>
<tr>
<td>2.9.1</td>
<td>Genomic DNA extraction</td>
</tr>
<tr>
<td>2.9.2</td>
<td>Assessing bacterial burden – 16S rRNA gene quantitative PCR</td>
</tr>
<tr>
<td>2.9.3</td>
<td>Quadruplicate PCR</td>
</tr>
<tr>
<td>2.9.4</td>
<td>Contamination check</td>
</tr>
<tr>
<td>2.9.5</td>
<td>Pooling of replicate PCR reactions</td>
</tr>
<tr>
<td>2.9.6</td>
<td>Purification (1st round)</td>
</tr>
<tr>
<td>2.9.7</td>
<td>PicoGreen® Quantification</td>
</tr>
<tr>
<td>2.9.8</td>
<td>Equimolar pooling and purification (2nd round)</td>
</tr>
<tr>
<td>2.9.9</td>
<td>Gel purification of concentrated pooled library</td>
</tr>
<tr>
<td>2.9.10</td>
<td>Library quantification qPCR</td>
</tr>
</tbody>
</table>
Illumina® MiSEQ sequencing .......................................................... 122

2.10 Sequence processing ........................................................................ 123

2.11 Data and statistical analyses .............................................................. 125
  2.11.1 Group comparisons .................................................................. 125
  2.11.2 Correlation and linear regression .............................................. 126
  2.11.3 Micobiota analysis .................................................................. 126

3 Clinical characteristics and outcomes .................................................. 129

3.1 Introduction ...................................................................................... 129

3.2 Naturally occurring colds .................................................................... 130
  3.2.1 Baseline clinical characteristics ................................................. 130
  3.2.2 Presentation of colds ................................................................. 131
  3.2.3 Respiratory virus detection ....................................................... 133
  3.2.4 Quantitative bacterial culture .................................................... 133
  3.2.5 Cold symptoms ...................................................................... 134
  3.2.6 Changes in lung function .......................................................... 138
  3.2.7 Pro-inflammatory cytokines ...................................................... 142
  3.2.8 Biomarker of viral infection ..................................................... 146
  3.2.9 Clinical management of colds ................................................... 148

3.3 Experimental RV-16 infection ............................................................ 148
  3.3.1 RV-16 infection and clinical demographics .............................. 148
  3.3.2 Upper respiratory symptoms ................................................... 149
  3.3.3 Lower respiratory symptom scores .......................................... 151
  3.3.4 Changes in lung function .......................................................... 153
  3.3.5 Changes in airway hyper-responsiveness ................................. 154
  3.3.6 Detection of RV-16 virus load ................................................... 155
  3.3.7 Biomarker of viral infection ..................................................... 157
5.4 Bacterial burden ................................................................. 186
5.4.1 Changes during stable chronic disease and naturally occurring cold ................. 186
5.4.2 Correlation with clinical parameters, pro-inflammatory cytokines and alpha diversity measures .................................................................................................................. 189
5.4.3 Summary .................................................................................. 190
5.5 Hierarchical clustering and indicator species analysis .............................................. 194
5.6 Cluster-based analyses ......................................................................................... 196
5.6.1 Baseline (Visit 1) ............................................................................. 196
5.6.2 Acute cold (Visit 2, day 1/2 post cold) ....................................................... 200
5.6.3 Middle of cold (Visit 3, day 4/5 post cold) ................................................... 202
5.6.4 End of cold (Visit 4) .............................................................................. 206
5.6.5 Summary of cluster-based analyses ............................................................... 206
5.7 Changes in microbial community structure following cold ..................................... 209
5.7.1 Alpha diversity ...................................................................................... 209
5.7.2 Beta diversity ....................................................................................... 212
5.7.3 Cluster stability in stable chronic disease and following natural cold .................. 216
5.7.4 Summary ............................................................................................. 219
5.8 Specific phyla, genera & OTUs ........................................................................... 220
5.8.1 Neisseria_2974 ..................................................................................... 221
5.8.2 Prevotella_6482 ................................................................................... 225
5.8.3 Veillonella_10839 ................................................................................... 229
5.8.4 Haemophilus at Genus level ..................................................................... 232
5.8.5 Proteobacteria ....................................................................................... 235
5.8.6 Summary ............................................................................................. 240
5.9 Discussion ................................................................................................. 241
5.9.1 Bacterial burden ........................................................................................................241
5.9.2 Hierarchical clusters and indicator species ...............................................................244
5.9.3 Clinical correlations with clusters and OTUs ............................................................245
5.9.4 Microbial community changes following natural cold .............................................249
5.10 Conclusions ................................................................................................................252
5.11 Summary of key findings ............................................................................................253

6 The airway microbiota in experimental rhinovirus infection ...........................................254
  6.1 Introduction ....................................................................................................................254
  6.2 Aims ..............................................................................................................................256
  6.3 Hypotheses ....................................................................................................................256
  6.4 Bacterial burden ..........................................................................................................256
    6.4.1 Changes following experimental RV-16 infection ..................................................256
    6.4.2 Correlation with clinical parameters, alpha diversity, Th-2 mediated and pro-
          inflammatory cytokines ...........................................................................................258
    6.4.3 Summary ................................................................................................................261
  6.5 Hierarchical clustering and indicator species analysis .................................................262
  6.6 Differences in microbiota composition between asthma and health ..........................264
    6.6.1 Differences in specific phyla and genera ................................................................266
    6.6.2 Summary ................................................................................................................270
  6.7 Microbial community changes following RV-16 infection .........................................270
    6.7.1 Alpha diversity ......................................................................................................270
    6.7.2 Beta diversity ........................................................................................................274
    6.7.3 Impact of RV-16 infection on beta diversity ..........................................................275
    6.7.4 Correlations with clinical outcomes and pro-inflammatory cytokines ..................277
    6.7.5 Summary ................................................................................................................279
  6.8 Specific phyla, genera and OTUs ..................................................................................280
6.8.1  Neisseria_2974 ......................................................... 280
6.8.2  Prevotella_6482 .......................................................... 285
6.8.3  Veillonella_10839 .......................................................... 295
6.8.4  Haemophilus at Genus level ............................................ 300
6.8.5  Summary ................................................................. 305

6.9  Discussion .................................................................... 306

6.9.1  Bacterial burden (BB) ................................................... 306
6.9.2  Hierarchical clustering and indicator species analysis in BAL reveal similar results to those observed in naturally-occurring colds ............................................... 308
6.9.3  No significant difference in microbiota composition between asthmatic and healthy subjects at baseline or following RV-16 infection ................................................................. 309
6.9.4  Microbiota community changes following RV-16 infection ................................................................. 310
6.9.5  Clinical correlations with specific OTUs and genera ................................................................. 314

6.10  Conclusions .................................................................. 315
6.11  Summary of key findings .................................................. 316

7  Discussion ........................................................................ 318

7.1  Introduction ..................................................................... 318
7.2  RV infection was not associated with secondary bacterial infection ................................................................. 318
7.3  The airway microbiota was more variable than previously proposed though distinct clusters were present ................................................................. 319
7.4  RV-16 viral load significantly correlated with beta diversity ................................................................. 320
7.5  Increased Neisseria_2974 relative abundance was associated with worse clinical outcomes ................................................................. 322
7.6  Increased Prevotella relative abundance was associated with improved clinical outcomes ................................................................. 322
7.7 Potential mechanisms by which the airway microbiota may play a role in virus-induced asthma exacerbations ........................................................................................................... 323

7.8 Limitations of study ..................................................................................................................... 324

7.9 Future directions .......................................................................................................................... 325

7.9.1 Selective quantitative culture to identify Neisseria_2974 and Prevotella species, in vitro and in vivo stimulation studies .......................................................................................................................... 326

7.9.2 Metagenomic sequencing to assess interactions between bacterial, viral and fungal communities ........................................................................................................................................ 326

7.9.3 Optimise future clinical studies .................................................................................................. 327

7.10 Concluding remarks .................................................................................................................... 327

8 References ...................................................................................................................................... 329

9 Appendix 1 – R Script ....................................................................................................................... 347

10 Appendix 2 – 16S rRNA gene sequencing barcodes ......................................................................... 356

11 Appendix 3 – Copyright clearance ................................................................................................. 357
Figures and tables

FIGURE 1.1 DIAGNOSTIC WORK FLOW OF PATIENTS WITH SUSPECTED ASTHMA. 36

FIGURE 1.2 APPROXIMATELY 1.5 KB 16S RNA GENE OF E.COLI SHOWING THE NINE VARIABLE REGIONS THAT MAKE IT AN IDEAL TARGET AS A PHYLOGENETIC MARKER GENE. 56

FIGURE 1.3 DISTRIBUTION OF THE PHYLA FROM BRONCHO-ALVEOLAR LAVAGE (BAL) IN CHILDREN WITH DIFFICULT ASTHMA AND CONTROLS. 60

FIGURE 1.4 CYCLE OF HOST INFLAMMATION AND RESPIRATORY DYSBIOSIS. 70

FIGURE 1.5 POTENTIAL MECHANISMS BY WHICH VIRAL INFECTIONS MAY PROMOTE BACTERIAL COLONISATION AND SUBSEQUENT INFECTION. 73

FIGURE 2.1 DESIGN OF NATURALLY OCCURRING COLD STUDY 84

FIGURE 2.2 THE WISCONSIN UPPER RESPIRATORY SYMPTOM SURVEY (WURRS-21) AS DESIGNED BY BARRETT AND CO-WORKERS. 88

FIGURE 2.3 (A) NASOSORPTION USING SYNTHETIC ABSORPTIVE MATRIX (SAM) BEING ADVANCED INTO THE RIGHT NOSTRIL (B) SAM STRIP DEPLOYED IN NOSE. 89

FIGURE 2.4 DESIGN OF EXPERIMENTAL RV-16 INFECTION STUDY 95

FIGURE 2.5 SYMPTOM DIARY CARD RECORDING UPPER AND LOWER RESPIRATORY TRACT SYMPTOMS AND HOME SPIROMETRY. 98

FIGURE 2.5 SAMPLING BRONCHIAL MUCOSAL LINING FLUID FROM THE RIGHT LOWER LOBE OF THE LUNG BY BRONCHOSORPTION. 100

FIGURE 2.7 INOCULATION ATOMISER (MODEL 286-RD, DEVILBISS HEALTHCARE) 101

FIGURE 2.8 OVERVIEW OF 16S RNA SEQUENCING AND DOWNSTREAM ANALYSIS PIPELINE 112

FIGURE 2.9 ORIENTATION OF INDEXED PRIMERS (LABELLED 701 – 712 AND 501 – 508) ON A 96-WELL PCR PLATE 116

FIGURE 2.10 GEL ELECTROPHORESIS FOR CONTAMINATION CHECK POST QUADRUPLICATE PCR. 117

FIGURE 2.11 GEL ELECTROPHORESIS ON CLINICAL SAMPLES TO ENSURE ADEQUATE PCR AMPLIFICATION. 118

FIGURE 2.12 EXAMPLE OF EQUIMOLAR POOLING CALCULATION. 120

FIGURE 2.13 BIOANALYSER RESULT SHOWING A PEAK AT 334BP (APPROPRIATE SIZE FOR LIBRARY) 121
FIGURE 2.14 FLOW DIAGRAM OF SEQUENCING PROCESSING USING QIIME

FIGURE 3.1 MONTH OF REPORTED Colds

FIGURE 3.2 SYMPTOMS FOLLOWING NATURALLY OCCURRING Colds.

FIGURE 3.3 TOTAL SYMPTOM SCORES OVER 28 DAYS IN SUBJECTS WITH RESPECT TO VIROLOGY AND QUANTITATIVE BACTERIAL CULTURE RESULTS.

FIGURE 3.4 TOTAL COLD SYMPTOMS OVER 28 DAYS SIGNIFICANTLY CORRELATED WITH ACQ AT BASELINE.

FIGURE 3.5 MAXIMAL CHANGE IN PEF FOLLOWING COLD IN SUBJECTS WITH DIFFERENT DISEASE SEVERITY.

FIGURE 3.6 CHANGE IN LUNG FUNCTION FOLLOWING COLD.

FIGURE 3.7 CORRELATION BETWEEN CHANGE IN PEF AND COLD SYMPTOMS.

FIGURE 3.8 LEVELS OF PRO-INFLAMMATORY CYTOKINES AT BASELINE (VISIT 1) AND FOLLOWING COLD (VISITS 2 – 4).

FIGURE 3.9 INDIVIDUAL SUBJECT TRENDS FOR CHANGES IN PRO-INFLAMMATORY CYTOKINE LEVELS.

FIGURE 3.10 IP-10 LEVELS AT BASELINE (VISIT 1) AND FOLLOWING COLD (VISITS 2 – 4).

FIGURE 3.11 INDIVIDUAL SUBJECT TRENDS FOR FOLD CHANGES IP-10 LEVELS.

FIGURE 3.12 DAILY UPPER RESPIRATORY TRACT SYMPTOM SCORES FOLLOWING RV-16 INOCULATION.

FIGURE 3.13 DAILY LOWER RESPIRATORY TRACT SYMPTOM SCORES FOLLOWING RV-16 INOCULATION.

FIGURE 3.14 TOTAL UPPER RESPIRATORY SYMPTOMS STRONGLY CORRELATED WITH TOTAL LOWER RESPIRATORY SYMPTOMS.

FIGURE 3.15 BASELINE ACQ AND RESPIRATORY SYMPTOM SCORES.

FIGURE 3.16 TOTAL LOWER RESPIRATORY SYMPTOMS SIGNIFICANTLY CORRELATED WITH MAXIMAL DECLINE IN PEF FROM BASELINE POST RV-16 INFECTION.

FIGURE 3.17 DAILY CHANGE IN PEF FROM BASELINE FOLLOWING RV-16 INOCULATION.

FIGURE 3.18 CHANGE IN AIRWAY HYPER-RESPONSIVENESS IN ASTHMATIC SUBJECTS FOLLOWING RV-16 INFECTION AS ASSESSED BY HISTAMINE PROVOCATION TEST (PC_{20} HISTAMINE).

FIGURE 3.19 VIRUS LOAD IN NASAL LAVAGE.

FIGURE 3.20 LEVELS OF IP-10 FOLLOWING RV-16 INFECTION.

FIGURE 3.21 LEVELS OF PRO-INFLAMMATORY CYTOKINES AT BASELINE (DAY 0), DAY 3 AND 8 POST RV-16 INFECTION.
FIGURE 3.22 LEVELS OF TH2-MEDIATED CYTOKINES AT BASELINE (DAY 0), PEAK LEVEL DURING RV-16 INFECTION (DAY 1 – 14) AND DAY 42 POST-INFECTION.

FIGURE 4.2 THE NUMBER OF SEQUENCE Reads PER SAMPLE FOR THE 100 SAMPLES WITH THE LEAST SEQUENCE Reads.

FIGURE 4.1 THE NUMBER OF SEQUENCE Reads PER SAMPLE.

FIGURE 4.3 READ LENGTH DISTRIBUTION ACROSS ALL SEVEN SEQUENCING RUNS.

FIGURE 4.4 CORRELATION BETWEEN BIOMASS/ BACTERIAL BURDEN AND SEQUENCE COUNTS.

FIGURE 4.5 CORRELATION BETWEEN BAL SAMPLE ALIQUOT VOLUME AND SEQUENCE COUNTS.

FIGURE 4.6 NMDS PLOT USING Bray-Curtis similarITy MEASURE OF ALL SAMPLES FROM NATURALLY OCCURRING COLD STUDY, MOCK COMMUNITIES AND NEGATIVE CONTROLS.

FIGURE 4.7 NMDS PLOT USING Bray-Curtis similarITy MEASURE OF ALL SAMPLES FROM EXPERIMENTAL RV-16 INFECTION STUDY, MOCK COMMUNITIES AND NEGATIVE CONTROLS.

FIGURE 4.8 DISTRIBUTION OF THE 27 MOST ABUNDANT GENERA IN THE MOCK COMMUNITIES ACROSS ALL SEVEN SEQUENCING RUNS.

FIGURE 4.9 SHOWS THE MOST DOMINANT AND PREVALENT OTUS IN STERILE SALINE FLUSHES THAT HAVE OVER 1000 READS POST-SEQUENCING.

FIGURE 5.1 SPUTUM BACTERIAL BURDEN DURING STABLE CHRONIC DISEASE AND NATURALLY OCCURRING COLD.

FIGURE 5.2 INDIVIDUAL SUBJECT TRENDS FOR BACTERIAL BURDEN DURING NATURALLY OCCURRING COLD AND STABLE CHRONIC DISEASE.

FIGURE 5.3 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 1 (BASELINE) AND COMMUNITY RICHNESS.

FIGURE 5.4 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 2 AND COMMUNITY RICHNESS.

FIGURE 5.5 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 3 AND IL-1β LEVEL AT VISIT 4.

FIGURE 5.6 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 3 AND COMMUNITY RICHNESS.
FIGURE 5.7 HIERARCHICAL CLUSTER ANALYSIS OF ALL SPUTUM SAMPLES USING BRAY-CURTIS SIMILARITY MEASURE.

FIGURE 5.8 CLUSTER-BASED ANALYSES ON CLINICAL PARAMETER AT BASELINE (VISIT 1).

FIGURE 5.9 CLUSTER-BASED ANALYSES ON PRO-INFLAMMATORY CYTOKINES AT BASELINE (VISIT 1).

FIGURE 5.10 CLUSTER-BASED ANALYSES ON CLINICAL OUTCOMES FOLLOWING COLD.

FIGURE 5.11 CLUSTER-BASED ANALYSIS ON CLINICAL OUTCOMES AT VISIT 2 (DAY 1/2 OF COLD ONSET).

FIGURE 5.12 CLUSTER-BASED ANALYSIS OF PRO-INFLAMMATORY CYTOKINES. PRO-INFLAMMATORY CYTOKINE (IL-1β, IL-6 AND IL-8) LEVELS OF THE DIFFERENT CLUSTERS AT VISIT 2 WERE ASSESSED

FIGURE 5.13 CLUSTER-BASED ANALYSIS ON CLINICAL OUTCOMES AT VISIT 3 (DAY 4/5 OF COLD ONSET).

FIGURE 5.14 CLUSTER-BASED ANALYSIS OF PRO-INFLAMMATORY CYTOKINES. PRO-INFLAMMATORY CYTOKINE (IL-1β, IL-6 AND IL-8) LEVELS OF THE DIFFERENT CLUSTERS AT VISIT 3 WERE ASSESSED

FIGURE 5.15 CLUSTER-BASED ANALYSIS ON CLINICAL OUTCOMES AT VISIT 4 (DAY 14 OF COLD ONSET).

FIGURE 5.16 CLUSTER-BASED ANALYSIS ON PRO-INFLAMMATORY CYTOKINE LEVELS AT VISIT 4 (DAY 14 OF COLD ONSET).

FIGURE 5.17 CHANGES IN ALPHA DIVERSITY MEASURES FOLLOWING NATURAL COLD AND DURING STABLE DISEASE.

FIGURE 5.18 BETA DIVERSITY DID NOT CHANGE SIGNIFICANTLY FOLLOWING NATURAL COLD, REGARDLESS OF VIROLOGY STATUS.

FIGURE 5.19 AT VISIT 3, UNWEIGHTED UNIFRAC DISTANCE NEGATIVELY CORRELATED WITH CHANGE IN PEF FROM BASELINE.

FIGURE 5.20 CLUSTER STABILITY FOLLOWING NATURAL COLD.

FIGURE 5.21 VIROLOGY POSITIVITY DID NOT IMPACT ON LIKELIHOOD OF CLUSTER CHANGE (VISIT 1 TO VISIT 2) FOLLOWING NATURAL COLD.

FIGURE 5.22 POSITIVE VIROLOGY DID NOT PREFERENTIALLY SELECT SPECIFIC CLUSTER FOLLOWING NATURAL COLD.

FIGURE 5.23 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT BASELINE AND MAXIMAL PEF CHANGE FROM BASELINE FOLLOWING NATURAL COLD.

FIGURE 5.24 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT VISIT 2 (ACUTE COLD, DAY 1/2 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS.
FIGURE 5.25 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT VISIT 3 (MIDDLE OF COLD, DAY 4/5 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS. 224

FIGURE 5.26 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT VISIT 4 (END OF COLD, DAY 14/15 POST COLD) AND CLINICAL OUTCOMES. 225

FIGURE 5.27 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT VISIT 1 AND MAXIMAL PEF CHANGE FOLLOWING NATURAL COLD. 225

FIGURE 5.28 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT VISIT 2 (ACUTE COLD, DAY 1/2 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS. 226

FIGURE 5.29 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT VISIT 3 (MIDDLE OF COLD, DAY 4/5 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS. 227

FIGURE 5.30 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT VISIT 4 (END OF COLD, DAY 14/15 POST COLD) AND CLINICAL OUTCOMES. 228

FIGURE 5.31 LINEAR REGRESSION BETWEEN PREVOTELLA RELATIVE ABUNDANCE AT VISIT 2 AND PEF CHANGE FROM BASELINE. 229

FIGURE 5.32 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT BASELINE (VISIT 1) AND MAXIMAL PEF CHANGE FOLLOWING NATURAL COLD. 229

FIGURE 5.33 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT VISIT 2 (ACUTE COLD, DAY 1/2 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS. 230

FIGURE 5.34 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT VISIT 3 (MIDDLE OF COLD, DAY 4/5 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS. 231

FIGURE 5.35 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT VISIT 4 (END OF COLD, DAY 14/15 POST COLD) AND CLINICAL OUTCOMES. 232

FIGURE 5.36 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT BASELINE (VISIT 1) AND MAXIMAL PEF CHANGE FOLLOWING NATURAL COLD. 233

FIGURE 5.37 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT VISIT 2 (ACUTE COLD, DAY 1/2 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS. 234

FIGURE 5.38 COMPARING PROTEOBACTERIA RELATIVE ABUNDANCE BETWEEN DIFFERENT DISEASE SEVERITY AT BASELINE. 235
FIGURE 5.39 LINEAR REGRESSION BETWEEN PROTEOBACTERIA RELATIVE ABUNDANCE AT BASELINE AND MAXIMAL PEF CHANGE FOLLOWING NATURAL COLD. 236

FIGURE 5.40 LINEAR REGRESSION BETWEEN PROTEOBACTERIA RELATIVE ABUNDANCE AT VISIT 2 (ACUTE COLD, DAY 1/2 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS 237

FIGURE 5.41 LINEAR REGRESSION BETWEEN PROTEOBACTERIA RELATIVE ABUNDANCE AT VISIT 3 (MIDDLE OF COLD, DAY 4/5 POST COLD) AND CLINICAL OUTCOMES/ PRO-INFLAMMATORY CYTOKINE LEVELS 238

FIGURE 5.42 LINEAR REGRESSION BETWEEN PROTEOBACTERIA RELATIVE ABUNDANCE AT VISIT 4 (END OF COLD, DAY 14/15 POST COLD) AND CLINICAL OUTCOMES. 239

FIGURE 6.1 CHANGES IN BRONCHOALVEOLAR LAVAGE (BAL) BACTERIAL BURDEN IN ASTHMATIC AND HEALTHY SUBJECTS FOLLOWING RV-16 INFECTION. 257

FIGURE 6.2 INDIVIDUAL SUBJECT TRENDS IN CHANGES IN BAL BACTERIAL BURDEN FOLLOWING RV-16 INFECTION. 257

FIGURE 6.3 SPEARMAN'S CORRELATION BETWEEN BAL BACTERIAL BURDEN AND FEV, % PREDICTED IN ASTHMATIC SUBJECTS AT BASELINE. 259

FIGURE 6.4 HIERARCHICAL CLUSTER ANALYSIS OF ALL BAL SAMPLES USING BRAY-CURTIS SIMILARITY MEASURE. 263

FIGURE 6.5 SAMPLE DISTRIBUTION AMONGST CLUSTERS ACROSS TIME POINTS (BASELINE, DAY 3 AND 8 POST RV-16 INFECTION) 264

FIGURE 6.6 NONMETRIC MULTIDIMENSIONAL SCALING ANALYSIS (NMDS) BASED ON BRAY-CURTIS DISTANCES SHOWING DIFFERENCES IN MICROBIOTA COMPOSITION BETWEEN ASTHMATIC AND HEALTHY SUBJECTS. 266

FIGURE 6.7 RELATIVE ABUNDANCES OF SPECIFIC PHYL A AND GENERA AT BASELINE. NO SIGNIFICANT DIFFERENCE WAS SEEN BETWEEN ASTHMA AND HEALTHY GROUP. 267

FIGURE 6.8 RELATIVE ABUNDANCES OF SPECIFIC PHYL A AND GENERA AT DAY 3 POST RV-16 INFECTION. 268

FIGURE 6.9 RELATIVE ABUNDANCES OF SPECIFIC PHYL A AND GENERA AT DAY 8 POST RV-16 INFECTION. 269

FIGURE 6.10 CHANGES IN ALPHA DIVERSITY MEASURES FOLLOWING RV-16 INFECTION. 271

FIGURE 6.11 COMPARING BETA DIVERSITY BETWEEN ASTHMATIC AND HEALTHY SUBJECTS FOLLOWING RV-16 INFECTION USING BRAY-CURTIS DISSIMILARITY 274

FIGURE 6.12 INDIVIDUAL SUBJECT TRENDS IN BETA DIVERSITY FOLLOWING RV-16 INFECTION. 275
FIGURE 6.13 LINEAR REGRESSION BETWEEN RV-16 VIRAL LOAD AT DAY 3 AND BETA DIVERSITY MEASURES AT DAY 3 (A & B) AND 8 (C & D) RESPECTIVELY.

FIGURE 6.14 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT BASELINE AND CLINICAL OUTCOMES/ BASELINE AIRWAY HYPER-RESPONSIVENESS (PC20).

FIGURE 6.15 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND CLINICAL OUTCOMES.

FIGURE 6.16 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES.

FIGURE 6.17 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND CLINICAL OUTCOMES.

FIGURE 6.18 LINEAR REGRESSION BETWEEN NEISSERIA_2974 RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES.

FIGURE 6.19 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT BASELINE AND CLINICAL OUTCOMES/ AIRWAY HYPER-RESPONSIVENESS (PC20).

FIGURE 6.20 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND CLINICAL OUTCOMES.

FIGURE 6.21 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES.

FIGURE 6.22 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND CLINICAL OUTCOMES.

FIGURE 6.23 LINEAR REGRESSION BETWEEN PREVOTELLA_6482 RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES.

FIGURE 6.24 LINEAR REGRESSION BETWEEN PREVOTELLA RELATIVE ABUNDANCE AT BASELINE AND CLINICAL OUTCOMES/ AIRWAY HYPER-RESPONSIVENESS (PC20).

FIGURE 6.25 LINEAR REGRESSION BETWEEN PREVOTELLA RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND CLINICAL OUTCOMES.

FIGURE 6.26 LINEAR REGRESSION BETWEEN PREVOTELLA RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES.
FIGURE 6.27 LINEAR REGRESSION BETWEEN PREVOTELLA RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND CLINICAL OUTCOMES. 294

FIGURE 6.28 LINEAR REGRESSION BETWEEN PREVOTELLA RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES. 295

FIGURE 6.29 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT BASELINE AND CLINICAL OUTCOMES/ AIRWAY HYPER-RESPONSIVENESS (PC_{20}). 296

FIGURE 6.30 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND CLINICAL OUTCOMES. 297

FIGURE 6.31 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES. 298

FIGURE 6.32 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND CLINICAL OUTCOMES. 299

FIGURE 6.33 LINEAR REGRESSION BETWEEN VEILLONELLA_10839 RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES. 300

FIGURE 6.34 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT BASELINE AND CLINICAL OUTCOMES/ AIRWAY HYPER-RESPONSIVENESS (PC_{20}). 301

FIGURE 6.35 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND CLINICAL OUTCOMES. 302

FIGURE 6.36 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT DAY 3 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES. 303

FIGURE 6.37 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND CLINICAL OUTCOMES. 304

FIGURE 6.38 LINEAR REGRESSION BETWEEN HAEMOPHILUS RELATIVE ABUNDANCE AT DAY 8 POST RV-16 INFECTION AND PRO-INFLAMMATORY CYTOKINES. 305

TABLE 2.1 DETAILS OF MATERIALS USED 79

TABLE 2.2 DETAILS OF CONSUMABLES USED 79

TABLE 2.3 DETAILS OF COMMERCIAL KITS USED 80
TABLE 2.4 DETAILS OF INSTRUMENTS USED
TABLE 2.5 DETAILS OF LABORATORY EQUIPMENT USED
TABLE 2.6 DETAILS OF SOFTWARES USED
TABLE 2.7 DETAILED SUBJECT VISIT PLAN OF EXPERIMENTAL RV-16 INFECTION STUDY
TABLE 2.8 VIROLOGY PCR PRIMERS
TABLE 2.9 QUALITATIVE VIROLOGY PCR MIX COMPOSITION, THERMAL CYCLE CONDITIONS AND PRODUCT SIZES
TABLE 2.10 SEQUENCING PRIMERS (EUROFINS GENOMICS, EBERSBERG, GERMANY)
TABLE 3.1 BASELINE CLINICAL CHARACTERISTICS OF RECRUITED SUBJECTS.
TABLE 3.2 CLINICAL CHARACTERISTICS OF SUBJECTS WHO PRESENTED WITH NATURALLY OCCURRING COLD.
TABLE 3.3 RESPIRATORY VIRUS DETECTION IN SUBJECTS WITH Colds.
TABLE 3.4 QUANTITATIVE BACTERIAL CULTURE IN SPUTUM SAMPLES.
TABLE 3.5 COLD SYMPTOMS.
TABLE 3.6 MEDIAN CHANGE IN LUNG FUNCTION FOLLOWING NATURAL COLD.
TABLE 3.7 SPEARMAN CORRELATION MATRIX OF PRO-INFLAMMATORY CYTOKINES.
TABLE 3.8 MEDIAN IL-6 LEVELS (PG/ML).
TABLE 3.9 CLINICAL MANAGEMENT OF Colds.
TABLE 3.10 CLINICAL DEMOGRAPHICS OF SUCCESSFULLY INFECTED SUBJECTS.
TABLE 3.11 SPEARMAN CORRELATION MATRIX OF PRO-INFLAMMATORY CYTOKINES.
TABLE 3.12 SPEARMAN CORRELATION MATRIX OF TH2-MEDIATED CYTOKINES.
TABLE 4.1 ILLUSTRATES THE NUMBER OF SAMPLES AVAILABLE POST-RAREFACTION AND NUMBER OF TAXA POST-PRUNING.
TABLE 5.1 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT BASELINE AND CLINICAL PARAMETERS, PRO-INFLAMMATORY CYTOKINES AND ALPHA DIVERSITY MEASURES.
TABLE 5.2 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 2 AND CLINICAL PARAMETERS, PRO-INFLAMMATORY CYTOKINES AND ALPHA DIVERSITY MEASURES.
TABLE 5.3 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 3 AND CLINICAL PARAMETERS, PRO-INFLAMMATORY CYTOKINES AND ALPHA DIVERSITY MEASURES.
TABLE 5.4 SPEARMAN’S CORRELATION BETWEEN SPUTUM BACTERIAL BURDEN AT VISIT 4 AND CLINICAL PARAMETERS, PRO-INFLAMMATORY CYTOKINES AND ALPHA DIVERSITY MEASURES. 193

TABLE 5.5 INDICATOR SPECIES ANALYSIS FOR CLUSTERS. 194

TABLE 5.6 CORRELATING ALPHA DIVERSITY (SHANNON DIVERSITY INDEX) WITH CLINICAL OUTCOMES AND PRO-INFLAMMATORY CYTOKINES. 211

TABLE 5.7 CORRELATING BRAY-CURTIS DISSIMILARITY WITH CLINICAL OUTCOMES AND PRO-INFLAMMATORY CYTOKINES. 214

TABLE 5.8 CORRELATING UNWEIGHTED UNIFRAC DISTANCE WITH CLINICAL OUTCOMES AND PRO-INFLAMMATORY CYTOKINES. 215

TABLE 5.9 CLUSTER STABILITY DURING STABLE CHRONIC DISEASE. 217

TABLE 5.10 CLUSTER STABILITY FOLLOWING NATURAL COLD. 218

TABLE 5.11 CORRELATION BETWEEN RELATIVE ABUNDANCE OF THE 5 MOST PREVALENT PHYLA AT BASELINE (VISIT 1). 240

TABLE 5.12 CORRELATION BETWEEN RELATIVE ABUNDANCE OF THE 5 MOST PREVALENT PHYLA DURING ACUTE COLD (VISIT 2). 240

TABLE 6.1 SPEARMAN’S CORRELATION BETWEEN BAL BACTERIAL BURDEN AT BASELINE AND CLINICAL PARAMETERS, ALPHA DIVERSITY MEASURES, TH-2 AND PRO-INFLAMMATORY CYTOKINES. 258

TABLE 6.2 SPEARMAN’S CORRELATION BETWEEN BAL BACTERIAL BURDEN AT DAY 3 POST RV-16 INFECTION AND CLINICAL PARAMETERS, ALPHA DIVERSITY MEASURES, TH-2 MEDIATED AND PRO-INFLAMMATORY CYTOKINES. 260

TABLE 6.3 SPEARMAN’S CORRELATION BETWEEN BAL BACTERIAL BURDEN AT DAY 8 POST RV-16 INFECTION AND CLINICAL PARAMETERS, ALPHA DIVERSITY MEASURES, TH-2 MEDIATED AND PRO-INFLAMMATORY CYTOKINES. 261

TABLE 6.4 INDICATOR SPECIES ANALYSIS FOR CLUSTERS. 262

TABLE 6.5 MULTIVARIATE ANOVA BASED ON DISSIMILARITIES (ADONIS) ASSESSING DIFFERENCES IN MICROBIOTA COMMUNITIES BETWEEN ASTHMATIC AND HEALTHY SUBJECTS. 265

TABLE 6.6 CORRELATING ALPHA DIVERSITY (SHANNON DIVERSITY INDEX) WITH CLINICAL OUTCOMES AND PRO-INFLAMMATORY CYTOKINES. 273
**TABLE 6.7** CORRELATING BRAY-CURTIS DISSIMILARITY WITH CLINICAL OUTCOMES AND PRO-INFLAMMATORY CYTOKINES.

**TABLE 6.8** CORRELATING UNWEIGHTED UNIFRAC DISTANCE WITH CLINICAL OUTCOMES AND PRO-INFLAMMATORY CYTOKINES.
Acknowledgements

Firstly, I wish to thank Professor Sebastian Johnston who gave me the opportunity to undertake this project, as well as providing insightful supervision and support throughout my PhD. I would also like to express my gratitude to my other supervisors Professor William Cookson and Professor Miriam Moffatt for their constant support, encouragement and guidance.

As a clinician with very little scientific background and laboratory knowledge, this project would not have been possible without the help and guidance of many people from the Johnston and Cookson/ Moffatt groups. Special thanks must go to Dr Michael Cox, Dr Phillip James and Dr Leah Cuthbertson for the many, many hours of teaching and support with ‘THE MiSEQ’ protocol (yes, I now know the difference between 20% and 80% ethanol); Jerico del Rosario and Belen Trujillo-Torralbo for their hard work in assisting me during clinical recruitment; Dr Jaideep Dhariwal, Dr Anny Sykes and Dr David Jackson for providing me with clinical samples; Tatiana Kebadze, Eteri Bakhsoiliani, Leila Gogsadze and Julia Aniscenko for their expert help in the Johnston lab, Dr Michael Edwards for his help in setting up the qPCR (and keeping me fit in the gym) and everyone else who kept me going when I lost my way (e.g. Aran, Jai, Mark and Hugo for all the banter in the office, Ross for answering my question of the day, Elena and Bushra for the afternoon tea on a very bad day at the office). Thank you everyone for making these three years so memorable.

I am forever grateful for my wonderful family, whose unconditional love and support have always spurred me on during difficult times. Writing up was a very difficult period as I was always tired and demotivated. I would like to thank Erina, my daughter, for contributing
towards my sleep deprivation but also making me smile everyday. I will make you read this thesis one day! Special mentions must also go to Bolt and Mochi for keeping me awake during the small hours of the morning. Finally, I would like to give my wholehearted thanks to my wife, Satomi, whose love, devotion and patience and love inspire me to be the best person I can be. I literally could not have done it without you.

~ For Satomi ~
Statement of personal contribution to this study

This study was not possible without the support of several scientific and clinical research staff at Imperial College London. I personally assessed the suitability of all clinical volunteers for the naturally-occurring cold study at screening visits, attended all the clinical study visits for the eligible volunteers and obtained clinical samples with the assistance of Jerico del Rosario, Belen Trujillo-Torralbo, Dr Sara Saturni and Dr Patrick Mallia. Clinical data curation was performed by myself with the assistance of Jerico del Rosario. I performed all subsequent clinical data analyses. For the experimental rhinovirus infection study, Dr Jaideep Dhariwal assessed the suitability of clinical volunteers and performed all subsequent clinical visits. I assisted Dr Jaideep Dhariwal in bronchoscopic sampling in this study and performed a number of bronchoscopies myself. Clinical data collation and analyses were performed by Dr Jaideep Dhariwal for this study.

Initial clinical sample processing and sorting were performed by Tatiana Kebadze, Julia Aniscenko and Leila Gogsadze. Virology PCR was performed by Julia Aniscenko. MSD on pro-inflammatory cytokines was performed by myself with the assistance of Eteri Bakhsoliani. IP-10 ELISA was performed by myself under the guidance of Dr Aran Singanayagam.

I performed all genomic DNA extraction, quantitative 16S rRNA PCR and sequencing and downstream analyses under the guidance of Dr Michael Cox, Dr Phillip James, Dr Leah Cuthbertson and Dr Michael Edwards.
Copyright declaration

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.
### Commonly used abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACQ</td>
<td>Asthma control questionnaire</td>
</tr>
<tr>
<td>AE</td>
<td>Acute asthma exacerbation</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyper-responsiveness</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BB</td>
<td>Bacterial burden</td>
</tr>
<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative For Asthma</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative for Chronic Obstructive Lung Disease</td>
</tr>
<tr>
<td>GP</td>
<td>General practitioner</td>
</tr>
<tr>
<td>GWASs</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-γ-induced protein 10</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting beta agonist</td>
</tr>
<tr>
<td>LRT</td>
<td>Lower respiratory tract</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso Scale Discovery</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PC&lt;sub&gt;20&lt;/sub&gt;</td>
<td>Provocative concentration of histamine resulting in a 20% reduction in FEV&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>PEF</td>
<td>Peak expiratory flow</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>SABA</td>
<td>Short-acting beta-agonist</td>
</tr>
<tr>
<td>SAM</td>
<td>Synthetic absorptive matrix</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper type 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>URT</td>
<td>Upper respiratory tract</td>
</tr>
<tr>
<td>WURSS</td>
<td>The Wisconsin Upper Respiratory Symptom Survey</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Asthma

1.1.1 Definition

Despite being one of the most common chronic airway diseases, there is no clear, universally accepted definition of asthma. The global initiative for asthma (GINA) provides an operational description: asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment(1).

1.1.2 Epidemiology and socio-economic burden

An estimated 300 million people are affected by asthma worldwide(2). Its global prevalence is increasing, with a predicted 400 million sufferers by 2025(2, 3). Prevalence, however, varies between 1-18% in different countries(4). According to a WHO estimate, 15 million disability-adjusted life-years are lost annually due to asthma, equating to 1% of the total global disease burden(5).

In the UK, approximately 5.4 million people currently receive treatment for asthma: 1.1 million children (1 in 11) and 4.3 million adults (1 in 12). The healthcare and socio-economic costs are unsurprisingly significant. The NHS spends an estimated £1 billion each year in the treatment and care of asthmatic patients(6). Despite this, asthma still accounts for 20 million
lost working days and approximately 1000 deaths each year(7, 8). Notably 50% of all asthma-related healthcare costs are attributed to the most severely affected 5% of asthmatic patients(7).

1.1.3 Aetiology

The aetiology of asthma is partially understood. It is complex with multiple influencing risk factors, which may be both heritable and environmental. Consequently, the natural history, severity and response to treatment may vary greatly between individual patients.

1.1.3.1 Genetics and epigenetics

Asthma is genetically heterogeneous. Genome-wide association studies (GWASs) have identified a number of asthma-associated loci including IL18R1, IL33, SMAD3, ORMDL3, HLA-DQ and IL2RB(9). Of these, only HLA-DQ was significantly associated with total serum immunoglobulin E (IgE) concentration, whilst other IgE-associated loci were not related to asthma. Furthermore, epigenetic modifications including DNA methylation, histone acetylation and micro-RNAs add to the complexity of asthma genetics. These may be inheritable and/or influenced by environmental factors such as cigarette smoking and pollution(10).

1.1.3.2 Environment

There is increasing evidence to support that environmental factors including bacteria, viruses and fungi play a role in asthma pathogenesis, though a causal relationship remains to be defined. Strachan postulated the ‘hygiene hypothesis’ when he observed that hay fever and eczema were less common in children from larger families, who were presumably exposed to
more infectious agents through their siblings, than in children from families with only one child(11). Several large-scale epidemiological studies have subsequently substantiated this(12-14). The most influential evidence was seen in children living on farms who had lower prevalence of asthma and atopy and were exposed to a wider range of microbes than those living in urban environment(13). Further supportive findings have come from experiments in mice, where intranasal exposure to *Escherichia coli* or *Acinetobacter lwaffi* str. F78 appeared to protect against allergic airway inflammation(15, 16). Significantly, recent studies have illustrated that early life modulation on the intestinal microbial community have profound effects on the maturation of the immune system and subsequent of allergic asthma(17). The ‘resident’ intestinal microbes and their metabolites such as short-chain fatty acids (SCFAs) constantly interact with the host immune system, thus regulating inflammation in peripheral tissues. In a murine study, a high fibre content diet altered the intestinal bacterial composition, increased circulating levels of SCFAs and was protective against allergic inflammation(18). Furthermore, SCFA propionate treatment led to changes in bone marrow haemopoiesis with enhanced macrophage and dendritic cell precursor generation and subsequent seeding to the lungs in addition to impaired T-helper type 2 (Th2) effector function(18). Similar host-microbial interactions may also occur within the lungs, inducing regulatory cells in early life(19).

Respiratory viruses, in particular respiratory syncytial virus (RSV) and human rhinoviruses (RV) are associated with recurrent wheezing in early infancy and subsequent development of asthma in later childhood. RSV accounts for approximately 70% of severe infantile viral bronchiolitis(20). Indeed, RSV infects nearly all children in their first two years of life but only about 40% exhibit clinical signs of a lower respiratory tract infection. A proportion of children
with RSV bronchiolitis progress to recurrent wheeze and ultimately develop asthma. The cause of this progression is unknown. Whether RSV infection directly leads to asthma or merely exposes individuals who are genetically predisposed is unclear and much debated. Recent studies suggest that these are not mutually exclusive and both may be important in asthma development(21). Perhaps the strongest evidence for a causal relationship derives from studies of Palivizumab, an anti-RSV monoclonal antibody(22, 23). A prospective study on preterm infants over a 24-month period showed a significant reduction (approximately 50%) in the rate of recurrent wheeze in those who received prophylactic Palivizumab(23). Similarly, Palivizumab prophylaxis in children aged two to five years with no family history of atopy reduced the relative risk of recurrent wheeze by 80%. However, Palivizumab did not have a significant effect in children with atopic family history, implying that RSV infection predisposes to recurrent wheeze in an atopy-independent fashion(22). Furthermore, birth cohort studies demonstrated that 90% of non-atopic wheezers have no symptoms of asthma by school age with normal lung function, suggesting virus-induced wheeze and asthma may be distinct conditions. Whilst much work has focused on RSV infection, recent studies have illustrated that RV may play an equally significant role in asthma development. Birth cohort studies have revealed that symptomatic RV infection in early life was the most important risk factor for asthma development(24, 25). However, as with RSV infection, no causal link has been identified. Early exposure to moulds (within the first year of life), specifically *Aspergillus ochraceus, Aspergillus unguis, and Penicillium variabile*, have also been associated with asthma development(26).

In summary, multiple environmental factors have been implicated in the development of asthma. Whilst associations may be considerable, no specific factor has been identified to
have a direct causal relationship as yet. It is likely that multiple factors contribute in a genetically susceptible individual and act across different time points.

1.1.4 Pathophysiology, phenotypes and endotypes

Classically, asthma is considered to be a Th2-cell dependent, IgE-mediated allergic disease. This was based on the observation that asthmatic subjects were more likely to be sensitised by local aeroallergens(27). CD4+ T cells expressing the Th2 cytokines IL-4, IL-5 and IL-13 help regulate the activation of eosinophils and mast cells(28). However, recent works have demonstrated that the epithelial innate immune response plays a pivotal role in asthma pathophysiology. Rather than a static structural barrier, the airway epithelium responds to environmental ‘insults’ such as viruses, bacteria, allergens and pollutants by releasing pro-inflammatory cytokines and antimicrobial peptides(28-30). In particular, IL-25, IL-33 and thymic stromal lymphoprotein (TSLP) stimulate the recently discovered type 2 innate lymphoid cells (ILC2s) which directly secrete Th2 cytokines, and dendritic cells (DCs) which induce Th2 responses(31).

Given the complexity of asthma pathophysiology, it is not surprising that conventional therapy is not effective in all patients, thus there is great urgency in identifying different phenotypes so that therapy can be ‘tailored’ for individual patients. Many classifications have been employed, commonly with respect to disease severity based on symptoms, medications required to alleviate symptoms and frequency of exacerbation. Another clinically-based classification split asthma into two phenotypes: ‘extrinsic’ asthma typically develops early in life and is associated with IgE-mediated allergic disease, whereas ‘intrinsic’ asthma tends to develop during adulthood without allergic sensitisation. Phenotyping based on the dominant
inflammatory cell type in sputum or peripheral blood has also been widely applied, including eosinophilic, neutrophilic, pauci-granulocytic. However, definition and stability of phenotype remain variable(32). More recently, statistical analyses such as cluster analysis have been used to further phenotype in an unbiased fashion(33). One such study explored the relationship between clinical symptoms and eosinophilic inflammation, demonstrating separate clusters of patients with distinct characteristics: early onset atopic, inflammation predominant and obese non-eosinophilic asthma(33). By combining these clusters with molecular signatures (e.g. cytokine gene expression profiles), potential endotypes of asthma are now emerging with an aim to identify suitable patients for targeted biological therapies(34).

1.1.5 Diagnosis

The diagnosis of asthma remains clinical. This is best supported by an obstructive pattern on spirometry as denoted by a forced expiratory volume in one second/ forced vital capacity ratio (FEV₁/FVC) of less than 0.7 when the patient is symptomatic. A normal spirometry reading in an asymptomatic patient does not exclude asthma. Furthermore, many respiratory (e.g. chronic obstructive pulmonary disease (COPD), bronchiectasis, sarcoidosis) and non-respiratory conditions (e.g. gastro-oesophageal reflux disease, heart failure) may mimic clinical features of asthma, thus cautious clinical assessment is vital. Additional objective measurements such as methacholine/ histamine provocation test (PC_{20}), exhaled nitric oxide concentration (FE_{NO}) and/or reversibility testing are indicated where diagnosis of asthma remains uncertain after clinical assessment and spirometry, though sensitivity and specificity vary between tests(35). Figure 1.1 illustrates a diagnostic workflow of patients with suspected asthma.
Diagnostic work flow of patients with suspected asthma

Clinical assessment and spirometry

High probability Intermediate probability Low probability

FEV/FVC <0.7 FEV/FVC >0.7

Clinical urgency to treat

Yes No

Trial of therapy BD reversibility test Bronchial provocation test

Positive Negative Positive Negative

Review asthma Probability*

Reassess for response Trial of therapy

Yes No

Continue therapy Assess compliance, inhaler technique

Improvement No improvement

Consider different diagnoses Consider referral

* If a bronchodilator (BD) reversibility test is negative, it is important to review the clinical probability of asthma and consider alternative diagnoses. If the probability remains high, further investigation including bronchial provocation testing and assessment of airways inflammation may be appropriate.

Figure 1.1 Diagnostic work flow of patients with suspected asthma. Figure copied from ‘Asthma: diagnosis and management’ by Wong and co-workers with permission(36)

1.1.6 Management

1.1.6.1 Prevention

Multiple non-pharmacological/ environmental interventions have been postulated in preventing asthma development. House dust mite (HDM) avoidance during pregnancy and
early infancy did not significantly prevent asthma development by age of eight (37, 38), whilst extensive environmental (e.g. avoidance of HDM, pets and tobacco smoke) and dietary (e.g. breast-feeding with delayed introduction of solid foods) interventions demonstrated a reduction in asthma development in infants with family history of allergic disease (39, 40). However, these results cannot be applied generally as the studies were limited by small numbers, high drop-out rates and biased population selection. Consequently, there is no current established strategy for primary prevention of asthma (35).

1.1.6.2 Pharmacological management

The aim of asthma management is to optimise disease control as defined by: absence of symptoms (daytime and nocturnal), no requirement for rescue medication, absence of exacerbations and no limitations on activity including exercise (35). Inhaled corticosteroids (ICS), with or without long-acting beta$_2$-agonists (LABA), have been the backbone of maintenance asthma therapy. ICS have been shown to reduce daily asthma symptoms, improve lung function and reduce risk of exacerbations (41, 42), though their efficacy appears to be less in patients with non-eosinophilic asthma (43). When symptoms acutely deteriorate, short-acting beta$_2$-agonists (SABA) are recommended as rescue medication (35). LABA have also been investigated as an alternative to SABA with a Cochrane review showing comparable efficacy (44). However, concerns linger about increased risk of serious adverse events with regular long term LABA use in patients not on ICS (45). Both GINA and British guidelines recommend a step-wise approach (1, 35). Patients with mild intermittent asthma are usually managed with SABA as required. As symptoms increase despite SABA use, regular ICS treatment is advised, with dose titrated to symptoms. In cases where symptoms persist despite high dose ICS, further adjunct therapy such as ICS/LABA combination inhaler,
leukotriene receptor and theophylline are considered. Patients with severe symptoms despite all the above measures are considered for long term oral corticosteroid treatment. Furthermore, these patients may be suitable for targeted therapies including anti-IgE monoclonal antibody (Omalizumab), anti-IL5 antibody and macrolide antibiotics, though the long term efficacy of these novel therapies remains to be determined(46).

1.1.6.3 Non-pharmacological management

Patient education and self-management play a vital role. Education in asthma self-management, involving self-monitoring of PEF and symptoms, coupled with a written action plan and regular medical review, has been shown to improve health outcomes in adult asthmatics(47).

Despite the consensus that the environment plays a role in the development and persistence of asthma, there is a paucity of evidence to support environmental control and allergen avoidance in managing established disease. One study investigating children living in the inner city identified that individualised, home-based, comprehensive environmental intervention reduced asthma-associated morbidity(48). However, allergen avoidance has not demonstrated significant positive effects in adult asthmatics(49). As with primary prevention, there is no established recommendation for allergen avoidance and other environmental intervention(35).
1.2  Acute asthma exacerbations

1.2.1  Definition and classification

GINA defines acute asthma exacerbations (AEs) as ‘transient worsening of asthma as a result of exposure to risk factors or triggers for asthma symptoms’(1). This is typically associated with a reduction in peak expiratory flow (PEF) and requires a change in intensity of treatment to prevent further deterioration in clinical status(35, 50). In clinical settings, AEs are classified in accordance to level of severity. This is determined by a combination of clinical signs (e.g. respiratory rate, heart rate, ability to complete sentences) and investigation parameters (e.g. PEF, partial pressure of oxygen and carbon dioxide), which consequently guide the intensity of treatment(35).

1.2.2  Epidemiology and socio-economic impact

AEs account for a sizable portion of asthma-related disease burden(51), with higher costs attributed to patients with moderate to severe disease(52). This burden may be related to the direct costs of healthcare use or the indirect costs associated with lost productivity. Furthermore, AE-related hospitalisations are intricately linked with a poorer quality of life(53). As many as 50% of asthma patients report having an AE over the course of one year, with more than a third of children and a quarter of adults requiring urgent medical care visits as a result(54, 55). In 2007 in the United States, asthma-related emergency department visits were estimated at 1.75 million (1.11 million adults and 0.64 children), in addition to 456,000 (299,000 adults and 157,000 children) hospital admissions(56). The true frequency of AEs, however, is difficult to ascertain as this depends on the definition applied, the severity and degree of chronic disease control. Large clinical trials estimate that patients on optimal
therapy experience an AE approximately once every three years (57). In one study involving patients with mild asthma, patients on low-dose ICS had an exacerbation rate of 0.92 per patient per year, compared with 0.36 in those on combination therapy of high-dose ICS and LABA (58). In moderate asthma, exacerbation incidence was similar to that of mild asthma, with exacerbation rates of 0.91 per patient per year in patients on low-dose ICS and 0.34 in those on high-dose ICS and LABA (59). In a trial investigating the efficacy of anti-IgE therapy in severe asthma, exacerbation rates over a 48-week period were 0.88 in the placebo group and 0.66 in the treatment group (60). In addition, the real incidence rates are likely to be underestimated as unstable, frequently exacerbating patients were excluded in clinical trials. However, it is well recognised that some asthmatic patients rarely or never experience AEs, reflecting the heterogeneity of asthma and AEs (57).

1.2.3 Predisposing factors

Multiple factors have been associated with predisposing patients to AEs. Disease severity and control appear to be linked with AE frequency. In one study, 54% of severe asthmatic patients had three or more AEs per year, compared with 5% and 13% in mild and moderate asthmatics respectively (61). Other factors associated with frequent or severe AEs include ethnic minority status, female sex, obesity, adverse psychosocial features (e.g. alcohol or drug abuse, psychosis, depression) and obstructive sleep apnoea (35, 50, 57). It remains unclear whether these factors are independent of asthma control.

1.2.4 Triggers of asthma exacerbations

AEs may be triggered by numerous factors including aeroallergens, cigarette smoking, nitrogen dioxide, ozone, exercise, medications (e.g. aspirin, non-steroidal anti-inflammatory
drugs) and pregnancy(57). However, the vast majority of AEs (up to 60-80%) are associated with respiratory viral infections, of which two thirds are accounted by RVs(62). Furthermore, there is increasing evidence supporting the notion of synergistic virus-allergen interactions which may amplify the severity of an AE(63). Bacteria, in particular the atypical bacteria *Chlamydophila pneumoniae* (*Cpn*) and *Mycoplasma pneumoniae* (*Mpn*), have been implicated in AEs, though the role remains to be defined(64-66). The role of viruses and bacteria in AE will be discussed in detail in a later section.

1.2.5 Management

Management depends on the severity of AE. However, treatment in clinical practice is primarily based on bronchodilators and corticosteroids, along with supportive measures such as oxygen for hypoxia(1, 35). Systemic corticosteroids, administered orally or intravenously, remain the mainstay of treatment. They reduce mortality, relapses, subsequent hospital admission and need of SABA use(67). SABA (e.g. salbutamol) is typically administered in nebulised form, driven through oxygen(68, 69). Nebulised ipratropium bromide, an anti-cholinergic, may be combined with salbutamol and significantly improves bronchodilation and hastens recovery time(70). In severe cases where patient is not responding well to the above measures, a single infusion of magnesium sulphate has been shown to significantly reduce hospital admissions and improve lung function(71). In extreme cases, deteriorating patients who fail to respond to therapy may require non-invasive or invasive ventilation.

Antibiotics are commonly prescribed for AEs, though the evidence for their benefits is variable(72). Macrolides, such as azithromycin, have anti-microbial, immunomodulatory and potential anti-viral properties and may be of benefit as adjunct therapy(73). This will be
further discussed in a later section. Given the substantial association between AEs and respiratory viral infections, there is a pressing need to develop targeted therapy to improve treatment of AEs.

1.3 The role of viruses in asthma exacerbations

Respiratory viruses have long been implicated as a major trigger of AEs. The association is perhaps best illustrated by epidemiological studies. The incidence of AEs in school-age children typically peak after school return from summer vacation during early September\(^{(74)}\), coinciding with the peak RV infection incidence\(^{(75)}\), commonly called the ‘September epidemic’\(^{(76)}\). In young children and adults, a similar albeit less dramatic trend is also observed\(^{(76)}\).

In conjunction, the advent of polymerase chain reaction (PCR) for respiratory virus detection has greatly advanced the understanding of virus-induced AEs. Numerous cohort studies have consistently illustrated the high prevalence of viral infection during AEs. In outpatient school age children with AE, approximately 62 to 85% show positivity based on PCR, compared with 12 to 41% in age and severity matched controls with stable disease\(^{(62, 74)}\). In one study, RVs were the most commonly detected (65% of cases), followed by coronaviruses (17%), influenza and parainfluenza viruses (9%) and RSV (5%)\(^{(62)}\). Similar findings have been demonstrated in adults with AE, though the prevalence of respiratory viruses is more variable, ranging from 40 to 65%\(^{(77-79)}\). In view of its high detection frequency, research has focused on RV-induced AEs and therefore these will be discussed in detail.
1.3.1 Human rhinoviruses

1.3.1.1 Classification and epidemiology

RVs are positive-sense, single stranded-RNA viruses that are members of the *Picornaviridae* family and the genus *Enterovirus* (80). RVs are classified in a number of ways. They may be classified according to serotype, of which there are more than 100 serotypes with no predictable pattern of infection based on serotype. RVs may also be classified in accordance to the receptor type used to gain host cell entry. The ‘major’ group uses the intercellular adhesion molecule 1 (ICAM-1) whilst the ‘minor’ group uses the low-density lipoprotein receptor (LDL-R) (81, 82). Furthermore, they may be classified based on genetic sequence similarity and susceptibility to anti-viral agents into RV-A, RV-B and the recently discovered RV-C. Some studies have suggested that the RV-C group may be intrinsically more virulent and have a greater propensity to trigger AEs (83, 84), though a recent study found no significant difference in infection severity between the different genotypes (85).

RVs are the most frequent cause of the common cold, accounting for approximately 70% of infectious agents detected in young adults with cold symptoms (86). In pre-school children, RV infection rate is as high as eight to 12 times per annum. This decreases to twice to four times per annum in adults, presumably related to development of immune memory (87). As mentioned previously, RV infections peak during spring and early autumn in temperate climates, though infections can occur throughout the year (75). Transmission is thought to be both aerosol inhalation and spread via contaminated surfaces (88, 89).
1.3.1.2 Viral replication

RV replication takes place in airway epithelial cells (AECs). Depending on the type of receptor (Intercellular Adhesion Molecule 1 (ICAM-1) or LDL-R or CDHR3(90)), virus uptake occurs via clathrin-dependent or -independent endocytosis or via macropinocytosis. This is followed by viral uncoating under low pH condition in the endosome. The negative strand (parent RNA) is replicated as well as translated into structural and non-structural proteins. The virion is then assembled, packaged and exported via cell lysis(80, 91, 92).

1.3.1.3 Innate and adaptive immune responses to human rhinovirus infection

Following uncoating within AEC, the release of viral RNA is recognised by pattern recognition receptors (PRRs). These include the endosomal toll-like receptors (TLR) 3, 7 and 8, and the intracellular RNA helicases retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5)(93-95). Subsequent to viral recognition, Toll/IL-1 receptor (TIR), located domain in cytoplasmic portion of the TLR, interacts with cytoplasmic adaptor molecules including MyD88, TIR-domain containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM),(96) whilst RIG-I and MDA5 interact with mitochondrial anti-viral signalling (MAVS) protein.(97) These interactions trigger a signalling cascade, resulting in the activation of several important transcription factors including nuclear factor (NF)-κB, interferon regulatory factor (IRF)-3, -7 and activating transcription factor (ATF)-2(98, 99). These activated transcription factors translocate into the nucleus and promote the transcription and subsequent translation of type 1 interferons (IFN)-α and – β(100). Furthermore, nuclear translocation of IRF-3 and NK-κB leads to the up-regulation of
primary anti-viral response genes including IFN-γ-inducible protein 10 (IP10, also called CXCL10) and RANTES (also called CCL5)(98).

This is followed by a secondary anti-viral response. The secreted type 1 IFNs bind to the type 1 IFN receptor complex (IFNAR-1 and -2), triggering the activation of janus kinase (JAK) and tyrosine kinase (TYK) proteins. These proteins in turn recruit and activate signal transducer and activator of transcription (STAT) proteins(98). The STAT proteins further combine with IRF-9, forming a heteroteremeric complex known as IFN-stimulating gene factor-3 (ISGF-3), which translocates to the nucleus and binds to promoters of IFN-stimulated genes (ISGs).(101) This results in transcriptional activation of ISGs, which encodes for an assortment of proteins that selectively interfere with viral replication, protein synthesis and trafficking(102). In addition, type I IFNs are potent inducers of natural killer (NK) cell-mediated cytotoxicity, which form an important part of the innate anti-viral defence(103).

More recently, a novel class of cytokines, IFN-λ (also known as type III IFN or IL-28/29), was discovered. IFN-λ possesses IFN-like anti-viral activity and the ability to induce typical IFN-inducible genes, thus may play an important role in the innate immune response against viruses.(104)

Both CD4+ and CD8+ T lymphocytes appear to play an important role in the host defence against respiratory viral infections, especially influenza and RSV(105, 106). The role of T cell responses in RV infections, however, is less well studied. There is an increase in submucosal lymphocytes with experimental RV infection(107), whilst pre-existing neutralising antibodies to RVs offer protection against infection and reduce symptoms(108).
1.3.1.4 Capacity to infect the lower respiratory tract

Given RV infections classically cause upper respiratory tract symptoms, there have been some debates as to how RVs trigger AEs, a predominantly lower respiratory tract condition. However, it is now evident that RVs do infect the lower respiratory tract. Following experimental infection, RV has been detected in the lower respiratory tract by immunostaining, PCR, and in situ hybridisation for positive-strand viral RNA(109-111). Furthermore, in experimental RV infection, increased viral load positively correlates with asthma symptoms, suggesting RV infection may initiate on lower respiratory tract symptoms(35).

1.3.2 Mechanism of human rhinovirus-induced asthma exacerbations

The exact mechanism of RV-induced AEs remains unclear. RVs cause minimal cytotoxicity(107), thus the amount of epithelial damage does not seem to correlate with the severity of symptoms. RV infection may trigger AEs in a number of ways:

1.3.2.1 Airway epithelial cell-induced inflammation

As mentioned previously, RV infection induces a host of pro-inflammatory cytokines and chemokines, including Interferon-γ-induced protein 10 (IP-10), RANTES, IL-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to recruitment of neutrophils, macrophages and eosinophils. This influx of inflammatory cells may further potentiate allergic inflammation within asthmatic airways(107, 112-114). Furthermore, airway epithelial damage and mucus metaplasia, both of which occur in asthma, may allow
RVs better access to more readily-infected basal and goblet cells, thus enhancing viral replication(115, 116).

### 1.3.2.2 Deficient interferon response

The innate immune response to viral infection may be altered in asthma patients, exemplified by a deficient Type 1 and III IFN production in response to RV infection(117-119). *Ex vivo* production of IFN-λ inversely correlated with severity of symptoms, BAL viral load and airway inflammation, whilst IFN-λ level was positively associated with reduction in lung function(118). The cause of this deficient interferon production is unclear, though the skewing of T-cell differentiation towards the Th2 phenotype in allergic asthma has been proposed. As the differentiation of Th1 and Th2 lineages is mutually antagonistic(120), the Th2-high asthmatic patients may have an impaired interferon response. In experimental RV infection, asthmatic patients who had a strong peripheral blood monocyte (PBMC) IFN-γ response showed milder symptoms and more efficient viral clearance(121). This may help explain why some asthmatics are more susceptible to viral infections than others. However, this phenomenon does not appear to be universal, as IFN production deficiency was not observed in RV-infected human bronchial epithelial cells (HBECs) of well-controlled asthmatic patients(122).

### 1.3.2.3 Effect on mucus hypersecretion and airway remodelling

Increased production and secretion of mucus is frequently observed in asthma and associated with AEs, accelerated decline in lung function and fatal asthma(123). The matrix of airway sputum consists of high molecular glycoproteins and mucins. Mucin5AC (MUC5AC) and MUC5B are the predominant components of respiratory mucus. RV infection has been shown
to upregulate MUC5AC production, thus potentially exacerbating airflow obstruction and ultimately airway remodelling (124).

1.4 The role of bacteria in asthma

In contrast to respiratory viruses, the significance of bacteria in chronic disease and AEs is less well defined. However, there is now growing evidence that bacteria may play a role in the development of asthma, persistence of chronic disease and AEs.

1.4.1 Development of asthma

Further to Strachan’s initial ‘hygiene hypothesis’ and large scale epidemiological studies (as discussed previously), it has been shown that colonisation of the hypopharyngeal region by *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenzae* (*H. influenzae*) or *Moraxella catarrhalis* (*M. catarrhalis*) during neonatal period confers a greater risk of developing recurrent wheeze and asthma early in life (125). Furthermore, colonisation of *H. influenza* and *M. catarrhalis* induced a mixed T helper cell response with high levels of IL-1β, tumour necrosis factor-α (TNF-α) and macrophage inflammatory protein-1β, inferring the active role of bacteria in disease pathogenesis (126).

1.4.1.1 Late onset asthma

The atypical bacteria, *Chlamydophila pneumoniae* (*C. pneumoniae*) and *Mycoplasma pneumoniae* (*M. pneumoniae*), have been linked with late onset/ adult onset asthma. Adult-onset asthmatic subjects were found to have higher *C. pneumoniae*-specific antibody levels compared with healthy controls (127, 128). Serological evidence of *C. pneumoniae* exposure has also been associated with the development of asthma with a dose-response relationship
between exposure and prevalence of asthmatic bronchitis (129). However, the findings of similar studies have been inconsistent and thus the strength of such association is variable.

1.4.2 Persistence of chronic asthma

1.4.2.1 Increased risk of invasive pneumococcal infection

Whilst colonisation of certain bacterial species appears to increase the risk of developing asthma during childhood, bacteria may also play a role in chronic disease state. Most notably, adults with stable asthma have been demonstrated to have a greater incidence of S. pneumoniae colonisation (130). Furthermore, invasive pneumococcal infection (IPI) is more frequent in both children and adults with asthma (131, 132) and asthma has been shown to be a independent risk factor for invasive pneumococcal disease. Patients with low-risk asthma (as defined by the need for maintenance therapy) had a 2.8-fold increase in risk of IPI compared with healthy controls. Astoundingly, subjects with high-risk asthma (as defined by asthma-related hospitalisation in the past 12 months) had an over 12-fold increase in risk (133). However, besides greater rate of colonisation, the reason behind the increased risk of IPI in asthma is unclear.

1.4.2.2 Mycoplasma pneumoniae and Chlamydia pneumoniae

Aside from their implications in late-onset asthma, M. pneumoniae and C. pneumoniae have also been heavily implicated in chronic asthma and AE. Johnston and Martin performed a comprehensive review on the role of M. pneumoniae and C. pneumoniae in chronic asthma. Of the 19 studies reviewed, 15 demonstrated an association between infection with these pathogens and asthma (66). For example, in a cohort of 55 stable asthmatic and 11 healthy
subjects, both *M. pneumoniae* and *C. pneumoniae* were more frequently detected by PCR in asthmatic subjects (56.4% vs 9%)(134). Interestingly, serological diagnosis of the same cohort revealed 19 positive subjects (18 asthmatic and 1 healthy) for *C. pneumoniae*, with the vast majority positive by IgG criteria, leading to the conclusion that patients with chronic asthma tended to have chronic infection. Furthermore, *C. pneumoniae* heat shock protein-60 IgA and IgG to *C. pneumoniae* levels have been shown to inversely correlate with predicted FEV$_1$(135, 136) , whilst elevated levels of IgA to *C. pneumoniae* correlated with daytime asthma symptoms(136).

Despite the growing evidence of such association, the precise mechanisms by which chronic infection of *C. pneumoniae* or *M. pneumoniae* could lead to worsening asthma symptoms remain unclear. In an *in vitro* setting, *C. pneumoniae* infection induced production of the pro-inflammatory cytokines IL-8 and TNF-α from PBMCs and alveolar macrophages(137, 138). In airway epithelial cells, *C. pneumoniae* infection appeared to activate TNF-α, IL-8, IFN-γ and NF-κB(139, 140).

1.4.2.3 The effects of macrolides in chronic asthma

If chronic *C. pneumoniae* and/ or *M. pneumoniae* infection does indeed play a role in the persistence of chronic asthma, then treatment with antimicrobials would seem logical to reduce the level of inflammation within the airways.

Macrolides, such as clarithromycin and azithromycin, have broad-range, bacteriostatic effects that are efficacious against a wide range of respiratory pathogens, including *C. pneumoniae* and *M. pneumoniae*. Macrolides inhibit bacterial protein synthesis by interaction with the 50S subunit of the 70S prokaryotic ribosome(141). Results from clinical trials in asthma, however,
have been variable, possibly due to the lack of optimal agent, dosage, duration and heterogeneity of asthma pathophysiology(73). A recent study by Brusselle and co-workers investigated the role of azithromycin in subjects with poorly controlled asthma (as defined by an ACQ score of greater than 1.4 despite high dose ICS and LABA therapy) with respect to preventing AEs. Whilst the overall study results were negative, the authors observed a significant beneficial effect in a pre-defined subgroup of subjects with non-eosinophilic asthma leading to the conclusion that macrolides may be effective in severe neutrophilic asthma(142). However, the mechanisms of such beneficial effects may not be entirely related to azithromycin’s antimicrobial effects as it also possesses immunomodulatory and potentially anti-viral properties(73).

1.4.3 Acute asthma exacerbations

Compared with chronic asthma, there is a paucity of studies investigating the role of bacteria in the context of acute asthma exacerbations (AEs).

1.4.3.1 Higher detection frequency of *M. pneumoniae* and *C. pneumoniae*

Similar to chronic asthma, various studies have reported an association between these atypical bacteria and AEs. In the review by Johnston and Martin, nine out of 12 studies (five paediatric and seven adult respectively) reported a significant association(66). In one study consisting of 100 adult asthmatic subjects with AE, serological evidence of acute *M. pneumoniae* infection was 18% in asthmatic subjects compared with 3% in matched healthy controls, though there was no difference in *C. pneumoniae* detection rate(143). Miyashita and co-workers also observed a significant increase in IgG and IgA to *C. pneumoniae* in asthmatic subjects compared with healthy controls, implying that acute or chronic infection
was associated with AEs(144). Interestingly, there was once again great discrepancy in detection rate between serological techniques and PCR, with the former achieving a much greater detection rate.

1.4.3.2 Limitations of serological diagnostic techniques

As illustrated above, serological diagnostic techniques for atypical bacteria often resulted in variable outcomes compared with PCR methods. Both \textit{C. pneumoniae} and \textit{M. pneumoniae} are fastidious in nature and thus quantitative culture is insensitive. A combination of serology and PCR is considered the current gold standard investigation(145, 146). Furthermore, distinction between acute and chronic infection remains challenging.

1.4.3.3 Low detection rate of pathogenic bacteria during AEs

Whilst bacteria were detected as frequently as viruses in children with acute wheezing episodes(147), pathogenic bacteria such as \textit{S. pneumoniae} and \textit{H. influenzae} are rarely detected during AEs in adults(148). In a recent UK study consisting of 171 asthmatic subjects admitted with AE, only 9 (5%) had positive bacterial detection by urinary antigen or quantitative culture, compared with 25% in COPD exacerbations (total of 304 subjects)(148). An Australian study investigating 96 asthmatic subjects with AEs showed matching detection rate (5%) of ‘pathogenic bacteria’ (e.g. \textit{S. pneumoniae}, \textit{H. influenza} and \textit{M. catarrhalis})(77). The low detection rate may be explained in part by the frequent prescription of antibiotic therapy thus limiting effectiveness of quantitative culture.
1.4.3.4 Efficacy of antibiotic therapy in AEs

Given the paucity of data on the role of bacteria in AEs, current BTS and GINA guidelines advise against the routine use of antibiotic therapy in AEs(1, 149). This advice was based on two previous double-blinded randomised controlled trials (RCTs), which did not observe any beneficial effect with amoxicillin treatment during AE(150, 151). However, in clinical practice the use of antibiotics is far greater, with one recent UK study quoting a rate of 59%(148).

A more recent study found that telithromycin, a ketolide antibiotic that is similar to macrolides, significantly reduced clinical symptoms during AE. However, the wider use of telithromycin was restricted due to concerns over hepatic toxicity(152). Of note, the authors did not identify a relationship between *C. pneumoniae/ M. pneumoniae* bacteriological status and response to treatment. The same authors recently performed a similar study investigating the use of azithromycin in AEs in a UK based multi-centre double-blinded randomised controlled trial and did not identify any additional benefit with azithromycin therapy(153). Importantly, this under-powered study was hampered by high exclusion rate at recruitment stage due to high rate of antibiotic prescription in the emergency department and primary care. For every one recruited subject, more than 10 were excluded as they were already on antibiotic. This major confounder not only obscures the clarity of the study, but greatly highlights the importance of evaluating the role of bacteria in AEs to prevent unwarranted antibiotic use.

1.4.4 Summary

There is now growing evidence that bacteria play a role in the development of asthma. Colonisation of *S. pneumoniae, H. influenzae or M. catarrhalis* during the neonatal period
appears to confer greater risk of developing asthma. The atypical bacteria, *M. pneumoniae* and *C. pneumoniae*, have been linked with late onset asthma. Furthermore, they are associated with chronic asthma and acute exacerbations, with higher frequency of detection than healthy controls. The mechanisms by which these organisms lead to chronic disease or exacerbation are unknown. Macrolide antibiotics may be of benefit in chronic severe non-eosinophilic asthma, whilst the ketolide, telithromycin, was shown to reduce clinical symptoms following acute exacerbation. However, it is unclear whether the beneficial effects are related to antimicrobial or immunomodulatory properties. There is currently no convincing evidence to justify the routine use of broad spectrum antibiotic in acute asthma exacerbations, though in clinical setting antibiotic therapy remains frequently prescribed.

Given the significant rise of antibiotic resistance, it is vital to better define the role of bacteria in acute exacerbations to rationalise and minimise the use of antibiotic therapy. Recent works using culture-independent techniques, however, have greatly advanced this understanding.

### 1.5 The concept of microbiota

Historically, research on microbial interactions in human diseases has focussed on single organisms. Those that were linked to diseased states were termed ‘pathogens’ and those that were not were called ‘commensals’. Pathogenic species were primarily examined by quantitative culture technique and little attention was paid to the commensal organisms. However, it is now known that 70% of bacterial species on human surfaces cannot be cultured, whilst the remaining 20 to 30% cannot be isolated easily (154, 155). It is related to the fact that many organisms are adapted to life in a community environment rather than a pure culture, therefore isolated selective culture is not possible. Culture-independent techniques
via next generation sequencing (NGS) have revealed complex microbial communities that interact and depend on each other, transforming the perception of ‘pathogens’ and ‘commensals’.

The term ‘microbiome’ literally means small biome, or rather the ecosystem that contains all microorganisms in a particular environment together with their genes and environmental interactions, whilst ‘microbiota’ refers to the assemblage of microorganisms themselves. This can include bacteria, archaea, viruses, phages, fungi and other microbial eukarya(156). As the bacteria are most researched, the term ‘microbiota’ frequently refers to the bacterial community only, though technically it also consists of the other organisms mentioned above. For the sake of clarity, in the current project, ‘microbiota’ solely refers to the bacterial community.

1.6 16S rRNA gene sequencing

1.6.1 A brief history of sequencing technology

Culture-independent studies assessing the microbiota community began almost 30 years ago and were based on targeted sequencing of the 16S ribosomal RNA (rRNA) gene(157), a highly conserved component of the transcriptional machinery of all DNA-based life forms. The conservative nature of this gene has allowed the development of more rapid methods of surveying the microbiota directly from DNA extracts(158).

The 16S rRNA gene consists of conserved and variable regions. It is the variable regions that allow discrimination between different microorganisms (Figure 1.2). The method relies on PCR using ‘universal’ primers targeted at the conserved regions and designed to amplify as
wide a range of different microorganisms as possible, followed by assaying the amplified fragment of the gene(159). This was initially performed using molecular fingerprinting approaches such as denaturing gradient gel electrophoresis(160) or by cloning and sequencing of the PCR products(161). An alternative approach is the use phylogenetic microarrays, which consist of multiple probes designed to distinguish sub-groups of organisms(162, 163). However, such microarrays needed to be pre-designed with target organisms thus limiting the capability of detecting novel organisms.

The coupling of 16S rRNA PCR with NGS has enabled surveying the entire microbiota community of multiple samples at low cost. There are a number of different NGS platforms available including the Roche-454 pyrosequencing and the Illumina® MiSEQ and HiSEQ. The current project employs the Illumina® MiSEQ platform.

![16S rRNA Gene](image)

**Figure 1.2** Approximately 1.5 kb 16S rRNA gene of E.coli showing the nine variable regions that make it an ideal target as a phylogenetic marker gene. Copied from ‘Sequencing the human microbiome in health and disease’ by Cox and co-workers with permission(156)

### 1.6.2 Operational taxonomic units (OTUs)

Once sequenced, the related sequences are clustered at a particular level of identity and the number of representatives of each cluster are calculated. As molecular methods for identifying bacteria do not map directly to the classical biological taxonomic classification, clusters of similar sequences are termed operational taxonomic units (OTUs). It is generally
accepted that a level of 95% sequence identity as being representative of a genus and 97% for species. However, some bacteria can only be identified at the level of genus or family, rather than species level (164). Finally, the OTUs are identified by matching the sequences to a known reference database (e.g. GenBank, GreenGenes, SILVA).

1.6.3 Technological variations and limitations

Whilst NGS has significantly advanced microbiota studies with respect to cost and sequencing efficiency, there are limitations to current technology that should be taken into consideration. Sequencing platforms vary with regards to sensitivity of taxa identification. For example, 454 pyrosequencing has a read length of approximately 400 bases. This means that only a portion of the 16S rRNA gene can be tested, targeting between one and three variable regions. Evaluating different variable regions will likely result in slightly different taxa identification. Various studies have examined different variable regions (or combinations), thus such results need cautious interpretation. Furthermore, sampling only a portion of the 16S rRNA gene will lead to loss of sensitivity, thus some taxa cannot be reliably distinguished at species level. The Illumina® MiSEQ platform has a read length of approximately 150 bases therefore further sensitivity is lost. However, this may be compensated with far greater sequencing depth and output.

A major limitation is the introduction of biases by PCR primer design, which may preferentially select for or against particular groups of organisms (165, 166). Furthermore, the number of copies of the 16S rRNA operon varies between bacterial species (between one and fifteen copies) and therefore influence the apparent relative abundance of an organism (167).
A recently recognised issue is that of contamination of reagents by soil and water bacterial species leading to erroneous identification of ‘novel’ species(168). This is especially the case in samples with low biomass (e.g. bronchoalveolar lavage). This potential confounder can be tackled by the use of concurrent negative reagent controls when obtaining clinical samples. Finally, the accuracy of taxa identification depends on database chosen. For example, GenBank (the NIH sequence database) has an error rate of approximately 5%(169). It may be preferable to use curated databases such as the Ribosomal Database Project(170), GreenGenes(171) and SILVA(172), where sequence quality and alignments are manually optimised.

With these limitations and potential confounders in mind, the ensuing sections will review the studies on airway microbiota to date with particular focus on asthma.

1.7 The airway microbiome in health and disease

1.7.1 Complex microbial community within healthy airways

The classical dogma that the lower respiratory tract (LRT) is sterile in health has been upheld until recently. Such was the belief that the Human Microbiome Project did not even include the LRT as one of its 18 sites at its indoctrination(173). In 2010, Hilty and co-workers first demonstrated that even in health there is a complex microbiota within the LRT, which differs from that in diseased states like asthma and COPD(174) (Figure 1.3). The LRT microbiota was populated by bacteria from five phyla (Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria respectively). The authors observed that healthy subjects had a greater relative abundance of the ‘commensals’ Bacteroidetes (in particular, Prevotella
spp.)(174). The same observation has been made in other studies since(175, 176), though the hypothesis of a distinct LRT microbiota in health was not universally supported(177).

Charlson and co-workers rebutted the ideation of a distinct LRT microbiota in a small study on six healthy non-smokers(177). The authors took serial samples of the upper respiratory tract (URT) and LRT using separate bronchosopes. They noted that there was no significant difference between the URT and LRT microbiota, with the LRT displaying a two to four log reduction in bacterial burden (as represented by total 16S rRNA gene copies). The authors argued that the microbial community structures should be exquisitely sensitive to differences in environment niche, such that the microbiota of one region (e.g. URT) should be evidently distinguishable from another (e.g. LRT). They therefore concluded that there was no distinct LRT microbiota, but rather a result of micro-aspiration/ transient carry-over from the URT.

Dickson and co-workers performed a similar study, taking sequential samples from lingula, right middle lobe (by BAL), right upper lobe, left upper lobe and supra-glottic spaces (by protected-specimen brush)(178). The authors postulated that the primary determinant of lung microbiota constitution in health is the balance of immigration and elimination of communities from the URT, coined “adapted island model of lung biogeography”, rather than differences in local environmental conditions. Whilst they did not find significant differences between the different sites, the right upper lobe community resembled the microbiota of the supra-glottic space more than any other site, supporting the hypothesis.

Currently, it is generally viewed that the LRT microbiota is a continuation of the URT/ oral microbiota and is influenced by several factors: the migration of microbes into the airways, the elimination of microbes from the airway and the relative reproduction rates of its community members, as determined by local growth conditions(179, 180).
1.7.2 The airway microbiota in chronic lung disease during stable disease and exacerbations

In the early study by Hilty and co-workers, it was observed that the airway microbiota in chronic lung diseases such as asthma and COPD was different from that in health (174) (Figure 1.3). The following section will summarise published studies on the airway microbiota in different chronic lung diseases during clinical stability and acute exacerbations. Studies on asthma will be discussed separately later.

*Figure 1.3 Distribution of the phyla from broncho-alveolar lavage (BAL) in children with difficult asthma and controls. Copied from ‘Disordered Microbial Communities in Asthmatic Airways’ by Hilty and co-workers with permission (174).*
1.7.2.1 Cystic fibrosis

Cystic fibrosis (CF) is perhaps the most studied chronic lung disease from the microbiota perspective, given its disease progression with recurrent respiratory infections. Classical views were founded on culture-based studies showing an evolution of dominant pathogenic species with age. *Staphylococcus aureus* (*S. aureus*) and *H. influenzae* dominate in early life, with *Pseudomonas aeruginosa* (*P. aeruginosa*) becoming increasingly prevalent with age. Furthermore, patients become susceptible to otherwise uncommon pathogens such as *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex. However, culture-independent studies have significantly advanced the understanding of the CF airway.

Instead of a single dominant organism, the CF airway consists of a complex community mainly consisting of *Streptococcus*, *Prevotella*, *Rothia*, *Veillonella* and *Actinomyces* at genus level. The CF-associated pathogens such as *P. aeruginosa* and *Burkholderia cepacia* are less prevalent but tend to dominate the community once present(181). This is reflected by the reduction in community diversity (known as alpha diversity) with age and is associated with progressive airway obstruction(182), though it may be secondary to the effects of antibiotic and systemic corticosteroid therapies rather than natural disease progression(183).

Longitudinal studies have demonstrated that the CF microbiota is relatively stable during stable chronic disease. During acute exacerbations, however, significant community structure changes occur in some but not all cases, suggesting the infective aetiology of CF exacerbations are heterogeneous(184). Moreover, the reduction in relative abundance of the dominant taxa during exacerbation implies the potential importance of less abundant taxa. Traditionally, antibiotic therapy was seen to be eradicative. However, rather than ‘clearing’ the infection,
antibiotic therapy appears to alter the structure and dynamics of the microbiota (185, 186). This may help explain in part the variable response to broad-spectrum antibiotic therapy in CF exacerbations.

1.7.2.2 COPD

In the initial study by Hilty and co-workers, subjects with COPD appeared to have a greater relative abundance of Proteobacteria (in particular *Haemophilus*) than healthy subjects though it was not statistically significant. This was followed up by Erb-Downward and co-workers, who compared the microbiota of 4 COPD subjects, 3 healthy non-smokers and 7 ‘healthy’ smokers (175). Again there was no significant community difference between the three groups though significant variations were observed between individual subjects. Whilst there was no significant community distinction in mild and moderate COPD subjects, the microbiota in severe disease (GOLD stage IV) COPD, appeared to be distinct with an increase in relative abundance of Firmicutes (in particular the genus *Lactobacillus*) with reduced alpha diversity (187). Interestingly, a more recent study by the same authors again demonstrated significant differences between the microbiota of severe COPD subjects and healthy controls, though on this occasion there was significant increase in relative abundance of Proteobacteria rather than Firmicutes (188).

The aetiology of COPD exacerbations is heterogeneous. Both respiratory viruses and bacteria have been implicated, with the latter being detected between 51 and 70% of cases (189-191), often as a new strain of pathogen previously not seen (191). To-date there have been three studies evaluating the COPD microbiota during exacerbations. Huang and co-workers conducted the first study to examine the dynamics of the microbiota during a naturally
occurring COPD exacerbation (192). The authors observed an increase in Proteobacteria acutely following exacerbation, though how the microbiota changes afterwards depended on the treatment received. Antibiotic monotherapy resulted in a prolonged reduction of Proteobacteria, whilst systemic corticosteroid therapy led to an enrichment of Proteobacteria. In addition, the authors performed predicted metagenomics. Those exacerbation-associated taxa were associated with impaired synthesis of antimicrobial and anti-inflammatory products. This differential response to antibiotic and corticosteroid therapy was again observed in a recent study by Wang and co-workers, though heterogeneous changes within the microbiota were noted during exacerbation (193). Finally, Molyneaux and co-workers examined the COPD microbiota using an experimental RV infection challenge model of RV-induced COPD exacerbation, demonstrating a significant increase in Proteobacteria (Haemophilus sp.) approximately two weeks following RV infection (194).

1.7.2.3 Idiopathic pulmonary fibrosis (IPF)

Compared with CF and COPD, the understanding of the airway microbiota in IPF is less well studied. In a recent study by Molyneaux and co-workers of 65 subjects with IPF and 27 healthy controls, IPF subjects had significantly increased relative abundance of a potentially pathogenic Haemophilus sp., Neisseria sp., Streptococcus sp. and Veillonella sp. compared with healthy controls (194). Of note, IPF subjects also exhibited increased bacterial burden compared with COPD and healthy subjects. In addition, subjects who had the greatest bacterial burden had lower survival rate at 30 months, suggesting that overall bacterial burden impacts on disease progression and decline.
1.7.3 Current understanding on the airway microbiota

It is now accepted that the healthy LRT consists a complex microbiota. The URT and LRT are viewed as a continuum with a gradual shift in local growth environment. The composition is further influenced by the rate of migration and elimination of microbes. This helps explain the similarities between the URT and LRT microbiota composition noted in previous studies (177), though there is spatial variation between different sites within the LRT (e.g. right upper lobe and left lower lobe). It should also be noted that there is significant inter-subject variation in microbiota composition.

The airway microbiota in chronic lung diseases appears to be distinct from that in health, with a greater proportion of potentially pathogenic species. This imbalanced state with respect to composition, diversity and overall bacterial burden is associated with adverse clinical outcomes. This imbalance seems to be further exaggerated during periods of disease exacerbation. The disrupted microbiota state has been termed ‘dysbiosis’ (186, 195). The proposed mechanisms by which a dysbiotic microbiota results in adverse clinical outcomes are not well defined, though increased airway inflammation secondary to greater abundance of ‘pathogenic’ species and reduced abundance of ‘commensals’ has been postulated. This will be discussed later.

The ensuing sections place focus on the published literature on the asthmatic airway microbiota and discuss the current gaps in knowledge.
1.8 The asthmatic airway microbiome

1.8.1 Imbalanced microbiota composition

In the initial report by Hilty and co-workers, the authors observed a greater relative abundance of Proteobacteria, in particular the ‘potentially pathogenic’ *Haemophilus* spp., in the microbiota of asthmatic subjects compared with healthy controls, who in turn exhibited a greater proportion of Bacteroidetes, particularly the ‘commensal’ *Prevotella* spp.(174) (Figure 1.3). The study was based on 11 asthmatic subjects of varying disease severity and treatment. Subsequent studies have examined the microbiota of various disease severity. Marri and co-workers examined 10 subjects with mild asthma and made similar observations(176). More recently, Zhang and co-workers performed a larger study consisting of 26 severe asthmatic and 18 non-severe asthmatic subjects. Interestingly, severe asthmatics exhibited reduced relative abundance of Proteobacteria compared with non-severe asthmatics(196). This finding was also observed in a study by Huang and co-workers(197).

The present findings do support the postulation of a distinct and imbalanced microbiota in asthma. However, it should be noted that, similar to other microbiome studies, there was significant inter-subject beta diversity, meaning subjects with the same disease may possess very different microbiota. It is therefore important to interpret such broad speculation on a heterogeneous disease with caution.

1.8.2 Microbiota composition/ structure associations with disease features

Currently it remains unclear if such differences in microbiota composition are related to disease severity or confounded by the effects of ICS and other phenotypic/ endotypic features.
A numbers of studies have attempted to investigate if certain taxa or community structure is associated with disease features.

1.8.2.1 ACQ score

In subjects with severe asthma, Proteobacteria dominant communities, consisting of families including Pasteurellaceae, Enterobacteriaceae, Neisseriaceae, Burkholderiaceae, and Pseudomonadaceae, were associated worsening ACQ score, whilst Actinobacteria dominant communities with families including Streptomycetaceae, Nocardiaceae, and Mycobacteriaceae were associated with improving or stable ACQ scores (197). Furthermore, greater bacterial burden also correlated with stable ACQ score (197), in contrast to previous findings in IPF and COPD that were associated with adverse outcomes (194, 198).

1.8.2.2 Bronchial hyper-responsiveness

In a cohort of sub-optimally controlled asthmatic subjects (average ACQ score of 1.8), greater relative abundance of Proteobacteria (including the families Comamonadaceae, Sphingomonadaceae, Nitrosomonadaceae, Oxalobacteraceae, and Pseudomonadaceae) were associated with greater bronchial hyper-responsiveness (199). In addition, greater alpha diversity was also associated with greater hyper-responsiveness (199).

1.8.2.3 Responsiveness to corticosteroid therapy

Thus far there have been two studies examining microbiota composition/structure and responsiveness to corticosteroid therapy. In a study by Huang and co-workers, Actinobacteria and to a certain extent Proteobacteria were associated with expression of FKBPS, a marker of steroid response, although the authors did not report identification of genus or species.
Furthermore, increased alpha diversity also significantly correlated with FKBP5 expression (197).

Goleva and co-workers addressed the same question in a different manner (200). Thirty-nine asthmatic subjects were given a course of systemic corticosteroid (Prednisolone 20mg twice a day for seven days). The cohort was then segregated into two groups: those who were ‘steroid responsive’ (as denoted by a 31% increase in FEV₁ post treatment) and those who were ‘steroid resistant’ (as defined by improvement in FEV₁ of less than 10% post treatment). The authors noted that within the steroid-resistant group, there was a subgroup of subjects (14 out of 29) who exhibited an expansion of Proteobacteria, including Haemophilus at genus level. BAL macrophages were then stimulated with either Haemophilus parainfluenzae (H. parainfluenzae), one of the uniquely expanded potential pathogens in the steroid-resistant subgroup, resulting in p38 mitogen-activated protein (MAPK) activation, increased IL-8, mitogen-activated kinase phosphatase 1 mRNA expression and ultimately reduced corticosteroid responses. In contrast, stimulation with the commensal Prevotella melaninogenica did not result with the same effect. The authors therefore concluded that specific Proteobacteria such as H. parainfluenzae may induce corticosteroid resistance.

1.8.2.4 Body mass index

In the study by Huang and co-workers, taxa from the phyla Firmicutes and Bacteroidetes were strongly associated with increased BMI, whilst Proteobacteria negatively correlated with body mass index (BMI) (197). Increased BMI was associated with lower eosinophil counts on bronchial biopsy. The authors speculated whether certain members of the Bacteroidetes and Firmicutes may be influential over the extent of eosinophilic inflammation or, alternatively,
stimulate non-eosinophil-associated immune responses. Further work is needed to address this issue.

1.9 The concept of dysbiosis in asthma

The understanding of the asthmatic airway microbiota has advanced significantly since the initial report by Hilty and co-workers(174). Multiple studies have illustrated that the airway microbiota in asthma is distinct from that in health, with a disproportionately high abundance of ‘potentially pathogenic’ Proteobacteria(174, 176, 196), though there was significant inter-subject variation (as noted in other microbiota studies). More recent studies have observed correlations between specific taxa/ community diversity and disease feature as discussed above. Thus far, increased abundance of species from the phylum Proteobacteria, in particular *Haemophilus*, has been associated with worse disease features, whilst species from the phyla Bacteroidetes and Actinobacteria are associated with less severe disease(197, 200). Interestingly, a similar pattern is observed within the gut microbiota in gastrointestinal diseases(201). Recent in vitro studies have illustrated that different bacterial species have the capacity to induce different immune responses. A study by Larsen and co-workers showed that recognised respiratory pathogens such as *H. influenzae* and *M. catarrhalis* induced approximately three to five-fold greater production of IL-23, IL-12p70 and IL-10 in human monocyte-derived dendritic cells (DCs) compared to the commensal bacteria (*Prevotella melaninogenica, Prevotella nanceiensis, Prevotella salivae, Veillonella dispar* and *Actinomyces graevenitzii*)(202). Remarkably, co-culture experiments found that *Prevotella* spp. could attenuate *H. influenzae*-induced IL-12p70 in DCs.
Whilst dysbiosis may be viewed as a marker of disease (18, 201), it remains unclear if it is cause or consequence of disease. However, recent animal studies have shed some light to support an active role of the microbiota in influencing the nature of airway inflammation. A study by Herbst and co-workers using germ-free mice demonstrated that absence of microbial colonisation resulted in increased allergic airway inflammation, which resolved following recolonization (203). In support of this, Nembrini and co-workers exposed mice to *Escherichia coli* and observed reduction of allergic Th2 type inflammation via a TLR-4 dependent mechanism (16). Conversely, a recent study by Yadava and co-workers created a murine model of chronic airway inflammation by repeated lipopolysaccharide (LPS)/ elastase challenge and evaluated the airway microbiota following establishment of chronic airway inflammation (204). Significantly the authors observed a reduction in alpha diversity, increased abundance of *Pseudomonas* and *Lactobacillus* with reduced abundance of *Prevotella*, suggesting the presence of host–microbial cross-talk in promoting chronic inflammation.

With respect to acute exacerbations, Dickson and co-workers proposed a cycle of host inflammation and respiratory dysbiosis (186) (Figure 1.4). A trigger such as a respiratory viral infection induces airway inflammation, resulting in alteration in the local environment via increased mucus secretion, inflammatory cell activation and inflammatory cytokine release. This new ‘inflammatory’ environment favours the growth of certain species and restricts growth of others, further worsening the state of dysbiosis which in turns promotes further airway inflammation.
1.10 Viral-bacterial interactions

Following on from the postulation of a cycle of host inflammation and microbiota dysbiosis, there is increasing evidence that viral infections may perturb the airway microbiota enhancing the risk of secondary bacterial infection. Conversely, there is also evidence to suggest the microbiota may influence the host immune response against viral infection(205).

*Figure 1.4 Cycle of host inflammation and respiratory dysbiosis.Copied from ‘The role of the microbiome in exacerbations of chronic lung diseases’ by Dickson and co-workers with permission(185)*
1.10.1 Viral infections perturb the airway microbiota

It has long been postulated that viral infection may enhance the risk of secondary bacterial infection. The early studies were predominantly animal models, demonstrating the ability of influenza virus to enhance the risk of secondary infection by *H. influenzae*, *S. aureus*, *Listeria monocytogenes* and *group B Streptococcus* (206-209).

This phenomenon has been mirrored clinically. In the 2009 influenza A pandemic virus (pH1N1) pandemic, significant morbidity and mortality was related to the strain’s capacity to replicate within the LRT, leading to epithelial destruction, viral pneumonia and subsequent secondary bacterial pneumonia (210). Leung and co-workers performed a study examining the oropharyngeal metagenome of subjects with and without pH1N1 infection (210). Infected subjects exhibited greater relative abundance of Firmicutes and Proteobacteria, particularly *Pseudomonas* and *Acinetobacter* species. Metagenomic sequencing further showed insights into the functional genes encoded by these organisms that were significantly augmented for motility, transcriptional regulation, metabolism and signalling genes, in addition to pathways involved in secondary metabolite biosynthesis and catabolism. Of note, these functional attributes were notably absent from the genera that significantly decreased in relative abundance (e.g. *Prevotella*, *Veillonella*, and *Neisseria*).

1.10.1.1 Microbiota composition alterations following RV infection

A number of clinical studies have investigated the impact of bacterial co-infection in RV-induced COPD and asthma exacerbations. Wilkinson and co-workers found that in subjects with COPD exacerbations, the presence of RV and pathogenic bacteria such as *H. influenzae* resulted in greater symptoms and FEV₁ decline (189). In a cohort of 308 children
asthmatic and 142 non-asthmatic), detection of RV greatly increased the likelihood of detecting a pathogenic bacterial species (S. pneumoniae, H. influenzae and M. catarrhalis by quantitative PCR)(211). In those with RV infection, detection of S. pneumoniae increased the risk of moderate AEs, whilst positive detection of M. catarrhalis was associated with greater cold and asthma symptoms compared with subjects with RV infection alone.

More recently, a number of studies have employed experimental RV infection challenge model. Molyneaux and co-workers observed a significant outgrowth of H. influenzae approximately two weeks following RV infection(198). Whilst the authors did not specifically report the effects of H. influenzae, overall bacterial burden was significantly associated with sputum inflammatory cells, neutrophils and neutrophil elastase levels. In another experimental RV infection study investigating the nasal microbiota of ten healthy subjects using nasal lavage, a significant increase in the relative abundance of Neisseria and Propionibacterium at genus level was noted following RV infection(212). In a study of six healthy subjects assessing the pharyngeal microbiota via throat swabs, a similar pattern was observed with an increase in H. parainfluenzae and Neisseria subflava following RV infection though it was not statistically significant(213).
1.10.2 Potential mechanisms by which viral infections may enhance bacterial colonisation and infection

There has been a great effort attempting to elucidate the mechanisms by which viral infections may promote secondary bacterial infections. The potential mechanisms appear to be numerous (214), which are summarised below and in Figure 1.5.

**Figure 1.5** Potential mechanisms by which viral infections may promote bacterial colonisation and subsequent infection. Adapted from 'Viral and bacterial interactions in the upper respiratory tract' by Bosch and co-workers (212).

1.10.2.1 Predisposition to bacterial adherence

Animal studies have illustrated that viral infection may predispose the epithelium to bacterial adherence and subsequent infection. *S. pneumoniae* adherence to the epithelial surface is enhanced following RSV infection (215). This may occur simultaneously with viral infection or after recovery. The risk of *S. pneumoniae* infection is increased even after recovery from
influenza infection, possibly related to increased IL-10 production resulting in dampened neutrophil function(216).

1.10.2.2 Disruption of epithelial barrier

Epithelial damage secondary to viral infection may expose underlying extracellular matrix proteins and facilitate bacterial entry. *S. pneumoniae, S. aureus* and *M. catarrhalis* have been shown to have strong affinity for extracellular matrix proteins such as fibronectin(217-219). Although RV infection does not generally result in extensive epithelial damage, it enhances bacterial adhesion by upregulating expression of fibronectin(220).

1.10.2.3 Upregulation of adhesion proteins

‘Major’ group RVs use ICAM-1 as their receptor, which is also utilised by *H. influenzae*. Conversely, *H. influenzae* make potentiate RV-induced immune response by inducing airway epithelial cell expression of ICAM-1 and TLR-3(221)

1.10.2.4 Dysfunction of innate immune response

In addition to enhancing bacterial adhesion and invasion, viral infections can blunt the innate immune response against bacteria. RSV has been shown to reduce bacterial clearance via altered neutrophil function(222), whilst influenza may enhance IL-10 secretion thus reducing neutrophil activation in addition to impairing neutrophil functions such as phagocytosis and intracellular reactive oxygen species generation(223). In COPD, rhinovirus infection induces neutrophil elastase resulting in increased degradation of anti-microbial peptides, potentially enabling outgrowth of pathogenic bacterial species(224). Finally, following a heightened inflammatory response against a viral infection, TLR desensitisation may occur in conjunction
with altered macrophage function, further enhancing risk of subsequent bacterial infection(225).

**1.10.3 Microbiota may modulate response to viral infection**

Whilst the majority of studies have investigated the impact of viral infection on the microbiota, there is now some evidence to suggest a bi-directional relationship. In a murine study, two strains of the commensal *Lactobacillus rhamnosus*, CRL1505 (Lr05) or CRL1506 (Lr06), protected against RSV infection(226). Interestingly, the mechanisms of ‘protection’ appeared to differ between the two strains. Lr05 induced IFN-γ and IL-10, whilst Lr06 modulated production of IFN-α, IFN-β and IL-6 expression. In both cases, TLR-3/retinoic acid-inducible gene 1 activation was associated with protection against RSV infection. These findings imply that different species may modulate the immune response via different mechanisms. Further work is needed to disentangle how the microbiota modulates the immune response as a community.

**1.11 Research rationale**

To date, all published studies on the asthmatic airway microbiota has been performed during chronic disease and there is a paucity of studies investigating the dynamics of the airway microbiota in the context of a virus-induced AE. There is an urgent clinical need to better define the role of bacteria in AEs as broad spectrum antibiotic therapy is often inappropriately prescribed. In light of the increasing evidence supporting viral-bacterial interactions and a dysbiotic microbiota in asthma, it seems plausible that a viral infection may further perturb an already imbalanced microbiota, resulting in increased airway inflammation and ultimately clinical symptoms.
1.12 Project aims

The project is divided to two parts. The first study aims to evaluate the dynamics of the asthmatic airway microbiota during a naturally occurring cold. The second study will focus on the impact of RV infection on the healthy and asthmatic airway microbiota using an experimental RV-16 challenge model.

1.12.1 Naturally occurring cold

- To investigate the dynamic changes within the airway microbiota of subjects with mild/moderate asthma during a naturally occurring cold.
- To examine if changes within the airway microbiota during a naturally occurring cold correlate with changes in clinical parameters (cold symptoms and lung function) and biomarkers of airway inflammation.

1.12.2 Experimental RV-16 infection

- To investigate the changes within the airway microbiota following experimental RV-16 infection in a cohort of moderate asthmatic and healthy subjects.
- To examine if changes within the airway microbiota following experimental RV-16 infection correlate with changes in clinical parameters (cold symptoms and lung function) and biomarkers of airway inflammation.
- To examine the differences in the airway microbiota between asthmatic and healthy subjects in period of stability and following experimental RV-16 infection.
1.13 Hypothesis

- Following RV-16 infection, the asthmatic airway microbiota is perturbed to a more pathogenic composition (compared with the airway microbiota in health) following a naturally occurring cold/ experimental RV-16 infection, leading to greater degree of airway inflammation ultimately resulting in increased airflow obstruction and clinical symptoms
2 Methods

2.1 Outline

The chapter will be divided into three main sections. The first section describes the clinical aspects of the project including subject recruitment and clinical sampling of each study. The second section depicts laboratory tests (virology, pro-inflammatory cytokine measurements). The final third section will focus on 16S rRNA gene sequencing and downstream analyses.

2.2 Materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
<th>Application</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose, molecular grade</td>
<td>As per manufacturer</td>
<td>Gel electrophoresis</td>
<td>Bioline, London, UK</td>
</tr>
<tr>
<td>Agencourt® AMPure® XP</td>
<td>As per manufacturer</td>
<td>16S rRNA amplicon purification</td>
<td>Beckman Coulter, High Wycombe, UK</td>
</tr>
<tr>
<td>Bovine Serum Albumin Powder (BSA)</td>
<td>1% BSA in 0.15M NaCl pH 7.0</td>
<td>ELISA</td>
<td>Sigma Aldrich, MO, USA</td>
</tr>
<tr>
<td>Diluent 1</td>
<td>RPMI-like medium. No other details supplied by manufacturer</td>
<td>MSD</td>
<td>MSD, USA</td>
</tr>
<tr>
<td>DNA ladder 1kb</td>
<td>As per manufacturer</td>
<td>Gel electrophoresis</td>
<td>Life Technologies, Paisley, UK</td>
</tr>
<tr>
<td>dNTP</td>
<td>100mM dATP, 100mM dCTP, 100mM dGTP, 100mM dTTP, 100mM dUTP</td>
<td>Virology PCR</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>ELISA stop solution</td>
<td>1M H₂SO₄</td>
<td>ELISA</td>
<td>Sigma Aldrich, MO, USA</td>
</tr>
<tr>
<td>ELISA/ MSD wash buffer</td>
<td>PBS, 0.05% Tween-20</td>
<td>ELISA, MSD</td>
<td>Sigma Aldrich, MO, USA</td>
</tr>
<tr>
<td>GoTaq Flexi DNA Polymerase</td>
<td>As per manufacturer</td>
<td>Virology PCR</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>Illumina Library Quantification DNA Standards</td>
<td>As per manufacturer</td>
<td>16S rRNA library quantification</td>
<td>KAPA BioSystems Ltd, London, UK</td>
</tr>
<tr>
<td>Low EDTA TE buffer</td>
<td>As per manufacturer</td>
<td>16S rRNA amplicon purification</td>
<td>Invitrogen, Thermo Fisher Scientific, MA, USA</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>As per manufacturer</td>
<td>qPCR</td>
<td>Promega, Southampton, UK</td>
</tr>
</tbody>
</table>
### Table 2.1 Details of materials used

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Application</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2ml Skirted 96-well PCR plate</td>
<td>Quadruplicate PCR</td>
<td>Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>Adhesive Sealing sheets</td>
<td>Quadruplicate PCR</td>
<td>Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>Costar 96-well black fluorometer plate</td>
<td>PicoGreen</td>
<td>Corning Inc, NY, USA</td>
</tr>
<tr>
<td>Eppendorf Safe-Lock tube 1.7ml</td>
<td>General use, DNA extraction</td>
<td>Eppendorf, Stevenage, UK</td>
</tr>
<tr>
<td>Eppendorf Safe-Lock tube 2.0ml</td>
<td>General use, DNA extraction</td>
<td>Eppendorf, Stevenage, UK</td>
</tr>
<tr>
<td>MicroAmp fast 96-well reaction plate</td>
<td>qPCR</td>
<td>Applied Biosystems, Paisley, UK</td>
</tr>
<tr>
<td>Nunc 96-well micro-plate</td>
<td>AmPure purification</td>
<td>ThermoScientific, MA, USA</td>
</tr>
<tr>
<td>Nunc MaxiSorp 96-well plate</td>
<td>ELISA</td>
<td>ThermoScientific, MA, USA</td>
</tr>
</tbody>
</table>

### Table 2.2 Details of consumables used

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Application</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (per tablet)</td>
<td>0.001M phosphate buffer, 0.0027M KCl, 0.137M NaCl in 200ml distilled water</td>
<td>ELISA Sigma Aldrich, MO, USA</td>
</tr>
<tr>
<td>PCR water</td>
<td>As per manufacturer</td>
<td>qPCR Cambio, Cambridge, UK</td>
</tr>
<tr>
<td>PhiX Control v3</td>
<td>As per manufacturer</td>
<td>16S rRNA sequencing control Illumina, CA, USA</td>
</tr>
<tr>
<td>Q5® High-Fidelity 2X Master Mix</td>
<td>As per manufacturer</td>
<td>16S rRNA quadruplicate PCR New England Biolabs, Herts, UK</td>
</tr>
<tr>
<td>Quantitect probe PCR Mastermix</td>
<td>As per manufacturer</td>
<td>RV-16 qPCR Qiagen Ltd, Crawley, UK</td>
</tr>
<tr>
<td>Random hexamers</td>
<td>As per manufacturer</td>
<td>Virology qPCR Promega, Southampton, UK</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>As per manufacturer</td>
<td>Virology PCR Invitrogen, MA, USA</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>As per manufacturer</td>
<td>16S rRNA amplicon library and PhiX denaturation Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Streptavidin-HRP (Horseradish Peroxidase)</td>
<td>In PBS pH 7.2 containing 0.01% Thimerosal and 40% glycerol</td>
<td>ELISA Biosource Inc, USA</td>
</tr>
<tr>
<td>SYBR Fast qPCR Kit Master Mix</td>
<td>As per manufacturer</td>
<td>16S rRNA qPCR KAPA BioSystems Ltd, London, UK</td>
</tr>
<tr>
<td>TMB</td>
<td>TMB (3,3’, 5,5’-tetramethylbenzidine) chromogen solution</td>
<td>ELISA Invitrogen, MA, USA</td>
</tr>
<tr>
<td>Tween-20</td>
<td>20 ethylene oxide units, 1 sorbitol unit, 1 lauric acid unit</td>
<td>ELISA, MSD Sigma Aldrich, MO, USA</td>
</tr>
</tbody>
</table>
### Table 2.3 Details of commercial kits used

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent High Sensitivity DNA Kit</td>
<td>Agilent Technologies, CA, USA</td>
</tr>
<tr>
<td>Duoset Human IP-10 Quantikine ELISA kit</td>
<td>R&amp;D Systems, Oxford, UK</td>
</tr>
<tr>
<td>Fast DNA™ SPIN Kit for Soil</td>
<td>MP Bio, CA, USA</td>
</tr>
<tr>
<td>MiSEQ v2 Reagent Kit</td>
<td>Illumina, CA, USA</td>
</tr>
<tr>
<td>MSD human proinflammatory-4 II Tissue Culture Kit</td>
<td>MSD, MD, USA</td>
</tr>
<tr>
<td>Omniscr ipt RT kit</td>
<td>Qiagen Ltd, Crawley, UK</td>
</tr>
<tr>
<td>QIAamp viral RNA mini kit</td>
<td>Qiagen Ltd, Crawley, UK</td>
</tr>
<tr>
<td>QIAquick® PCR Purification Kit</td>
<td>Qiagen Ltd, Crawley, UK</td>
</tr>
<tr>
<td>Quant-iT™ PicoGreen® dsDNA Assay Kit</td>
<td>Life Technologies, CA, USA</td>
</tr>
</tbody>
</table>

### Table 2.4 Details of instruments used

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchosorption</td>
<td>Mucosal Diagnostics, Hunt Developments Ltd, Midhurst, UK</td>
</tr>
<tr>
<td>Flocked throat swab</td>
<td>VWR, Lutterworth, UK</td>
</tr>
<tr>
<td>MicroMedical MicroLab spirometer</td>
<td>MicroMedical, Rochester, UK</td>
</tr>
<tr>
<td>Olympus PF260 bronchoscope</td>
<td>Olympus, UK</td>
</tr>
<tr>
<td>Piko-1 device</td>
<td>nSpire, UK</td>
</tr>
<tr>
<td>RV-16 atomiser, model 286-RD</td>
<td>DeVilbiss Healthcare, Heston, UK</td>
</tr>
<tr>
<td>Synthetic absorptive matrix (SAM)</td>
<td>Leukosorb, Pall Life Sciences, Hampshire, UK</td>
</tr>
</tbody>
</table>

### Table 2.5 Details of laboratory equipment used

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Biosystems 2720 Thermal Cycler</td>
<td>Applied Biosystems, Paisley, UK</td>
</tr>
<tr>
<td>Biosystems ABI Prism 7700 sequence detection system</td>
<td>Applied Biosystems, Paisley, UK</td>
</tr>
<tr>
<td>Eppendorf 5415R centrifuge</td>
<td>Eppendorf, Stevenage, UK</td>
</tr>
<tr>
<td>Illumina® MiSEQ sequencer</td>
<td>Illumina, CA, USA</td>
</tr>
<tr>
<td>Infinite® 200 Pro</td>
<td>Tecan Group Ltd, Männedorf, Switzerland</td>
</tr>
<tr>
<td>MSEO QuickPlex SQ 120 imager</td>
<td>MSD, MD, USA</td>
</tr>
<tr>
<td>Precellys24 homogeniser</td>
<td>Bertin Instruments, Montigny-le-Bretonneux, France</td>
</tr>
<tr>
<td>Spectramax Plus plate reader</td>
<td>MDS Analytical Technologies, Wokingham, UK</td>
</tr>
<tr>
<td>Stuart Tube Rollers SRT6D Rotator</td>
<td>Bibby Scientific Limited, Staffordshire, UK</td>
</tr>
<tr>
<td>ViiA™ 7 Real-Time PCR System</td>
<td>Thermo Fisher Scientific Inc, MA, USA</td>
</tr>
</tbody>
</table>
Table 2.6 Details of softwares used

<table>
<thead>
<tr>
<th>Software</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discovery Workbench® 4.0 Software</td>
<td>MSD, MD, USA</td>
</tr>
<tr>
<td>GraphPad Prism6</td>
<td>GraphPad Software Inc, La Jolla, USA</td>
</tr>
<tr>
<td>HRM Software Module for ViiA™ 7 System</td>
<td>Thermo Fisher Scientific Inc, MA, USA</td>
</tr>
<tr>
<td>QIIME</td>
<td>QIIME</td>
</tr>
<tr>
<td>RStudio</td>
<td>RStudio, MA, USA</td>
</tr>
<tr>
<td>Softmax Pro software, version 5.2</td>
<td>Molecular Devices, Sunnyvale, USA</td>
</tr>
</tbody>
</table>

2.3 The asthmatic airway microbiota in naturally occurring colds

2.3.1 Study aim

The chief aim of the study was to investigate the dynamic changes within the airway microbiota of subjects with mild/ moderate asthma during a naturally occurring cold. Furthermore, the study examines if changes within the airway microbiota during a naturally occurring cold correlate with changes in clinical parameters (cold symptoms and lung function) and biomarkers of airway inflammation.

2.3.2 Study cohort

2.3.2.1 Sample size

A sample size of 47 asthmatic subjects was targeted. This was based on the power calculation that a sample size of 47 has 80% power and an alpha significance level of 5% to detect a range from 30-70% of secondary bacterial infections.

2.3.2.2 Inclusion criteria

- Age 18 - 65
Clinical diagnosis of asthma (by GP or respiratory physician)

Symptoms of asthma for at least 2 years prior to the screening visit confirmed by a medical history and

- ≥12% and 200mL bronchodilator reversibility at screening or documented in the past, OR,
- Evidence of bronchial hyper-responsiveness at screening or documented in the past (as defined by histamine PC_{20} < 8 µg/ml), OR,
- A documented hospital admission (including an Emergency department admission) for asthma since the age of 18, OR.
- Documented evidence that they have attended their GP surgery, out-of-hours clinic (or alternative health care provider) for worsening of asthma symptoms, since the age of 18

Pre-bronchodilator FEV₁ ≥40% and post-bronchodilator FEV₁ ≥ 50% predicted at screening

No other chronic respiratory condition or systemic disease

No respiratory tract infection or exacerbation for six weeks

Asthmatics may be on treatment including:

- SABA i.e. Salbutamol
- ICS
- LABA
- Combination LABA and ICS
- Up to step 4 BTS guidelines / GINA moderate asthma(4, 149)
2.3.2.3 Exclusion criteria

- Current smokers
- Current significant other chronic respiratory or systemic disease
- Severe or uncontrolled asthmatics (GINA - moderate persistent or severe persistent, BTS - step 5)(4, 149)
- Significant other abnormalities on lung function testing i.e. restrictive defect etc.

2.3.2.4 Recruitment method

Potential participants were recruited via newspaper advertisement (Metro and London Evening Standard). Additional adverts were placed on the Imperial College Website and on noticeboards around the Imperial College Campus. Potential participants contacted the research team via telephone and email. They were then sent patient information sheet and invited to a screening visit (Visit 1).

2.3.3 Ethics and consent

All clinical work and sampling was approved by the London Bridge Research Ethics Committee (reference 12/LO/0312) and was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all subjects prior to their participation.

2.3.4 Study design

At screening visit (Visit 1), informed consent was obtained and inclusion and exclusion criteria were reviewed. If eligible, subject would undertake baseline sampling (Visit 1), after which
subject would contact the research team again as soon as possible on developing cold symptoms. Subject was reviewed and sampled within 48 hours of developing cold symptoms (Visit 2). Further follow-up visits took place on day 4/5 (Visit 3) and on day 14/15 (Visit 4) post cold symptoms. Figure 2.1 illustrates the study design.

![Figure 2.1 Design of naturally occurring cold study](image)

2.3.5 Visit breakdown

2.3.5.1 Visit 1 (Baseline)

The purpose of this visit was to obtain informed consent, assess eligibility and obtain baseline samples. The following sequence was performed:

- Informed consent – signing of study consent form after addressing any questions from participant
• General medical history and clinical examination to exclude significant co-morbidities.
  Medication history and allergy status obtained
• Asthma control questionnaire (ACQ) to assess symptom control(227)
• Spirometry including measurement of PEF, FEV₁ and FVC
• Clinical observations including blood pressure, heart rate, respiratory rate, oxygen saturation and temperature
• Histamine challenge test (PC<sub>20</sub>) to confirm airway hyper-responsiveness (AHR)
• Blood tests including full blood count (FBC), urea and electrolytes (U&Es), C-reactive protein (CRP) and IgE
• Nasosorption for pro-inflammatory cytokines
• Nasal lavage for virology
• Throat swab for virology
• Sputum induction. Sample was split into two portions for quantitative culture and 16S rRNA sequencing respectively

2.3.5.2 Visit 2 (Acute phase of natural cold)

• Clinical history and examination to confirm the diagnosis of naturally occurring cold, change in medication/ allergy status
• Spirometry including measurement of PEF, FEV₁ and FVC
• Clinical observations including blood pressure, heart rate, respiratory rate, oxygen saturation and temperature
• Blood tests including full blood count (FBC), urea and electrolytes (U&Es), C-reactive protein (CRP) and IgE
• Nasosorption for pro-inflammatory cytokines
• Nasal lavage for virology
• Throat swab for virology
• Sputum induction. Sample was split into two portions for quantitative culture and 16S rRNA sequencing respectively
• Confirmation of commencement of symptom diary. The Wisconsin Upper Respiratory Symptom Survey (WURRS-21) was used

2.3.5.3 Visit 3 (Mid phase of natural cold)

Visit breakdown was as per Visit 2 as described above. Cold symptom diary compliance was checked.

2.3.5.4 Visit 4 (End of natural cold)

Visit breakdown was as per Visit 2. Cold symptom diary compliance was again checked. All subjects continued with symptom diaries for a duration of 28 days. Diaries were subsequently returned to research team.

Detailed descriptions of spirometry, cold symptom score and sampling techniques are presented in the following section.

2.3.5.5 Longitudinal sampling in chronic stable disease

In addition to subjects with naturally-occurring colds, a further eight subjects with stable chronic disease (in absence of naturally occurring cold or any other acute illness) were invited back for longitudinal sampling to evaluate changes in the airway microbiota over time in stable chronic disease. The same sampling procedures were followed as described above.
2.3.6 Spirometry

Spirometry was performed using a MicroMedical MicroLab spirometer (MicroMedical, Rochester, UK) in accordance to the BTS/ARTP guidelines(228).

2.3.7 Histamine challenge test

AHR is defined as an abnormal increase in airflow limitation due to an increased sensitivity of the airways to inhaled stimuli(229). Histamine diphosphate, a bronchoconstrictor with direct actions on airways smooth muscle, was used in the current study(229). Assessment of AHR was performed using the two-minute tidal breathing method in accordance to published guidelines(229). PC20, the provocative concentration of histamine resulting in a 20% reduction in FEV1, was subsequently calculated.

2.3.8 The Wisconsin Upper Respiratory Symptom Survey (WURRS-21)

The WURRS-21 is a validated illness-specific health-related quality-of-life questionnaire outcomes instrument(230). It comprises of 21 questions divided into clinical symptoms and functional impairments (Figure 2.2). It is a shortened form of the original WURRS-44 and has been demonstrated to have similar performance in respect to reliability, responsiveness, importance-to-patients, and convergence with other measures(230). Importantly, the WURRS-21 score has been illustrated to predict subsequent changes in asthma control (as represented by changes in mini-ACQ score) especially in the acute phase of cold(231).
<table>
<thead>
<tr>
<th></th>
<th>Not sick</th>
<th>Very mildly</th>
<th>Mildly</th>
<th>Moderately</th>
<th>Severely</th>
</tr>
</thead>
<tbody>
<tr>
<td>How sick do you feel today?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Please rate the average severity of your cold symptoms over the last 24 hours for each symptom:

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not have this symptom</th>
<th>Very mild</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runny nose</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Plugged nose</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Sneezing</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Sore throat</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Scratchy throat</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Cough</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Hoarseness</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Head congestion</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Chest congestion</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Feeling tired</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Over the last 24 hours, how much has your cold interfered with your ability to:

<table>
<thead>
<tr>
<th>Activity</th>
<th>Not at all</th>
<th>Very mildly</th>
<th>Mildly</th>
<th>Moderately</th>
<th>Severely</th>
</tr>
</thead>
<tbody>
<tr>
<td>Think clearly</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Sleep well</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Breathe easily</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Walk, climb stairs, exercise</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Accomplish daily activities</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Work outside the home</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Work inside the home</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Interact with others</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Live your personal life</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Compared to yesterday, I feel that my cold is…

<table>
<thead>
<tr>
<th>Improvement</th>
<th>Very much better</th>
<th>Somewhat better</th>
<th>A little better</th>
<th>The same</th>
<th>A little worse</th>
<th>Somewhat worse</th>
<th>Very much worse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compared to yesterday</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

WURSS -21 © (Wisconsin Upper Respiratory Symptom Survey) 2004
Created by Bruce Barrett MD PhD et al., UW Department of Family Medicine, 777 S. Milis St. Madison, WI 53715, USA

Figure 2.2 The Wisconsin Upper Respiratory Symptom Survey (WURRS-21) as designed by Barrett and co-workers.
2.3.9 Nasosorption

Nasosorption was used for the measurement of pro-inflammatory cytokines. This was performed prior to nasal lavage. A single strip of synthetic absorptive matrix (SAM) (Leukosorb, Pall Life Sciences, Hampshire, UK) measuring 7mm x 35mm was placed into each nostril for a total duration of two minutes to obtain samples of neat nasal mucosal lining fluid (Figure 2.3). The strips were then removed and inserted into a single labelled Spin-X Centrifuge Tube with Filter (Sigma-Aldrich, USA), which has been pre-filled with 200µl of pre-chilled assay buffer (phosphate buffered saline, bovine serum albumin (1%); Tween®-20 (0.05%); sodium azide (0.05%)). The sample was transported to the laboratory on ice and centrifuged at 16 000g for 5 minutes at 4°C. The eluate was then aliquoted into a 0.65mL labelled Eppendorf tube and stored at -80°C until further analysis.

![Figure 2.3](image)

**Figure 2.3** (A) Nasosorption using synthetic absorptive matrix (SAM) being advanced into the right nostril (B) SAM strip deployed in nose.

2.3.10 Nasal lavage

5 mL of sterile 0.9% saline was drawn into a 10mL syringe. A nasal olive was attached to the end of the syringe and this was employed to block the nostril to prevent leakage of lavage
fluid. With the subject’s head tilted forward, the saline was instilled into the nose. The fluid was aspirated by manual suction through the syringe and then gently instilled back into the nose. This process was repeated a total of 20 times in 1 minute before removal of the olive from the nose. The nasal lavage sample was aliquoted into 1 ml samples (1.7ml sterile labelled Eppendorf tubes) and stored at -80°C until further analysis (respiratory virus detection).

2.3.11 Throat swab

With the subject’s head tilted back, a sterile flocked throat swab (VWR, Lutterworth, UK) was inserted into the mouth (avoiding the tongue with the aid of a tongue depressor). The central posterior pharynx and tonsillar area was sampled by gentle rotation for approximately 5 seconds. The swab was then immediately removed from mouth. The flocked tip was cut using a pair of sterile scissors and placed into a labelled sterile 5ml universal tube. The sample was stored at -80°C until further analysis (respiratory virus detection).

2.3.12 Sputum induction

Sputum induction was performed using a two-minute tidal breathing method using nebulised 5% saline for a maximum of four cycles. Spirometry was monitored after each cycle to ensure subject safety. Induced sputum was expectorated into sterile universal container. Sample was transported back to laboratory on ice within 30 minutes. Sputum plugs were isolated using sterile forceps and placed into labelled 2ml Eppendorf tube. Sample was aliquoted into two portions for quantitative culture and 16S rRNA gene sequencing respectively. Samples for 16S rRNA gene sequencing were stored at -80°C until further analysis.
2.4  The airway microbiota in experimental rhinovirus infection

2.4.1  Study aim

This study focuses on the dynamic changes within the lower airway microbiota of healthy subjects and subjects with moderate asthma during an experimental RV-16 infection. In addition, the study examines if changes within the lower airway microbiota during an experimental RV-16 infection correlate with changes in clinical parameters (clinical symptoms and lung function) and biomarkers of airway inflammation.

Given the scale and cost of such investigations, this study was part of a larger project and additional samples (including blood test, nasal curettage, bronchial brushings and biopsies) were obtained from the participants and utilised for other studies. The following sections focus on the current study only.

2.4.2  Study cohort

2.4.2.1  Sample size

A total number of 15 healthy and 15 asthmatic subjects were targeted. Whilst this was not based on power calculation, previous similar studies using experimental RV-16 infection have yielded meaningful results.

2.4.2.2  Inclusion criteria for asthmatic subjects

- Age 18-55 years
- Clinical diagnosis of asthma (by GP or respiratory physician)
• Airway hyper-responsiveness as defined by histamine PC20 < 8 µg/ml (or < 12 µg/ml and bronchodilator response ≥ 12%) and worsening asthma symptoms with infection since last change in asthma therapy

• Atopic on skin prick allergen testing

• Treatment comprising ICS or combination inhaler (LABA+ICS). Subjects on ICS were on a daily dose of 400 µg fluticasone or equivalent

• ACQ score of ≥ 0.75

2.4.2.3 Exclusion criteria for asthmatic subjects

• Smoking history over 6 months prior to study entry

• Negative skin-prick tests

• Current symptoms of allergic rhinitis

• Current or previous history of significant respiratory disease (other than asthma)

• Any clinically relevant abnormality on screening or detected significant systemic disease

• Pregnant or nursing women

• Contact with infants or elderly at home or at work

• Asthma exacerbation or viral illness within 6 weeks prior to study entry

• Current treatment with oral corticosteroid therapy or in 3 months prior to study entry
• Use of nasal spray, anti-histamine, anti-leukotrienes

• Antibodies to RV-16 in a titre ≥ 1:2

2.4.2.4 Inclusion criteria for healthy subjects

• Age 18-55 years

• No history or clinical diagnosis of asthma

• No history of allergic rhinitis or eczema

• Negative responses on skin prick testing

• Histamine PC$_{20}$ ≥ 8 µg/ml and bronchodilator response <12%

• Absence of current or previous history of significant respiratory disease

• Absence of significant systemic disease

2.4.2.5 Exclusion criteria for healthy subjects

• Any clinically relevant abnormality on screening or detected significant systemic disease

• A current or previous diagnosis of asthma

• Any positive skin-prick test or current symptoms of allergic rhinitis

• History of eczema or allergic rhinitis

• Pregnant or nursing women
• Common cold within the previous 8 weeks

• Treatment with oral or inhaled corticosteroids at time of recruitment or in the previous 3 months; current use of LABA, nasal spray, anti-histamine, leukotrienes or tiotropium

• Shortness of breath score at screening over 1 or total lower respiratory tract score over 7

• Antibodies to RV-16 in a titre $\geq 1:2$

2.4.2.6 Recruitment

Subjects were recruited in the same fashion as previously described (see Section 2.2.2.4).

2.4.3 Ethics and consent

All clinical work and sampling was approved by the London Bridge Research Ethics Committee (reference 12/LO/1278) and was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all subjects prior to their participation.

2.4.4 Study design

Potential participants were given a patient information leaflet and invited to the initial screening visit. Informed consent was obtained and inclusion and exclusion criteria were reviewed. In addition, venepuncture was performed to assess for serum neutralising antibodies to RV-16. Subjects who were identified as seronegative subsequently returned for
a further screening visit and underwent histamine challenge to assess bronchial hyper-
responsiveness.

Eligible subjects were formally enrolled for the study and underwent baseline bronchoscopic sampling. Subjects were nasally inoculated with RV-16 14 days later and further underwent bronchoscopic sampling 3 and 8 days following RV-16 infections (Figure 2.4). All subjects completed daily spirometry and clinical symptom diary following baseline bronchoscopy up to 14 days following RV-16 infection. Additional sampling including nasosorption and nasal lavage was performed in further visits. A more detailed study plan is described in Table 2.7

![Figure 2.4 Design of experimental RV-16 infection study](image-url)
2.4.5 Histamine challenge

This was performed in identical manner to previous study (see Section 2.3.7).

2.4.6 Portable spirometry

Subjects were instructed to perform daily home spirometry using a Piko-1 device (nSpire, UK).

Spirometry technique was assessed prior to use. Home spirometry was performed on waking, with the best of three attempts for both FEV\textsubscript{1} and PEF being recorded. Subjects recorded home spirometry for a total duration of 8 weeks.

2.4.6.1 Assessment of change in lung function following RV-16 infection

The percentage change in morning FEV\textsubscript{1} and PEF from baseline was calculated for each subject as the percentage change from the mean result of the five-day period just prior to RV-16 inoculation. To correct for potential confounding changes in lung function as a result of bronchoscopy, these values were expressed as the three-day rolling mean percentage change.

The same methods were also applied in previous published studies\,(35, 232). The maximal
change from baseline was termed as the greatest observed change from baseline in the initial two-week period following RV-16 inoculation.

2.4.7 Symptom scores

Subjects were instructed to complete daily upper (URT) and lower respiratory tract (LRT) symptom diary cards for the duration of the study period (Figure 2.5). This was commenced 14 days prior to RV-16 inoculation to derive a baseline symptom score and continued for six weeks post-inoculation. URT symptom scoring was based on the Jackson criteria(233). The same symptom diary system was used in previous published studies(35, 232). Importantly, daily symptoms were recorded on waking and prior to any sampling procedures including bronchoscopy.

Total URT and LRT symptom scores were expressed as the summation of the daily scores for the two-week period post RV-16 inoculation. Peak symptom scores were calculated as the maximal daily score observed during the same two-week period.
2.4.8 Bronchoscopic sampling

Bronchoscopies were performed in accordance with the BTS guidelines in the endoscopy unit of St. Mary’s Hospital (London, UK)(234). Bronchoscopies were performed using an Olympus PF260 bronchoscope. All subjects were administered nebulised salbutamol (2.5mg) prior to the procedure and sedation was achieved with midazolam and fentanyl. The bronchoscope...
was inserted via the mouth to minimise trauma to the nasal cavity which may impact on severity of upper respiratory tract symptoms. In addition, oral intubation minimised potential artificial contamination of RV-16 from the nose to the lower respiratory tract. Bronchoscopy was performed by the same two operators for the entire study to minimise inter-operator differences as a confounder.

2.4.8.1 Negative control for bronchoscopy

To assess for potential microbial contamination by the actual bronchoscope, 10ml of sterile 0.9% saline was flushed through the working channel port and collected distally into a sterile universal container. This sample was processed through the 16S rRNA sequencing protocol in the same manner as actual clinical samples.

2.4.8.2 Bronchosorption

Bronchosorption (Mucosal Diagnostics, Hunt Developments Ltd, Midhurst, UK) was used to sample bronchial mucosal lining fluid that was used to measure pro-inflammatory cytokine levels. The distal end of the bronchosorption device contains a strip of SAM enclosed in a sheath. The device was passed down the operating port of the bronchoscope and deployed in the right lower lobe (inferior lobar bronchus). The SAM strip is placed against the bronchial mucosa for 20-30 seconds prior to withdrawal into its sheath and removed from the bronchoscope (Figure 2.6). The distal tip containing the SAM strip is then cut and placed into a spin filter tube and processed in the same manner to nasosorption samples.
2.4.8.3 Bronchoalveolar lavage

BAL was performed by first inserting and wedging the bronchoscope into a segmental bronchus of the right middle lobe. The same location was used for all subjects. Up to 240 ml of sterile 0.9% saline was then instilled in 30ml aliquots with a dwell time of 10 seconds prior to suction being applied and fluid aspirated into a sterile plastic collection chamber. Aliquots of 5 – 10ml of BAL were used for 16S rRNA sequencing.

2.4.9 Nasosorption and nasal lavage

Both procedures were performed and processed in the same fashion as previously described (Sections 2.2.9 and 2.2.10 respectively).

2.4.10 RV-16 inoculation

RV-16 inoculation was performed in a single clinical room in the Imperial Clinical Respiratory Research Unit at St. Mary’s Hospital (London, UK). RV-16 was prepared at a dose of 100 TCID$_{50}$ (50% tissue culture infective dose) in a total volume of 250µL of 0.9% saline solution.
RV-16 was inoculated as a spray into both nostrils via an atomiser (model 286-RD, DeVilbiss Healthcare, Heston, UK) (Figure 2.7). With the nozzle of the atomiser was placed into the nose, subjects were asked to sniff on actuation to encourage delivery of virus particles to the lower airway. In addition, subjects were instructed to refrain from swallowing during the procedure and blowing their noses for one hour to maximise virus pharyngeal contact.

![Figure 2.7 Inoculation atomiser (model 286-RD, DeVilbiss Healthcare)](image)

2.4.11 Diagnosis of clinical cold

A clinical cold was diagnosed if two or more of the following three criteria were met:

1. A cumulative URT score of 14 or greater over a six-day period.
2. Nasal discharge (rhinorrhoea) present on three or more days.
3. A subjective impression of a cold.

2.4.12 Confirmation of RV-16 infection

RV-16 infection was confirmed by at least one of the following tests:

1. Positive nasal lavage for rhinovirus by standard or quantitative PCR (qPCR)
2. A rise in serum neutralising antibodies to RV-16 of ≥ 1:4 at six weeks post inoculation
2.5 **Virology testing**

The following section will describe the serological and PCR techniques used in both studies.

### 2.5.1 Neutralising antibodies to RV-16

Serum samples were placed in a water bath for 30 minutes at 56°C thus heat inactivated. Serology was then performed by the microneutralisation test for neutralising antibody to RV-16 utilising HeLa cell monolayers in a 96-well plate.

50 µL of serum was serially diluted from concentrations of 1:2 to 1:128. 50 µL of RV-16 stock virus containing 100 TCID$_{50}$ was then instilled into each well. The 96-well plate was then shaken for 1 hour at room temperature. 100 µL of freshly stripped HeLa cells (2x10$^5$ cells/mL) were added and the plates incubated for 72 hours at 37°C prior to analysis.

Six wells were reserved for positive controls (RV-16 and cells in the absence of serum) and negative controls (media and cells, in the absence of serum). The plate was assessed for cytopathic effect (CPE), where the antibody titre was defined as the greatest dilution with no identifiable CPE. Seroconversion in previously seronegative subjects was defined as a titre of RV-16 neutralising antibody of ≥ 1:4 in the day 42 serum sample.

### 2.5.2 Quantitative PCR for RV-16

RNA was extracted from nasal lavage and BAL using QIAamp viral RNA mini kit (Qiagen Ltd, Crawley, UK) as per manufacturer’s instructions and reverse transcribed to cDNA with the Omniscript RT kit (Qiagen Ltd, Crwaley, UK) with random hexamers (Promega, Southampton, UK). Taqman quantitative PCR was then performed using the Biosystems ABI Prism 7700
sequence detection system (Applied Biosystems, Paisley, UK). A PCR master mix was made up consisting of Quantitect probe PCR Mastermix (Qiagen Ltd, Crawley, UK), RV-16 specific forward primer (50nM), reverse primer (300nM) (Table 2.8), FAM/TAMRA labelled RV-16 probe (FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA, 100nM) and RNase inhibitor. 23µL of PCR master mix and 2µL cDNA were added to each well of a MicroAmp fast 96-well reaction plate (Applied Biosystems, Paisley, UK). Equivalent volume of negative extraction (nuclease-free water (Promega, Southampton, UK)), negative PCR (template only) and positive extraction (RV-16 stock) were also included. The following thermal cycle conditions were set: 50°C for 2 minutes, 95°C for 10 mins then 45 cycles of 95°C for 15 seconds, 55°C for 20 seconds followed by 72°C for 40 seconds. All samples were analysed in duplicate. Fluorescence data was collected for each cycle and the cycle number (Ct) at which fluorescence rose above threshold was calculated (threshold for RV in data analysis was 0.11). A standard curve was produced by using 2µL of RV plasmid serially diluted 10 fold from 10⁷ to 100 copies. Results were expressed as copies/ml with a PCR sensitivity of 102 copies/ml.

2.5.3 Qualitative PCR for respiratory viruses

Qualitative PCR for respiratory viruses was performed on nasal lavage and throat swab samples. The following viruses were investigated: adenovirus, coronavirus, human bocavirus, human metapneumovirus, influenza (AH1, AH3, B), parainfluenza 1-3, picornavirus and RSV (Table 2.8). RNA was extracted using QIAamp viral RNA mini kit (Qiagen Ltd, Crawley, UK as per manufacturer’s instructions.

For picornavirus, reverse transcription (RT) and PCR were performed. 5µl RNA was converted to cDNA using OL27 as primer and the following RT mix: 2µl of 5xFirst-Strand Buffer (250mM
Tris-HCl, 375mM KCl, 15mM MgCl₂, 1µl 0.1M DTT, 0.2µl dNTPs (100mM dATP, 100mM dCTP, 100mM dGTP, 100mM dTTP, 100mM dUTP), 1.55µl OL27 (25µmolar, Invitrogen, MA, USA) and 0.25µl (10 000U) Reverse Transcriptase (Invitrogen, MA, USA). This cDNA was then used to detect picornaviruses in a single round PCR of 32 cycles(235). Differentiation of RVs from enteroviruses is achieved by restriction enzyme digestion of the PCR product(236).

For all other viruses, RT and PCR were performed using a total of 18µl RNA. 6.5µl of UHQ (nuclease-free water, Promega, Southampton, UK) with 2.5µl of random hexamer (0.5µg/µl, Promega, Southampton, UK) was added to re-suspended RNA and denatured at 70°C for 10 min. After quenching on ice, 6µl of UHQ, 10µl of 5x First-Strand Buffer, 5µl 0.1M DTT, 1.25µl dNTPs, 2µl (200U) of Reverse Transcriptase were added. The mixture was incubated at 37°C for 60 minutes to yield cDNA.

4µl of this cDNA was then used for each PCR in a panel of RT-PCR assays based on previously published methods(65, 237-241). PCR was performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Paisley, UK). All primers were 25µM stock (Invitrogen, MA, USA). GoTaq Flexi DNA Polymerase (Promega, Southampton, UK) was used for all PCRs. PCR products were fractionated on 2% agarose gels and visualised by ethidium bromide staining and photographed. Any visible band of appropriate size was reported as a positive result. Positive and negative control samples were included in all PCR assays. Details of specific viral PCR reaction mix and thermal cycle conditions are described in Table 2.9.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Primers</th>
</tr>
</thead>
</table>
| RV-16                       | **Forward:** 5’GTG AAG AGC CSC RTG TGC T-3’  
|                             | **Reverse:** 5’-GCT SCA GGG TTA AGG TTA GCC-3’                           |
| Adenovirus                  | **Ad1:** GCC GAG AAG GGC GTG CGC AGG TA  
|                             | **Ad2:** TAC GCC AAC TCC GCC CAC GCG CT                                         |
| Coronavirus                 | **1st Round**  
|                             | **229E**  
|                             | 0637: GGT ACT CCT AAG CCT TCT CG  
|                             | 0647: TGC ACT AGG GTT AA GAA GAG G                                          |
|                             | **OC43**  
|                             | 1A: AGG AAG GTC TGC TCC TAA TTC C  
|                             | 1B: TGC AAA GAT GGG GAA CTG TGG G                                          |
|                             | **2nd Round**  
|                             | **229E**  
|                             | 0657: TTT GGA AGT GCA GGT GTT GTG G  
|                             | 0667: GAC TAT CAA ACA GCA TAG CAG C                                          |
|                             | **OC43**  
|                             | 2A: GTT CTG GCA AAA CTT GGC AAG G  
|                             | 2B: TTA TTG GGG CTC CTC TTC CGG C                                          |
| Human Bocavirus             | **HBOV01.1:** TAT GGC CAA GGC AAT CGT CCA AG  
|                             | **HBOV01.2:** GCC GCG TGA ACA TGA GAA ACA GA                                 |
| Human Metapneumo Virus      | **RF930:** CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC  
|                             | **RF931:** CCT ATT TCT GCA GCA TAT TTG TAA TCA G                              |
| Influenza                   | **1st Round**  
|                             | **Influenza AH3**  
|                             | AH3A: CAG ATT GAA GTG ACT AAT GC  
|                             | AH3D11: GTT TCT CTG GTA CAT TCC GC                                           |
|                             | **Influenza AH1**  
|                             | AH1A: CAG ATG CAG ACA CAA TAT GT  
|                             | AH1F11: AAA CCG GCA ATG GCT CCA AA                                            |
|                             | **Influenza B**  
|                             | BHAA: GTG ACT GGT GTG ATA CCA CT  
|                             | BHAD11: TGT TTT CAC CCA TAT TGG GC                                           |
|                             | **2nd Round**  
|                             | **Influenza AH3**  
|                             | AH3B: AGC AAA GCT TTC AGC AAC TG  
|                             | AH3C11: GCT TCC ATT TGG AGT GAT GC                                           |
|                             | **Influenza AH1**  
|                             | AH1B: ATA GGC TAC CAT GCG AAC AA  
|                             | AH1E11: CTT AGT CCT GTA ACC ATC CT                                           |
|                             | **Influenza B**  
<p>|                             | BHAAB: CAT TTT GCA AAT CTC AAA GG                                          |</p>
<table>
<thead>
<tr>
<th>Virus</th>
<th>PCR mix composition</th>
<th>Thermal cycle condition</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>4µl hexamer cDNA</td>
<td>94C 120s 94C 20s 60C 20s 40 cycles 72C 30s 72C 240s 4C “forever”</td>
<td>161bp</td>
</tr>
<tr>
<td></td>
<td>5µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7µl MgCl₂ (3.5 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5µl dNTP (3 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5µl Ad 1 (0.25 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5µl Ad 2 (0.25 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5µl TAQ (2000U)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32µl UHQ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.8 Virology PCR primers**

**Parainfluenza (1-3)**
- **PF1/01**: CAG AAT TAA TCA GAC AAG AAG T
- **PF1/02**: AGG ATA CAT ATC TGA ATT TAA G
- **PF2/01**: GGA TAA TAC AAC AAT CTG CTG
- **PF2/02**: CAC AGG TTA TGT TGG GAT G
- **PF3/01**: CTC GAG GTT GTC AGG ATA TAG
- **PF3/02**: CTT TGG GAG TGT AAC ACA GTT

**Picornavirus**
- **OL26**: GCA CTT CTG TTT CCC C
- **OL27**: CGG ACA CCC AAA GTA G

Hemi-nested
- **JWA-1B**: CAT TCA GGG GCC GGA GGA

**RSV**
1
- **22K1**: ATG TCA CGA AGG AAT CCT TGC
- **22K2**: TAG CTC TTC ATT GTC CCT CAG
2
- **22K3**: GAG GTC ATT GCT TAA ATG G
- **22K4**: GCA ACA CAT GCT GAT TGT
<table>
<thead>
<tr>
<th><strong>Coronavirus</strong></th>
<th>1st Round</th>
<th>2nd Round</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µl hexamer cDNA</td>
<td>4 µl 1st round PCR product</td>
<td></td>
</tr>
<tr>
<td>5 µl Buffer (10x Buffer)</td>
<td>36 µl UHQ</td>
<td></td>
</tr>
<tr>
<td>4 µl MgCl₂ (2.5 µm)</td>
<td>5 µl Buffer (10x Buffer)</td>
<td></td>
</tr>
<tr>
<td>0.5 µl dNTP (2 µm)</td>
<td>0.5 µl MgCl₂ (2.5 µm)</td>
<td></td>
</tr>
<tr>
<td>0.5 µl 0637,</td>
<td>0.5 µl dNTP (2 µm)</td>
<td></td>
</tr>
<tr>
<td>0.5 µl 0647 (0.25 µm)</td>
<td>0.5 µl 0657,</td>
<td></td>
</tr>
<tr>
<td>0.5 µl 1A,</td>
<td>0.5 µl 0667 (0.25 µm)</td>
<td></td>
</tr>
<tr>
<td>0.5 µl 1B (0.25 µm)</td>
<td>0.5 µl 2A,</td>
<td></td>
</tr>
<tr>
<td>0.5 µl TAQ (2000U)</td>
<td>2B (0.25 µm)</td>
<td></td>
</tr>
<tr>
<td>34 µl UHQ</td>
<td>0.5 µl TAQ (2000U)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>94C 120s</td>
<td>94C 120s</td>
<td></td>
</tr>
<tr>
<td>94C 20s</td>
<td>94C 20s</td>
<td></td>
</tr>
<tr>
<td>60C 20s</td>
<td>60C 20s</td>
<td></td>
</tr>
<tr>
<td>30 cycles</td>
<td>25 cycles</td>
<td></td>
</tr>
<tr>
<td>72C 30s</td>
<td>72C 30s</td>
<td></td>
</tr>
<tr>
<td>72C 240s</td>
<td>72C 240s</td>
<td></td>
</tr>
<tr>
<td>4C “forever”</td>
<td>4C “forever”</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Human Bocavirus</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µl hexamer cDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 µl MgCl₂ (2.5 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 dNTP (2 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl HBOV01.2 (0.25 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl HBOV02.2 (0.25 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl TAQ (2000U)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 µl UHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95C 15min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>94C 20s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56C 20s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72C 30s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72C 240s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4C “forever”</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Human Metapneumo Virus</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µl hexamer cDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 µl MgCl₂ (2.5 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85 dNTP (2 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl RF930 (0.25 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl RF931 (0.25 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85 µl TAQ (2000U)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.3 µl UHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>94C 120s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>94C 30s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54C 30s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72C 120s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72C 240s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4C “forever”</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Influenza</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 µl hexamer cDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µl MgCl₂ (2.5 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl dNTP (2 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl AHIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>94C 120s</td>
<td>B 767bp</td>
<td></td>
</tr>
<tr>
<td>94C 20s</td>
<td>AH3 591bp</td>
<td></td>
</tr>
<tr>
<td>50C 20s</td>
<td>AH1 944bp</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>229E 116bp</strong></th>
<th><strong>OC43 100bp</strong></th>
</tr>
</thead>
</table>

|**354bp**      |**171bp**       |

|**30 cycles**  |**40 cycles**   |

|**72C 100s**   |**72C 240s**    |

|**72C 600s**   |**4C “forever”**|

|**4C “forever”|**4C “forever”**|

|**AH3 591bp** |**AH1 944bp** |

|**B 767bp**   |**72C 600s**   |

|**72C 240s**  |**72C 240s**   |

<p>|<strong>AH1 944bp</strong> |<strong>72C 240s</strong>   |</p>
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5µl AH1 F11</td>
<td>(0.25µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25µl AH3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25µl AH3 D11</td>
<td>(0.125µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl BHAA,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 BHA D11</td>
<td>(0.25µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl TAQ(2000U)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32.5µl UHQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Round</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2µl 1st round PCR product</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5µl MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl dNTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl AHIB,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl AH1 E11(0.25µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25µl AH 3B,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25µl AH 3C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11(0.125µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl BHAB,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl BH AC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11(0.25µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl TAQ(2000U)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34.5µl UHQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parainfluenza (1-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4µl hexamer cDNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5µl Buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4µl MgCl&lt;sub&gt;2&lt;/sub&gt; (2.5µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl dNTP(2µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl pf1/01,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 pf1/02(0.25µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl pf2/01,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 pf2/02(0.25µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5µl pf3/01,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 pf3/02(0.25µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 TAQ (2000U)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33µl UHQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picornavirus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10µl OL 27 cDNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3µl MgCl&lt;sub&gt;2&lt;/sub&gt; (1.5µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85µl dNTP (3.4µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3µl OL26 (1.5µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3µl OL27 (1.5µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85µl TAQ(2000U)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.3µl UHQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picornavirus Hemi-nested</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1µl 1st round product</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33.3µl UHQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5µl Buffer (10x Buffer)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**nC “forever”**

- Parainfluenza (1-3)
  - 94C 120s
  - 94C 20s
  - 50C 20s
  - 30 cycles
  - 72C 100s
  - 72C 600s
  - 4C “forever”

- Picornavirus
  - 94C 120s
  - 94C 30s
  - 50C 30s
  - 32 cycles
  - 72C 120s
  - 72C 240s
  - 4C “forever”

- Picornavirus Hemi-nested
  - 94C 120s
  - 94C 30s
  - 50C 30s

**PF1 430bp**

**PF2 370bp**

**PF3 180bp**

**Picornavirus**

**380bp**
Table 2.9 Qualitative virology PCR mix composition, thermal cycle conditions and product sizes

<table>
<thead>
<tr>
<th>1st Round</th>
<th>2nd Round</th>
</tr>
</thead>
<tbody>
<tr>
<td>3µl MgCl₂</td>
<td>3µl MgCl₂</td>
</tr>
<tr>
<td>0.85µl DNTP</td>
<td>(2.5µm) k2</td>
</tr>
<tr>
<td>3µl OL26</td>
<td>(0.25µm)</td>
</tr>
<tr>
<td>3µl Jwa 1b</td>
<td>0.5µl Taq</td>
</tr>
<tr>
<td>0.85µl Taq</td>
<td>0.5µl TAQ(2000U)</td>
</tr>
<tr>
<td>20-32 cycles</td>
<td>94C 120s</td>
</tr>
<tr>
<td>72C 120s</td>
<td>94C 20s</td>
</tr>
<tr>
<td>72C 240s</td>
<td>50C 20s</td>
</tr>
<tr>
<td>4C “forever”</td>
<td>30 cycles</td>
</tr>
<tr>
<td>94C 120s</td>
<td>72C 30s</td>
</tr>
<tr>
<td>94C 20s</td>
<td>72C 240s</td>
</tr>
<tr>
<td>50C 20s</td>
<td>4C “forever”</td>
</tr>
<tr>
<td>259bp</td>
<td>25 cycles</td>
</tr>
<tr>
<td>72C 30s</td>
<td>72C 240s</td>
</tr>
<tr>
<td>72C 240s</td>
<td>4C “forever”</td>
</tr>
</tbody>
</table>

2.6 Quantitative bacterial culture

Quantitative bacterial culture on sputum samples were performed by the microbiology department of Imperial College Healthcare NHS Trust. Positive culture was defined as a quantitative culture of \( \geq 10^6 \) cfu/ml.

2.7 Pro-inflammatory cytokine measurement

IL-1β, IL-6, IL-8 and TNF-α levels from nasosorption and bronchosorption samples were quantified using the Meso Scale Discovery (MSD) platform (Meso Scale Discovery, Gaithersburg, MD, USA).
The MSD human proinflammatory-4 II Tissue Culture Kit (K11025B-2, MSD, MD, USA) was used as per manufacturer’s protocol. Serial 1:4 dilutions of human Proinflammatory-4 II Calibrator Blend were made in Diluent 1 (MSD, MD, USA) at 10 000pg/ml to 0pg/ml. Clinical samples were diluted 1:10 in Diluent 1. 25µl of calibrator (in duplicate) or diluted sample was added to each well of the MSD plate. The plate was sealed and incubated for two hours at room temperature with vigorous plate shaking. Following incubation, the plate was washed three times with 0.05% Tween-20/PBS. 25µl of SULFO-TAG Anti-human ProInflammatory 4-Plex II Antibody (diluted to a final concentration of 1x in Diluent 1) was added to each well. The plate was sealed again and further incubated for 90 minutes at room temperature with vigorous shaking, after which it was washed three times with 0.05% Tween-20/PBS. 150µl of 2x Read Buffer T (MSD) was added to each well and the plate was immediately processed on the MESO QuickPlex SQ 120 imager. Data was analysed using the Discovery Workbench® 4.0 Software (MSD, MD, USA). The sensitivities were 0.4pg/ml (IL-1β), 0.7pg/ml (IL-6), 0.7pg/ml (IL-8) and 1.0pg/ml (TNF-α) respectively.

2.8 IP-10 measurement

IP-10 level was measured from nasosorption samples by enzyme-linked immunosorbent assay (ELISA). This was carried out using the Duoset Human IP-10 Quantikine ELISA kit (R&D Systems, Oxford, UK) as per manufacturer’s instructions.

All steps were done at room temperature and plates were washed three times with ELISA wash buffer between steps. The primary antibody was incubated in a 96-well plate (Nunc MaxiSorp, ThermoScientific, MA, USA) overnight followed by blocking with the ELISA reagent diluent (1% BSA in PBS) for two hours. 100µl of diluted clinical sample (1:5 in reagent buffer)
or standard (recombinant protein serially diluted 1:2 in reagent diluent) was instilled to each well and incubated for two hours. Biotin conjugated detection (secondary) antibody in reagent diluent was added and incubated for 90 minutes. Next, streptavidin-HRP (Invitrogen, MA, USA) was added at 0.2μg/ml in reagent diluent and incubated for 15 minutes without direct light exposure. 75μl of TMB substrate (Invitrogen, MA, USA) was then added and colour development monitored before adding 75μl of ELISA stop solution.

Plates were immediately read using a Spectramax Plus plate reader (MDS Analytical Technologies, Wokingham, UK) at 450nm with corrections made for plate and background absorbance at 540nm and concentrations calculated from the standard curve. Data were analysed using Softmax Pro software (version 5.2; Molecular Devices, Sunnyvale, USA).

2.9 16S rRNA gene sequencing

16S rRNA gene sequencing was performed using the Illumina® MiSEQ system (Illumina, CA, USA). An overview of the sequencing and downstream analysis pipeline is shown on Figure 2.8.
Genomic DNA extraction was performed using the Fast DNA™ SPIN Kit for Soil (MP Bio, CA, USA). All extraction was performed in a sterile laminar flow hood. Clinical samples (sputum or BAL) were thawed and kept on ice until use. For BAL, the sample was centrifuged at top speed (21,000 x g) for 30 minutes, after which the supernatant was discarded and the BAL pellet re-suspended with 978µl of Sodium Phosphate Buffer and transferred to the Lysing Matrix E tube. For sputum samples, the sputum plug was re-suspended with 978µl of Sodium Phosphate Buffer and transferred to the Lysing Matrix E tube.

A further 122µl of MT buffer was added into the Lysing Matrix E tube and the sample was homogenised using the Precellys24 instrument (Bertin Instruments, Montigny-le-Bretonneux, France) under the settings of 6800 rpm for two cycles of 30 seconds each. The sample was
further centrifuged at 14,000 x g for 10 minutes to pellet debris. The resulting supernatant was transferred to a sterile 2.0ml microcentrifuge tube containing 250µl of PPS (protein precipitation solution) and mixed by manual shaking (inversion 10 times), followed by further centrifugation at 14,000 x g for five minutes. Once again, the supernatant was transferred to a sterile Falcon™ 15mL conical centrifuge tube containing 1ml of Binding Matrix (re-suspended by pipette prior to addition). The tube was placed on a rotator (Stuart Tube Rollers SRT6D, Bibby Scientific Limited, Staffordshire, UK) for 20 minutes to enable DNA binding.

Following rotation, the tube was placed vertically for three minutes to facilitate sedimentation of silica matrix, after which 800µl of supernatant was discarded. The Binding Matrix mixture was then re-suspended thoroughly and 600µl transferred to a SPIN Filter, followed by centrifugation at 14,000 x g for one minute. The residual fluid was discarded and the remaining Binding Matrix mixture was added to the SPIN Filter. This was once more centrifuged at 14,000 x g for one minute and residual fluid discarded. 500µl of SEWS-M was then added to the remaining pellet and gently re-suspended. Once thoroughly re-suspended, the mixture was centrifuged at 14,000 x g for one minute and residual fluid discarded. The pellet was centrifuged at 14,000 x g for a further minute. Once again, the residual fluid was discarded and the filter was transferred to a clean SPIN Filter, which was air dried for two minutes. 100µl of DES (DNase/Pyrogen-Free Water) was added to the pellet and gently re-suspended, followed by incubation at 55°C for five minutes using a heat block. Finally, the SPIN Filter was centrifuged at 14,000 x g for one minute and the eluted genomic DNA was collected and instilled into a labelled sterile 1.5ml Eppendorf tube. The sample was swiftly stored at -80°C until further downstream processing was performed.
2.9.2 Assessing bacterial burden – 16S rRNA gene quantitative PCR

SYBRGreen Quantitative PCR (qPCR) of the 16S rRNA gene was performed using the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific Inc, MA, USA) to evaluate bacterial burden. All qPCR experiments were carried out in a sterile PCR fume hood.

2.9.2.1 Primer preparation

The following primers targeting the hypervariable region 4 (v4) region of the 16S rRNA gene were used:

- 520F (5‘- AYT GGG YDT AAA GNG -3’)
- 802R (5’- TAC NVG GGT ATC TAA TCC -3’)

Primers were ordered from Eurofins MWG Operon (Eurofins Genomics, Ebersberg, Germany) and were HPLC Purified. Concentration for both primers were 10µM.

2.9.2.2 Standard preparation – Vibrio natregens

Amplified Vibrio natregens full length of 16S rRNA gene was cloned into TOPO TA Cloning vector, grown up before hand, quantified and diluted down to a working concentration (2 x 10^7 per µl). Standard was serially diluted in 1:10 with PCR water (Cambio, Cambridge, UK).

2.9.2.3 SYBRGreen qPCR reaction

The following qPCR reaction mix (total volume 10µl) was added to each well of a MicroAMP Fast 96-well reaction plate (Applied Biosystems, Paisley, UK): 5µl of SYBR Fast qPCR Kit Master Mix (KAPA BioSystems Ltd, London, UK), 0.2µl of 10µM Forward primer, 0.2µl of 10µM Reverse primer, 3.6µl of PCR water and 1µl of sample (genomic DNA). In addition, standards
(as described above) and negative controls (PCR water) were also included. All samples were analysed in triplicates. The plate was sealed using an Adhesive Seal Applicator to ensure that there were no air bubbles and briefly spun. The following thermal cycle conditions were set: 90°C for 3 minutes then 40 cycles of 95°C for 20 seconds, followed by 50°C for 30 seconds and 72°C for 30 seconds.

The results were analysed using the HRM Software Module for ViiA™ 7 System (Thermo Fisher Scientific Inc, MA, USA). The Ct was set at 0.069 for all qPCR experiments. The lowest threshold for detection was 1,000 copies per ml.

2.9.3 Quadruplicate PCR

To amplify the bacterial 16S rRNA genes from individual clinical samples, quadruplicate PCR using indexed primers was performed. All PCR experiments were performed in PCR hoods that had been irradiated by UV for at least 35 minutes prior to use. 14µl of diluted Q5® High-Fidelity 2X Master Mix (New England Biolabs, Herts, UK) (5ml of Q5 Master Mix diluted with 600µl of PCR water (Cambio, Cambridge, UK)) was pipetted into each well of a 0.2ml Skirted 96-well PCR plate (Thermo Scientific, MA, USA).

5µl of indexed primers (labelled 701 – 712 and 501 – 508 respectively) at 1.5µM was pipetted into their corresponding wells as shown on Figure 2.9. The primers targeted the v4 region of the 16S rRNA gene as shown previously, in addition to specific barcode sequence. This enabled the unique identification of individual clinical samples during 16S rRNA sequencing. The plate was then transferred to a separate PCR hood, where 1µl of sample (extracted genomic DNA) was added to each well. Each PCR plate consisted of 94 individual samples, in addition to positive control (mock community) and negative control (PCR water). The same
procedure was performed four times for each experiment (i.e. quadruplicate PCR using four PCR plates).

![Diagram of PCR plate orientation](image)

*Figure 2.9 Orientation of indexed primers (labelled 701 – 712 and 501 – 508) on a 96-well PCR plate*

The four PCR plates were then carefully sealed with Adhesive Sealing sheets (Thermo Scientific, MA, USA) and spun down briefly. PCR was then performed under the following thermal cycling conditions: 95°C for 2 minutes, then 35 cycles of 95°C for 20 seconds, 50°C for 20 seconds, 72°C for 5 minutes. The plates were cooled and maintained at 10°C thereafter until collection.

**2.9.4 Contamination check**

To check for potential cross contamination by mis-pipetting, 5µl of each positive (mock community) and negative controls underwent gel electrophoresis (1.2% Agarose gel with 5µl
gel red) run at 120 V for 35 minutes. This was then visualised under UV to confirm correct amplicon size in positive controls and absence of amplification in negative controls.

<table>
<thead>
<tr>
<th>Ladder</th>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Plate 3</th>
<th>Plate 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Figure 2.10* Gel electrophoresis for contamination check post quadruplicate PCR. + indicates mock community and – indicates negative PCR control.

### 2.9.5 Pooling of replicate PCR reactions

If the contamination check was satisfactory, 96 samples (94 clinical samples and 2 controls) were pooled into a single 96-well PCR plate. To ensure adequate PCR amplification, gel electrophoresis was performed on a randomly selected column (from 96-well plate) of samples.
2.9.6 Purification (1\textsuperscript{st} round)

The 16S rRNA amplicons were purified using Agencourt\textregistered AMPure\textsuperscript{®} XP (Beckman Coulter, High Wycombe, UK) as per manufacturer’s instructions. A uniform volume of amplicons (70 - 80\(\mu\)l) from each well was transferred to a round bottom plate (Thermo Fisher Scientific, MA, USA). Next, AMPure was added to each well and mixed with samples by gentle pipetting (20 times). Of note, the AMPure was at room temperature (for at least 30 minutes) and re-suspended thoroughly (by vortex) prior to use. The volume of AMPure added was at a 0.7:1 ratio to volume of sample (e.g. for 100\(\mu\)l of sample, 70\(\mu\)l of AMPure would be added). The plate was left at room temperature for 10 minutes then placed on the magnetic stand for 5 minutes. The supernatant was then discarded carefully without disturbing the pellet of congregated beads. The plate was removed from the magnetic stand and 100\(\mu\)l of freshly prepared 80\% ethanol was added to each well and gently re-suspended. The plate was placed back on the magnetic stand until the solution became clear (i.e. beads have congregated) and the ethanol was discarded carefully without disturbing the beads. This ethanol wash step was repeated for a second time, after which the pellet was left to air dry for approximately 5 minutes. 30\(\mu\)l of low EDTA TE buffer (Invitrogen, Thermo Fisher Scientific, MA, USA) was added to each well and re-suspended with pellet. After 5 minutes, the plate was placed back...
on the magnetic stand until the solution was clear and the supernatant (purified 16S rRNA amplicons) was transferred to a new 96-well round bottom plate.

2.9.7 PicoGreen® Quantification

Next, amplicon quantification was performed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, CA, USA) as per manufacturer’s instructions. 100µl of DNA standards, serially diluted at 1:2 with 1x TE (Invitrogen, Thermo Fisher Scientific, MA, USA), was placed into a 96-well black fluorometer plate (Costar, Corning Inc, NY, USA). The top standard had a concentration of 1ng/µl and bottom standard was a negative control. Standards were performed in duplicates. For 96 samples, two black fluorometer plates were needed. 99µl of 1x TE was added to the remaining wells, after which 1µl of purified 16S rRNA amplicon from each sample was added and gently re-suspended. 100µl of 1:200 dilution of PicoGreen reagent was then added to each well and gently re-suspended. The plates were then covered from light and amplicon concentrations were determined using the Infinite® 200 Pro (Tecan Group Ltd, Männedorf, Switzerland).

2.9.8 Equimolar pooling and purification (2nd round)

With the concentration of 16S rRNA amplicons from each clinical sample calculated, equimolar pooling was performed. The collection of the 96 samples (94 clinical samples, mock community and negative control) was termed the ‘library’. The desired pooled library concentration was termed by the operator, ranging from 25 to 40ng. The aim was to achieve as high a concentration as possible with a total pooled library volume of approximately 1ml. Once library concentration was determined, the specific volume of 16S rRNA amplicon from
Each sample was added to a single 1.5ml Eppendorf tube. An example of equimolar pooling calculation is shown on Figure 2.12.

<table>
<thead>
<tr>
<th>Plate 1 Fluorescence values</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1354164</td>
<td>734626</td>
<td>3851693</td>
<td>997856</td>
<td>1262538</td>
<td>686959</td>
<td>1297829</td>
<td>2112158</td>
<td>2393086</td>
<td>1799424</td>
<td>7979150</td>
<td>8012072</td>
</tr>
<tr>
<td>B</td>
<td>1353577</td>
<td>2845729</td>
<td>2005162</td>
<td>1278029</td>
<td>1681478</td>
<td>544098</td>
<td>1618238</td>
<td>3204124</td>
<td>5246260</td>
<td>2064005</td>
<td>4318122</td>
<td>4027094</td>
</tr>
<tr>
<td>C</td>
<td>331707</td>
<td>1547747</td>
<td>749600</td>
<td>1153766</td>
<td>324763</td>
<td>704868</td>
<td>1356062</td>
<td>503430</td>
<td>1035413</td>
<td>155151</td>
<td>1978580</td>
<td>1953668</td>
</tr>
<tr>
<td>D</td>
<td>988052</td>
<td>339338</td>
<td>968837</td>
<td>146288</td>
<td>417275</td>
<td>1036604</td>
<td>1942364</td>
<td>2560226</td>
<td>5554645</td>
<td>825948</td>
<td>1007281</td>
<td>1015963</td>
</tr>
<tr>
<td>E</td>
<td>1026214</td>
<td>1639395</td>
<td>1034112</td>
<td>177819</td>
<td>432431</td>
<td>414244</td>
<td>2632549</td>
<td>1720769</td>
<td>192914</td>
<td>519772</td>
<td>519659</td>
<td>522686</td>
</tr>
<tr>
<td>F</td>
<td>185231</td>
<td>144722</td>
<td>320229</td>
<td>424777</td>
<td>388653</td>
<td>1708004</td>
<td>1302402</td>
<td>213197</td>
<td>586995</td>
<td>125050</td>
<td>283746</td>
<td>276792</td>
</tr>
<tr>
<td>G</td>
<td>404078</td>
<td>145287</td>
<td>953389</td>
<td>1026912</td>
<td>166524</td>
<td>475257</td>
<td>2579027</td>
<td>258254</td>
<td>243566</td>
<td>245205</td>
<td>143092</td>
<td>149861</td>
</tr>
<tr>
<td>H</td>
<td>435380</td>
<td>507620</td>
<td>153557</td>
<td>701955</td>
<td>318167</td>
<td>397282</td>
<td>1637106</td>
<td>125466</td>
<td>401863</td>
<td>1137581</td>
<td>16065</td>
<td>12335</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate 2 Fluorescence values</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>624430</td>
<td>689527</td>
<td>7143282</td>
<td>7620229</td>
<td>356661</td>
<td>3608060</td>
<td>1735941</td>
<td>1789708</td>
<td>48964</td>
<td>930881</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2374183</td>
<td>340678</td>
<td>4642268</td>
<td>4501507</td>
<td>1373105</td>
<td>122880</td>
<td>252911</td>
<td>248503</td>
<td>73628</td>
<td>313904</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>138435</td>
<td>113287</td>
<td>446228</td>
<td>551058</td>
<td>16117</td>
<td>13069</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR product ng/µl</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
</table>

Figure 2.12 Example of equimolar pooling calculation. The fluorescence values of PicoGreen quantification (highlighted in light blue) were translated into PCR product concentration (3rd table from top). The desired pool concentration was determined (30ng in this example). The volume required from each 16s rRNA amplicon sample to achieve the desired pool concentration of 30ng was calculated and shown in the bottom table. Volumes of > 20µl were highlighted in red and where the volume required exceeded the volume of amplicon available, the entire amplicon volume was added to the pooled library. Please note that H12 is negative control.

Once equimolar pooling was completed, the library underwent a further round of AMPure purification. A set volume of the pooled library was transferred to a new 1.5ml Eppendorf tube. The volume transferred depended on the pooled library volume available. For example,
if total volume of library was 851µl, then 800µl was taken. AMPure purification was then performed as previously described, except on this occasion, 200µl of freshly prepared ethanol was used (instead of 100µl). After the removal of ethanol (at the end of second ethanol wash step), 30µl of low EDTA TE Buffer (Invitrogen, Thermo Fisher Scientific, MA, USA) was re-suspended with the bead pellet. The tube was placed on the magnetic stand until the solution was clear. The supernatant (the concentrated pooled library) was retained.

2.9.9 Gel purification of concentrated pooled library

The concentrated pooled library was then further purified by gel electrophoresis with 1.8% Agarose gel and 6µl of Gel Red running at 100V for 45 minutes. Under UV visualisation, the concentrated library band was cut and extracted using the QIAquick® PCR Purification Kit (Qiagen Ltd, Crawley, UK) as per manufacturer’s instructions. The size of purified library was confirmed by bioanalyser using the Agilent High Sensitivity DNA Kit (Agilent Technologies, CA, USA). This was performed as per manufacturer’s protocol with the targeted peak at approximately 330 to 350 base pairs (Figure 2.13).

**Figure 2.13** Bioanalyser result showing a peak at 334bp (appropriate size for library)
2.9.10 Library quantification qPCR

To assess concentrated pooled library concentration (in picomolar(pM)), qPCR of diluted sample libraries was performed using the Illumina Library Quantification DNA Standards (KAPA BioSystems Ltd, London, UK). There were six DNA standards in total, each with known concentration. The sample library was diluted in 1:1,000, 1:2,000, 1:4,000 and 1:8,000 respectively. Quantitative PCR was performed and analysed using the ViiA™ 7 System (Thermo Fisher Scientific Inc, MA, USA under the following conditions: 95°C for 1 minute, then 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and melt curve of 95°C for 15 seconds, 60°C for 60 seconds and 95°C for 15 seconds. Quantification of the sample library concentration enabled the accurate calculation of library dilution to achieve an 8pM library required for Illumina® MiSEQ sequencing.

2.9.11 Illumina® MiSEQ sequencing

Once the concentrated sample library has been purified and quantified as described above, 16S rRNA sequencing on the Illumina® MiSEQ platform was performed as per manufacturer’s protocol. The MiSEQ v2 Reagent Kit (Illumina, CA, USA) was used for the current project. A maintenance wash using 0.5% Tween-20 was performed prior to each sequencing run. The new Incorporation Buffer was slotted into the MiSEQ sequencer. The flow-cell was washed thoroughly using molecular grade water until all salt solution excess was removed.

The reagent kit was defrosted. The reagent cartridge was placed in a 20°C water bath for approximately one hour. 4µl of sequencing primers (Read 1, Index and Read 2 sequencing primers respectively) (Table 2.10) were then loaded into the cartridge into the appropriate wells (Wells 12, 13 and 14 respectively).
Table 2.10 Sequencing primers (Eurofins Genomics, Ebersberg, Germany)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Read 1 Sequencing Primer</td>
<td>5’-TCC GGT GCG GGC CAY TGG YD TAA AGN G-3’</td>
</tr>
<tr>
<td>Index Sequencing Primer</td>
<td>5’-GGA TTA GAT ACC CBN GTA ACG CGG CCG CCT-3’</td>
</tr>
<tr>
<td>Read 2 Sequencing Primer</td>
<td>5’-AGG CGG CGG CGT TAC NVG GGT ATC TAA TCC-3’</td>
</tr>
</tbody>
</table>

PhiX Control library (PhiX Control v3, Illumina, CA, USA), which acts as a sequencing run control, was prepared. 2µl of 10M PhiX (with 3µl of EBT buffer (Illumina, CA, USA)) was denatured using 5µl of 0.2N Sodium hydroxide (Sigma-Aldrich, USA) and incubated for five minutes. This was further diluted to 8pM using HT1 Buffer. Next, the sample library was denatured in similar fashion by adding 10µl of 0.2N Sodium hydroxide to 10µl of sample library. This was incubated for five minutes and diluted with 980µl of HT1 Buffer. The denatured library was diluted to a concentration of 8pM using HT1 Buffer. The volume of denatured library was determined by the library concentration previously calculated during library qPCR (Section 2.8.9). 200µl of 8pM PhiX was ‘spiked’ into 800µl of 8pM library and the solution was termed ‘Library Spike’. Finally, 600µl of Library Spike was added into the appropriate well (Well 17) in the reagent cartridge. The cartridge was loaded into the sequencer and sequencing was commenced after appropriate checks.

2.10 Sequence processing

The raw reads from the MiSEQ machine were compressed and transferred to codon, the bioinformatics server at Imperial College London. Further downstream processing was performed using QIIME (Quantitative Insights Into Microbial Ecology), an open-source
bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. Figure 2.14 illustrates the processing steps.

In summary, the forward and reverse barcodes were joined to make a 16 base-pair long identifier. The sequence reads were then trimmed to remove reverse primer and index that
had been sequenced through and the forward and reverse reads were paired. The sequence reads from all sequencing runs were then demultiplexed (i.e. mapping each read back to its original sample). PhiX contamination was then removed and OTUs were picked using the SILVA rRNA gene database(172). Representative sequences were picked from each OTU and the sequences were aligned. Chimeric sequences were identified and removed. Finally, a phylogenetic tree was created, along with assignment of taxonomy resulting in an OTU table. The phylogenetic tree, representative sequences and OTU table were formatted and imported to RStudio (RStudio, MA, USA) for data analysis.

2.11 Data and statistical analyses

Data analysis was performed using R Studio, an open source and professional software for R (RStudio, MA, USA). Microbiota analysis was primarily performed using the phyloseq package(243). However, other R packages that were also used included vegan(244), Biostrings(245), reshape2(246), stringr(247), plyr(248), ggplot2(249), stats(250) and indicspecies(251). Statistical analyses were performed using GraphPad Prism6 (GraphPad Software Inc, La Jolla, USA) unless otherwise stated. Continuous data were presented as mean with standard deviation (or standard error of the mean) and categorical data were presented as median with interquartile ranges.

2.11.1 Group comparisons

Data were tested for normality using GraphPad Prism 6 and the appropriate statistical tests were applied. The Kruskal-Wallis test was used when comparing non-normally distributed groups at a single time point and One-way ANOVA was used for normally distributed data. Two-way ANOVA was used to compare multiple groups (two or more) across multiple time
points. To correct and account for multiple comparisons, the Tukey or Dunnett test was used. The Tukey test compares every mean with every other mean whilst the Dunnett test compares every mean to a control mean.

2.11.2 Correlation and linear regression

Spearman’s rank correlation was used for correlation testing in data that were not normally distributed. All P values were two-tailed P values. Where linear regression was performed, log-transformation was performed on non-parametric data prior to testing. For multiple comparisons, P values were adjusted using the false discovery rate (also known as Benjamini and Hochberg method)(252). As GraphPad Prism 6 did not automatically adjust P values for multiple testing, this was performed on R studio using the stats package(250).

2.11.3 Micobiota analysis

2.11.3.1 Rarefaction

To account for different sequencing depth between samples (i.e. some samples would have sequenced better than others, thus the number of sequence reads would differ), the samples underwent rarefaction to ‘even out’ sequence read lengths though there remains some debate as to whether this is necessary(253). This meant that some samples with low sequence reads were lost. The ‘threshold’ for rarefaction was tested between 500 and 1000 reads and the impact of rarefying at a lower read length on community results was examined by the Procrustes test (from the vegan package)(254). Further details of this test are described in Chapter 4.
2.11.3.2 Hierarchical clustering and indicator species analysis

Hierarchical cluster analysis is an algorithmic approach to find discrete groups with varying degrees of dissimilarity in a data set. In the current project, dissimilarity was assessed using the Bray-Curtis dissimilarity distance (255). Hierarchical clustering was performed using the ‘hclust’ function of the stats package (250). A dendrogram and barplot (showing the 25 most abundant OTUs) were produced.

Once the clusters had been identified, indicator species analysis was performed to identify the most prevalent and unique species within each cluster. The indicspecies package was used for this test (251). The test provides a number of outputs: ‘A’ denotes the specificity or positive predictive value of the representative OTU as an indicator of the cluster (i.e. the probability that the representative OTU belongs to the designated cluster given the fact that the OTU is found). ‘B’ denotes the fidelity or sensitivity of the representative OTU (i.e. the probability of finding the representative OTU in the designated cluster). Finally, ‘Stat’ denotes the overall strength of association between the representative OTU and cluster, where 0 = no association and 1 = perfect association. Further explanations are found in Chapters 5 and 6.

These distinct clusters with their respective indicator species were then examined with respect to clinical outcomes and pro-inflammatory cytokine levels.

2.11.3.3 Alpha diversity measurements

Alpha diversity is a function of species richness (the number of different species present) and evenness (relative abundance of each species presence) in a given environment/sample (156).

In this project, Shannon diversity and Inverse Simpson indices were used as measurement of
alpha diversity (256, 257). The Shannon diversity varies such that a sample with minimal diversity (i.e. one dominant OTU present in the sample) will have a near zero value, and a very diverse sample (i.e. many OTUs with similar abundances) will have a high value (maximal value of 1). In contrast, the Simpson's index is a similarity index (the higher the value the lower in diversity). The Inverse Simpson index merely ‘inverses’ the value to allow to easier interpretation when performed alongside Shannon diversity. Whilst both indices provide a measure of alpha diversity, the Simpson's index is more weighted on dominant species compared to Shannon diversity.

2.11.3.4 Beta diversity measurements

Beta diversity is a measure of the difference in species composition between two environments/samples. In the current project, the Bray-Curtis dissimilarity and unweighted Unifrac distance were used as measurements of beta diversity (255, 258). The Bray-Curtis dissimilarity is calculated between 0 and 1, where 0 indicates that the two samples have exactly the same composition (i.e. they share all the species), and 1 indicates the two samples do not share any species (i.e. completely different composition). Whilst the unweighted Unifrac distance provides similar information on beta diversity, it differs from Bray-Curtis dissimilarity in that it integrates information on the relative relatedness of community members by involving phylogenetic distances between observed organisms in its computation. Moreover, the unweighted Unifrac distance is a qualitative measure (i.e. the mere presence or absence of particular species).
3 Clinical characteristics and outcomes

3.1 Introduction

Acute asthma exacerbations (AEs) result in significant morbidity and healthcare cost(259). Approximately 60-85% of AEs are associated with respiratory viruses, in particular rhinovirus (RV)(260). The role of bacteria in AEs, however, is not well understood and current GINA guidelines recommend against the routine use of antibiotics in AEs(1). Nonetheless, there is evidence that bacteria may play an important role in AEs. Asthmatic patients were observed to have significantly greater risk of invasive pneumococcal infection compared with healthy subjects(133). In neonates, airway colonisation of *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenza* (*H. influenza*) and *Moraxella catarrhalis* (*M. catarrhalis*) is associated with increased risk of pneumonia and bronchiolitis in early life(261). In both children and adults with asthma, atypical bacterial infection/ reactivation with *Mycoplasma pneumoniae* (*M. pneumoniae*) and *Chlamydia pneumoniae* (*C. pneumoniae*) have also been frequently detected during AEs, with serological positivity rates as high as 40-60%(66, 262, 263). Furthermore, there is mounting evidence that respiratory viral infections may enhance the risk of secondary bacterial infections(214), with co-infection leading to greater AE symptoms(211).

More recently, culture-independent studies have challenged the classical dogma of ‘sterility within the healthy lower airways’ by exhibiting a complex microbial community within the lower respiratory tract in both health and disease(174). Importantly, the airway microbiota in asthmatic subjects appear to have a greater abundance of potentially pathogenic species such as *Haemophilus* spp.(174). Furthermore, specific communities appear to correlate with the
degree of bronchial hyper-reactivity\(^{(199)}\) and corticosteroid responsiveness\(^{(200)}\). It is therefore conceivable that the microbiota may play a role in virus-induced AEs.

To date, all publications on the asthmatic airway microbiota have been cross-sectional studies during stable chronic disease. This project therefore represents the first study to examine the dynamic changes within the airway microbiota in the context of a virus-induced AE. The project is divided into two sections. The first section investigates the changes during naturally occurring colds, whilst the second section specifically examines RV-induced AEs.

### 3.2 Naturally occurring colds

#### 3.2.1 Baseline clinical characteristics

Ninety-three subjects were screened, with 81 subjects recruited after fulfilling the inclusion/exclusion criteria. Table 3.1 illustrates the baseline clinical characteristics. The mean age was 39.8 years, with a slight female predominance (54% female, 46% male). Ninety-five percent of subjects had mild to moderate asthma (BTS treatment step 1 – 3) and were evenly distributed between groups. The median Asthma Control Questionnaire (ACQ) score was 0.83 (IQR 0.42 – 1.50).
Over a 30-month period (November 2012 to April 2015), 46 (57%) out of 81 recruited subjects presented with naturally occurring colds and proceeded to complete the study. The clinical characteristics of these subjects remained similar to those at baseline and are shown on Table 3.1.

### Table 3.1 Baseline clinical characteristics of recruited subjects.
The results are displayed as mean with standard deviation or median with inter-quartile range (IQR) where appropriate. ACQ = Asthma control questionnaire, BTS = British Thoracic Society, FEV$_1$ = forced expiratory volume in one second, FVC = forced vital capacity, ICS = inhaled corticosteroid, IgE = immunoglobulin E, PEF = peak expiratory flow, SABA = short-acting beta-agonist.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>39.8 +/- 13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male (n, %)</td>
<td>37 (46%)</td>
</tr>
<tr>
<td>Female (n, %)</td>
<td>44 (54%)</td>
</tr>
<tr>
<td>Baseline FEV$_1$% predicted</td>
<td>90.8 +/- 15.7</td>
</tr>
<tr>
<td>Baseline PEF% predicted</td>
<td>100.3 +/- 16.6</td>
</tr>
<tr>
<td>Baseline FEV$_1}$/FVC</td>
<td>0.74 +/- 0.09</td>
</tr>
<tr>
<td>Blood Neutrophil (x 10$^9$/L)</td>
<td>3.30 (IQR 2.63 – 4.00)</td>
</tr>
<tr>
<td>Blood Eosinophil (x 10$^9$/L)</td>
<td>0.2 (IQR 0.1 to 0.3)</td>
</tr>
<tr>
<td>IgE (IU/mL)</td>
<td>179 (IQR 84.5 - 461)</td>
</tr>
<tr>
<td>ACQ</td>
<td>0.83 (IQR 0.42 – 1.50)</td>
</tr>
</tbody>
</table>

### BTS treatment step

<table>
<thead>
<tr>
<th>Step</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28 (35%)</td>
</tr>
<tr>
<td>2</td>
<td>20 (25%)</td>
</tr>
<tr>
<td>3</td>
<td>29 (35%)</td>
</tr>
<tr>
<td>4</td>
<td>4 (5%)</td>
</tr>
</tbody>
</table>

### Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SABA only</td>
<td>28 (35%)</td>
</tr>
<tr>
<td>ICS (µg/day)</td>
<td></td>
</tr>
<tr>
<td>&lt; 400</td>
<td>21 (26%)</td>
</tr>
<tr>
<td>400 – 800</td>
<td>27 (33%)</td>
</tr>
<tr>
<td>&gt; 800</td>
<td>5 (6%)</td>
</tr>
</tbody>
</table>

### 3.2.2 Presentation of colds

Over a 30-month period (November 2012 to April 2015), 46 (57%) out of 81 recruited subjects presented with naturally occurring colds and proceeded to complete the study. The clinical characteristics of these subjects remained similar to those at baseline and are shown on Table 3.1.
3.2. The majority of reported colds were between the months of October to March (Figure 3.1).

<table>
<thead>
<tr>
<th>Data</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40.35 +/- 12.4</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male (n, %)</td>
<td>21 (46%)</td>
</tr>
<tr>
<td>Female (n, %)</td>
<td>25 (54%)</td>
</tr>
<tr>
<td>Baseline FEV₁% predicted</td>
<td>89.2 +/- 16.3</td>
</tr>
<tr>
<td>Baseline PEF% predicted</td>
<td>99.4 +/- 16.2</td>
</tr>
<tr>
<td>Baseline FEV₁/FVC</td>
<td>0.73 +/- 0.09</td>
</tr>
<tr>
<td>Blood Neutrophil (x 10⁹/L)</td>
<td>3.4 (IQR 2.80 – 3.93)</td>
</tr>
<tr>
<td>Blood Eosinophil (x 10⁹/L)</td>
<td>0.2 (IQR 0.1 – 0.2)</td>
</tr>
<tr>
<td>IgE (IU/mL)</td>
<td>146 (IQR 61.7 – 411.5)</td>
</tr>
<tr>
<td>ACQ</td>
<td>0.83 (0.5 - 1.5)</td>
</tr>
<tr>
<td>BTS treatment step</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19 (41%)</td>
</tr>
<tr>
<td>2</td>
<td>9 (20%)</td>
</tr>
<tr>
<td>3</td>
<td>15 (33%)</td>
</tr>
<tr>
<td>4</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
</tr>
<tr>
<td>SABA only</td>
<td>19 (41%)</td>
</tr>
<tr>
<td>ICS (µg/day)</td>
<td></td>
</tr>
<tr>
<td>&lt; 400</td>
<td>9 (20%)</td>
</tr>
<tr>
<td>400 – 800</td>
<td>14 (30%)</td>
</tr>
<tr>
<td>&gt; 800</td>
<td>4 (9%)</td>
</tr>
</tbody>
</table>

Table 3.2 Clinical characteristics of subjects who presented with naturally occurring cold.

Figure 3.1 Month of reported colds
3.2.3 Respiratory virus detection

Respiratory viruses were detected by PCR in 35 sputum, 20 nasal lavage and 7 throat swab samples respectively. This resulted in a positive virus detection rate of 21 out of 46 (46%) subjects with reported colds; the vast majority being RVs (Table 3.3). Of these 21 subjects, 18 were positive within five days following cold symptoms (i.e. at Visit 2 (days 1/2 of cold) or 3 (days 4/5). The remaining three subjects (two RV and one coronavirus) only exhibited positive virology after 14 days (Visit 4). One subject had detectable RV and coronavirus concurrently.

<table>
<thead>
<tr>
<th>Virus</th>
<th>PCR positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picornavirus</td>
<td>18</td>
</tr>
<tr>
<td>-RV</td>
<td>14*</td>
</tr>
<tr>
<td>-Other</td>
<td>4</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>4*</td>
</tr>
<tr>
<td>RSV</td>
<td>0</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>0</td>
</tr>
<tr>
<td>Bocavirus</td>
<td>0</td>
</tr>
<tr>
<td>Influenza</td>
<td>0</td>
</tr>
<tr>
<td>hMPV</td>
<td>0</td>
</tr>
</tbody>
</table>

*One subject had detectable RV and coronavirus concurrently.

Table 3.3 Respiratory virus detection in subjects with colds. 21 subjects had positive virology. hMPV = human metapneumovirus, RV = rhinovirus, RSV = respiratory syncytial virus.

3.2.4 Quantitative bacterial culture

There were 242 sputum samples in total; of which 203 (84%) had adequate volume for quantitative culture. Twenty-six (13%) samples were positive for bacteria known to be respiratory pathogens, identified in 18 subjects (Table 3.4). Six subjects had positive culture at baseline (Visit 1), whilst two subjects had positive culture during stable chronic disease.
Fourteen out of 46 (30%) subjects had positive culture following a cold (Visits 2 to 4 (days 1 to 14 of cold). Two subjects cultured two pathogens concurrently. Furthermore, eight subjects were positive for both viral PCR and bacterial culture.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>No of positive quantitative culture (&gt; $10^6$ cfu/ml)</th>
<th>No of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>5</td>
<td>5**</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Neisseria flavescens</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>2</td>
<td>2**</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>4</td>
<td>4*</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>1</td>
<td>1*</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Other Coliform</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 3.4 Quantitative bacterial culture in sputum samples. *1 subject cultured *S. aureus* and *S. maltophilia* concurrently. **1 subject cultured *K. oxytoca* and *P. aeruginosa* concurrently.*

### 3.2.5 Cold symptoms

Severity of cold symptoms were assessed using the Wisconsin upper respiratory symptom score (WURSS). Forty-three of 46 subjects (93%) had at least 80% symptom diary completion (i.e. at least 23 days completed). The remaining three subjects had 0%, 21% and 75% completion rates respectively and were excluded from further symptom score analysis. There was significant inter-subject heterogeneity (mean total WURSS score = 658, SD = 677).
Subjects with colds had significantly greater symptoms than controls during the first five days of cold symptoms (P < 0.0001 days 1-2, P = 0.0006 day 3, P = 0.0017 day 4, P = 0.047 day 5. Two-way ANOVA with Bonferroni’s correction) (Figure 3.2). There was no significant difference between subjects with positive virology and those with negative virology (P > 0.05 across all days. Two-way ANOVA).

Figure 3.2 Symptoms following naturally occurring colds. Scores are expressed means. Error bars represent standard errors of the mean.
Table 3.5 Cold symptoms. Two-way ANOVA with Dunnett’s correction of subjects with positive and negative virology (against controls). Subjects with positive virology experience more prolonged symptoms than those with negative virology.

<table>
<thead>
<tr>
<th>Day</th>
<th>Virology status</th>
<th>Mean Diff.</th>
<th>95% CI</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>-61.07</td>
<td>-86.51 to -35.63</td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-52.39</td>
<td>-76.91 to -27.87</td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>-57.80</td>
<td>-83.24 to -32.36</td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-48.11</td>
<td>-72.63 to -23.59</td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>-51.15</td>
<td>-76.59 to -25.71</td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-39.51</td>
<td>-64.02 to -14.99</td>
<td>0.0007 ***</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>-46.20</td>
<td>-71.64 to -20.76</td>
<td>0.0001 ***</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-39.07</td>
<td>-63.58 to -14.55</td>
<td>0.0009 ***</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>-37.18</td>
<td>-62.43 to -11.92</td>
<td>0.0023 **</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-29.52</td>
<td>-54.03 to -4.96</td>
<td>0.0149 *</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>-34.03</td>
<td>-59.28 to -8.774</td>
<td>0.0056 **</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-26.13</td>
<td>-50.90 to -1.349</td>
<td>0.0368 *</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>-28.25</td>
<td>-53.50 to -2.999</td>
<td>0.0252 *</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-22.03</td>
<td>-46.81 to 2.744</td>
<td>0.0892</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>-30.73</td>
<td>-55.98 to -5.474</td>
<td>0.0136 *</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-22.83</td>
<td>-47.61 to 1.945</td>
<td>0.0758</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>-28.68</td>
<td>-53.93 to -3.424</td>
<td>0.0228 *</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>-23.30</td>
<td>-48.08 to 1.478</td>
<td>0.0688</td>
</tr>
</tbody>
</table>

**Figure 3.3** Total symptom scores over 28 days in subjects with respect to virology and quantitative bacterial culture results. Positive quantitative bacterial culture did not
However, subjects with positive virology had more prolonged symptoms than those with negative virology, when compared with controls (Table 3.5). In contrast, positive quantitative bacterial culture did not affect total symptom scores, regardless of virology (P = 0.20. Kruskal-Wallis) (Figure 3.3). Furthermore, the total symptom score significantly correlated with baseline ACQ score (R2 0.25, P = 0.0001. Linear regression) (Figure 3.4).

**Figure 3.4** Total cold symptoms over 28 days significantly correlated with ACQ at baseline.
3.2.6 Changes in lung function

Lung function was assessed by FEV₁ and PEF respectively. There was significant inter-subject variability. The maximal drop in PEF from baseline was 38%, whilst four subjects did not reduce their PEF throughout the study. Median change in lung function in the different groups (all subjects, positive and negative virology) are illustrated in Table 3.6. Disease severity (as denoted by BTS treatment step) was not associated with the maximal decline in PEF following cold (P = 0.069. One-way ANOVA) (**Figure 3.5**). PEF strongly correlated with FEV₁ across all time points in all subjects (R² 0.48, P < 0.0001. Linear regression).

PEF significantly reduced from baseline on day 4/5 (Visit 3) in subjects with positive virology (P = 0.0003. Two-way ANOVA with Tukey’s correction), and day 1 (visit 2) and day 4 in those with negative virology (P = 0.0012, P = 0.0002 respectively. Two-way ANOVA with Tukey’s correction) (**Figure 3.6**). Change in FEV₁ following cold was not statistically significant in either group (P = 0.11. Two-way ANOVA). During the acute phase of colds (Visits 2 and 3), PEF change did not correlate with cold symptoms (Visit 2 R² = 0.054, P = 0.14, Visit 3 R² = 5.64e-005, P = 0.96. Linear regression). Likewise, maximal change in PEF did not correlate with overall cold symptoms (R² = 0.012, P = 0.49. Linear regression) (**Figure 3.7**).
Median change in FEV\textsubscript{1} from baseline (%)

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Positive Virology</th>
<th>Negative Virology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 2 (Day 1/2)</td>
<td>-2.7 (-9.7 – 1.2)</td>
<td>-2.6 (-13 – 1.6)</td>
<td>-2.7 (-8.2 – 1)</td>
</tr>
<tr>
<td>Visit 3 (Day 4/5)</td>
<td>-1.8 (-9.8 – 2.5)</td>
<td>-1.8 (-12 – 4)</td>
<td>-1.8 (-9.8 – 1.9)</td>
</tr>
<tr>
<td>Visit 4 (Day 14/15)</td>
<td>0 (-.7 – 3.9)</td>
<td>0.35 (-9.4 – 5.1)</td>
<td>-0.71 (-6.9 – 2.4)</td>
</tr>
</tbody>
</table>

Median change in PEF from baseline (%)

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Positive Virology</th>
<th>Negative Virology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 2 (Day 1/2)</td>
<td>-8.4 (-13 – 1.3)</td>
<td>-8.9 (-13 – 1.6)</td>
<td>-9 (-15 – -0.24)</td>
</tr>
<tr>
<td>Visit 3 (Day 4/5)</td>
<td>-8.2 (-16 – -2.5)</td>
<td>-8.3 (-17 – -0.95)</td>
<td>-7.4 (-15 – -2.6)</td>
</tr>
<tr>
<td>Visit 4 (Day 14/15)</td>
<td>-3 (-8.6 – 1.9)</td>
<td>-2.2 (-8 – 2.2)</td>
<td>-6.8 (-13 – 1.7)</td>
</tr>
</tbody>
</table>

**Table 3.6** Median change in lung function following natural cold. Inter-quartile range is expressed in brackets.

---

One-way ANOVA

P = 0.069

**Figure 3.5** Maximal change in PEF following cold in subjects with different disease severity.
**Figure 3.6** Change in lung function following cold. (A) FEV<sub>1</sub> (B) PEF. Line indicates median. ** P < 0.005 *** P < 0.0005. Two-way ANOVA test with Tukey’s correction.

Visit 1 = baseline, Visit 2 = day 1/2 post cold, Visit 3 = day 4/5 post cold, Visit 4 = day 14/15 post cold.
**Figure 3.7** Correlation between change in PEF and cold symptoms. There was no significant correlation at Visit 2 (day 1/2 post cold) or 3 (day 4/5 post cold). Maximal change in PEF did not correlate with total cold symptoms.
3.2.7 Pro-inflammatory cytokines

Pro-inflammatory cytokines were measured using nasosorption. IL-1β, IL-6, IL-8 and TNF-α strongly correlated with each other (Table 3.8). There was no significant change in IL-1β, IL-6, IL-8 and TNF-α levels during stable disease. Following cold, there was no significant change in IL-1β, IL-8 and TNF-α levels regardless of virology status (P > 0.05. Two-way ANOVA with Tukey’s correction). IL-6 levels, however, were significantly increased following cold in subjects with positive virology (Visit 1 (baseline) vs Visit 2 (day 1/2): P = 0.001, Visit 2 (day 1/2) vs Visit 4 (day 14/15): P = 0.0023. Two-ANOVA with Tukey’s correction). Table 3.7 illustrates the median IL-6 level at baseline and following cold.

At Visit 2 (day 1/2), subjects with positive virology also had significantly greater levels of IL-6 compared with the negative virology group and controls (P = 0.014 in both groups. Two-way ANOVA with Tukey’s correction) (Figure 3.8).

There was significant inter-subject variability as demonstrated by Figure 3.9. Changes in IL-1β were generally modest; though six subjects had increase of > 100 pg/ml following cold. In contrast, three subjects had reduction in IL-1β of > 200 pg/ml following cold. Most subjects with positive virology had an increase in IL-6 level, either at Visit 2 or 3, and subsequently return to near-baseline level by Visit 4. Only four subjects with negative virology had induction of IL-6 of > 100 pg/ml. IL-8 response in cold appeared to be the most variable. In contrast, TNF-α was less commonly detected, though frequency of detection increased with cold, especially in those with negative virology.
### Table 3.7 Median IL-6 levels (pg/ml).
Inter-quartile range is expressed in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Median IL-6 level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 1 (baseline)</td>
<td>1.4 (0.1 – 4.2)</td>
</tr>
<tr>
<td>Visit 2 (day 1/2 post cold)</td>
<td>21 (3.9 – 112)</td>
</tr>
<tr>
<td>Visit 3 (day 4/5 post cold)</td>
<td>19 (7.5 – 87)</td>
</tr>
<tr>
<td>Visit 4 (day 14/15)</td>
<td>6.3 (0.69 – 17)</td>
</tr>
</tbody>
</table>

### Table 3.8 Spearman correlation matrix of pro-inflammatory cytokines.

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>R 0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 6.05e-28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>R 0.83</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0.00</td>
<td></td>
<td>2.32e-9</td>
</tr>
<tr>
<td>TNF-α</td>
<td>R 0.44</td>
<td>0.48</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>P 2.54e-11</td>
<td>4.69e-13</td>
<td>5.97e-12</td>
</tr>
</tbody>
</table>

*Interquartile range is expressed in brackets.*
Visit 1 = baseline, Visit 2 = day 1/2 post cold, Visit 3 = day 4/5 post cold, Visit 4 = day 14/15 post cold.
**Figure 3.9**
Individual subject trends for changes in pro-inflammatory cytokine levels. Each line denotes an individual subject across the four study visits.
3.2.8 Biomarker of viral infection

Interferon-γ-induced protein 10 (IP-10) (also known as CXCL-10) from nasosorption was used as biomarker marker of viral infection. IP-10 was frequently detected even at baseline. However, IP-10 levels significantly increased at Visit 2 and 3 in subjects with positive virology (P < 0.0001 for both visits. Two-way ANOVA with Tukey’s correction). This was not the case in the negative virology groups and controls. Furthermore, IP-10 levels were significantly higher in the positive virology group compared with negative virology and control at both Visit 2 and 3 (Positive vs negative virology: Visit 2 P = 0.011, Visit 3 P < 0.0001. Positive virology vs control: Visit 2 P = 0.0025, Visit 3 P = 0.0006. Two-way ANOVA with Tukey’s correction). Eleven out of 21 (57%) subjects with positive virology had an increase in IP-10 of > 10-fold, whilst two out of 25 (8%) subject with negative virology had > 9-fold increase in IP-10.

![Figure 3.10](image-url)  
*Figure 3.10* IP-10 levels at baseline (Visit 1) and following cold (Visits 2 – 4). Line represents median. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0005. Two-way ANOVA with Tukey’s correction.
**Figure 3.11** Individual subject trends for fold changes IP-10 levels. Each line denotes an individual subject across the four study visits.
3.2.9 Clinical management of colds

The vast majority of colds were self-managed by subjects. Out of the 46 subjects, four attended their GPs for assessment, whilst one was assessed by his community asthma nurse specialist. There was no emergency department attendance or in-patient hospital admission. All subjects reported increased use of short-acting beta agonist (SABA) (at least two more puffs per day from usual dose). Five subjects had a 5-day course of oral prednisolone, with one subject extending the course to 10 days. Six subjects had antibiotic therapy. With regards to the timing of treatment instigation, one subject commenced prednisolone treatment before Visit 2. All other subjects commenced treatment (prednisolone and/or antibiotic) after Visit 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>SABA (Increased dosage)</td>
<td>46</td>
</tr>
<tr>
<td>ICS (Increased dosage)</td>
<td>3</td>
</tr>
<tr>
<td>Prednisolone 30mg od 5 days</td>
<td>5*</td>
</tr>
<tr>
<td>Antibiotic</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin 500mg tds 5-7 days</td>
<td>5</td>
</tr>
<tr>
<td>Doxycycline 100mg bd 7 days</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 3.9 Clinical management of colds. ICS = inhaled corticosteroid, SABA = short-acting beta agonist. od = once daily, bd = twice daily, tds = three times a day. * One subject extended prednisolone course to 10 days.*

3.3 Experimental RV-16 infection

3.3.1 RV-16 infection and clinical demographics

Fifteen asthmatic and 14 healthy subjects were recruited and inoculated with RV-16. Infection was defined as increase in serum neutralising antibody to RV-16 of $\geq 1:4$ six weeks post-
inoculation and detectable RV-16 viral load in nasal lavage or bronchoalveolar lavage (BAL) at any time point post-inoculation. Overall, six subjects (four asthmatics and two healthy) failed to develop an infection. Consequently, 11 asthmatic and 12 healthy subjects were successfully infected. Table 3.10 illustrates the clinical demographics of successfully infected subjects. Both healthy and asthma groups are evenly matched in age and sex, with a slight female predominance. Asthmatic subjects had significantly lower FEV$_1$ and higher serum IgE levels than healthy subjects at baseline. With regards to asthma symptom control in accordance to ACQ score, eight subjects were poorly controlled and three were partially controlled respectively.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Asthma</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 12)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27 +/- 3</td>
<td>31 +/- 3</td>
<td>-</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n, %)</td>
<td>8 (67%)</td>
<td>7 (64%)</td>
<td>-</td>
</tr>
<tr>
<td>Female (n, %)</td>
<td>4 (33%)</td>
<td>4 (36%)</td>
<td>-</td>
</tr>
<tr>
<td>Baseline FEV$_1$ % predicted</td>
<td>99 +/- 3</td>
<td>90 +/- 6</td>
<td>0.03</td>
</tr>
<tr>
<td>Baseline PC$_{20}$ (mg/ml)</td>
<td>-</td>
<td>1.63 +/- 0.69</td>
<td>-</td>
</tr>
<tr>
<td>Baseline ACQ</td>
<td>-</td>
<td>1.59 +/- 0.14</td>
<td>-</td>
</tr>
<tr>
<td>ICS (µg/day)</td>
<td>-</td>
<td>873 +/- 213</td>
<td>-</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>28 (IQR 10-58)</td>
<td>201 (IQR 152-352)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Table 3.10** Clinical demographics of successfully infected subjects. Results are expressed as mean +/- SD unless stated otherwise.

3.3.2 Upper respiratory symptoms

Cold symptoms became apparent in most healthy and asthmatic subjects within 48 hours post inoculation, with sore throat being the most common presentation. Compared with baseline,
symptoms were significantly raised on days 2-6, and day 8 in the healthy group (Day 2, 6, and 8 P <0.05. Days 3 – 5 P<0.01). Asthmatic subjects had significantly elevated symptoms on days 2-6 and days 8-10 (Days 2 -5 P<0.01. Day 6, days 8 - 10 P<0.05). Peak symptoms were observed on day 3 and 4 post inoculation in healthy and asthmatic subjects respectively. There was a second crescendo of symptoms on day 8. This was apparent in both healthy and asthma groups. Asthmatic subjects had significantly greater symptoms than healthy subjects on days 1, 2 and 4-14 (Figure 3.12). Furthermore, total upper respiratory symptom scores over the 28-day study period were significantly greater in asthma compared with healthy controls (P < 0.0001).

![Figure 3.12](image)

**Figure 3.12** Daily upper respiratory tract symptom scores following RV-16 inoculation. Line indicates mean symptom score in each group. Asthmatic subjects experienced significantly greater symptoms on days 1, 2 and 4 – 14. * P < 0.05, ** P < 0.01.
3.3.3 Lower respiratory symptom scores

A similar pattern is observed in lower respiratory symptoms. Asthmatic subjects experienced significantly greater lower respiratory symptoms on days 1, 2-5, 7-11 and days 13 and 14 (P<0.05 on all days) (Figure 3.13). Peak symptoms were seen on day 3. A second peak was again observed on day 8, though this was limited to the asthmatic group. Total lower respiratory scores over the 14-day period following RV-inoculation was greater in asthma (P<0.001).

![Figure 3.13](image)

**Figure 3.13** Daily lower respiratory tract symptom scores following RV-16 inoculation. Line indicates mean symptom score in each group. Asthmatic subjects experienced significantly greater symptoms on days 1, 2–5, 7–11, 13 and 14. * P < 0.05, ** P < 0.01, *** P < 0.001.

Total lower respiratory symptoms strongly correlated with upper respiratory symptoms (R2 = 0.71, P < 0.0001. Linear regression) (Figure 3.14). Neither upper nor lower respiratory
symptoms correlated with ACQ at baseline (Upper respiratory: R² 0.0002, P = 0.98. Lower respiratory: R² 0.22, P = 0.18. Linear regression) (Figure 3.15).

**Figure 3.14** Total upper respiratory symptoms strongly correlated with total lower respiratory symptoms.

**Figure 3.15** Baseline ACQ and respiratory symptom scores. There was no significant correlation between symptoms and ACQ at baseline.
3.3.4 Changes in lung function

Lung function was assessed by changes in PEF and FEV\textsubscript{1} from baseline. Following RV-16 infection, the mean PEF decline in asthmatic subjects was greatest on day 5, followed by a second trough on day 11, whilst the greatest decline in healthy subjects was seen on day 5 without the second trough. The maximal PEF decline from baseline was 26.6% in asthma and 15.6% in healthy. Maximal decline in PEF from baseline significantly correlated with total lower respiratory symptoms ($R^2 = 0.25$, $P = 0.020$. Linear regression) (Figure 3.16). Asthmatic subjects had significant decline in the PEF, compared to baseline, on days 3-14 ($P<0.05$ on all days). Furthermore, the asthma group experienced significantly greater decline in morning PEF than the healthy group on days 3, 7, 9-13 ($P<0.05$ on all days) (Figure 3.17). A similar pattern was observed with changes in FEV\textsubscript{1}. The maximal FEV\textsubscript{1} declines from baseline were 35.4% and 14.2% in asthma and healthy respectively. Healthy subjects did not experience significant decline in FEV\textsubscript{1} from baseline on any day post infection. In contrast, asthmatic subjects had significant decline on days 4 - 7 and day 11 ($P <0.05$ on all days). Asthmatic

![Graph showing correlation between total lower respiratory symptoms and maximal change in PEF% from baseline.](image)

*Figure 3.16 Total lower respiratory symptoms significantly correlated with maximal decline in PEF from baseline post RV-16 infection.*
subjects had significantly greater decline in FEV$_1$ compared to healthy subjects on days 1 and 4 (P <0.05 on both days).

![Figure 3.17](image)

**Figure 3.17** Daily change in PEF from baseline following RV-16 inoculation. Line indicates mean PEF change in each group. Compared with healthy subjects, asthmatic subjects experienced significantly greater PEF decline on days 3, 7 and 9 – 13. * P < 0.05, ** P < 0.01, *** P < 0.001.

### 3.3.5 Changes in airway hyper-responsiveness

Airway hyper-responsiveness (AHR) was assessed by histamine provocation test (PC$_{20}$ histamine). All subjects underwent histamine provocation test at baseline and day 6 post RV-16 infection. There was no change in AHR in healthy subjects. Two out of 11 asthmatic subjects required the rescue inhaler use on day 6 prior to histamine provocation test due to respiratory symptoms and were subsequently excluded from PC$_{20}$ analysis. Six out of nine
asthmatic subjects had increased AHR (i.e. reduction in \( \text{PC}_{20} \)) following RV-16 infection Figure 3.18).

**Figure 3.18** Change in airway hyper-responsiveness in asthmatic subjects following RV-16 infection as assessed by histamine provocation test (\( \text{PC}_{20} \) histamine). Red lines indicate subjects with \( \text{PC}_{20} \) reduction. Blue lines indicate subjects with no \( \text{PC}_{20} \) change.

### 3.3.6 Detection of RV-16 virus load

RV-16 virus load was measured on days 1-8, 11 and 15 post inoculation in nasal lavage and on days 3 and 8 in BAL. In nasal lavage, virus load peaked during day 3 and 4 post inoculation in both asthmatic subjects and healthy volunteers. On day 4, virus load was significantly higher in asthmatic subjects (40-fold) compared with healthy subjects (Median day 4 virus load: Asthma \( 5.81 \times 10^6 \) copies/ml [IQR \( 2.79 \times 10^5 - 1.803 \times 10^7 \)]. Healthy \( 1.55 \times 10^5 \) copies/ml [IQR \( 1.38 \times 10^4 - 8.35 \times 10^5 \)], \( P <0.05 \)). There was no significant difference in virus load between healthy and asthmatic subjects at all other time-points.

Virus load did not significantly correlate with change in PEF or lower respiratory symptom score on days 3 (PEF: \( r = -0.11 \), \( P = 0.68 \), lower respiratory symptom score: \( r = 0.52 \), \( P = 0.08 \) Spearman’s correlation with FDR correction) and 8 post infection (PEF: \( r = -0.24 \), \( P = 0.33 \))
Lower respiratory symptom score: \( r = -0.28, \ P = 0.33 \) Spearman’s correlation with FDR correction) respectively.

**Figure 3.19** Virus load in nasal lavage. Asthmatic subjects had significantly greater virus load than healthy subjects on day 4 post inoculation. There was no significant difference in peak virus load between asthmatic and healthy subjects. * \( P<0.05 \)
3.3.7 Biomarker of viral infection

IP-10 levels were measured via bronchosorption. IP-10 significantly increased following RV-16 infection in asthma, peaking at day 8 (P = 0.0099. Two-way ANOVA with Dunnett’s correction).

![Figure 3.20](image) 

*Figure 3.20 Levels of IP-10 following RV-16 infection. Line denotes median. ** P < 0.01*

3.3.8 Pro-inflammatory and T-helper 2 (Th2)-mediated cytokines

Pro-inflammatory cytokines were measured via bronchosorption. Th2-mediated cytokines were measured using nasosorption. IL-1β, IL-6 and IL-8 significantly correlated with each other (Table 3.11). In asthmatic subjects, there were significant inductions of IL-1β, IL-6 and IL-8 from baseline on day 8 post infection (IL-1β: P = 0.014. IL-6: P = 0.0013. IL-8: P = 0.027. Two-way ANOVA with Dunnett’s correction). Asthmatic subjects had significantly higher levels of IL-6 on day 8 than healthy subjects (P = 0.0089. Two-way ANOVA with Dunnett’s correction) (Figure 3.21).
The Th2-mediated cytokines (IL-4, IL-5 and IL-13) also significantly correlated with each other (Table 3.12). IL-4 and IL-6 levels were significantly increased from baseline on day 3 post-infection in asthma only (IL-4: P = 0.046, IL-13: P = 0.0076. Two-way ANOVA with Dunnett’s correction), whilst asthmatic subjects had significantly greater levels of IL-5 on day 3 compared with healthy subjects (P = 0.048. Two-way ANOVA with Dunnett’s correction) (Figure 3.22).

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>R</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0031</td>
</tr>
<tr>
<td>IL-8</td>
<td>R</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1.00 e-5</td>
</tr>
</tbody>
</table>

Table 3.11 Spearman correlation matrix of pro-inflammatory cytokines.

<table>
<thead>
<tr>
<th></th>
<th>IL-4</th>
<th>IL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>R</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1.00 e-5</td>
</tr>
<tr>
<td>IL-13</td>
<td>R</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>6.55 e-9</td>
</tr>
</tbody>
</table>

Table 3.12 Spearman correlation matrix of Th2-mediated cytokines.
Figure 3.21 Levels of Pro-inflammatory cytokines at baseline (day 0), day 3 and 8 post RV-16 infection. Line represents median. * $P < 0.05$, ** $P < 0.01$. 

A

B

C
Figure 3.22 Levels of Th2-mediated cytokines at baseline (day 0), peak level during RV-16 infection (day 1 – 14) and day 42 post-infection. * P < 0.05, ** P < 0.01
3.4 Discussion

3.4.1 Naturally occurring colds

3.4.1.1 Clinical characteristics and frequency of colds

The recruited cohort represents a mix of mild and moderate asthmatic subjects. Disease severity, symptom control and management were evenly spread. Out of 81 recruited subjects, 46 completed the study, one short of the targeted 47. It is difficult to assess the true frequency of colds in the recruited cohort. Whilst some subjects never contracted a cold during the study period, multiple factors also affected the completion rate. These include subject withdrawal from study, migration and unavailability for clinical visits. Nonetheless, the current cohort likely represents ‘real life’ asthmatic patients who are mostly managed in primary care.

3.4.1.2 Detection of respiratory viruses and pathogenic bacteria

Respiratory viruses were detected in 46% of subjects. The detection rate was lower than expected as previous paediatric and adult studies have reported detection rate as high as 70%(264, 265). A recent UK study involving 171 adult asthmatic subjects with acute respiratory illness requiring hospital admission showed a positive virology rate of 58%(148). Picornaviruses, in particular RV, were by far the most frequently detected. This is consistent with other published studies (148, 264). The lower than expected virus detection rate in the current study may be due to a number of factors. The majority of viruses was detected in sputum sample. As sputum samples were split three-ways for virology, quantitative culture and 16S sequencing (sample prioritised for 16S sequencing where sample was insufficient), the quality of sputum sample for virology might have been sub-optimal in some cases. In a
small proportion of cases no sputum sample was available. Detection rates in throat swab and nasal lavage were poor, the latter possibly related to low yield rate from nasal olives. Other published studies utilised nasopharyngeal swabs, which may provide a higher detection rate. It is therefore plausible that a proportion of ‘virology negative’ subjects did in fact have a viral infection.

As many as 20% of subjects may have detectable virology when stable(264). It is therefore important to interpret virology result with caution. The significant increase in IP-10 level in the virology positive group in the context of acute symptoms, therefore, provides reassurance of an acute viral infection. Furthermore, a small proportion of virology negative subjects had significant induction of IP-10 following cold symptoms, supporting the possibility of a viral infection that was undetectable by PCR. In the current study, 30% of subjects cultured respiratory bacterial pathogens, consistent with previous publications showing a detection of 19 – 30%(77, 266).

3.4.1.3 Symptom severity and changes in lung function

Overall, most subjects did not experience a severe cold. However, the striking finding was the great inter-subject variability in both WURSS score and PEF change. Subjects with or without detectable respiratory virus did not differ significantly with respect to symptoms and change in lung function, though those with positive virology appeared to have prolonged symptoms. As discussed previously, it is not possible to definitively exclude an underlying viral infection in the negative virology group. Cold symptoms did not significantly correlate with PEF change. Whilst increasing WURSS score may predict worsening asthma symptom control(231), it primarily focuses on upper respiratory and functional symptoms. It therefore may not correlate with lung function changes, which mainly reflect lower airway obstruction. With the
benefit of hindsight, the addition of repeated ACQs might have provided more asthma-specific information in relation to an acute cold. Baseline ACQ, however, did significantly correlate with total cold symptoms. This finding suggests that whilst subjects with worse asthma control experienced greater and more prolonged cold symptoms, they did not necessarily suffer greater lung function decline during cold.

3.4.1.4 Pro-inflammatory cytokines

In similar fashion to cold symptoms and lung function changes, the most remarkable observation was inter-subject variability. In in-vitro setting, IL-1β, IL-6, IL-8 and TNF-α are induced by RV infection. In the current study, only IL-6 showed significant induction following cold symptoms in the positive virology group. Although there was no significant change in IL-1β, IL-8 and TNF-α levels following cold as a cohort, a number of individuals did exhibit significant induction. It is therefore likely that other factors in addition to virus contribute to pro-inflammatory cytokine levels during cold.

3.4.2 Experimental RV-16 infection

3.4.2.1 Clinical characteristics and RV-16 infection

Whilst sex distribution was similar to that of the naturally occurring cold study, the subjects were younger. Furthermore, the asthmatic subjects were all moderate with respect to disease severity with worse asthma control at baseline (as denoted by a higher ACQ score) in general compared with subjects in the naturally occurring cold study. It is noteworthy that six subjects failed to ‘develop’ an infection following inoculation. The precise reason for this observation is unclear, though it is possible that these subjects had sub-detectable RV-16 infection.
3.4.2.2 Symptom severity and changes in lung function

RV-16 caused both upper and lower respiratory symptoms, which significantly correlated with each other. Asthmatic subjects experienced significantly greater symptoms than healthy subjects as a whole, consistent with previous studies(35, 232). However, there were two peaks in symptoms on days 3 – 4 and 8 respectively, which were more prominent in the asthma group. Whilst these peaks may reflect genuine clinical symptoms, it is conceivable that they are confounded by bronchoscopic sampling. A similar pattern is also seen in PEF change following RV-16 infection. This again may be related to repeated bronchoscopic sampling, creating an ‘artificial’ trough in PEF. However, the absence of a second trough in PEF in healthy subjects suggests that asthmatic subjects may have genuinely experienced prolonged decline in PEF following RV-16 infection compared with healthy subjects. This finding would be consistent with previous experimental RV-16 challenge studies, which demonstrated similar patterns in PEF decline without the third bronchoscopic sampling on day 8(35, 232).

Both upper and lower respiratory symptom scores correlated with each other. Furthermore, total lower respiratory symptom scores correlated with maximal decline in PEF, an observation that was not apparent in naturally occurring cold. A possible explanation is that WURSS score includes functional deficits in addition to clinical symptoms, whereas the respiratory symptom score in this study focuses solely on clinical symptoms.

3.4.2.3 Virus load and IP-10

Consistent with previous work(232), virus load peaked between day 3/ 4 post-inoculation, with virus load significantly greater on day 4 in the asthma group. IP-10 levels also increased
significantly in asthma on day 8 following RV-16 infection, suggesting a time lag between virus load and host interferon-mediated response. Previous work has shown significant correlations between peak virus load, chest symptoms and change in AHR (35). However, this was not seen in the current study.

3.4.2.4 Pro-inflammatory and Th2-mediated cytokines

There were significant increases in pro-inflammatory and Th2-mediated cytokine levels following RV-16 infection, particularly in asthmatic subjects. Similar to naturally occurring colds, there was gross inter-subject variability.

3.5 Conclusions

At the time of writing, this project is the first study to investigate the asthmatic airway microbiota in the context of a viral infection. Ideally, such study should be performed on subjects that have clinically-relevant disease (i.e. subjects who have sub-optimally controlled disease and more likely to require medical intervention), whilst minimising potential factors that could alter the microbiota such as antibiotic and oral corticosteroid use. The current project has endeavoured to address these issues. The naturally occurring cold study provided a more ‘real-life’ picture whilst the experimental infection model offered a controlled infection setting to specifically examine the impact of viral infection.

The recruited subjects ranged from healthy to moderate disease. Although severe disease (as denoted by BTS treatment step 5) would have been most clinically relevant (as this group carries the greatest healthcare burden), it was extremely likely that such subjects will require antibiotic and oral corticosteroid treatment thus confounding microbiota assessment. Age has been speculated to affect the airway microbiota (181). The age difference between the
naturally occurring cold and experimental RV-16 infection studies was related to the degree of invasive sampling required for each study.

As the current project is the first of its kind, it was not possible to calculate an accurate sample size estimation. The naturally occurring cold study was one subject short of its target of 47, which would provide power of 80% and an alpha-significance level of 5%. Given the lack of similar studies, results of this project should be viewed as exploratory in nature, which may kindle larger, more focused studies in the future.

The clinical outcomes in both studies were generally consistent. Acute respiratory viral infections in both healthy and asthma mostly led to mild – moderate symptoms, though a minority of subjects experienced significant symptoms and lung function decline. Clinical outcomes of the experimental infection challenge were consistent with previous work, though the effects of multiple bronchoscopic sampling should be taken into account. Perhaps the most striking finding was the great inter-variability with respect to symptoms, lung function and cytokine response, highlighting the complexity of individuals’ response to an acute respiratory viral illness. The following chapters will explore the airway microbiota during such event and examine how the microbiota may account for inter-subject variability.

3.6 Summary of key findings

- 46 mild/ moderate asthmatic subjects experienced natural colds, with a viral detection rate of 46%.
- Most subjects experienced mild/ moderate symptoms, though PEF declined by as much as 38% in a proportion of subjects. ACQ at baseline positively correlated with total cold symptoms.
• Eleven moderate asthmatic and 12 healthy subjects were experimentally infected with RV-16.

• In experimental RV-16 infection, lower respiratory symptoms significantly correlated with PEF decline.

• IP-10 and IL-6 were significantly induced by respiratory viral infection.

• There was significant inter-subject variability with respect to clinical symptoms, lung function change and cytokine response in both studies.
4 16S rRNA sequencing outcomes and contamination checks

4.1 Introduction

Culture-independent studies using 16S rRNA sequencing have challenged the traditional paradigm of sterile airways in health, revealing complex microbial communities within the airways in both health and disease (174). Furthermore, differential species relative abundance, community diversity and inter-species interactions have been shown to relate to disease features, providing new understanding in pathophysiology of respiratory diseases and potential novel therapeutic targets (186, 267). However, 16S rRNA sequencing has a number of technical limitations and caveats, which if overlooked may lead to spurious results and interpretation. Different methods of sample collection and preservation, DNA extraction, sequencing technology, read length, depth and bioinformatics analysis techniques can all substantially affect the eventual data (268, 269). These issues are addressed in details in Chapter 2 Methods and materials.

However, a further source of error derives from contamination of samples during sampling collection and library preparation (168). Potential sources include saline used for BAL, molecular biology grade water, PCR reagents and DNA extraction kit. This is especially important in low biomass samples such as BAL, sputum and throat swabs (168). The current study endeavoured to minimise contamination by including a number of ‘negative control’ samples, in addition to performing 16S rRNA qPCR to assess sample biomass prior to sequencing.

This chapter presents the ‘raw’ sequencing results, quality control checks and negative control results to ensure accurate downstream analysis and data interpretation.
4.2 Sequence counts and length distribution

Seven runs were performed (labelled as Runs 1 – 7). Run 7 was a redundancy run, consisting of ‘repeat’ samples and 43 samples from another study that was not part of the current project. Samples underwent ‘repeat’ sequencing if initial sequencing was suboptimal (< $10^3$ reads/sample) despite adequate biomass (16S rRNA qPCR > $10^4$ copies/µl of extracted genomic DNA). In total, 12 sputum, 8 throat swabs and 20 BAL samples underwent repeat sequencing. After removing the 43 samples from the other study, there were a total of 629 samples for the current project. Figure 4.2 illustrates the number of sequence reads in each sample, whilst Figure 4.1 highlights the 100 samples with the least number of sequence reads. After sequence processing, there were 25,818,979 high-quality sequence reads for the entire project. The sample with the most number of sequence reads had 418,948, whilst the sample with the least sequence reads had 24. The sequence reads per sample distribution was non-parametric. The median number of sequence reads per sample was 24410. Sequence read distributions were similar across all runs (Figure 4.3).
Figure 4.2 The number of sequence reads per sample. The x-axis represents all samples in project; sorted by number of sequence reads per sample.

Figure 4.1 The number of sequence reads per sample for the 100 samples with the least sequence reads. The x-axis represents the samples; sorted by number of sequence reads per sample.
Figure 4.3 Read length distribution across all seven sequencing runs.
4.3 Impact of biomass on sequence counts

Biomass or bacterial burden, estimated by 16S rRNA gene copy/µl of extracted genomic DNA, significantly correlated with number of sequence reads per sample (R² = 0.36, P < 0.0001. Linear regression) (Figure 4.4). Furthermore, BAL sample aliquot volume also significantly correlated with number of sequence reads per sample, though the strength of correlation was weak (R² = 0.10, P = 0.0035. Linear regression) (Figure 4.5).

**Figure 4.4** Correlation between biomass/bacterial burden and sequence counts. **** P < 0.0001. Linear regression

**Figure 4.5** Correlation between BAL sample aliquot volume and sequence counts. ** P < 0.01. Linear regression
4.4 Mock community assessment

Each sequencing run contains one ‘mock community’ sample to act as ‘positive’ control. **Figure 4.6** is a non-metric multidimensional scaling (NMDS) plot using Bray-Curtis similarity measure of all samples from the naturally occurring cold study and control samples. This illustrates that the mock communities are grouped together (i.e. each mock community is similar to each other). Furthermore, the mock communities are dissimilar to actual samples and other negative controls (negative PCR control, DNA extraction kit, throat swab control).
Figure 4.6 NMDS plot using Bray-Curtis similarity measure of all samples from naturally occurring cold study, mock communities and negative controls. Mock = mock community, Negative = negative PCR control, Reagent = DNA extraction kit, Saline = sterile saline, Sputum = sputum sample, TS = throat swab sample, TS_CNT = throat swab control.
Figure 4.7 shows the NMDS plot using Bray-Curtis similarity measure of all BAL samples from the experimental RV-16 infection study (rarefied to 1000 reads) and control samples. Once again, mock communities are grouped together and distinct from BAL samples and negative controls.

![Figure 4.7 NMDS plot using Bray-Curtis similarity measure of all samples from experimental RV-16 infection study, mock communities and negative controls. BAL = bronchoalveolar lavage sample, Mock = mock community, Negative = negative PCR control, Saline = sterile saline.](image)

Finally, Figure 4.8 illustrates the bar plot of the most abundant 27 genera of all seven mock communities (rarefied to $10^5$ reads), showing fair consistency across the seven runs.
As discussed earlier, multiple sources of contamination may be present, from sample collection to library preparation. To take these potential sources into account, different ‘negative controls’ were performed, including 7 ‘blank’ negative PCR controls, 11 DNA extraction kits, 3 sterile throat swabs and 3 sterile saline aliquots (that was flushed through the sterile bronchoscope prior to use).

16S rRNA qPCR was performed to assess biomass/bacterial burden. All negative control samples were below the qPCR detection limit (10³ copies/µl). Furthermore, negative samples...
clustered separately from actual samples on both ordination plots (Figure 4.6 & Figure 4.7).

Figure 4.9 shows the most dominant and prevalent OTUs in sterile saline flushes that have over 1000 reads after sequencing. Three OTUs (Veillonella_10839, Fusibacterium_8486 and Haemophilus_7393) had greater than 1000 reads. There were no OTU that had greater than 1000 reads in negative PCR controls, DNA extraction kits or sterile throat swabs.

4.6 Pre-processing – pruning of low abundant OTUs and rarefaction

Low abundant OTUs that have less than 20 reads in total were removed (pruned). Samples were rarefied to a threshold of 1000 reads (i.e. samples with less than 1000 reads were
removed). Table 4.1 shows the number of samples available for further downstream analysis post-rarefaction.

<table>
<thead>
<tr>
<th></th>
<th>Number of samples pre-sequencing</th>
<th>Number of samples post-rarefaction</th>
<th>Number of taxa post-pruning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>242</td>
<td>210 (87%)</td>
<td>1780</td>
</tr>
<tr>
<td>Throat swab</td>
<td>245</td>
<td>233 (95%)</td>
<td>1780</td>
</tr>
<tr>
<td>BAL</td>
<td>91</td>
<td>72 (79%)</td>
<td>356</td>
</tr>
</tbody>
</table>

*Table 4.1 illustrates the number of samples available post-rarefaction and number of taxa post-pruning. % denotes the percentage of initial samples that were available post-rarefaction. BAL = bronchoalveolar lavage.*

### 4.6.1 Evaluating different rarefaction levels

In order to boost the number of BAL samples for downstream analysis, rarefaction threshold was reduced to 500 reads, resulting in 78 BAL samples (six more than rarefying at 1000 reads). The Procrustes test was performed to assess if the microbial community distribution of each sample would be significantly altered due to the lower rarefaction threshold (254), showing significant correlation between the two rarefaction thresholds (Procrustes Sum of Squares (m12 squared) = 0.3103. Correlation in a symmetric Procrustes rotation = 0.8305. P = 0.001).

In other words, reducing rarefaction threshold to 500 reads had no significant impact on microbial community distribution compared with a threshold of 1000 reads.

After careful considerations, however, the threshold of 1000 reads was retained for BAL samples for the sake of consistency across all samples within the project.
4.7 Discussion

4.7.1 Sequence counts and the impact of bacterial burden

Over the seven sequencing runs, the vast majority (86%) of samples produced greater than 1000 reads. The 14% which had less than 1000 reads were mostly negative control samples; though some actual samples failed to sequence adequately despite multiple attempts. As observed by Salter and colleagues(168), a significant influential factor on the sequence counts per sample was biomass/ bacterial burden, as demonstrated by the strong correlation between bacterial burden and number of sequence reads ($R^2 = 0.36$, $P < 0.0001$. Linear regression). To that end, it is unsurprising that BAL aliquot volume also correlated with sequence counts, despite a weak correlation ($R^2 0.10$, $P = 0.0035$. Linear regression). This phenomenon was noticed midway through the project. As a result, target BAL aliquot volume was increased from 4ml to 10ml, with improved sequencing outcome.

4.7.2 Consistency between sequencing runs

Overall, there was good consistency between sequencing runs as demonstrated by the comparable sequence distribution lengths and mock communities across the seven runs. This was further exhibited by clustering of the mock communities on the ordination plot, confirming similarity between the mock communities.

4.7.3 Potential contamination

Whilst it is not possible to completely exclude contamination, it was reassuring to observe that all negative controls had very low biomass (below 16S rRNA qPCR detection limit). Furthermore, negative controls clustered separately from actual samples on ordination plot,
indicating that the microbial communities of the negative controls were dissimilar to those of actual samples (which clustered together). Within the sterile saline flush, however, three OTUs, Veillonella_10839, Fusibacterium_8486 and Haemophilus_7393 respectively, had greater than 1000 reads. All three OTUs have previously been identified in the lower respiratory tract samples. This is likely to represent that these species are present in very low abundance within the ‘clean’ bronchoscope as overall bacterial burden remains low. This will need to be taken into consideration when determining if such OTUs correlate with clinical parameters in further analyses.

4.7.4 Rarefaction

Rarefaction is commonly performed in microbiome analyses. The process allows for a more unbiased approach of assessing samples with varying sequence counts. However, some authors argue that the rarefaction process actually limits the view of the overall community profile and should not be performed at all (253). For the current project, rarefaction was performed in view of the great variation in sample sequence counts. The threshold was set at 1000 reads as it is accepted as an acceptable level in published works (using the same sequencing platform) (270) whilst allowing retention of the majority of samples. BAL samples had lower bacterial burden and thus had lower sequence counts on average. The rarefaction threshold was therefore reduced to 500 reads in an attempt to retain more samples for downstream analysis. The Procrustes test confirmed that the reduction of rarefaction threshold to 500 reads did not alter the OTU distribution significantly. However, given the desire to compare and contrast the two studies of the project, the rarefaction threshold was maintained at 1000 reads. This resulted in a sample retention rate of 80 – 95%.
4.8 Conclusions

Overall, the majority of samples produced adequate sequence counts (> 1000 reads per sample). Biomass/bacterial burden had a significant impact on sequence counts. This was particularly relevant to BAL, which generally had a lower bacterial burden compared with sputum and throat swab samples. As a result, BAL aliquot volume was increased. There was also satisfactory consistency across the seven sequencing runs as demonstrated by the mock community relative abundance and clustering on NMDS ordination. Negative controls, including negative PCR controls, DNA extraction kits, sterile throat swabs and sterile saline flushes, had low bacterial burden below detection limit. Furthermore, they clustered independently from actual samples on NMDS ordination, indicating that they have more dissimilar microbiota than actual samples. Three OTUs, which are known to be present in lower airways, were identified in sterile saline flushes pre-bronchoscopy although they were in low abundance. The clinical significance of these OTUs will require careful consideration during further analyses. Finally, samples were rarefied at a threshold of 1000 reads with a sample retention rate of 80 – 95%.

4.9 Summary of key findings

- A total of 629 sputum, throat swab and BAL samples underwent 16S rRNA sequencing in seven sequencing runs, resulting in 25,818,979 high-quality sequence reads.
- Sample bacterial burden significantly correlated with sequence counts.
- There was reasonable consistency between sequencing runs.
• All negative control samples had low bacterial burden (below qPCR detection limit). Three OTUs were identified in sterile saline flushes. This will need further consideration during further analyses.

• Samples were rarefied at 1000 reads, resulting in a retention rate of 80 – 95%. 210 sputum, 233 throat swabs and 72 BAL samples were eligible for further analyses.
5 The asthmatic airway microbiota in naturally occurring colds

5.1 Introduction

Acute asthma exacerbations (AEs) cause significant morbidity and healthcare burden(259). Respiratory viruses, in particular rhinoviruses (RVs), are associated with 60 – 80% of AEs(260). In a recent UK survey of 1600 asthmatic patients, 73% reported that their asthma symptoms exacerbate in the presence of a cold(271). The role of bacteria in AEs, however, is unclear. From quantitative culture-based and serological studies, chronic infection and re-activation of the atypical bacteria, *Mycoplasma pneumoniae* (*M. pneumoniae*) and *Chlamydophila pneumoniae* (*C. pneumoniae*), have been associated with AEs in adults and children(66, 262), though detection rates were variable. In a recent UK study involving 171 asthmatic patients hospitalised with acute respiratory illness, only 5% had detectable bacteria in sputum, blood culture or urine (urinary antigen test of *Streptococcus pneumoniae*)(148). Current guidelines recommend against the routine use of antibiotics in AEs(1, 149). However, antibiotic therapy is frequently prescribed in ‘real-life’ clinical practice. In the same UK study, 25% of patients received antibiotics prior to admission, and 59% were prescribed antibiotics during admission despite the low bacterial detection rate(148). In view of significant increase in antimicrobial resistant organisms, the role of bacteria in AEs needs to be addressed.

More recently, culture-independent based studies using 16S rRNA gene sequencing have revealed complex microbial communities (termed microbiota) in both health and disease(174, 177). Furthermore, the communities appeared to be distinct from those within healthy airways in a range of chronic lung diseases including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and non-CF bronchiectasis(186), raising the possibility of
the role of the microbiota impacting pathogenesis and disease progression via modulation of immune responses. Recently, the hypothesis of dysbiosis has been postulated(186, 195). In health, the airways microbiota consists of a diverse community of ‘commensal’ species as well as ‘potentially pathogenic’ species, which are tolerate by the immune system. However, the balance is shifted in disease, with a greater abundance of potentially pathogenic species leading to reduced immune tolerance and increased inflammation.

In chronic asthma, numerous studies have reported increased relative abundance of bacteria in the Proteobacteria phylum, which consists of pathogenic species such as *Haemophilus influenzae* (*H.*influenzae) and *Moraxella catarrhalis*(174, 176, 200), while the relative abundance of commensal species such as *Prevotella* spp. are reduced(174). Furthermore, *in vivo* murine and *in vitro* studies have shown that Proteobacteria such as *H.*influenzae and *Haemophilus parainfluenzae* (*H.*parainfluenzae) and Bacteroidetes such as *Prevotella* spp. induce differential immune responses(200, 202). Pre-incubation of asthmatic airway macrophages with *H.*parainfluenzae resulted in p38 mitogen-activated protein kinase (MAPK) activation, increased IL-8, mitogen-activated kinase phosphatase 1 mRNA expression and inhibited response to corticosteroid. In contrast, this was not observed with *Prevotella melaninogenica*(200). In an *in vivo* murine lung model, *H.*influenzae induced TLR-2 independent inflammation resulting in airway neutrophilia, whereas *Prevotella nanceiensis* induced a diminished neutrophilic airway inflammation(202). Results from such studies support the ideation that a ‘dysbiotic’ microbiota may lead to increased inflammation and tissue damage, though the reality is likely to be considerably more complex.

There is a growing wealth of evidence demonstrating the effects of respiratory viral infections on the airway microbiota and the enhancement of secondary bacterial infections(205, 214).
Experimental RV infection in a cohort of 14 subjects with mild COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) Stage 2) was associated with significant outgrowth of *H. influenzae* compared with healthy subjects (198). A similar observation was made in a recent longitudinal study of 12 subjects with GOLD Stage 2 COPD with naturally occurring exacerbations, showing significant expansion of Proteobacteria, in particular *H. influenzae* (192).

Given a significant proportion of AEs is related to RV infections, it seems plausible that RV infection may further perturb an already imbalanced and dysregulated microbiota, resulting in greater airway inflammation and ultimately increased severity of AE. As yet, there is no longitudinal study assessing the airway microbiota during AEs. The current project aims to address this question.

This chapter will investigate the dynamic changes within the asthmatic airway microbiota during a naturally occurring cold and examine if specific community alterations or species are associated with worse outcomes.

### 5.2 Aims

- To investigate the dynamic changes within the airway microbiota of subjects with mild/moderate asthma during a naturally occurring cold.
- To examine if changes within the airway microbiota during a naturally occurring cold correlate with changes in clinical parameters (cold or asthma symptoms and lung function) and biomarkers of airway inflammation.
5.3 Hypothesis

- The asthmatic airway microbiota is altered to a more pathogenic composition following a naturally occurring cold, leading to worsened airway inflammation, lung function and clinical symptoms.

5.4 Bacterial burden

5.4.1 Changes during stable chronic disease and naturally occurring cold

Total bacterial burden (BB) was assessed by 16S rRNA qPCR. Baseline sputum samples demonstrated high copy numbers, though there was great variability amongst subjects (median bacterial burden $7.1 \times 10^8$ copies/g, IQR $2.6 \times 10^8 – 1.6 \times 10^9$). Repeated longitudinal sampling in stable chronic asthma showed no significant change in BB. Following a naturally-occurring cold, there was no significant change in BB, regardless of virology status (Time $P = 0.79$, Virology $P = 0.15$. Two-way ANOVA with Dunnett’s correction) (Figure 5.1). However, three subjects had between 100 - 1000-fold increases in bacterial burden following a cold, while changes during stable chronic disease remain modest (Figure 5.2).
Figure 5.1 Sputum bacterial burden during stable chronic disease and naturally occurring cold. Line denotes median bacterial burden. No significant change in bacterial burden was observed during stable chronic disease or following cold regardless of virology status.

Visit 1 = baseline, Visit 2 = day 1/2 post cold, Visit 3 = day 4/5 post cold, Visit 4 = day 14/15 post cold
Figure 5.2 Individual subject trends for bacterial burden during naturally occurring cold and stable chronic disease. Changes are expressed as fold-change from baseline.

Visit 1 = baseline, Visit 2 = day 1/2 post cold, Visit 3 = day 4/5 post cold, Visit 4 = day 14/15 post cold
5.4.2 Correlation with clinical parameters, pro-inflammatory cytokines and alpha diversity measures

At baseline, BB did not correlate with clinical symptoms, lung function or pro-inflammatory cytokines (P > 0.05 for all variables. Spearman correlation with FDR adjustment). There was no significant difference in BB between BTS treatment steps (P = 0.24. Kruskal-Wallis with Dunn’s multiple comparison test). Whilst BB did not correlate with community diversity (as assessed by Shannon and Inverse Simpson index), it positively correlated with richness (i.e. number of OTUs observed in a given sample) (r = 0.39, P = 0.023. Spearman’s correlation with FDR adjustment) (Table 5.1 & Figure 5.3).

<table>
<thead>
<tr>
<th>Visit 1</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1/FVC</td>
<td>0.26</td>
<td>0.0028 to 0.48</td>
<td>0.27</td>
</tr>
<tr>
<td>Maximal change in PEF from baseline</td>
<td>0.12</td>
<td>-0.20 to 0.42</td>
<td>0.74</td>
</tr>
<tr>
<td>Serum IgE (IU/ml)</td>
<td>-0.16</td>
<td>-0.40 to 0.10</td>
<td>0.61</td>
</tr>
<tr>
<td>Total WURSS score over 28 days</td>
<td>-0.12</td>
<td>-0.37 to 0.15</td>
<td>0.74</td>
</tr>
<tr>
<td>Peak WURSS score</td>
<td>-0.022</td>
<td>-0.34 to 0.30</td>
<td>0.89</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>0.19</td>
<td>-0.13 to 0.47</td>
<td>0.61</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.23</td>
<td>-0.085 to 0.51</td>
<td>0.59</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>-0.033</td>
<td>-0.34 to 0.28</td>
<td>0.89</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.078</td>
<td>-0.24 to 0.38</td>
<td>0.81</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>0.068</td>
<td>-0.19 to 0.32</td>
<td>0.81</td>
</tr>
<tr>
<td>Pielou evenness</td>
<td>-0.098</td>
<td>-0.34 to 0.16</td>
<td>0.74</td>
</tr>
<tr>
<td>Richness</td>
<td>0.39</td>
<td>0.15 to 0.58</td>
<td><strong>0.023</strong> *</td>
</tr>
<tr>
<td>Inverse Simpson diversity</td>
<td>0.04</td>
<td>-0.22 to 0.29</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 5.1 Spearman’s correlation between sputum bacterial burden at baseline and clinical parameters, pro-inflammatory cytokines and alpha diversity measures. P values are adjusted using false discovery rate (FDR) (alpha = 0.05). Significant correlation is highlighted in bold. CI = confidence interval.
During the acute phase of naturally occurring cold (Visits 2 & 3), a similar pattern was observed. BB did not correlate with cold symptoms or PEF change, though it significantly correlated with community richness (Visit 2: \( r = 0.47 \), \( P = 0.048 \). Visit 3: \( r = 0.43 \), \( P = 0.038 \). Spearman’s correlation with FDR adjustment) (Figure 5.4, Figure 5.6, Table 5.2 & Table 5.3 respectively). In addition, BB on Visit 3 significantly correlated with IL-1β level on Visit 4 (i.e. a 10-day lag) \( (r = 0.43, P = 0.038 \). Spearman’s correlation with FDR adjustment) (Figure 5.5).

Finally, BB did not correlate with symptoms, PEF change, pro-inflammatory cytokines or alpha diversity measures at Visit 4 (Table 5.4).

5.4.3 Summary

Bacterial burden did not significantly alter following a natural cold, though there was a dramatic increase (> 10 fold) in a small proportion of subjects. Whilst bacterial burden did not significantly correlate with cold symptoms or lung function following a cold, it positively correlated with IL-1β (at Visit 3) and richness.
<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>0.32</td>
<td>-0.015 to 0.58</td>
<td>0.28</td>
</tr>
<tr>
<td>WURSS score (Visit)</td>
<td>-0.21</td>
<td>-0.51 to 0.13</td>
<td>0.59</td>
</tr>
<tr>
<td>Total WURSS score over 28 days</td>
<td>-0.18</td>
<td>-0.48 to 0.16</td>
<td>0.59</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 2</td>
<td>0.099</td>
<td>-0.24 to 0.41</td>
<td>0.72</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 3</td>
<td>-0.068</td>
<td>-0.39 to 0.27</td>
<td>0.73</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 2</td>
<td>0.095</td>
<td>-0.26 to 0.43</td>
<td>0.72</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 3</td>
<td>-0.16</td>
<td>-0.48 to 0.21</td>
<td>0.64</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 2</td>
<td>0.15</td>
<td>-0.19 to 0.45</td>
<td>0.64</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 3</td>
<td>-0.081</td>
<td>-0.40 to 0.25</td>
<td>0.72</td>
</tr>
<tr>
<td>TNF-α (pg/ml) Visit 2</td>
<td>0.028</td>
<td>-0.30 to 0.35</td>
<td>0.87</td>
</tr>
<tr>
<td>TNF-α (pg/ml) Visit 3</td>
<td>-0.18</td>
<td>-0.48 to 0.16</td>
<td>0.59</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>0.3</td>
<td>-0.031 to 0.57</td>
<td>0.28</td>
</tr>
<tr>
<td>Pielou evenness</td>
<td>0.12</td>
<td>-0.22 to 0.43</td>
<td>0.72</td>
</tr>
<tr>
<td>Richness</td>
<td>0.47</td>
<td>0.16 to 0.69</td>
<td><strong>0.048</strong> *</td>
</tr>
<tr>
<td>Inverse Simpson diversity</td>
<td>0.29</td>
<td>-0.040 to 0.57</td>
<td>0.28</td>
</tr>
</tbody>
</table>

**Table 5.2** Spearman’s correlation between sputum bacterial burden at Visit 2 and clinical parameters, pro-inflammatory cytokines and alpha diversity measures. P values are adjusted using false discovery rate (FDR) (alpha = 0.05). Significant correlation is highlighted in bold. CI = confidence interval.

**Figure 5.4** Spearman’s correlation between sputum bacterial burden at Visit 2 and community richness.
### Table 5.3

Spearman’s correlation between sputum bacterial burden at Visit 3 and clinical parameters, pro-inflammatory cytokines and alpha diversity measures. P values are adjusted using false discovery rate (FDR) (alpha = 0.05). Significant correlation is highlighted in bold. CI = confidence interval.

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>0.1</td>
<td>-0.22 to 0.40</td>
<td>0.62</td>
</tr>
<tr>
<td>WURSS score (Visit)</td>
<td>-0.048</td>
<td>-0.37 to 0.28</td>
<td>0.83</td>
</tr>
<tr>
<td>Total WURSS score over 28 days</td>
<td>0.019</td>
<td>-0.30 to 0.33</td>
<td>0.91</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 3</td>
<td>0.23</td>
<td>-0.090 to 0.51</td>
<td>0.31</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 4</td>
<td>0.43</td>
<td>0.14 to 0.66</td>
<td><strong>0.038</strong>*</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 3</td>
<td>0.15</td>
<td>-0.22 to 0.48</td>
<td>0.51</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 4</td>
<td>0.19</td>
<td>-0.14 to 0.48</td>
<td>0.40</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 3</td>
<td>0.19</td>
<td>-0.0084 to 0.57</td>
<td>0.19</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 4</td>
<td>0.31</td>
<td>-0.0048 to 0.57</td>
<td>0.19</td>
</tr>
<tr>
<td>TNF-α (pg/ml) Visit 3</td>
<td>0.31</td>
<td>-0.092 to 0.51</td>
<td>0.31</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>0.25</td>
<td>-0.072 to 0.52</td>
<td>0.31</td>
</tr>
<tr>
<td>Richness</td>
<td>0.43</td>
<td>0.13 to 0.66</td>
<td><strong>0.038</strong>*</td>
</tr>
<tr>
<td>Pielou evenness</td>
<td>0.15</td>
<td>-0.18 to 0.44</td>
<td>0.49</td>
</tr>
<tr>
<td>Inverse Simpson diversity</td>
<td>0.17</td>
<td>-0.16 to 0.46</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**Figure 5.5** Spearman’s correlation between sputum bacterial burden at Visit 3 and IL-1β level at Visit 4.
**Figure 5.6** Spearman’s correlation between sputum bacterial burden at Visit 3 and community richness.

![Graph showing Spearman's correlation between sputum bacterial burden and community richness.](image)

**Table 5.4** Spearman’s correlation between sputum bacterial burden at Visit 4 and clinical parameters, pro-inflammatory cytokines and alpha diversity measures. *P* values are adjusted using false discovery rate (FDR) (alpha = 0.05). Significant correlation is highlighted in bold. CI = confidence interval.

<table>
<thead>
<tr>
<th>Visit 4</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>0.064</td>
<td>-0.24 to 0.36</td>
<td>0.7865</td>
</tr>
<tr>
<td>WURSS score (Visit)</td>
<td>-0.089</td>
<td>-0.39 to 0.23</td>
<td>0.7865</td>
</tr>
<tr>
<td>Total WURSS score over 28 days</td>
<td>-0.11</td>
<td>-0.40 to 0.19</td>
<td>0.7865</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>0.063</td>
<td>-0.24 to 0.35</td>
<td>0.7865</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>-0.045</td>
<td>-0.37 to 0.29</td>
<td>0.7906</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>-0.072</td>
<td>-0.36 to 0.23</td>
<td>0.7865</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.2</td>
<td>-0.11 to 0.47</td>
<td>0.7865</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>-0.055</td>
<td>-0.35 to 0.25</td>
<td>0.7865</td>
</tr>
<tr>
<td>Richness</td>
<td>0.1</td>
<td>-0.20 to 0.39</td>
<td>0.7865</td>
</tr>
<tr>
<td>Pielou evenness</td>
<td>-0.091</td>
<td>-0.38 to 0.21</td>
<td>0.7865</td>
</tr>
<tr>
<td>Inverse Simpson diversity</td>
<td>-0.076</td>
<td>-0.37 to 0.23</td>
<td>0.7865</td>
</tr>
</tbody>
</table>
5.5 Hierarchical clustering and indicator species analysis

Hierarchical clustering analysis using Bray-Curtis similarity method was performed on all 210 samples, regardless of subject and time points. Five distinct clusters (Cluster A – E) were observed. As Cluster A was substantially larger than other clusters, it was further divided into three sub-clusters (Cluster A1 – A3)(Figure 5.7).

To identify the most prevalent and unique species in each cluster, indicator species analysis was performed for each cluster. A number of OTUs were identified as representative for Clusters A1, A2, B, C, D and E. No specific OTU (indicator species) was identified as representative for Cluster A3. From each set of representative OTUs, the OTU with the highest abundance was assigned to represent the cluster (Table 5.5).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>A</th>
<th>B</th>
<th>Stat</th>
<th>P value</th>
<th>Representative OTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.2873</td>
<td>1.000</td>
<td>0.536</td>
<td>0.021</td>
<td>Prevotella_6482</td>
</tr>
<tr>
<td>A2</td>
<td>0.3290</td>
<td>1.000</td>
<td>0.574</td>
<td>0.002</td>
<td>Veillonella_10839</td>
</tr>
<tr>
<td>B</td>
<td>0.4817</td>
<td>1.000</td>
<td>0.694</td>
<td>0.001</td>
<td>Neisseria_2974</td>
</tr>
<tr>
<td>C</td>
<td>0.3180</td>
<td>1.000</td>
<td>0.564</td>
<td>0.001</td>
<td>Streptococcus_12165</td>
</tr>
<tr>
<td>D</td>
<td>0.9228</td>
<td>1.000</td>
<td>0.961</td>
<td>0.001</td>
<td>Haemophilus_7534</td>
</tr>
<tr>
<td>E</td>
<td>0.3466</td>
<td>1.000</td>
<td>0.589</td>
<td>0.013</td>
<td>Veillonella_10850</td>
</tr>
</tbody>
</table>

Table 5.5 Indicator species analysis for clusters. Column A denotes the specificity or positive predictive value of the representative OTU as an indicator of the cluster (i.e. the probability that the representative OTU belongs to the designated cluster given the fact that the OTU is found). Column B denotes the fidelity or sensitivity of the representative OTU (i.e. the probability of finding the representative OTU in the designated cluster). Stat denotes the overall strength of association between the representative OTU and cluster, where 0 = no association and 1 = perfect association.
Figure 5.7 Hierarchical cluster analysis of all sputum samples using Bray-Curtis similarity measure. Samples are arranged and clustered along the x-axis. The white bar at the top represents the remaining genera. Above the bar plot is a dendrogram illustrating the different clusters (A1 – E).
5.6 Cluster-based analyses

Cluster-based analyses were performed to ascertain if certain microbiota composition was associated with greater disease burden or adverse outcomes following colds.

5.6.1 Baseline (Visit 1)

5.6.1.1 Clinical characteristics and pro-inflammatory cytokines at baseline

At baseline (Visit 1), there was no significant difference in age, asthma control (ACQ), lung function (FEV₁/FVC), disease severity (BTS treatment step), serum IgE level or pro-inflammatory cytokines between clusters (P > 0.05 for all variables, Kruskal-Wallis test) (Figure 5.8 & Figure 5.9).

5.6.1.2 Clinical outcomes following cold

There was a significant difference between clusters in the lung function changes following cold (as denoted by the maximal change in PEF% from baseline), with subjects in Cluster B at baseline ("Neisseria" cluster) exhibiting the greatest PEF decline (P = 0.034. Kruskal-Wallis test). However, cold symptoms did not differ significantly between clusters (P = 0.53. Kruskal-Wallis test) (Figure 5.10).
Figure 5.8 Cluster-based analyses on clinical parameter at baseline (Visit 1). There was no significant difference between clusters. Line denotes median. In D, bar represents mean and error bars represent standard deviation.
Figure 5.9 Cluster-based analyses on pro-inflammatory cytokines at baseline (Visit 1). There was no significant difference between clusters. Line denotes median.
Figure 5.10 Cluster-based analyses on clinical outcomes following cold. Line denotes median. There was significant difference between clusters at baseline with regards to PEF change but not cold symptoms.
5.6.2 Acute cold (Visit 2, day 1/2 post cold)

5.6.2.1 Clinical outcomes

Similar to Visit 1, there was significant difference between clusters in PEF change during acute cold (P = 0.017. Kruskal-Wallis test). Cluster B (“Neisseria” cluster) appeared to have the most dramatic PEF decline, whilst clusters A1, A2 and E (“Prevotella” and “Veillonella” clusters) had the least decline. There was no significant inter-cluster difference with regards to cold symptoms (P = 0.38. Kruskal-Wallis test) (Figure 5.11).

5.6.2.2 Pro-inflammatory cytokines

Pro-inflammatory cytokines at both Visits 2 and 3 were examined to assess if changes in the pro-inflammatory cytokines lag behind changes within the microbiota. There was no significant difference between the clusters at either Visits 2 or 3 (P > 0.05 for all cytokines on both visits. Kruskal-Wallis test) (Figure 5.12).
Figure 5.11 Cluster-based analysis on clinical outcomes at Visit 2 (day 1/2 of cold onset). Line denotes median. There was significant difference between clusters with regards to PEF change but not cold symptoms.
Figure 5.12 Cluster-based analysis of pro-inflammatory cytokines. Pro-inflammatory cytokine (IL-1β, IL-6 and IL-8) levels of the different clusters at Visit 2 were assessed (A). To assess if changes in pro-inflammatory cytokines lagged behind changes in microbiota, pro-inflammatory cytokine levels at Visit 3 were assessed using clusters at Visit 2 (B). Line denotes median.
5.6.3 Middle of cold (Visit 3, day 4/5 post cold)

5.6.3.1 Clinical outcomes

There was no significant difference between clusters in either PEF change (P = 0.87 Kruskal-Wallis test) or cold symptoms (P = 0.78. Kruskal-Wallis test) (Figure 5.13).

5.6.3.2 Pro-inflammatory cytokines

Pro-inflammatory cytokine levels at Visits 3 and 4 were examined to evaluate if pro-inflammatory cytokines lag behind changes within the microbiota (a time lag of 10 days between Visit 3 and Visit 4). There was no significant inter-cluster difference at either Visit 3 or 4 (P > 0.05 for all cytokines at both visits. Kruskal-Wallis test) (Figure 5.14).
Figure 5.13 Cluster-based analysis on clinical outcomes at Visit 3 (day 4/5 of cold onset). Line denotes median. There was no significant difference between clusters with regards to PEF change or cold symptoms. * There was no subject in Cluster D at Visit 3.
Figure 5.14 Cluster-based analysis of pro-inflammatory cytokines. Pro-inflammatory cytokine (IL-1β, IL-6 and IL-8) levels of the different clusters at Visit 3 were assessed (A). To assess if changes in pro-inflammatory cytokines lagged behind changes in microbiota, pro-inflammatory cytokine levels at Visit 4 were assessed using clusters at Visit 3 (B). Line denotes median. * There was no subject in Cluster D at Visit 3.
5.6.4 End of cold (Visit 4)

5.6.4.1 Clinical outcomes and pro-inflammatory cytokines

At Visit 4, there was no significant inter-cluster difference in either PEF change, cold symptoms (Figure 5.15) or pro-inflammatory cytokine levels (Figure 5.16) (P > 0.05 for all variables. Kruskal-Wallis test).

5.6.5 Summary of cluster-based analyses

At baseline, there was no significant inter-cluster difference with respect to age, disease severity, symptom control, lung function and IgE level. However, subjects in Cluster B ("Neisseria" cluster) at baseline appeared to have greater PEF decline following a cold. Similarly, during the acute cold, subjects in Cluster B had significantly greater PEF decline whilst subjects in Clusters A1 and A2 ("Prevotella" and "Veillonella" clusters) had the least decline in PEF. A similar pattern (statistically insignificant) was observed with respect to IL-1β and IL-8 levels following cold. Pro-inflammatory cytokine levels appeared to be greater in Cluster B and lower in Clusters A1 and A2. There was no apparent inter-cluster difference with respect to cold symptoms at any stage.

The ensuing section will evaluate changes in microbial community following a cold, including cluster stability/ migration, alpha-diversity measures and beta-diversity.
Figure 5.15 Cluster-based analysis on clinical outcomes at Visit 4 (day 14 of cold onset). Line denotes median. There was no significant difference between clusters with regards to PEF change or cold symptoms.
Figure 5.16 Cluster-based analysis on pro-inflammatory cytokine levels at Visit 4 (day 14 of cold onset). Line denotes median. There was no significant inter-cluster difference.
5.7 Changes in microbial community structure following cold

5.7.1 Alpha diversity

Shannon diversity and Inverse Simpson indices were used to assess alpha diversity. For both indices, the greater the value, the more diverse the microbial community. There was no significant change in alpha diversity following natural colds in both indices (all $P > 0.05$. Two-way ANOVA with Tukey’s multiple comparison test). Furthermore, there was no significant difference between controls (subjects sampled longitudinally during stable disease), subjects with positive virology and those with negative virology at any time point (all $P > 0.05$. Two-way ANOVA with Tukey’s multiple comparison test). A similar trend was observed for richness ($P > 0.05$ for both time and subject group. Two-way ANOVA with Tukey’s multiple comparison test) (Figure 5.17).

5.7.1.1 Correlations with clinical outcomes and pro-inflammatory cytokines

Alpha diversity (assessed using Shannon diversity index) did not significantly correlate with PEF change, cold symptoms or pro-inflammatory cytokines at any time point ($P > 0.05$ for all variables. Spearman’s correlation with FDR adjustment).
Figure 5.17 Changes in alpha diversity measures following natural cold and during stable disease. Dot denotes mean and error bars denote standard deviation. There was no significant difference between the four visits. In addition, positive virology following cold did not significantly impact on alpha diversity (Two-way ANOVA with Tukey’s multiple comparison test).

Visit 1 = baseline, Visit 2 = day 1/2 post cold, Visit 3 = day 4/5 post cold, Visit 4 = day 14/15
<table>
<thead>
<tr>
<th>Visit 1</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal PEF change from baseline (%)</td>
<td>-0.37</td>
<td>-0.63 to -0.039</td>
<td>0.13</td>
</tr>
<tr>
<td>Total WURSS score over 28 days</td>
<td>-0.0077</td>
<td>-0.34 to 0.33</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>0.028</td>
<td>-0.31 to 0.36</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.0096</td>
<td>-0.40 to 0.41</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>0.091</td>
<td>-0.25 to 0.42</td>
<td>0.96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Visit 2</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>0.26</td>
<td>-0.071 to 0.54</td>
<td>0.66</td>
</tr>
<tr>
<td>WURSS score</td>
<td>-0.21</td>
<td>-0.51 to 0.12</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 2</td>
<td>0.0065</td>
<td>-0.3 to 0.33</td>
<td>0.97</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 3</td>
<td>-0.014</td>
<td>-0.34 to 0.31</td>
<td>0.97</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 2</td>
<td>-0.2</td>
<td>-0.51 to 0.15</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 3</td>
<td>0.018</td>
<td>-0.33 to 0.36</td>
<td>0.97</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 2</td>
<td>-0.02</td>
<td>-0.34 to 0.31</td>
<td>0.97</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 3</td>
<td>-0.12</td>
<td>-0.43 to 0.21</td>
<td>0.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Visit 3</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>0.034</td>
<td>-0.28 to 0.34</td>
<td>0.83</td>
</tr>
<tr>
<td>WURSS score</td>
<td>-0.16</td>
<td>-0.46 to 0.17</td>
<td>0.57</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 3</td>
<td>0.053</td>
<td>-0.26 to 0.36</td>
<td>0.83</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 4</td>
<td>0.13</td>
<td>-0.19 to 0.42</td>
<td>0.57</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 3</td>
<td>0.14</td>
<td>-0.2 to 0.45</td>
<td>0.57</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 4</td>
<td>0.25</td>
<td>-0.11 to 0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 3</td>
<td>0.23</td>
<td>-0.088 to 0.51</td>
<td>0.42</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 4</td>
<td>0.28</td>
<td>-0.035 to 0.54</td>
<td>0.42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Visit 4</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>-0.11</td>
<td>-0.43 to 0.25</td>
<td>0.90</td>
</tr>
<tr>
<td>WURSS score</td>
<td>0.0091</td>
<td>-0.35 to 0.37</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 4</td>
<td>0.062</td>
<td>-0.29 to 0.40</td>
<td>0.90</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 4</td>
<td>0.22</td>
<td>-0.19 to 0.56</td>
<td>0.90</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 4</td>
<td>0.12</td>
<td>-0.23 to 0.44</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Table 5.6 Correlating alpha diversity (Shannon diversity index) with clinical outcomes and pro-inflammatory cytokines. There was no significant correlation between any variables across all time points (Spearman’s correlation with FDR adjustment).
5.7.2 Beta diversity

Bray-Curtis dissimilarity and unweighted Unifrac distance were used to assess beta diversity. Even during longitudinal sampling in stable chronic disease, microbial communities differ across different time points, though these changes were not statistically significant (P > 0.05 for all visits. Two-way ANOVA with Tukey’s multiple comparison test). During colds, beta diversity did not change significantly, regardless of virology status (P > 0.05 across all visits. Two-way ANOVA with Tukey’s multiple comparison test). Furthermore, the degree of change within the microbiota did not significantly differ between colds and stable disease (P > 0.05. Two-way ANOVA with Tukey’s multiple comparison test) (Figure 5.18).

5.7.2.1 Correlations with clinical outcomes and pro-inflammatory cytokines

There was no significant correlation between beta diversity measures and clinical outcomes/pro-inflammatory cytokines at Visit 2 (day 1/2 post cold) and Visit 4 (day 14/15 post cold) (P > 0.05 for all variables. Spearman’s correlation with FDR adjustment) (Table 5.7 & Table 5.8).

At Visit 3 (day 4/5 post cold), unweighted Unifrac distance negatively correlated with change in PEF from baseline (P = 0.022. Spearman’s correlation with FDR adjustment) (Table 5.8 & Figure 5.19). In other words, the greater the change within the microbiota (from baseline), the greater the decline in PEF at Visit 3. However, this was not observed with Bray-Curtis dissimilarity (P = 0.71. Spearman’s correlation with FDR adjustment). Neither beta diversity indices significantly correlated with cold symptoms or pro-inflammatory cytokines (P > 0.05. Spearman’s correlation with FDR adjustment).
Figure 5.18  Beta diversity did not change significantly following natural cold, regardless of virology status. Furthermore, beta diversity did not significantly differ between natural cold and stable chronic disease ($P > 0.05$ for all variables. Two-way ANOVA with Tukey’s multiple comparison test).

Visit 2 = day 1/2 post cold, Visit 3 = day 4/5 post cold, Visit 4 = day 14/15 post cold
Table 5.7 Correlating Bray–Curtis dissimilarity with clinical outcomes and pro-inflammatory cytokines. There was no significant correlation between any variables across all time points (Spearman’s correlation with FDR adjustment).
<table>
<thead>
<tr>
<th>Visit 2</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>-0.31</td>
<td>-0.60 to 0.050</td>
<td>0.21</td>
</tr>
<tr>
<td>WURSS score</td>
<td>-0.089</td>
<td>-0.44 to 0.28</td>
<td>0.72</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 2</td>
<td>0.24</td>
<td>-0.13 to 0.55</td>
<td>0.37</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 3</td>
<td>0.37</td>
<td>0.0093 to 0.64</td>
<td>0.16</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 2</td>
<td>0.047</td>
<td>-0.34 to 0.42</td>
<td>0.81</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 3</td>
<td>0.2</td>
<td>-0.20 to 0.55</td>
<td>0.48</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 2</td>
<td>0.17</td>
<td>-0.20 to 0.50</td>
<td>0.48</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 3</td>
<td>0.36</td>
<td>0.0076 to 0.64</td>
<td>0.16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Visit 3</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>-0.5</td>
<td>-0.72 to -0.18</td>
<td>0.022 *</td>
</tr>
<tr>
<td>WURSS score</td>
<td>0.2</td>
<td>-0.17 to 0.52</td>
<td>0.45</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 3</td>
<td>0.29</td>
<td>-0.069 to 0.58</td>
<td>0.27</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 4</td>
<td>0.32</td>
<td>-0.027 to 0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 3</td>
<td>0.0059</td>
<td>-0.37 to 0.38</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 4</td>
<td>-0.0031</td>
<td>-0.41 to 0.40</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 3</td>
<td>0.24</td>
<td>-0.12 to 0.54</td>
<td>0.37</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 4</td>
<td>0.12</td>
<td>-0.24 to 0.45</td>
<td>0.68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Visit 4</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF change from baseline (%)</td>
<td>-0.21</td>
<td>-0.55 to 0.18</td>
<td>0.95</td>
</tr>
<tr>
<td>WURSS score</td>
<td>-0.013</td>
<td>-0.41 to 0.39</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Visit 4</td>
<td>-0.088</td>
<td>-0.45 to 0.30</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Visit 4</td>
<td>-0.022</td>
<td>-0.45 to 0.41</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Visit 4</td>
<td>-0.052</td>
<td>-0.42 to 0.33</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 5.8 Correlating unweighted Unifrac distance with clinical outcomes and pro-inflammatory cytokines. At Visit 3, unweighted Unifrac distance negatively correlated with PEF change from baseline (Spearman’s correlation with FDR adjustment).
5.7.3 Cluster stability in stable chronic disease and following natural cold

Cluster stability was assessed by comparing clusters at Visit 1 and Visit 2 (subject matched). During stable chronic disease, three out of eight subjects (37.5%) changed clusters on repeated sampling, though this was limited to Cluster C ("Streptococcus" cluster) and Cluster A ("Prevotella" and "Veillonella" clusters) (Table 5.9).

Following natural colds, 10 out of 34 subjects (29.4%) changed clusters (Table 5.10). Of note, all six subjects in Cluster B ("Neisseria" cluster) remained in the same cluster following cold (Figure 5.20). Furthermore, virology positivity did not impact on likelihood of cluster change (Figure 5.21) nor did it preferentially select specific cluster(s) (Figure 5.22).

Figure 5.19 At Visit 3, unweighted Unifrac distance negatively correlated with change in PEF from baseline (Spearman’s correlation with FDR adjustment).
<table>
<thead>
<tr>
<th>Visit 1 Cluster</th>
<th>Number of subjects</th>
<th>Cluster change during stable disease</th>
<th>Visit 2 Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>Yes = 0</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>Yes = 0</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>Yes = 3</td>
<td>A = 3</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>Yes = 0</td>
<td></td>
</tr>
</tbody>
</table>

No = 2

No = 2

No = 0

No = 0

Table 5.9 Cluster stability during stable chronic disease. Three out of eight subjects (37.5%) changed clusters following longitudinal sampling.

Figure 5.20 Cluster stability following natural cold. None of the subjects in Cluster B (“Neisseria” cluster. n = 6) changed cluster.
<table>
<thead>
<tr>
<th>Visit 1 Cluster</th>
<th>Number of subjects</th>
<th>Cluster change following cold</th>
<th>Visit 2 Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16</td>
<td>Yes = 4</td>
<td>C = 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No = 12</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>Yes = 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No = 6</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>Yes = 4</td>
<td>A = 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No = 5</td>
<td>B = 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D = 1</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>Yes = 1</td>
<td>C = 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No = 0</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>Yes = 1</td>
<td>C = 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No = 1</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.10 Cluster stability following natural cold. Ten out of 34 subjects (29.4%) changed clusters.

Figure 5.21 Virology positivity did not impact on likelihood of cluster change (Visit 1 to Visit 2) following natural cold.
5.7.4 Summary

Alpha diversity did not alter significantly following natural colds and did not significantly correlate with clinical outcomes or pro-inflammatory cytokines. Compared with stable chronic disease, beta-diversity did not significantly increase following natural colds. However, increased beta-diversity (as measured by unweighted Unifrac distance) following natural colds was associated with greater PEF decline. Cluster stability following natural cold was similar to that of longitudinal sampling during stable chronic disease, regardless of virology positivity. However, Cluster B (“Neisseria” cluster) appeared to be more stable compared with other clusters.

Figure 5.22 Positive virology did not preferentially select specific cluster following natural cold.
5.8 Specific phyla, genera & OTUs

Cluster-based analyses revealed that Cluster B ("Neisseria" cluster) was associated with greater PEF decline following natural colds. In contrast, Clusters A ("Prevotella" cluster) and B ("Veillonella" cluster) appeared to be associated with less PEF decline. A similar trend was observed in pro-inflammatory cytokines (in particular IL-1β and IL-8), though it was not statistically significant. In addition, previous published studies have observed an increased relative abundance of Proteobacteria in asthma, particularly *Haemophilus* spp (174, 199). The role of Proteobacteria (and *Haemophilus* spp.) in chronic disease or AE is unclear.

This section follows up the results of the cluster-based analyses, paying particular attention to the individual OTUs identified from indicator species analysis: *Neisseria_2974, Prevotella_6482* and *Veillonella_10839*. The role of Proteobacteria (and *Haemophilus* spp.) were also evaluated. The relative abundance of the above phyla, genera or OTUs were correlated with cold symptoms, PEF changes and pro-inflammatory cytokine levels. It was previously observed that bacterial burden in the middle of the cold (Visit 3, day 4/5 post cold) significantly correlated with IL-1β level at the end of cold (Visit 4, day 14/15 post cold) (Table 5.3), suggesting that changes in pro-inflammatory cytokine levels may lag behind changes in bacterial burden. To further evaluate this, relative abundance of specific phyla, genera or OTUs were correlated with pro-inflammatory cytokine levels measured at the subsequent visit (e.g. relative abundance of a specific OTU at Visit 2 was correlated with pro-inflammatory cytokine levels at Visit 3).
There was no significant linear relationship between the relative abundance of Neisseria_2974 at baseline (Visit 1) and the maximal change in PEF following natural colds ($R^2 = 0.016$, $P = 0.47$. Linear regression) (Figure 5.23).

However, during the acute cold (Visit 2, day 1/2 post cold), Neisseria_2974 relative abundance exhibited a statistically significant linear relationship with PEF change from baseline ($R^2 = 0.13$, $P = 0.046$. Linear regression with FDR adjustment). Furthermore, similar linear relationships were observed with IL-1β and IL-8 levels at Visit 3 (i.e. a 3-4 day time lag between Neisseria_2974 relative abundance and pro-inflammatory cytokine level) (IL-1β: $R^2 = 0.14$, $P = 0.046$. IL-8: $R^2 = 0.16$, $P = 0.046$. Linear regression with FDR adjustment). There was no significant linear relationship between Neisseria_2974 relative abundance and cold symptoms and IL-6 level (WURSS score: $R^2 = 0.023$, $P = 0.37$. IL-6: $R^2 = 0.066$, $P = 0.19$. Linear regression with FDR adjustment) (Figure 5.24).

**Figure 5.23** Linear regression between Neisseria_2974 relative abundance at baseline and maximal PEF change from baseline following natural cold. Relative abundance was normalised by log transformation.
During the middle of the cold (Visit 3, day 4/5 post cold), there was no significant linear relationship between *Neisseria_2974* relative abundance and clinical outcomes or pro-inflammatory cytokine levels (*P* > 0.05 for all variables. Linear regression with FDR adjustment) (Figure 5.25). At the end of cold (Visit 4, day 14/15 post cold), however, there was a significant linear relationship between *Neisseria_2974* relative abundance and total cold symptoms (over 28 days) (*R*² = 17, *P* = 0.030. Linear regression with FDR adjustment) (Figure 5.26). There was no significant relationship with PEF change on Visit 4 (*R*² = 0.00089, *P* = 0.86. Linear regression with FDR adjustment).
Figure 5.24 Linear regression between Neisseria_2974 relative abundance at Visit 2 (acute cold, day 1/2 post cold) and clinical outcomes/ pro-inflammatory cytokine levels.
Figure 5.25 Linear regression between Neisseria_2974 relative abundance at Visit 3 (middle of cold, day 4/5 post cold) and clinical outcomes/pro-inflammatory cytokine levels.
5.8.2 *Prevotella_6482*

Relative abundance of *Prevotella_6482* at baseline (Visit 1) was not associated with greater PEF decline following natural cold ($R^2 = 0.0036$, $P = 0.73$. Linear regression) (Figure 5.27).

*Figure 5.26* Linear regression between Neisseria_2974 relative abundance at Visit 4 (end of cold, day 14/15 post cold) and clinical outcomes. Total WURSS score was measured as a summation of WURSS score over a 28-day period.

*Figure 5.27* Linear regression between *Prevotella_6482* relative abundance at baseline (Visit 1) and maximal PEF change following natural cold.
Visit 2

\[ R^2 = 0.084 \quad P = 0.23 \]

**PEF change**

\[ R^2 = 0.0057 \quad P = 0.65 \]

**WURSS score**

**IL-1\( \beta \)**

\[ R^2 = 0.0036 \quad P = 0.23 \]

**IL-6**

\[ R^2 = 0.052 \quad P = 0.32 \]

**IL-8**

\[ R^2 = 0.025 \quad P = 0.42 \]

*Figure 5.28* Linear regression between *Prevotella_6482* relative abundance at Visit 2 (acute cold, day 1/2 post cold) and clinical outcomes/ pro-inflammatory cytokine levels.
Figure 5.29 Linear regression between Prevotella_6482 relative abundance at Visit 3 (middle of cold, day 4/5 post cold) and clinical outcomes/ pro-inflammatory cytokine levels.
Following natural cold (Visits 2 – 4), there was no statistically significant linear relationship between *Prevotella_6482* relative abundance and clinical outcomes or pro-inflammatory cytokine levels (P > 0.05 for all variables at all visits. Linear regression with FDR adjustment) (Figure 5.28, Figure 5.29 & Figure 5.30).

![Graph](image)

*Figure 5.30* Linear regression between *Prevotella_6482* relative abundance at Visit 4 (end of cold, day 14/15 post cold) and clinical outcomes. Total WURSS score was measured as a summation of WURSS score over a 28-day period.

5.8.2.1 *Prevotella* at Genus level

Whilst *Prevotella_6482* relative abundance did not significantly correlate with PEF change following natural colds acutely (Visit 2, day 1/2 post cold), there was a statistically significant linear relationship between *Prevotella* relative abundance at Visit 2 and PEF change ($R^2 = 0.14$, P = 0.019. Linear regression) (Figure 5.31).
5.8.3 *Veillonella_10839*

At baseline (Visit 1), *Veillonella_10839* relative abundance did not significantly correlate with maximal PEF change following natural cold ($R^2 = 0.028$, $P = 0.33$. Linear regression) (Figure 5.32).

**Figure 5.31** Linear regression between *Prevotella* relative abundance at Visit 2 and PEF change from baseline.

**Figure 5.32** Linear regression between *Veillonella_10839* relative abundance at baseline (Visit 1) and maximal PEF change following natural cold.
Visit 2

PEF change

$R^2 = 0.061$
$P = 0.22$

WURSS score

$R^2 = 0.028$
$P = 0.35$

**Figure 5.33** Linear regression between *Veillonella_10839* relative abundance at Visit 2 (acute cold, day 1/2 post cold) and clinical outcomes/ pro-inflammatory cytokine levels.
**Visit 3**

**PEF change**  
R² = 0.0010  
P = 0.84

**WURSS score**  
R² = 0.026  
P = 0.40

**IL-1β**  
R² = 0.049  
P = 0.40

**IL-6**  
R² = 0.034  
P = 0.40

**IL-8**  
R² = 0.13  
P = 0.094

*Figure 5.34* Linear regression between *Veillonella_10839* relative abundance at Visit 3 (middle of cold, day 4/5 post cold) and clinical outcomes/pro-inflammatory cytokine levels.
At acute cold (Visit 2, day 1/2 post cold), *Veillonella_10839* relative abundance negatively correlated with IL-1β and IL-8 levels (IL-1β: $R^2 = 0.16$, $P = 0.031$. IL-8: $R^2 = 0.20$, $P = 0.031$. Linear regression with FDR adjustment). *Veillonella_10839* relative abundance, however, did not correlate with PEF change or cold symptoms ($P > 0.05$ for both variables. Linear regression with FDR adjustment) (Figure 5.33). There were no significant correlations observed at Visits 3 (day 4/5 post cold) and 4 (day 14/15 post cold) (Figure 5.34 & Figure 5.35).

### 5.8.4 *Haemophilus* at Genus level

At baseline (Visit 1), there was no significant linear relationship between *Haemophilus* relative abundance and maximal PEF change following cold ($R^2 = 0.075$, $P = 0.11$. Linear regression) (Figure 5.36).
During the acute cold (Visit 2, day ½ post cold), *Haemophilus* relative abundance did not correlate with clinical outcomes or pro-inflammatory cytokines (P > 0.05 for all variables). Linear regression with FDR adjustment) (Figure 5.37).
Figure 5.37 Linear regression between Haemophilus relative abundance at Visit 2 (acute cold, day 1/2 post cold) and clinical outcomes/ pro-inflammatory cytokine levels.
5.8.5 Proteobacteria

At baseline (Visit 1), subjects with mild disease (as denoted by BTS treatment step 1) had significantly greater relative abundance of Proteobacteria than subjects with more severe disease (BTS treatment step 4) ($P = 0.016$. Kruskal-Wallis test with Dunn’s multiple comparison) (Figure 5.38).

![Figure 5.38 Comparing Proteobacteria relative abundance between different disease severity at baseline. Severity was classified in accordance to British Thoracic Society (BTS) treatment step. Kruskal-Wallis test with Dunn’s multiple comparison. * $P < 0.05.$](image)

A similar trend to Neisseria_2974 was observed. At baseline (Visit 1), there was no significant correlation between Proteobacteria relative abundance and maximal PEF change following cold ($R^2 = 0.052$, $P = 0.18$. Linear regression) (Figure 5.39). During the acute cold (Visit 2, day 1/2 post cold), Proteobacteria relative abundance negatively correlated with PEF change ($R^2$
= 0.14, \( P = 0.043 \). Linear regression with FDR adjustment) and positively correlated with IL-1\( \beta \) level (\( R^2 = 0.15, \ P = 0.043 \). Linear regression with FDR adjustment) (Figure 5.40). There was no significant correlation at Visit 3 (middle of cold, day 4/5 post cold) (Figure 5.41). At Visit 4 (end of cold, day 14/15 post cold), Proteobacteria relative abundance did not significantly correlate with total cold symptoms after FDR adjustment (\( R^2 = 0.13, \ P = 0.063 \). Linear regression with FDR adjustment) (Figure 5.42).

Figure 5.39 Linear regression between Proteobacteria relative abundance at baseline and maximal PEF change following natural cold.
Figure 5.40 Linear regression between Proteobacteria relative abundance at Visit 2 (acute cold, day 1/2 post cold) and clinical outcomes/ pro-inflammatory cytokine levels
**Visit 3**

Observations:
- **PEF change**
  - $R^2 = 0.0077$
  - $P = 0.83$
  ![Graph showing linear regression between PEF change and log(Proteobacteria relative abundance)]

- **WURSS score**
  - $R^2 = 0.0013$
  - $P = 0.83$
  ![Graph showing linear regression between WURSS score and log(Proteobacteria relative abundance)]

- **IL-1β**
  - $R^2 = 0.0027$
  - $P = 0.83$
  ![Graph showing linear regression between log(IL-1β at Visit 4) and log(Proteobacteria relative abundance)]

- **IL-6**
  - $R^2 = 0.0015$
  - $P = 0.83$
  ![Graph showing linear regression between log(IL-6 at Visit 4) and log(Proteobacteria relative abundance)]

- **IL-8**
  - $R^2 = 0.024$
  - $P = 0.83$
  ![Graph showing linear regression between log(IL-8 at Visit 4) and log(Proteobacteria relative abundance)]

**Figure 5.41** Linear regression between Proteobacteria relative abundance at Visit 3 (middle of cold, day 4/5 post cold) and clinical outcomes/pro-inflammatory cytokine levels
5.8.5.1 Relationship with other phyla

In the current study, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria were identified as the five most prevalent phyla within the airway microbiota. At baseline (Visit 1), Proteobacteria relative abundance negatively correlated with Actinobacteria and Firmicutes (Actinobacteria: $R = -0.48$, $P = 6.33 \times 10^{-5}$, Firmicutes: $R = -0.76$, $P = 2.79 \times 10^{-13}$, Spearman’s correlation) (Table 5.11). A similar pattern was observed during the acute cold (Visit 2, day 1/2 post cold), in addition to Bacteroidetes (Actinobacteria: $R = -0.49$, $P = 0.0020$, Bacteroidetes: $R = -0.37$, $P = 0.020$, Firmicutes: $R = -0.67$, $P = 2.86 \times 10^{-6}$, Spearman’s correlation) (Table 5.12).
5.8.6 Summary

At baseline (Visit 1), Proteobacteria relative abundance was significantly greater in subjects on BTS treatment step 1 than those on step 4, though greater relative abundance of Proteobacteria at baseline did not correlate with worse PEF decline following a cold. During the acute cold (Visit 2, day 1/2 post cold), however, *Neisseria_2974* relative abundance was associated with greater PEF decline and subsequent IL-1β and IL-8 levels (Visit 3). In contrast,
Veillonella_10839 relative abundance was associated with lower subsequent IL-1β and IL-8 levels (Visit 3). Prevotella relative abundance (at Genus level) was associated with less PEF decline. Of note, Haemophilus relative abundance at Visit 2 negatively correlated with IP-10 level. Finally, greater Neisseria_2974 relative abundance at Visit 4 was associated with greater total cold symptoms over a 28-day period.

5.9 Discussion

5.9.1 Bacterial burden

5.9.1.1 Inter-subject variability in bacterial burden

In the current study, a cohort of mild and moderate asthmatic subjects with variable disease control (as denoted by ACQ) were examined. There was great variability in bacterial burden (BB) between subjects (four log difference). This observation appears to be consistent with other published studies of asthma, COPD and idiopathic pulmonary fibrosis microbiota (IPF)(194, 198, 199). The reason for this variability remains unclear. Sampling technique, sample quality, qPCR reagents and protocol may all contribute, though the current study has attempted to minimise such confounding issues by employing the same sampling protocol, qPCR reagents and protocol throughout the study.

5.9.1.2 No significant correlation with clinical outcomes at baseline

At baseline (Visit 1), BB did not significantly differ with disease severity (as classified by BTS treatment steps), nor did it correlate with lung function or pro-inflammatory cytokine levels. Furthermore, increased BB at baseline did not predict more severe cold symptoms or greater PEF decline following the colds. The results would suggest that BB does not impact on disease
severity and lung function in chronic disease. A recent study evaluating a cohort of severe asthmatic subjects (on maintenance daily ICS dose of > 1000 mcg, mean ACQ score 2.7) reported that greater BB at baseline was associated with less ACQ change (i.e. better symptom control) over a 2 – 3 week period(197). This would suggest that increased BB is coupled with favourable clinical outcome. The current study did not assess ACQ score longitudinally and thus was unable to make comparable assessment. In contrast, a study in IPF observed that increased BB was associated with greater lung function decline and mortality over a 30-month period(194), though the mechanism was unclear. Such long-term observation in asthma would be of interest though it was beyond the scope of the current study.

5.9.1.3 No significant shift in bacterial burden following natural cold

Overall, BB did not significantly alter during stable chronic disease or following a natural cold. However, BB did drastically increase in a small proportion of subjects (by nearly 1000-fold) following natural colds, whilst changes in subjects with stable chronic disease were less dramatic (by eight fold). Moreover, increase in BB was not observed more frequently in subjects with detectable virology.

The results would suggest that a natural cold, regardless of whether it is virus-induced or not, does not necessarily predispose to secondary bacterial infection in all subjects (following the classical view that bacterial infection is associated with an increase in BB). This observation is consistent with an experimental RV infection challenge study involving COPD subjects, where a small proportion of subjects had significant increase in BB following RV infection(198). It is plausible that natural colds may predispose secondary bacterial infections in a small
A proportion of susceptible subjects. An alternative explanation is that a proportion of natural colds may be bacteria-induced.

The exact impact of respiratory viral infection (specifically RV) on BB remains uncertain, as subjects with negative virology may yet have contracted viral infection that was not detected by the virology PCR for the study. This issue will be evaluated in the ensuing chapter.

During natural colds, BB did not correlate with cold symptoms or lung function changes, though it did positively correlate with IL-1β level on day 4/5. This is consistent with a finding in the mentioned COPD study, where BB positively correlated with sputum inflammatory cell counts, neutrophil counts and neutrophil elastase levels (198). The current study’s finding implies that BB does contribute to increased airway inflammation, though not to the extent of impacting on clinical outcomes. This highlights the complexity of airway inflammation and its relationship with clinical symptoms and lung function.

In summary, BB did not significantly alter following natural colds, though a small proportion of subjects had a dramatic increase in BB. The influence of respiratory viral infection on BB is uncertain. BB appeared to contribute towards increased airway inflammation but it did not impact on clinical outcomes. More recently the concept of ‘dysbiosis’ has been postulated, where an imbalance between the different groups of bacteria within the microbiota leads to increased airway inflammation (186, 195). It is therefore conceivable that in the context of a natural cold in asthma, a dysbiosis/imbalance of microbiota rather than an absolute increase in BB contributes to airway inflammation and ultimately clinical outcomes. The following section will discuss the changes within the microbiota following natural colds.
5.9.2 Hierarchical clusters and indicator species

Previous publications have reported that asthmatic airway microbiota consists of greater relative abundance of bacteria from the Proteobacteria phylum (e.g. *Haemophilus* spp. and *Neisseria* spp.), whilst the airway microbiota in health is more diverse and predominantly consists of ‘commensal’ bacteria from the phyla Bacteroidetes (e.g. *Prevotella* spp.) and Firmicutes (e.g. *Veillonella* spp. and *Streptococcus* spp.)(174, 176).

5.9.2.1 The airway microbiota in asthma is more variable than previously proposed

Hierarchical clustering provides an unbiased method of collating samples that have similar microbial community composition, regardless of subject identity, clinical background or time point. It became apparent that there were several distinct clusters. Indicator species analysis was performed to select the ‘representative’ species in each cluster and showed that some clusters were represented by Proteobacteria (Clusters B “*Neisseria*” and D “*Haemophilus*”), whilst others were represented by Bacteroidetes (Cluster A1 “*Prevotella*”) and Firmicutes (e.g. Clusters A2 and E “*Veillonella*”). This finding suggests that the asthmatic airway microbiota is not a fixed pattern as previously proposed but highly individualised.

The current study’s contrasting findings with previous works may be related to a number of issues. Previous studies did not apply the same analytical approach as subjects were segregated in accordance to clinical classifications (such as disease severity). Furthermore, microbiota compositions were presented as ‘average’ relative abundance, thus individual subject’s microbiota may not be evident. Sample population would likely have affected hierarchical clustering outcome. The current study recruited a range of asthmatic subjects
who had varying disease severity, symptom control and treatment, whereas previous studies had chosen different inclusion criteria. Given the heterogeneity of asthma, it is conceivable that the microbiota profiles vary between different studies. In addition, all studies on the asthmatic airway microbiota (including the current study) have been relatively small with respect to sample size, thus results may not reflect that of the population. Despite these potential limitations, however, the current study provides a novel understanding of the asthmatic airway microbiota.

5.9.3 Clinical correlations with clusters and OTUs

Having identified distinct clusters within the cohort, the next step was to examine if the clusters correlated with clinical characteristics or biomarkers of airway inflammation at baseline and following colds.

5.9.3.1 No significant correlation at baseline

There was no significant correlation between the clusters and baseline clinical characteristics (including age, disease severity, ACQ, lung function and IgE) or pro-inflammatory cytokine levels. Based on current findings, the cause and significance of the different clusters during stable chronic disease remains unclear. The airway microbiota may be affected by an extensive array of environmental or even genetic factors that are yet to be explored. This is consistent with a previous study that also did not show significant correlation between microbiota composition and clinical characteristics(199). However, the same study reported that several taxa (at Family level) from the Proteobacteria phylum (including Comamonadaceae, Sphingomonadaceae and Oxalobacteraceae) significantly correlated with bronchial hyper-responsiveness(199).
5.9.3.2 Subjects with more severe disease had lower Proteobacteria relative abundance

Given the focus on an outgrowth of Proteobacteria in asthma in previous studies, the current study also assessed the relative abundance of Proteobacteria in its cohort. As seen in cluster-based analyses, there was significant variability in Proteobacteria relative abundance amongst the subjects. Furthermore, subjects with the most severe disease (BTS treatment step 4) had significantly less Proteobacteria than subjects with mild disease (BTS treatment step 1). There were only seven subjects on BTS treatment step 4, thus a larger study is needed to confirm the finding. However, a recent study on severe asthmatic subjects also observed a lower Proteobacteria relative abundance in severe asthmatics (BTS treatment step 5) compared with non-severe asthmatic subjects (196). It is not possible to distinguish whether the lower relative abundance of Proteobacteria in severe disease is secondary to actual disease severity or effects of inhaled corticosteroids (ICS). Further work is needed to disentangle this question. Of note, *Haemophilus parainfluenzae*, a Proteobacteria, has been shown to inhibit corticosteroid responses *in vitro* (200), though in reality interactions between the microbiota and ICS-induced immune responses are likely to be far more complex.

5.9.3.3 *Neisseria_2974* is associated with greater PEF decline and IL-1β and IL-8 levels following colds

Whilst there was no significant correlation with clinical features at baseline, subjects in the *Neisseria* cluster at baseline had significantly great PEF decline following cold. These findings led to the postulation that a *Neisseria*-dominant microbiota predisposes to increased airway inflammation leading to greater airflow obstruction during a cold. As *Neisseria_2974* was the
most prevalent *Neisseria* OTU thus was employed to evaluate the influence of *Neisseria* at an OUT/species level.

Unlike the *Neisseria* cluster, *Neisseria_2974* relative abundance at baseline did not correlate with PEF decline following cold. Furthermore, there was no significant correlation even at phyla level (Proteobacteria). These findings suggest that if the baseline microbiota does indeed influence PEF changes during a natural cold, it is likely to do so as a community, rather than a single species or phylum.

*Neisseria_2974*, however, significantly correlated with PEF decline during the acute phase of colds (Visit 2, day 1/2 post cold). In addition, *Neisseria_2974* relative abundance at Visit 2 significantly correlated with IL-1β and IL-8 levels at Visit 3 (middle of cold, day 4/5 post cold), which took place 3–4 days after Visit 2. This would suggest that pro-inflammatory cytokine responses react to *Neisseria_2974* exposure, rather than *Neisseria_2974* reflecting a pro-inflammatory environment. This time-lag response in pro-inflammatory cytokine is consistent with the findings of an *ex vivo* model of lipopolysaccharide (LPS) induced acute inflammation using human lung tissue, where IL-8 level was still increasing 48-hours after exposure to LPS(272).

The mechanism of pro-inflammatory cytokine stimulation by *Neisseria_2974* is unclear, though potential mechanisms are discussed in a later section. Moreover, even if *Neisseria_2974* does induce IL-1β and IL-8 production, it remains uncertain how this will result in increased airflow obstruction and ultimately PEF decline, though nasal IL-8 levels have been shown to correlate with the degree of nasal resistance(273).
Whilst *Neisseria_2974* relative abundance did not correlate with cold symptoms during the acute phase of colds (Visit 2, day 1/2 post cold), its relative abundance at the end of cold (Visit 4, day 14/15 post cold) did correlate with total cold symptoms. Although WURSS is well validated and predicts subsequent asthma control(231), it is a subjective measure and primarily reports cold symptoms. Its correlation with PEF changes is likely to be sub-optimal. However, it is possible that greater *Neisseria_2974* relative abundance may lead to prolonged inflammation and ultimately greater cold symptoms.

Despite the paucity of mechanistic evidence, the current study shows for the first time that a *Neisseria* species exhibits a linear relationship with PEF decline and pro-inflammatory cytokine levels in asthmatic subjects during a naturally occurring cold.

**5.9.3.4 Veillonella_10839 and Prevotella correlate with lower pro-inflammatory cytokine levels and PEF decline respectively**

Both *Veillonella* and *Prevotella* are considered to be ‘normal commensals’ of the respiratory tract and have been shown to be in high abundance within the airways of healthy subjects(174, 196). During the acute phase of cold, *Veillonella_10839* was associated with lower IL-1β and IL-8 levels. Although *Prevotella_6482* did not exhibit significant correlations with clinical outcomes or pro-inflammatory cytokines, *Prevotella* at genus level was significantly associated with less PEF decline.

Similar to *Neisseria_2974*, it is not possible to decipher whether *Veillonella* and *Prevotella* actively promote reduced airway inflammation and improve PEF or simply reflect a less inflamed airway environment. However, the temporal lag in correlation between *Veillonella_10839* and pro-inflammatory cytokines would support the former case. In an in
vitro setting, *Prevotella* has been shown to induce significantly weaker TLR4 dependent inflammatory responses compared with Proteobacteria such as *Haemophilus influenzae* (202). However, a recent murine model of chronic pulmonary inflammation induced by multiple LPS challenges observed a reduction of *Prevotella* (204). Finally, the observation that *Prevotella* correlated with PEF change at genus level also raise the possibility of a group of related species functioning in a similar fashion. It is therefore plausible that the microbiota has significant interactions and cross-talks with the local environment and immune system.

5.9.4 Microbial community changes following natural cold

5.9.4.1 The airway microbiota is dynamic during stable chronic disease

To date, there has been no longitudinal study assessing the stability of the airway microbiota in asthma. In a recent study, the human salivary microbiota was shown to be relatively stable over the course of a 24-hour period and after one week, with no significant diurnal variation. However, similar to the findings of current study, there was significant variations between subjects, all of whom were healthy (274).

In the current study, there was variability in both alpha and beta diversity measures even in stable chronic disease, though these variations were not statistically significant. These changes in microbial community may be attributed to numerous factors. These may be external, such as change in medication (e.g. increase in ICD dose), season/temperature and living environment. Other factors include sampling techniques, sequencing reagents and analysis protocols. As mentioned previously, the current study has attempted to minimise such confounders by employing the same sampling technique (and personnel), sequencing reagents and analysis protocol for the duration of the study. Previous studies on the airway
microbiota have also reported variations in community profile at different locations of the respiratory tract (177, 178). Whilst it was not possible to locate the biogeographical origin of induced sputum samples, each sample was collected using the same technique with multiple cycles of sample collection, therefore the sample should provide a general representation of the airway microbiota.

The current study demonstrates the airway microbiota in asthma is dynamic even during stable chronic disease. Moreover, longitudinal sampling of stable subjects has highlighted the importance of the clinical context during data interpretation.

5.9.4.2 Alpha and beta diversity did not alter significantly with natural cold

Previous studies on airway microbiota have demonstrated that alpha diversity (as measured by Shannon diversity index) correlates with clinical features. In COPD, reduced alpha diversity (i.e. community dominated by one or few species) was associated with emphysematous destruction, bronchiolar and alveolar tissue remodelling (188), whilst increased alpha diversity was associated with increased bronchial hyper-responsiveness in asthma (199).

In the current study, alpha diversity did not significantly alter during natural colds compared with stable chronic disease. Furthermore, it did not correlate with clinical features or pro-inflammatory cytokine levels. Based on these findings, community diversity does not appear to impact on clinical outcomes during natural cold.

Similarly, beta diversity did not significantly alter during natural colds. However, increased beta diversity (as measured by unweighted Unifrac distance) significantly correlated with PEF decline. In other words, the greater the change in microbial community from baseline, the worse the PEF decline. In cystic fibrosis (CF), acute exacerbations are coupled with significant
change in microbial community profiles (i.e. significant increase in beta diversity) compared with period of stable disease (184). Whilst the pathophysiology of asthma and CF are vastly different, one might expect a more dramatic change in community profile during acute disease exacerbations. The modest change in microbial community profile seen in the current study may be attributed to the fact that most subjects had relatively mild symptoms that did not necessitate emergency medical care. The correlation between unweighted Unifrac distance and PEF change would support that in more severe exacerbations, beta diversity may be associated with clinical outcomes. Further work is needed to examine this postulation, though such severe exacerbations may increase the risk of other confounding issues such as use of oral corticosteroid and antibiotic therapy.

5.9.4.3 Respiratory viral infection did not significantly alter microbial community

There was no significant difference in beta diversity between subjects with positive virology and those with negative virology. Furthermore, as observed in cluster-based analyses, respiratory viral infection did not favour any specific microbial community profile. However, these findings do not conclusively substantiate that respiratory viral infections do not impact on the airway microbiota. The observation that the microbiota correlates with clinical features during acute colds but not stable disease may indicate the role of respiratory viral infection in disrupting the airway epithelium and facilitating the potentially harmful interactions between microbiota and immune system. It was of interest to note that subjects in the Neisseria cluster did not change cluster following cold, though they had greater PEF decline. This observation may suggest the postulation that respiratory viral infection
facilitates increased microbiota-related airway inflammation in subjects who already possess an ‘hostile’ microbiota at baseline.

The current study is sub-optimal in assessing the impact of respiratory viral infection on the microbiota as not every subject had detectable virology. The range of viruses detected and variable and uncertain timing of contraction of viral infection increased the difficulty of assessing the temporal relationship between viral infection and microbiota. Chapter 6, however, places focus on the impact of RV infection on the microbiota.

5.10 Conclusions

In contrast to previous publications, the current study observed that the asthmatic airway microbiota is highly individualised, though distinct clusters were seen in the sample cohort. The cause for such clustering is unclear. At baseline, clusters did not correlate with clinical characteristics. However, subjects with *Neisseria*-dominated microbial communities at baseline experienced significantly greater PEF decline following natural colds.

Alpha and beta diversities did not alter significantly following natural colds compared with stable chronic disease. Respiratory viral infection did not significantly impact on alpha or beta diversity. However, greater *Neisseria_2974* relative abundance during acute colds was associated with greater PEF decline, IL-1β and IL-8 levels. Conversely, greater relative abundance of *Prevotella* and *Veillonella_10839* correlated with lower PEF decline and pro-inflammatory cytokine levels respectively. It was not possible to confirm whether the microbiota actively promotes airway inflammation or is simply reflective of airway inflammation. However, the temporal lag between microbial abundance and pro-inflammatory cytokine levels suggests the former may be plausible.
Overall, the findings support the postulation of a dysbiotic/imbalanced microbiota leading to greater airway inflammation and ultimately airflow obstruction. The impact of respiratory viral infection could not be accurately assessed, though it may stimulate immune responses that further initiate microbial-related inflammation.

Chapter 6 will focus on the impact of RV infection on the airway microbiota and investigate if similar observations are made in a different cohort of asthmatic and healthy subjects.

5.11 Summary of key findings

- The asthmatic airway microbiota is more individualised than previously proposed; though distinct clusters were observed.
- Subjects with a Neisseria-dominated community experienced greater PEF decline following natural colds.
- Alpha and beta diversity did not alter significantly following natural cold compared with stable chronic disease. However, increased beta diversity was associated with greater PEF decline.
- During acute cold, Neisseria_2974 relative abundance was significantly associated with greater PEF decline, IL-1β and IL-8 levels. Prevotella and Veillonella_10839 were associated with lower PEF decline and IL-1β and IL-8 levels respectively.
The airway microbiota in experimental rhinovirus infection

6.1 Introduction

As discussed previously, respiratory viruses, in particular rhinoviruses (RVs), may be associated with 60 – 80% of acute asthma exacerbations (AEs)(260). The role of bacteria in virus-induced AEs is currently unexplored. However, previous studies using quantitative culture and specific pathogen polymerase chain reaction (PCR) have demonstrated the propensity for respiratory viral infections to enhance the risk of secondary bacterial infections, resulting in increased disease severity and morbidity. In influenza, bacterial super-infection, particularly Staphylococcus aureus or Streptococcus pneumoniae (S.pneumoniae), results in prolonged hospital stay and increased mortality rate(275). In the case of chronic airway diseases, secondary bacterial infections following RV infection have also been shown to result greater morbidity. In COPD, experimental RV infection led to secondary bacterial infections in 60% of subjects (as denoted by quantitative culture). Furthermore, subjects with secondary bacterial infection experienced significantly greater lung function decline compared with those with no detectable bacteria by quantitative culture(224). In children with asthma, co-detection of RV and potentially pathogen bacteria including S.pneumoniae and Moraxella catarrhalis resulted in greater cold and asthma symptoms(211).

Viral infection may precipitate secondary bacterial infection via multiple mechanisms(214, 225). In the case of RV, secondary bacterial colonisation and subsequent infection may arise due to up-regulation of the adhesion protein intercellular adhesion molecule 1 (ICAM-1), which is used by Haemophilus influenzae (H. influenzae) in adhering to the epithelial
surface(276). Furthermore, RV infection has been shown to increase the degradation of antimicrobial peptides(224).

It is therefore plausible that RV infection may perturb the microbiota, leading to greater inflammation and ultimately greater airflow obstruction and disease severity. This has been recently observed in COPD, where experimental RV infection was followed an expansion of *H.influenzae*(198). Significantly, several studies have observed that there is a significantly greater relative abundance of Proteobacteria (which consist of potentially pathogenic bacteria including *H. influenzae*) in the airways of asthmatic subjects compared with healthy subjects(174, 197, 200). Asthmatic subjects may therefore have an increased risk of developing secondary bacterial infections following RV infection. Conversely, microbiota composition may modulate immune response to viral infections. In a murine model, enrichment of the airway microbiota with two strains of *Lactobacillus rhamnosus* by nasal inoculation differentially modulated immune responses following poly(I:C) challenge(226).

Currently the impact of RV infection on the airway microbiota in asthma is unknown. In the previous chapter, a greater relative abundance of *Neisseria_2974* during the acute phase of a naturally occurring cold was associated with greater peak flow decline, whilst greater relative abundance of *Prevotella* and *Veillonella_10839* were associated with less peak flow decline.

Given the heterogeneity in time of cold presentation and virus detection, it was not possible to examine if RV infection was associated with these observations. The current chapter aims to address the question using a human experimental RV infection challenge model, whilst also examining potential differences between the asthmatic and healthy airway microbiota.
6.2 Aims

- To investigate the changes within the airway microbiota following experimental RV-16 infection in a cohort of moderate asthmatic and healthy subjects.
- To examine if changes within the airway microbiota following experimental RV-16 infection correlate with changes in clinical parameters (cold and asthma symptoms and lung function) and biomarkers of airway inflammation.
- To examine the differences in the airway microbiota between asthmatic and healthy subjects in period of stability and following experimental RV-16 infection.

6.3 Hypotheses

- The airway microbiota in asthma consists of a more potentially pathogenic composition than that in health at baseline.
- Following RV-16 infection, the asthmatic airway microbiota is perturbed to a more pathogenic composition (compared with the airway microbiota in health), resulting in greater airway inflammation and airflow obstruction and clinical symptoms.

6.4 Bacterial burden

6.4.1 Changes following experimental RV-16 infection

The median bacterial burden (BB) in all subjects (healthy and asthmatic) at baseline was 5.7 x 10^6 copies of 16S rRNA gene/ml of bronchoalveolar lavage (BAL) (IQR 2.4 x 10^6 - 1.1 x 10^7). Following RV-16 infection, there was no significant change in BB in either asthma or healthy subjects (P = 0.35. Two-way ANOVA with Tukey’s multiple comparison test). Furthermore, BB did not differ significantly between asthmatic and healthy subjects at any time point (P =
0.072. Two-way ANOVA with Bonferroni’s multiple comparison test) (Figure 6.1). BB in the vast majority of subjects (asthma and healthy) did not change significantly, though BB did increase by 40-fold in one healthy subject following RV-16 infection (Figure 6.2).

**Figure 6.1** Changes in bronchoalveolar lavage (BAL) bacterial burden in asthmatic and healthy subjects following RV-16 infection. There was no significant change in bacterial burden following RV-16 infection in either asthma or healthy subjects. There was no significant difference in bacterial burden between asthma and healthy subjects at any time point (P > 0.05. Two-way ANOVA test)

**Figure 6.2** Individual subject trends in changes in BAL bacterial burden following RV-16 infection.
6.4.2 Correlation with clinical parameters, alpha diversity, Th-2 mediated and pro-inflammatory cytokines

At baseline, BB did not significantly correlate with lung function, alpha diversity, Th-2 or pro-inflammatory cytokines (P > 0.05 for all variables. Spearman’s correlation with FDR adjustment). Furthermore, greater BB at baseline was not associated with greater clinical symptoms or PEF decline following RV-16 infection (P > 0.05 for all variables. Spearman’s correlation with FDR adjustment) (Table 6.1).

<table>
<thead>
<tr>
<th>Baseline</th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1% predicted</td>
<td>-0.51</td>
<td>-0.78 to -0.088</td>
<td>0.25</td>
</tr>
<tr>
<td>Maximal change in PEF from baseline</td>
<td>-0.31</td>
<td>-0.68 to 0.18</td>
<td>0.69</td>
</tr>
<tr>
<td>Serum IgE (IU/ml)</td>
<td>0.051</td>
<td>-0.40 to 0.48</td>
<td>0.96</td>
</tr>
<tr>
<td>Peak viral load (copies/ml)</td>
<td>0.073</td>
<td>-0.38 to 0.50</td>
<td>0.96</td>
</tr>
<tr>
<td>Total URT score</td>
<td>-0.11</td>
<td>-0.55 to 0.38</td>
<td>0.96</td>
</tr>
<tr>
<td>Total LRT score</td>
<td>0.033</td>
<td>-0.43 to 0.48</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>0.08</td>
<td>-0.38 to 0.50</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>-0.048</td>
<td>-0.48 to 0.40</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>0.45</td>
<td>0.0041 to 0.74</td>
<td>0.30</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>0.0065</td>
<td>-0.44 to 0.45</td>
<td>0.98</td>
</tr>
<tr>
<td>IL-5 (pg/ml)</td>
<td>-0.14</td>
<td>-0.55 to 0.32</td>
<td>0.96</td>
</tr>
<tr>
<td>IL-13 (pg/ml)</td>
<td>-0.18</td>
<td>-0.58 to 0.29</td>
<td>0.96</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>0.24</td>
<td>-0.26 to 0.63</td>
<td>0.93</td>
</tr>
<tr>
<td>Richness</td>
<td>0.38</td>
<td>-0.11 to 0.72</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Table 6.1 Spearman’s correlation between BAL bacterial burden at baseline and clinical parameters, alpha diversity measures, Th-2 and pro-inflammatory cytokines. P values are adjusted using false discovery rate (FDR) (alpha = 0.05). Note that Th-2 mediated and pro-inflammatory cytokine levels were measured using nasosorption and bronchosorption respectively. LRT = lower respiratory tract, URT = upper respiratory tract.
When asthmatic subjects were assessed separately, greater BB was significantly associated with lower FEV$_1$ predicted (P = 0.0016. Spearman’s correlation with FDR adjustment) (Figure 6.3). However, BB did not correlate with ACQ score or airway hyper-responsiveness (AHR) (P = 0.52, P = 0.95 respectively. Spearman’s correlation with FDR adjustment).

Following RV-16 infection, BB did not significantly correlate with PEF change, symptoms, alpha diversity measures or cytokine levels at day 3 or day 8 (P > 0.05 for all variables. Spearman’s correlation with FDR adjustment).
<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>95% CI</th>
<th>P value (FDR adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF% change from baseline</td>
<td>-0.021</td>
<td>-0.47 to 0.44</td>
<td>0.93</td>
</tr>
<tr>
<td>URT score</td>
<td>0.076</td>
<td>-0.40 to 0.52</td>
<td>0.88</td>
</tr>
<tr>
<td>LRT score</td>
<td>-0.11</td>
<td>-0.55 to 0.37</td>
<td>9.88</td>
</tr>
<tr>
<td>Peak infection IL-4 (pg/ml)</td>
<td>-0.56</td>
<td>-0.80 to -0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>Peak IL-5 (pg/ml)</td>
<td>-0.4</td>
<td>-0.72 to 0.050</td>
<td>0.25</td>
</tr>
<tr>
<td>Peak IL-13 (pg/ml)</td>
<td>-0.42</td>
<td>-0.73 to 0.032</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-1B Day 3 (pg/ml)</td>
<td>0.47</td>
<td>0.018 to 0.76</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-1B Day 8 (pg/ml)</td>
<td>0.11</td>
<td>-0.36 to 0.54</td>
<td>0.88</td>
</tr>
<tr>
<td>IL-6 Day 3 (pg/ml)</td>
<td>-0.35</td>
<td>-0.69 to 0.13</td>
<td>0.31</td>
</tr>
<tr>
<td>IL-6 Day 8 (pg/ml)</td>
<td>-0.27</td>
<td>-0.64 to 0.21</td>
<td>0.51</td>
</tr>
<tr>
<td>IL-8 Day 3 (pg/ml)</td>
<td>-0.029</td>
<td>-0.48 to 0.43</td>
<td>0.93</td>
</tr>
<tr>
<td>IL-8 Day 8 (pg/ml)</td>
<td>-0.084</td>
<td>-0.52 to 0.38</td>
<td>0.88</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>0.16</td>
<td>-0.32 to 0.57</td>
<td>0.88</td>
</tr>
<tr>
<td>Richness</td>
<td>0.38</td>
<td>-0.086 to 0.71</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Table 6.2 Spearman’s correlation between BAL bacterial burden at Day 3 post RV-16 infection and clinical parameters, alpha diversity measures, Th-2 mediated and pro-inflammatory cytokines. P values are adjusted using false discovery rate (FDR) (alpha = 0.05). Note that Th-2 and pro-inflammatory cytokine levels were measured using nasosorption and bronchosorption respectively. LRT = lower respiratory tract, URT = upper respiratory tract.
BAL bacterial burden (BB) did not significantly differ between asthmatic and healthy subjects. Furthermore, BB did not significantly alter following RV-16 infection in either group, though one (healthy) subject exhibited a 40-fold increase in BB. At baseline, BB in asthmatic subjects negatively correlated with FEV₁% predicted. However, following RV-16 infection, BB did not correlate with clinical symptoms, lung function, alpha diversity measures, Th-2 mediated or pro-inflammatory cytokine levels.
6.5 Hierarchical clustering and indicator species analysis

To examine if the airway microbiota from BAL resembled that of sputum, Hierarchical clustering analysis using Bray-Curtis similarity method was performed on all 72 BAL samples, regardless of subject, disease state and time points. Six distinct clusters were identified (Clusters A – F). Indicator species analysis was performed to examine representative OTUs in each cluster (Table 6.4). No representative OTU was identified for Cluster F (i.e. microbiota in Cluster F was not dominated by any particular OTU).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>A</th>
<th>B</th>
<th>Stat</th>
<th>P value</th>
<th>Representative OTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.6350</td>
<td>1.0000</td>
<td>0.797</td>
<td>0.001</td>
<td>Neisseria_2974</td>
</tr>
<tr>
<td>B</td>
<td>0.3592</td>
<td>1.0000</td>
<td>0.599</td>
<td>0.002</td>
<td>Streptococcus_12165</td>
</tr>
<tr>
<td>C</td>
<td>0.7322</td>
<td>1.0000</td>
<td>0.856</td>
<td>0.001</td>
<td>Selenomonas_9853</td>
</tr>
<tr>
<td>D</td>
<td>0.3846</td>
<td>1.0000</td>
<td>0.620</td>
<td>0.001</td>
<td>Veillonella_10839</td>
</tr>
<tr>
<td></td>
<td>0.3579</td>
<td>1.0000</td>
<td>0.598</td>
<td>0.004</td>
<td>Prevotella_6482</td>
</tr>
<tr>
<td>E</td>
<td>0.5546</td>
<td>1.0000</td>
<td>0.745</td>
<td>0.005</td>
<td>Fusobacterium_8489</td>
</tr>
</tbody>
</table>

Table 6.4 Indicator species analysis for clusters. Column A denotes the specificity or positive predictive value of the representative OTU as an indicator of the cluster (i.e. the probability that the representative OTU belongs to the designated cluster given the fact that the OTU is found). Column B denotes the fidelity or sensitivity of the representative OTU (i.e. the probability of finding the representative OTU in the designated cluster). Stat denotes the overall strength of association between the representative OTU and cluster, where 0 = no association and 1 = perfect association.

The indicator species analysis demonstrated that similar representative OTUs were observed in BAL samples as those identified in sputum samples (e.g. Neisseria_2974, Veillonella_10839 and Prevotella_6482).
Figure 6.4 Hierarchical cluster analysis of all BAL samples using Bray-Curtis similarity measure. Samples are arranged and clustered along the x-axis. Bar plot illustrates the 25 most-abundant genera. Y-axis denotes abundance of genera within each sample rarefied at 1000 reads. The white bar at the top represents the remaining genera. Above the bar plot is a dendrogram illustrating the different clusters (A – F). Below the bar plot, a colour bar denotes the origin of the sample (red = asthmatic subject, blue = healthy subject).
6.6 Differences in microbiota composition between asthma and health

Based on hierarchical clustering, there was no apparent distinction between asthmatic and healthy samples (Figure 6.4). Cluster A (Neisseria cluster) only consisted of asthmatic samples, though there were only four samples in total. In contrast, samples from healthy subjects

**Figure 6.5** Sample distribution amongst clusters across time points (baseline, day 3 and 8 post RV-16 infection)
generally congregated in Clusters D (Veillonella and Prevotella cluster) and F respectively, regardless of time points (Figure 6.5).

To formally investigate if the microbiota of asthmatic subjects were significantly different to that of healthy subjects, multivariate ANOVA based on dissimilarities (adonis) was used. Dissimilarity was assessed using Bray-Curtis distance. The microbiota communities between asthma and healthy subjects were not significantly different at any time points (Table 6.5).

<table>
<thead>
<tr>
<th></th>
<th>R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.038</td>
<td>0.52</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.046</td>
<td>0.54</td>
</tr>
<tr>
<td>Day 8</td>
<td>0.074</td>
<td>0.13</td>
</tr>
</tbody>
</table>

**Table 6.5** Multivariate ANOVA based on dissimilarities (adonis) assessing differences in microbiota communities between asthmatic and healthy subjects. R² denotes the degree of sample clustering that is explained by the variable of interest (asthma vs healthy). P value denotes the statistical significance of test based on permutations performed. There was no significant difference between asthma and healthy subjects at any time points.

**Figure 6.6** visually illustrates the absence of distinct sample clustering of asthmatic and healthy samples at the three time points.
Differences in specific phyla and genera

Previous studies have observed differences in microbiota composition between asthma and health. A greater abundance of Proteobacteria (e.g. *Haemophilus*) was seen in asthma whilst a greater abundance of Bacteroidetes (e.g. *Prevotella*) was observed in health\(^{(174)}\).

Figure 6.6 Nonmetric Multidimensional Scaling analysis (NMDS) based on Bray-Curtis distances showing differences in microbiota composition between asthmatic and healthy subjects. Each dot or triangle represents the overall microbiota composition of each subject. Similar microbiota compositions cluster together, whilst distinct microbiota compositions are distant from each other. There was no apparent clustering of samples from asthmatic or healthy subjects at any time point.
In the current study, there was no significant difference in relative abundance of either Proteobacteria or Bacteroidetes between asthma and health at baseline (P > 0.05. Mann-Whitney test).

**Figure 6.7 Relative abundances of specific phyla and genera at baseline. No significant difference was seen between asthma and healthy group. (Mann-Whitney test)**
However, on day 3 after RV-16 infection, healthy subjects had significantly greater relative abundance of Bacteroidetes and *Prevotella* compared with asthmatic subjects ($P = 0.048$ and $0.016$ respectively. Mann-Whitney test) though no significant difference was seen in Proteobacteria ($P = 0.15$. Mann-Whitney test).

**Figure 6.8** Relative abundances of specific phyla and genera at day 3 post RV-16 infection. Healthy subjects had significantly greater relative abundance of Bacteroidetes and *Prevotella* compared with asthmatic subjects. (Mann-Whitney test)
A similar pattern was apparent on day 8, though only *Prevotella* relative abundance was significantly different (P = 0.043. Mann-Whitney test).

![Diagram showing relative abundances of specific phyla and genera at day 8 post RV-16 infection. Healthy subjects exhibited significantly greater relative abundance of *Prevotella* compared with asthmatic subjects. (Mann-Whitney test)]
6.6.2 Summary

In the current study, the airway microbiota did not significantly differ between asthmatic and healthy subjects at baseline or following RV-16 infection. Asthmatic subjects did not have a greater relative abundance of Proteobacteria (including *Neisseria* and *Haemophilus*) compared with healthy subjects at baseline or following RV-16 infection. However, healthy subjects exhibited a significantly greater relative abundance of Bacteroidetes (including *Prevotella*) following RV-infection.

6.7 Microbial community changes following RV-16 infection

6.7.1 Alpha diversity

Alpha diversity was assessed by Shannon diversity and Inverse Simpson indices. The larger the index value, the greater the microbial community diversity. Following RV-16 infection, there was no significant change in alpha diversity in either asthmatic or healthy subjects (P > 0.05 for both Shannon and Inverse Simpson indices. Two-way ANOVA with Tukey’s multiple comparison test). Furthermore, there was no significant difference in alpha diversity between asthmatic and healthy subjects at baseline or following RV-16 infection (P > 0.05 for all time points for both Shannon and Inverse Simpson indices. Two-way ANOVA with Tukey’s multiple comparison test) (Figure 6.10).
Figure 6.10 Changes in alpha diversity measures following RV-16 infection. Dot denotes mean and error bars denote standard deviation. There was no significant difference following RV-16 infection in either asthma or healthy group. In addition, there was no significant difference between asthmatic and healthy subjects. (Two-way ANOVA with Tukey’s multiple comparison test).
6.7.1.1 Correlations with clinical outcomes and pro-inflammatory cytokines

At baseline, alpha diversity (as measured by Shannon diversity index) did not significantly correlate with age, FEV$_1$% predicted, IgE or pro-inflammatory cytokine levels. Furthermore, it was not correlated with clinical symptoms or maximal PEF change following RV-16 infection (P > 0.05 for all variables. Spearman’s correlation with FDR adjustment). Within the asthma group, increased alpha diversity was not associated with increased AHR or ACQ score (P > 0.05 for both variables. Spearman’s correlation with FDR adjustment). Following RV-16 infection, alpha diversity did not significantly correlate with clinical symptoms, PEF change or pro-inflammatory cytokines (P > 0.05 for all variables. Spearman’s correlation with FDR adjustment) (Table 6.6).
Table 6.6 Correlating alpha diversity (Shannon diversity index) with clinical outcomes and pro-inflammatory cytokines. There was no significant correlation between any variables across all time points. (Spearman’s correlation with FDR adjustment). LRT = lower respiratory tract, URT = upper respiratory tract, PC<sub>20</sub> = histamine challenge test.

<table>
<thead>
<tr>
<th>Baseline</th>
<th>r</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.17</td>
<td>-0.54 to 0.26</td>
<td>0.77</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;% predicted</td>
<td>-0.017</td>
<td>-0.42 to 0.39</td>
<td>0.94</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>-0.2</td>
<td>-0.57 to 0.25</td>
<td>0.77</td>
</tr>
<tr>
<td>Total URT score</td>
<td>-0.29</td>
<td>-0.68 to 0.24</td>
<td>0.77</td>
</tr>
<tr>
<td>Total LRT score</td>
<td>-0.39</td>
<td>-0.73 to 0.10</td>
<td>0.77</td>
</tr>
<tr>
<td>Maximal change in PEF from baseline (%)</td>
<td>0.13</td>
<td>-0.39 to 0.58</td>
<td>0.93</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>-0.31</td>
<td>-0.68 to 0.19</td>
<td>0.77</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.023</td>
<td>-0.45 to 0.48</td>
<td>0.94</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>0.067</td>
<td>-0.41 to 0.52</td>
<td>0.94</td>
</tr>
<tr>
<td>ACQ (Asthma only)</td>
<td>0.48</td>
<td>-0.085 to 0.81</td>
<td>0.16</td>
</tr>
<tr>
<td>PC&lt;sub&gt;20&lt;/sub&gt; (Asthma only)</td>
<td>-0.33</td>
<td>-0.74 to 0.26</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 3</th>
<th>r</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URT score Day 3</td>
<td>-0.16</td>
<td>-0.59 to 0.34</td>
<td>0.78</td>
</tr>
<tr>
<td>URT score Day 4</td>
<td>-0.21</td>
<td>-0.63 to 0.30</td>
<td>0.78</td>
</tr>
<tr>
<td>LRT score Day 3</td>
<td>-0.1</td>
<td>-0.55 to 0.40</td>
<td>0.78</td>
</tr>
<tr>
<td>LRT score Day 4</td>
<td>-0.032</td>
<td>-0.50 to 0.45</td>
<td>0.90</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 3</td>
<td>-0.13</td>
<td>-0.57 to 0.37</td>
<td>0.78</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 4</td>
<td>0.15</td>
<td>-0.36 to 0.58</td>
<td>0.78</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 8</td>
<td>-0.21</td>
<td>-0.62 to 0.28</td>
<td>0.78</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 8</td>
<td>-0.2</td>
<td>-0.61 to 0.29</td>
<td>0.78</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 8</td>
<td>-0.27</td>
<td>-0.65 to 0.22</td>
<td>0.78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 8</th>
<th>r</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URT score Day 8</td>
<td>0.12</td>
<td>-0.42 to 0.59</td>
<td>0.85</td>
</tr>
<tr>
<td>URT score Day 9</td>
<td>-0.044</td>
<td>-0.54 to 0.47</td>
<td>0.87</td>
</tr>
<tr>
<td>LRT score Day 8</td>
<td>0.047</td>
<td>-0.47 to 0.54</td>
<td>0.87</td>
</tr>
<tr>
<td>LRT score Day 9</td>
<td>-0.19</td>
<td>-0.63 to 0.36</td>
<td>0.85</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 8</td>
<td>0.15</td>
<td>-0.39 to 0.61</td>
<td>0.85</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 9</td>
<td>0.32</td>
<td>-0.22 to 0.71</td>
<td>0.85</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 8</td>
<td>0.16</td>
<td>-0.38 to 0.62</td>
<td>0.85</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 8</td>
<td>-0.12</td>
<td>-0.59 to 0.41</td>
<td>0.85</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 8</td>
<td>0.19</td>
<td>-0.35 to 0.64</td>
<td>0.85</td>
</tr>
</tbody>
</table>
6.7.2 Beta diversity

Beta diversity was examined by assessing Bray-Curtis dissimilarity and unweighted Unifrac distance. For both indices, the larger the value, the more distinct the microbiota is from the microbiota at baseline. Beta diversity did not differ significantly between asthma and healthy subjects at day 3 or 8 following RV-16 infection (P > 0.05 for both measures at both time points. Two-way ANOVA). In addition, beta diversity did not differ significantly between day 3 and 8 (P > 0.05 for both measures. Two-way ANOVA) (Figure 6.11). However, there was also apparent intra-subject variation in beta-diversity (Figure 6.12).

**Figure 6.11** Comparing beta diversity between asthmatic and healthy subjects following RV-16 infection using Bray-Curtis dissimilarity (A) and unweighted Unifrac distance (B). There was no significant difference between asthma and healthy group. Furthermore, there was no significant difference in beta diversity between day 3 and day 8. (P > 0.05 for all. Two-way ANOVA)
To assess if RV-16 infection impacted on changes with the microbiota, RV-16 viral load (on day 3) was correlated with beta diversity at day 3 and 8 respectively. RV-16 viral load did not significantly correlate with beta diversity at day 3 (\(P > 0.05\) for both Bray-Curtis dissimilarity and unweighted Unifrac distance. Linear regression with FDR adjustment). On day 8, however, RV-16 viral load strongly correlated with Bray-Curtis dissimilarity (\(R^2 = 0.61, P = 0.011\). Linear

---

**Figure 6.12** Individual subject trends in beta diversity following RV-16 infection.

---

**6.7.3 Impact of RV-16 infection on beta diversity**

...
regression with FDR adjustment) but not unweighted Unifrac distance \( (R^2 = 0.0028, P = 0.97) \). Linear regression with FDR adjustment (Figure 6.13).

**Figure 6.13** Linear regression between RV-16 viral load at day 3 and beta diversity measures at day 3 (A & B) and 8 (C & D) respectively. Viral load was normalised by log transformation. Linear regression with FDR adjustment.
6.7.4 Correlations with clinical outcomes and pro-inflammatory cytokines

Neither Bray-Curtis dissimilarity nor unweighted Unifrac distance significantly correlated with clinical symptoms, PEF change or pro-inflammatory cytokines on day 3 or 8 following RV-16 infection (Table 6.7 and Table 6.8 respectively).

<table>
<thead>
<tr>
<th>Day 3</th>
<th>r</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URT score Day 3</td>
<td>-0.13</td>
<td>-0.65 to 0.47</td>
<td>0.71</td>
</tr>
<tr>
<td>URT score Day 4</td>
<td>0.11</td>
<td>-0.48 to 0.64</td>
<td>0.71</td>
</tr>
<tr>
<td>LRT score Day 3</td>
<td>0.31</td>
<td>-0.31 to 0.74</td>
<td>0.59</td>
</tr>
<tr>
<td>LRT score Day 4</td>
<td>0.25</td>
<td>-0.36 to 0.71</td>
<td>0.63</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 3</td>
<td>-0.51</td>
<td>-0.83 to 0.039</td>
<td>0.25</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 4</td>
<td>-0.52</td>
<td>-0.83 to 0.029</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 3</td>
<td>0.48</td>
<td>-0.089 to 0.81</td>
<td>0.27</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 8</td>
<td>0.23</td>
<td>-0.35 to 0.69</td>
<td>0.63</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 3</td>
<td>0.55</td>
<td>0.006 to 0.84</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 8</td>
<td>0.12</td>
<td>-0.45 to 0.62</td>
<td>0.71</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 3</td>
<td>0.40</td>
<td>-0.19 to 0.77</td>
<td>0.38</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 8</td>
<td>0.20</td>
<td>-0.38 to 0.67</td>
<td>0.65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 8</th>
<th>r</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URT score Day 8</td>
<td>0.21</td>
<td>-0.40 to 0.69</td>
<td>0.99</td>
</tr>
<tr>
<td>URT score Day 9</td>
<td>0.14</td>
<td>-0.46 to 0.65</td>
<td>0.99</td>
</tr>
<tr>
<td>LRT score Day 8</td>
<td>0.1</td>
<td>-0.49 to 0.63</td>
<td>0.99</td>
</tr>
<tr>
<td>LRT score Day 9</td>
<td>0.039</td>
<td>-0.54 to 0.59</td>
<td>1.00</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 8</td>
<td>0.51</td>
<td>-0.081 to 0.83</td>
<td>0.99</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 9</td>
<td>0.16</td>
<td>-0.44 to 0.66</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 8</td>
<td>0.17</td>
<td>-0.44 to 0.67</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 8</td>
<td>-0.082</td>
<td>-0.62 to 0.50</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 8</td>
<td>0.16</td>
<td>-0.44 to 0.66</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 6.7 Correlating Bray-Curtis dissimilarity with clinical outcomes and pro-inflammatory cytokines. There was no significant correlation between any variables across all time points (Spearman’s correlation with FDR adjustment).
Table 6.8 Correlating unweighted Unifrac distance with clinical outcomes and pro-inflammatory cytokines. There was no significant correlation between any variables across all time points (Spearman’s correlation with FDR adjustment).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 3</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>95% CI</td>
</tr>
<tr>
<td>URT score Day 3</td>
<td>0.033</td>
<td>-0.52 to 0.57</td>
</tr>
<tr>
<td>URT score Day 4</td>
<td>0.099</td>
<td>-0.47 to 0.61</td>
</tr>
<tr>
<td>LRT score Day 3</td>
<td>-0.11</td>
<td>-0.61 to 0.46</td>
</tr>
<tr>
<td>LRT score Day 4</td>
<td>0.21</td>
<td>-0.37 to 0.68</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 3</td>
<td>-0.34</td>
<td>-0.73 to 0.23</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 4</td>
<td>-0.36</td>
<td>-0.74 to 0.2</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 3</td>
<td>0.12</td>
<td>-0.43 to 0.61</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 8</td>
<td>0.24</td>
<td>-0.33 to 0.68</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 3</td>
<td>0.20</td>
<td>-0.36 to 0.66</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 8</td>
<td>0.064</td>
<td>-0.48 to 0.57</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 3</td>
<td>0.086</td>
<td>-0.46 to 0.58</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 8</td>
<td>0.057</td>
<td>-0.48 to 0.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 3</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>95% CI</td>
</tr>
<tr>
<td>URT score Day 8</td>
<td>-0.27</td>
<td>-0.72 to 0.35</td>
</tr>
<tr>
<td>URT score Day 9</td>
<td>-0.31</td>
<td>-0.74 to 0.31</td>
</tr>
<tr>
<td>LRT score Day 8</td>
<td>-0.12</td>
<td>-0.64 to 0.47</td>
</tr>
<tr>
<td>LRT score Day 9</td>
<td>-0.042</td>
<td>-0.59 to 0.53</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 8</td>
<td>-0.34</td>
<td>-0.76 to 0.28</td>
</tr>
<tr>
<td>PEF change from baseline (%) Day 9</td>
<td>-0.3</td>
<td>-0.74 to 0.32</td>
</tr>
<tr>
<td>IL-1β (pg/ml) Day 8</td>
<td>-0.15</td>
<td>-0.66 to 0.45</td>
</tr>
<tr>
<td>IL-6 (pg/ml) Day 8</td>
<td>0.46</td>
<td>-0.14 to 0.81</td>
</tr>
<tr>
<td>IL-8 (pg/ml) Day 8</td>
<td>-0.088</td>
<td>-0.62 to 0.50</td>
</tr>
</tbody>
</table>
6.7.5 Summary

Alpha diversity did not significantly alter following RV-16 infection. There was no difference in alpha diversity between asthmatic and healthy subjects at any time point. Furthermore, alpha diversity did not significantly correlate with clinical outcomes or pro-inflammatory cytokines following RV-16 infection.

Similarly, beta diversity did not significantly differ between asthmatic and healthy subjects following RV-16 infection. However, viral load at day 3 significantly correlated with Bray-Curtis dissimilarity at day 8. Beta diversity did not significantly correlate with clinical outcomes or pro-inflammatory cytokines.
6.8 Specific phyla, genera and OTUs

In view of the low sample size, cluster-based analysis was not performed as it was unlikely to yield any meaningful results. In naturally-occurring colds, specific OTUs/ genera such as *Neisseria_2974* and *Prevotella* appeared to correlate with lung function decline. Furthermore, the relative abundance of such OTUs/ genera appeared to correlate with pro-inflammatory cytokine levels at a later time point (e.g. relative abundance of *Neisseria_2974* at day 1/2 post cold correlated with IL-1β level at day 4/5 post cold). In the following section, these specific phyla, genera and OTUs of interest are examined in a similar fashion.

6.8.1 *Neisseria_2974*

*Neisseria_2974* relative abundance at baseline did not significantly correlate with maximal PEF decline, total upper (URT) and lower respiratory tract (LRT) symptoms following RV-16 infection. In other words, greater relative abundance of *Neisseria_2974* at baseline was not associated with greater PEF decline or clinical symptoms. In asthmatic subjects at baseline, *Neisseria_2974* relative abundance did not correlate with airway hyper-responsiveness. (P > 0.05 for all variables. Linear regression with FDR adjustment) (Figure 6.14). At day 3 post RV-16 infection, *Neisseria_2974* relative abundance did not significantly correlate with PEF, clinical symptoms or pro-inflammatory cytokines (at day 8) (P > 0.05 for all variables. Linear regression with FDR adjustment) (Figure 6.15 & Figure 6.16).
Figure 6.14 Linear regression between Neisseria_2974 relative abundance at baseline and clinical outcomes/ baseline airway hyper-responsiveness (PC_{20}). Linear regression with FDR adjustment.
Figure 6.15 Linear regression between Neisseria_2974 relative abundance at day 3 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
However, at day 8, greater *Neisseria_2974* relative abundance was associated with greater PEF decline (Day 8: $R^2 = 0.38$, $P = 0.050$, Day 9: $R^2 = 0.41$, $P = 0.050$. Linear regression with FDR adjustment) though it did not significantly correlate with either clinical symptoms or pro-inflammatory cytokines ($P > 0.05$ for all variables. Linear regression with FDR adjustment) (Figure 6.17).
Figure 6.17 Linear regression between Neisseria_2974 relative abundance at day 8 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
6.8.2 *Prevotella_6482*

*Prevotella_6482* relative abundance at baseline did not significantly correlate with airway hyper-responsiveness or clinical outcomes following RV-16 infection ($P > 0.05$ for all variables). Linear regression with FDR adjustment) (Figure 6.19). Furthermore, *Prevotella_6482* relative abundance did not significantly correlate with PEF changes, clinical symptoms or pro-

![Graphs showing linear regression for IL-1β, IL-6, and IL-8](image_url)
inflammatory cytokine levels at day 3 (Figure 6.20 & Figure 6.21) and 8 following RV-16 infection (P > 0.05 for all variables. Linear regression with FDR adjustment) (Figure 6.22 & Figure 6.23).

![Baseline](image)

**Figure 6.19** Linear regression between *Prevotella_6482* relative abundance at baseline and clinical outcomes/ airway hyper-responsiveness (*PC*<sub>20</sub>). Linear regression with FDR adjustment.
Figure 6.20 Linear regression between Prevotella_6482 relative abundance at day 3 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
Day 3

IL-1β

$R^2 = 0.0013$

$P = 0.99$

IL-6

$R^2 = 0.16$

$P = 0.38$

Figure 6.21 Linear regression between Prevotella_6482 relative abundance at day 3 post RV-16 infection and pro-inflammatory cytokines. Linear regression with FDR adjustment.
Figure 6.22: Linear regression between Prevotella_6482 relative abundance at day 8 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
6.8.2.1 Prevotella at Genus level

Similar to *Prevotella_6482*, Prevotella relative abundance at baseline did not significantly correlate with airway hyper-responsiveness or clinical outcomes following RV-16 infection (P > 0.05 for all variables. Linear regression with FDR adjustment) (Figure 6.24). However, on day 3 post RV-16 infection, *Prevotella* relative abundance negatively correlated with upper
respiratory tract symptoms ($R^2 = 0.56$, $P = 0.0036$. Linear regression with FDR adjustment) (Figure 6.25), though it did not significantly correlate with lower respiratory tract symptoms, PEF change or pro-inflammatory cytokine levels ($P > 0.05$ for all variables. Linear regression with FDR adjustment) (Figure 6.26). There was no significant correlation on day 8 post RV-16 infection ($P > 0.05$ for all variables. Linear regression with FDR adjustment).

**Figure 6.24** Linear regression between Prevotella relative abundance at baseline and clinical outcomes/ airway hyper-responsiveness (PC$_{20}$). Linear regression with FDR adjustment.
Figure 6.25 Linear regression between Prevotella relative abundance at day 3 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
Day 3

\[ R^2 = 0.030 \quad \text{IL-1} \beta \quad R^2 = 0.21 \quad \text{IL-6} \]

\[ P = 0.62 \quad P = 0.15 \]

**Figure 6.26** Linear regression between Prevotella relative abundance at day 3 post RV-16 infection and pro-inflammatory cytokines. Linear regression with FDR adjustment.
Figure 6.27 Linear regression between Prevotella relative abundance at day 8 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
There was no significant correlation between *Veillonella_10839* relative abundance and clinical outcomes/ pro-inflammatory cytokine levels at baseline (*Figure 6.29*), day 3 (*Figure 6.30* & *Figure 6.31*) and day 8 post RV-16 infection (P > 0.05 for all variables. Linear regression with FDR adjustment) (*Figure 6.32* & *Figure 6.33*).
Figure 6.29 Linear regression between Veillonella_10839 relative abundance at baseline and clinical outcomes/airway hyper-responsiveness (PC_{20}). Linear regression with FDR adjustment.
**Figure 6.30** Linear regression between Veillonella_10839 relative abundance at day 3 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment
Figure 6.31 Linear regression between Veillonella_10839 relative abundance at day 3 post RV-16 infection and pro-inflammatory cytokines. Linear regression with FDR adjustment.
Figure 6.32 Linear regression between Veillonella_10839 relative abundance at day 8 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
6.8.4 *Haemophilus* at Genus level

At baseline, *Haemophilus* relative abundance did not correlate with airway hyper-responsiveness \((R^2 = 0.36, P = 0.062\). Linear regression with FDR adjustment) (Figure 6.34).

*Haemophilus* relative abundance did not correlate with clinical symptoms, PEF change or pro-
inflammatory cytokines at baseline, day 3 or 8 following RV-16 infection (P > 0.05 for all variables. Linear regression with FDR adjustment).

**Figure 6.34** Linear regression between Haemophilus relative abundance at baseline and clinical outcomes/airway hyper-responsiveness (PC_{20}). Linear regression with FDR adjustment.
**Figure 6.35** Linear regression between Haemophilus relative abundance at day 3 post-RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
Figure 6.36 Linear regression between Haemophilus relative abundance at day 3 post RV-16 infection and pro-inflammatory cytokines. Linear regression with FDR adjustment.
Figure 6.37 Linear regression between Haemophilus relative abundance at day 8 post RV-16 infection and clinical outcomes. LRT = lower respiratory tract symptom score, URT = upper respiratory tract symptom score. Linear regression with FDR adjustment.
6.8.5 Summary

At day 3 following RV-16 infection, *Prevotella* relative abundance significantly correlated with reduced upper respiratory tract symptom scores; though it did not correlate with lower respiratory tract symptoms, PEF change or pro-inflammatory cytokines. At day 8 post-RV-16 infection, greater *Neisseria_2974* relative abundance was associated with greater PEF decline.

**Figure 6.38** Linear regression between Haemophilus relative abundance at day 8 post RV-16 infection and pro-inflammatory cytokines. Linear regression with FDR adjustment.
6.9 Discussion

The following section will discuss the findings of the current study. Furthermore, where appropriate the findings will be compared with those observed in the naturally-occurring cold study (Chapter 5).

6.9.1 Bacterial burden (BB)

6.9.1.1 No significant difference in BB between asthmatic and healthy subjects

In the current study consisting of 12 healthy and 11 asthmatic subjects, there was no significant difference in BB between asthmatic and healthy subjects. A previous study had observed greater BB in asthmatic subjects compared with healthy subjects(199). Although this study was larger (47 subjects in total) than the current study, the number of asthmatic subjects heavily outweighed healthy subjects. It is therefore possible that the significant difference in BB between asthma and health may be abolished if a greater number of healthy subjects were examined. In a recent study, subjects with idiopathic pulmonary fibrosis has significantly greater BB than healthy subjects, whilst those with COPD had similar BB to healthy subjects(194). Based on the current study’s findings, if microbiota in asthma were significantly different to that in health, the distinction would likely lie at a community relative abundance level rather than overall BB.

6.9.1.2 No significant change in BB following RV-16 infection

Overall, there was no significant change in BB following RV-16 infection in both asthma and healthy groups on days 3 and 8. At an individual level, the BB of asthmatic subjects was generally stationary (within one-fold difference). A similar pattern was observed amongst
healthy subjects, though one subject did experience a 40-fold increase in BB on day 3 post RV-16 infection. The findings would suggest that RV-16 infection does not generally lead to secondary bacterial infection as hypothesised (following the classical view that bacterial infection would be associated with an increase in total BB). This is consistent with the observation made in the naturally-occurring cold study, where BB increased in only a small proportion of asthmatic subjects regardless of virology positivity. Furthermore, in a recent study examining the BB in sputum samples of COPD and healthy subjects, only a small proportion of subjects (two out of 31) exhibited a significant increase in BB following RV-16 infection, though both had COPD(198). Finally, as previously discussed, sampling location and technique may also contribute to changes in BB, though substantial effort was made to standardise sampling.

6.9.1.3 No correlation with clinical parameters or pro-inflammatory cytokines

A recent study had observed that increased BB correlated with worsening asthma control (as represented by increasing ACQ score) in patients with severe asthma(197). The current study did not examine change in ACQ score. However, greater BB at baseline did significantly correlate with reduced FEV₁ in asthmatic subjects; a finding that has not been previously observed. This would be consistent with previous speculation that increased BB is associated with worse clinical characteristics. However, similar findings were not demonstrated in natural colds. One explanation may be related to the small cohort size in the current study and such association was observed by chance. However, it may be possible that BB only plays in role in airflow obstruction within lower airways, thus BB in induced sputum may not reflect such effect. Further investigation is required to verify this finding.
Following RV-16 infection, BB did not correlate with clinical symptoms, PEF changes or pro-inflammatory cytokine levels. A study using the same experimental RV-16 infection challenge model in COPD and healthy subjects demonstrated significant correlations between BB and sputum inflammatory cell count, neutrophil count and neutrophil elastase levels(198). This is likely reflecting the differing pathophysiology of COPD compared with asthma in addition to different sampling technique (induced sputum versus BAL).

In conclusion, the findings of this study suggest that overall BB does not impact on clinical outcomes following RV-16 infection in either health or asthma. These findings are consistent with those seen in naturally-occurring colds. It is therefore possible that the composition and relative abundances of specific species may impact on clinical outcomes rather than overall BB. This will be discussed in detail in forthcoming sections.

6.9.2 Hierarchical clustering and indicator species analysis in BAL reveal similar results to those observed in naturally-occurring colds

Whilst the sample size in the current study is appreciably smaller than that of the natural cold study, the same indicator species were identified following hierarchical clustering, in particular *Neisseria_2974, Prevotella_6482* and *Veillonella_10839*. This observation suggests that the dominant (with regards to prevalence and uniqueness) species are the same in the microbiota community of BAL and sputum samples. Furthermore, it enables direct comparisons of the two studies at OTU level.
6.9.3 No significant difference in microbiota composition between asthmatic and healthy subjects at baseline or following RV-16 infection

Previous studies have demonstrated significant differences in the airway microbiota between asthmatic and healthy individuals (174, 176). At the phylum level, there was greater relative abundance of Proteobacteria in asthma, whilst Bacteroidetes and Actinobacteria were more prevalent in healthy individuals.

However, the current study did not identify a statistically significant difference between asthmatic and healthy subjects when the microbiota communities were assessed as a whole using multivariate ANOVA based on dissimilarities (adonis). This was further illustrated by both hierarchical clustering and NMDS plots which showed no substantial distinction between the microbiota communities of asthmatic and healthy subjects. This remained the case following RV-16 infection with no significant distinction between the two groups. When the cohort was evaluated at phylum and genus level, however, healthy subjects did exhibit a significantly greater relative abundance of Bacteroidetes, particularly *Prevotella*, following RV-16 infection. There was no significant difference in Proteobacteria; though a small proportion of asthmatic subjects exhibited a relative abundance of Proteobacteria, in particular *Neisseria*, at baseline and following RV-16 infection.

The current study’s findings appear to contradict previously published findings. This may well be secondary to the small sample size, however, it is increasingly apparent that the airway microbiota is variable in different individuals. This is best illustrated by hierarchical clustering showing distinct microbiota communities in asthmatic subjects. This observation is again evident in naturally-occurring colds (Chapter 5). Previous studies had presented the
microbiota communities as an average of all recruited subjects thus obscuring such distinct clustering.

The differences in communities may be related to a number of factors including the underlying pathophysiological state (e.g. Th2 high or low in asthmatic subjects), pharmacological interventions (e.g. high dose ICS) and external environment (allergen exposure). The impact of such factors on the airway microbiota was not evaluated in the current study and further investigations are needed.

**6.9.4 Microbiota community changes following RV-16 infection**

**6.9.4.1 No significant difference in alpha diversity between asthmatic and healthy subjects**

Previous studies have demonstrated that the asthmatic subjects had greater alpha diversity than healthy subjects(176, 199). The current study did not observe such differentiation. This may be related to the small sample size of the current study. However, the study by Marri and co-workers had comparable sample size (10 asthmatic and 10 healthy subjects)(176), whilst Huang and co-workers had a larger number of asthmatic subjects (65 in total) but only 10 healthy subjects(199). Sampling techniques were also different (induced sputum, bronchial brushing and BAL). Finally, disease severity differed between studies, though a recent study showed no significant difference in alpha diversity between mild, moderate and severe asthma(196). Further examination is needed to distinguish if a difference in alpha diversity exists between healthy and asthmatic subjects.
6.9.4.2 Alpha diversity did not significantly change or correlate with clinical outcomes and pro-inflammatory cytokine levels

In the current study, there was no significant alteration in alpha diversity following RV-16 infection in either asthmatic or healthy subjects. The finding is similar to that observed in naturally-occurring colds (Chapter 5). To date, there has been a limited number of studies investigating the impact of RV infection on the airway microbiota and the findings of these studies have been varying. Allen and co-workers observed a significant reduction in alpha diversity whilst Hofstra and co-workers did not demonstrate any significant change (212, 213).

The discrepancy between studies may be due to a number of factors. Hofstra and co-workers employed the same RV-16 infection model as the current study whilst Allen and co-workers used a RV-39 infection model. Allen and co-worker assessed alpha diversity using the Chao1 diversity index, which is more sensitive for rarer OTUs thus will likely enhance alpha diversity in the presence of numerous low abundance OTUs. Shannon diversity index was used in the current study, which takes into account evenness as well as richness. Whilst both indices are acceptable measures of alpha diversity, the outputs are likely to differ, thus may in part explain the differences observed in the studies. Furthermore, the study by Allen and co-workers directly compared individuals who were infected with RV-39 compared to those who were ‘uninfected’ (based on undetectable RV-39 viral load following inoculation). Given the high variability in alpha diversity amongst individuals, the result of such direct comparison will likely differ from longitudinal infection study.
The impact of alpha diversity on clinical outcomes remains unclear. Increased alpha diversity has been linked with greater airway hyper-responsiveness (199). However, increased alpha diversity also has been associated with improved airway hyper-responsiveness after clarithromycin treatment (199) and sensitivity to steroid therapy (197). The current study did not demonstrate significant correlations between alpha diversity and clinical outcomes or pro-inflammatory cytokine levels at baseline or following RV-16 infection.

In summary, alpha diversity did not significant alter following RV-16 infection or naturally occurring colds (regardless of positive virology). In conjunction with the absence of significant increase in overall bacterial burden, it is apparent that RV-16 infection does not lead to secondary bacterial infection as postulated, whereby one species dominates the microbiota community. The following section will discuss if RV-16 infection perturbs the microbiota community.

6.9.4.3 No significant difference in microbiota community change between asthmatic and healthy subjects

Beta diversity measures the extent of overall community change from baseline following RV-16 infection. In the current study, beta diversity, as assessed by Bray-Curtis dissimilarity and unweighted Unifrac distance, did not differ significantly between asthmatic and healthy subjects. This would suggest that RV-16 does not perturb the airway microbiota differentially between asthma and health. There was also inter-subject variability, implying individuals are affected by RV-16 to various degrees. By day 8, the microbiota of some subjects appeared to be returning to baseline (as represented by lower Bray-Curtis dissimilarity or unweighted
Unifrac distance), whilst others seemed to drift away. No obvious pattern of change was seen. These findings are similar to those seen in natural colds (Chapter 5).

6.9.4.4 RV-16 viral load directly correlated with beta diversity in linear fashion

Given the variable response to RV-16 infection, further evaluation was performed to assess if subjects with more severe infection (as represented by higher RV-16 viral load) had greater microbiota community change.

On day 3, RV-16 viral load directly correlated with Bray-Curtis dissimilarity on day 8 but not day 3. This would indicate that the more severe RV-16 infection, the greater the change in the microbiota community. Furthermore, the temporal lag implies that RV-16 infection perturbs the microbiota rather than the microbiota affecting the severity of RV-16 infection. However, this linear relationship was not observed when beta diversity was assessed using unweighted Unifrac distance. This may be explained by the differences between the two measures of beta diversity. Bray-Curtis is based on abundance of species that are present or absent(255), whereas unweighted Unifrac distance is a qualitative measure that takes into account the relative relatedness of community members(258). In the naturally-occurring cold study, greater unweighted Unifrac distance was associated with greater peak flow decline in the acute phase of cold. In the current study, beta diversity also negatively correlated with PEF change on days 3 and 8; though it was statistically insignificant following FDR adjustment.

In summary, RV-16 infection appears to impact on the extent of microbiota community alteration in a viral-load dependent manner. There was no significant difference between asthma and health. However, the true impact of RV-16 infection on the microbiota remains
unclear in the absence of ‘control’ subjects undergoing multiple bronchoscopic sampling without RV-16 infection and further work is needed to quantify such effect.

6.9.5 Clinical correlations with specific OTUs and genera

6.9.5.1 Increased *Neisseria_2974* relative abundance was associated with greater PEF decline following RV-16 infection

In the naturally-occurring cold study (Chapter 5), increased *Neisseria_2974* relative abundance was associated with greater PEF decline in the acute phase of the cold. A similar pattern is observed in the current study, where *Neisseria_2974* relative abundance (on day 8 post-infection) negatively correlated with PEF change on days 8 and 9. The difference in timing between the two studies may be related to the heterogeneity of the naturally-occurring cold study with respect to timing of subject presentation to clinic, aetiology of respiratory virus infection and sampling technique (induced sputum vs BAL). However, overall it seems evident that in the increased *Neisseria_2974* relative abundance is associated with greater PEF decline in the context of RV-16 infection or naturally-occurring cold.

On day 8, *Neisseria_2974* relative abundance did not correlate with IL-1β and IL-8 levels as observed in natural colds. In that study, pro-inflammatory cytokine levels correlated with *Neisseria_2974* relative abundance from the previous visit (3 – 4 days ago). In the current study, there was no further sampling time point beyond day 8 thus it may be conceivable that pro-inflammatory cytokine levels may correlate with *Neisseria_2974* relative abundance at a later stage.
6.9.5.2 Increased *Prevotella* relative abundance was associated with reduced upper respiratory tract symptoms

On day 3, greater *Prevotella* relative abundance was strongly associated with reduced upper respiratory tract symptoms. There were also similar non-significant tendencies with respect to lower respiratory tract symptoms (day 4) and pro-inflammatory cytokines. In the natural cold study, increased *Prevotella* relative abundance significantly correlated with less PEF reduction and lower pro-inflammatory cytokine levels. In both studies increased *Prevotella* relative abundance was associated with better clinical outcome. Nonetheless remained unclear as to why in the current study *Prevotella* relative abundance within the lower respiratory tract correlated with upper respiratory tract symptoms, though upper respiratory symptoms strongly correlated with lower respiratory tract symptoms and maximal PEF change (Chapter 3). As discussed in Chapter 5, further work is needed to assess if *Prevotella* actively plays a role in remediating airway inflammation.

6.10 Conclusions

The lower airway microbiota (as analysed using BAL) was similar in composition to that of induced sputum. Furthermore, hierarchical clustering and indicator species analysis revealed comparable clusters and dominant OTUs, thus allowing comparison between the two studies.

In contrast to published works, the lower airway microbiota of asthmatic and healthy subjects did not differ significantly at baseline or following RV-16 infection, though at genus level healthy subjects exhibited greater relative abundance of *Prevotella* post infection. This may be related to small sample size and further work is needed to confirm this. Overall bacterial burden and community alpha diversity did not alter significantly post-infection or correlate
with clinical outcomes. The findings imply that RV-16 infection does not lead to secondary bacterial infections as hypothesised. However, greater RV-16 viral load strongly correlated with greater community change (as denoted by increased beta diversity index measures) in a linear manner. Furthermore, the time lag in correlation between viral load and beta diversity suggests that RV-16 infection perturbs the microbiota though further work is needed to verify this.

Consistent with findings of the natural cold study, increased *Neisseria_2974* relative abundance was associated with greater PEF decline post infection, whilst increased *Prevotella* relative abundance was associated with reduced upper respiratory symptoms. The results of this study further supports the notion of an imbalanced/ dysbiotic microbiota that is associated with worse clinical outcomes in the context of a RV-16 infection. However, it remains uncertain whether the microbiota actively contributes to the airway inflammation or merely reflects an inflamed airway environment.

In the final chapter, the main findings of the two studies are summarised. Potential mechanisms by which the microbiota may contribute to the overall airway inflammatory state are proposed with discussion on future directions to evaluate these hypotheses.

### 6.11 Summary of key findings

- The microbiota composition of BAL and induced sputum are similar as illustrated by hierarchical clustering and indicator species analysis.
- The airway microbiota of asthmatic and healthy subjects did not differ significantly at baseline or following RV-16 infection, though at genus level healthy subjects had greater relative abundance of *Prevotella* following RV-16 infection.
• Bacterial burden and alpha diversity did not significantly alter following RV-16 infection and did not correlate with clinical outcomes or pro-inflammatory cytokine levels.

• RV-16 viral load significantly correlated with the degree of microbiota community change (beta diversity).

• Increased *Neisseria_2974* relative abundance was associated with greater PEF decline, whilst increased *Prevotella* relative abundance was associated with reduced upper respiratory tract symptoms.
7 Discussion

7.1 Introduction

The current project is the first to investigate the dynamics of the airway microbiota in adult subjects in the context of a virus-induced asthma exacerbation (AE). The project was divided into two studies: the first examined a cohort of 46 mild/moderate asthmatic subjects during a naturally-occurring cold and the second involved 11 moderate asthmatic and 12 healthy subjects in an experimental RV-16 infection study. This chapter reviews the key findings from both studies, the potential mechanisms by which the airway microbiota may contribute to airway inflammation and clinical symptoms and future directions.

7.2 RV infection was not associated with secondary bacterial infection

Whilst there has been no other study examining changes in BB in virus-induced asthma exacerbations, studies in COPD had demonstrated a significant increase in BB following RV infection peaking approximately 14 days after initial infection(198, 277). This initiated the hypothesis that RV infection increases the risk of secondary bacterial infection in asthma. However, bacterial burden (BB) did not alter significantly in neither natural colds nor RV-16 infection suggesting that in asthma RV infection does not lead to secondary bacterial infection as hypothesised. Furthermore, BB did not correlate with neither clinical outcomes nor pro-inflammatory cytokine levels. These findings imply that if RV infection were to perturb the airway microbiota, it was more likely to disrupt the community balance rather than causing an increase in overall BB (i.e. a dysbiotic state).
This speculation was further supported by the absence of significant changes in alpha diversity and lack of correlation with clinical outcomes, suggesting that the airway microbiota was not dominated by a single OTU/ genus as observed in other respiratory infections such as ventilator-associated pneumonia(278).

7.3 The airway microbiota was more variable than previously proposed though distinct clusters were present

Previous publications have suggested distinctions within the airway microbiota between health and asthma. Healthy individuals were observed to have greater relative abundance of ‘commensals’ such as Actinobacteria, Bacteroidetes and Firmicutes (at phylum level), whilst asthmatic subjects possessed communities with greater abundance of ‘potentially pathogenic’ Proteobacteria(174, 176). In the current project, hierarchical clustering and indicator species analysis revealed several distinct clusters, showing significant variation in community structure in asthma. Furthermore, similar clusters were identified across the two studies, indicating that community structures assessed using induced sputum samples were similar to BAL samples. It is noteworthy that previous studies presented the average relative abundance of microbiota community of all recruited subjects thus such clustering might be obscured.

Contrary to published studies, the current study did not identify a significant difference in the airway microbiota between healthy and asthmatic subjects, though this may be related to small cohort size. When specific phyla/ genera were examined, however, healthy subjects were observed to have greater abundance of Bacteroidetes/ Prevotella than asthmatic subjects following RV-16 infection.
The aetiology of such clustering is currently unclear and is beyond the scope of the current project. However, the relative abundance of Proteobacteria in more severe disease (BTS treatment step 4) was significantly lower compared with those with mild disease (BTS treatment step 1). This observation is consistent with recent published studies in severe asthma(196, 197), though whether this is associated with increased inhaled corticosteroid intake is unknown.

There was no correlation between clusters and clinical parameters at baseline, suggesting that the microbiota community does not impact on clinical symptoms and disease control during chronic stable disease. However, during a natural cold, the Neisseria cluster was associated with greater PEF decline. The potential significance of Neisseria spp. will be discussed in later section.

7.4 RV-16 viral load significantly correlated with beta diversity

Beta diversity was not significantly different following natural cold compared with chronic stable disease. To date, there has been no published study assessing the stability of the asthmatic airway microbiota over time, though the oral microbiota was shown to be relatively stable over the course of one week(274). Changes to the microbiota community over stable disease may be related to a number of factors including sampling location, technique, sequencing variability and changes to the local airway environment (e.g. medications). A number of steps were taken to minimise such issues in the study protocol though confounders could not be completely excluded. It is therefore important to interpret beta diversity measures in the appropriate clinical context.
There was no significant difference in beta diversity between asthmatic and healthy subjects following RV-16 infection though there was great inter-subject variability in both studies. In other words, the microbiota did not alter to a greater extent in asthma compared with health and the degree of change varied considerably amongst individuals. In natural colds, greater beta diversity was associated greater PEF decline, implying that greater change from baseline community was linked to greater airway inflammation. However, this was not the case with experimental RV-16 infection. In light of inconsistency between the two studies, further work is required to examine this issue.

To evaluate if the severity of RV infection would impact on the degree of microbiota change, RV-16 viral load was correlated with beta diversity, demonstrating a significant linear relationship between the two. Furthermore, there was a time delay between viral load and beta diversity. These findings support the postulation that the severity of RV infection (as represented by viral load) directly influences the degree of change within the microbiota community. The mechanisms by which RV infection may perturb the microbiota are unclear.

Finally, neither RV infection nor a naturally occurring cold was associated with any specific alteration within the microbiota, in contrast to a previous study in COPD illustrating an outgrowth of *Haemophilus influenza* following RV-16 infection(198). However, *Neisseira* dominant communities appeared to be more stable following natural colds. Interestingly, *Neisseria* dominant communities at baseline predicted worse PEF decline following natural colds.
7.5 Increased *Neisseria_2974* relative abundance was associated with worse clinical outcomes

As mentioned above, subjects with a *Neisseria* dominated (specifically the OTU *Neisseria_2974*) community were associated with worse PEF decline during natural colds. When *Neisseria_2974* was specifically examined, it significantly correlated with worsened PEF decline. The same observation was replicated in experimental RV-16 infection, providing further confidence on the validity of the initial finding. The exact identity of *Neisseria_2974* is unknown currently and further work (by selective culture) is required to identify this OTU as a species.

It is also undetermined whether *Neisseria_2974* actively contributes to airway inflammation (hence worse PEF) or merely reflects the local airway inflammation. *Neisseria_2974* abundance was associated with greater levels of IL-1β and IL-8 with a temporal lag, inferring that *Neisseria_2974* may lead to greater pro-inflammatory cytokine release. This will need further investigation to establish causality.

7.6 Increased *Prevotella* relative abundance was associated with improved clinical outcomes

*Prevotella* dominant (specifically the OTU *Prevotella_6482*) communities appeared to have reduced PEF decline during natural colds. At OTU level, *Prevotella_6482* did not significantly correlate with clinical outcomes. However, *Prevotella* at Genus level was associated with reduced PEF decline. This was not observed in experimental RV-16 infection; though *Prevotella* was associated with reduced symptoms. These findings imply that multiple
*Prevotella* species may be associated with reduced airway inflammation (resulting in less PEF decline and clinical symptoms). Of note, asthmatic subjects exhibited less relative abundance of *Prevotella* than healthy subjects following RV-16 infection, who had significantly less PEF decline and symptoms. As with *Neisseria_2974*, it is not possible to decipher whether *Prevotella* plays an active role in dampening airway inflammation based on current findings.

### 7.7 Potential mechanisms by which the airway microbiota may play a role in virus-induced asthma exacerbations

From the findings, it appears that the airway microbiota is relatively quiescent during stable chronic disease. However, when an acute event such as RV infection occurs within the airways, homeostasis is disrupted enabling the microbiota to influence the degree of inflammation and ultimately airflow obstruction and clinical symptoms. The processes by which RV infection may propagate microbiota interaction are unknown. A possible mechanism may be related to RV’s induction of the adhesion protein ICAM-1(6), which is also used by some pathogenic *Neisseria* species (e.g. *Neisseria meningitides*) for epithelial adherence(279).

RV infection does not generally result in secondary bacterial infections. Rather, it alters the microbiota community structure and balance leading to a state of ‘dysbiosis’. The extent of microbiota disruption is related to the severity of RV infection (as represented by viral shedding/ load), though RV infection does not appear to preferentially favour or discriminate specific genus or species.

It is evident that the airway microbiota is highly individualised and the extent of microbiota-induced airway inflammation may depend the community composition. Communities with a greater abundance of *Neisseria* (in particular the OTU *Neisseria_2974*) are associated with
worse airflow obstruction. The exact mechanisms are unclear though *Neisseria* abundance seems to correlate with IL-1β and IL-8 levels. IL-1β has been shown to play a role in airway hyper-responsiveness in a murine model(280), whilst IL-8 and subsequent neutrophil activation have been shown to accompany eosinophil activation in asthmatic children(113).

Conversely, greater *Prevotella* (at genus level) abundance was associated with improved clinical outcomes. The mechanisms are again unknown, though *Prevotella* has been illustrated to induce weaker TLR-4 dependent response than other ‘potential’ pathogens such as *Haemophilus influenzae*(202). It may be possible that several *Prevotella* species function similarly to achieve this effect.

Ultimately, individuals with an ‘imbalanced’ microbiota (i.e. disproportionately high abundance of *Neisseria*) experience greater PEF decline thus exacerbating severity of acute asthma exacerbation whilst those with a more ‘balanced’ community (i.e. greater abundance of ‘commensals’ such as *Prevotella*) experience less symptoms.

### 7.8 Limitations of study

Despite a number of steps that have been taken to minimise confounding factors in subject recruitment, sampling techniques and sequencing analysis, a number of limitations exist.

The experimental RV-16 infection study had a small cohort size, whilst the naturally occurring cold study was one short of its intended target of 47 (and therefore was marginally under powered). In light of this issue, the findings of this project need to be interpreted with a degree of caution and serve as exploratory data for a larger scale study. Nonetheless, the consistency across the two independent studies provides confidence on the validity of
observations made. Asthma is a heterogeneous condition with different phenotypes and endotypes. It is currently unknown if such phenotypes/endotypes would impact on the airway microbiota and its changes following RV infection. The current study recruited asthmatic subjects based on disease severity alone, thus would not be able to account for such potential impact.

Whilst the project aimed to evaluate the effects of a virus-induced asthma exacerbation, the majority of subjects in both studies only experienced a mild/moderate exacerbation. This allowed for safe repeated sampling and minimised confounders such as systemic steroid and antibiotic use, though the findings were less clinical relevant.

Previous studies have demonstrated spatial variation in the airway microbiota(177, 178) thus obtaining samples from different segments/lobes of the lung may have an impact on the results. Furthermore, the impact of repeated bronchoscopic sampling (BAL) remains unknown. Every effort was made to maintain sampling consistency but slight variations in location and sampling techniques could not be completely excluded.

Finally, it is important to acknowledge that based on the current findings, it is not possible to ascertain if the airway microbiota influences airway inflammation. Whilst the correlations demonstrate a temporal relationship, causality cannot be validated. However, the findings should stimulate further targeted investigations into the role of the airway microbiota.

7.9 Future directions

The current project’s findings show for the first time that an imbalanced airway microbiota is associated with worsened PEF decline in the context of naturally occurring colds and RV-16 infection. Whilst the findings were generally consistent across the two studies, further work
is needed to investigate the identity of the OTU *Neisseria_2974* and whether *Neisseria_2974* and *Prevotella* actively contribute to the local airway inflammation or not. Conversely, if the microbiota merely reflects the local inflammatory state, it may be a potential novel biomarker of severity of disease or exacerbation.

**7.9.1 Selective quantitative culture to identify *Neisseria_2974* and *Prevotella* species, *in vitro* and *in vivo* stimulation studies**

To accurately identify *Neisseria_2974*, subjects with high abundance of *Neisseria_2974* may be re-sampled and *Neisseria_2974* can then be cultured via selective media. Identity can then be confirmed by 16S rRNA sequencing. Once verified, *in vitro* and *in vivo* RV infection challenge studies can be performed to assess if the cultured *Neisseria* species does indeed lead to increased induction of pro-inflammatory cytokines. The same technique may also be applied to identify *Prevotella* species to evaluate if *Prevotella* species exert an ‘immunomodulatory’ effect. If this is the case, further investigations may be performed to assess if introduction of *Prevotella* or subtraction of *Neisseria* will result in reduced inflammation.

**7.9.2 Metagenomic sequencing to assess interactions between bacterial, viral and fungal communities**

To date, studies on microbiota have focused on bacteria. However, other organisms such as viruses, fungi and phages also populate the airway. It is likely that these organisms interact with each other and may play a role in the pathophysiology of asthma and acute asthma exacerbations. Metagenomic sequencing may enable assessment of physiological capability
between these different communities and environmental conditions and construct metabolic and biogeochemical pathways, providing a depiction of an airway ecosystem.

7.9.3 Optimise future clinical studies

Given the delicate nature and potential pitfalls of 16S rRNA sequencing, future clinical studies should be planned to minimise confounders. The benefits of obtaining genuine lower airways samples must be weighed up against the feasibility and safety of repeated sampling. Multiple control samples (e.g. bronchoscope, DNA extraction agents) are compulsory and effects of multiple sampling remains to be explored. Subject selection should reflect real life clinical practice, though the heterogeneity of asthma should be considered.

7.10 Concluding remarks

The current project investigated the dynamics within the airway microbiota in the context of a naturally occurring cold and experimental RV-16 infection. It has shown that the airway microbiota is highly individualised, though distinct clusters exist. Specific to RV infection, viral load significantly correlates with the degree of change within the microbiota. During infection, increased *Neisseria* abundance is associated with worse outcomes, whilst increased *Prevotella* abundance seems to correlate with improved outcomes. The project has offered a glimpse of the complex interactions that occur within the airway microbial ecosystem and provides the basis for further studies. Whilst the mentioned future directions require significant sequencing effort and bioinformatics capability, rapid technological advances will likely make such progress feasible in the near future. Ultimately, the goal is to explore whether the microbiota plays an active role in acute asthma exacerbations and if specific
modulation/ manipulation of the microbiota would translate into novel therapeutic avenues with clinical benefit.
8 References

7. SIGN/BTS. Asthma priorities: influencing the agenda. 2013.
44. Welsh EJ, Cates CJ. Formoterol versus short-acting beta-agonists as relief medication for adults and children with asthma. The Cochrane database of systematic reviews. 2010(9):Cd008418.


Adults hospitalised with acute respiratory illness rarely have detectable bacteria in the absence of COPD or pneumonia; viral infection predominates in a large prospective UK sample. The Journal of infection. 2014;69(5):507-15.


223. McNamee LA, Harmsen AG. Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection. Infection and immunity. 2006;74(12):6707-21.
229. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and


247. Wickham H. stringr: Make it easier to work with strings. 2012.


9 Appendix 1 – R Script

The following forms the basis of the script applied in R studio. In the current project, two separate studies were analysed: AS study (naturally occurring cold study) and JD study (experimental RV-16 infection study). To avoid duplication, script below is that of the AS study, except for the Adonis and Procrustes test where JD study was used.

Set directory

```r
setwd("~/AS study")
```

Load phyloseq and other programmes needed

```r
library(phyloseq)
library(Biostrings) ##Note that Biostrings also has a distance function - load phyloseq second so its distance function is not masked.
library(ggplot2)
library(plyr)
library(stringr)
library(gridExtra)
library(reshape2)
library(knitr)
library(vegan)
```

Pre-processing

Import data to R

```r
all_data <- import_biom(BIOMfilename = "otu_table_json.biom",
                        treefilename = "AS.tre",
                        refseqfilename = "rep_set_for_r.fasta") #import the files

map <- read.csv("AS_overall_map.csv", row.names =1) #import the mapping file as .csv
map <- sample_data(map) # turn that into sample data

all_data <- merge_phyloseq(all_data, map) # merge these objects into 1

all_data

```r
tail(sample_data(all_data)) # view tail of map file
```

Sorting and re-naming OTUs into something that makes sense

```r
all_data = subset_taxa(all_data, Rank1 != "Unclassified")
```
all_data = subset_taxa(all_data, Rank2 != "p__Cyanobacteria")

makeTaxLabel <- function(OTU, mydata){
  # Makes a string label using the lowest informative tax level
  #
  # Args:
  # OTU: OTU number
  # mydata: the phyloseq object with the tax table
  #
  # Returns:
  # a tax name
  OTU <- as.character(OTU) # the OTU numbers are stored as character not integer!
  taxstrings <- as.character(tax_table(mydata)[OTU])
  empty_strings <- c("k__", "p__", "c__", "o__", "f__", "g__", "s__")
  tax_name <- NA
  tax_level <- length(taxstrings) # start at lowest tax level
  while(is.na(tax_name) |
    (tax_name %in% empty_strings)){
    tax_name <- taxstrings[tax_level]
    tax_level <- tax_level -1
  }
  tax_name
}

tax_table(all_data) = gsub("s__uncultured_bacterium",
  as.character(NA),
  tax_table(all_data))
tax_table(all_data) = gsub("s__uncultured_organism",
  as.character(NA),
  tax_table(all_data))
tax_table(all_data) = gsub("g__uncultured",
  as.character(NA),
  tax_table(all_data))

mynames = NULL
for (i in 1:length(taxa_names(all_data))){
  mynames <- rbind(mynames, c(makeTaxLabel(taxa_names(all_data)[i], all_data)))
}
mynames = gsub("s__", ",", mynames)
mynames = gsub("g__", ",", mynames)
mynames = gsub("f__", ",", mynames)
mynames = gsub("o__", ",", mynames)
mynames = gsub("c__", ",", mynames)
mynames = gsub("p__", ",", mynames)

OTUID = str_c(mynames[,1], ",", seq(1,length(taxa_names(all_data)),by=1))
tax_table(all_data) <- cbind(tax_table(all_data), mynames=OTUID)
#tax_table(all_data)
## Rename tax table headings to something real
colnames(tax_table(all_data)) = c("Kingdom",

348
"Phylum", "Class", "Order", "Family", "Genus", "Species", "OTUID")

tax_table(all_data) <- gsub("k__", "", tax_table(all_data))
tax_table(all_data) <- gsub("p__", "", tax_table(all_data))
tax_table(all_data) <- gsub("c__", "", tax_table(all_data))
tax_table(all_data) <- gsub("o__", "", tax_table(all_data))
tax_table(all_data) <- gsub("f__", "", tax_table(all_data))
tax_table(all_data) <- gsub("g__", "", tax_table(all_data))
tax_table(all_data) <- gsub("s__", "", tax_table(all_data))

Rename the taxa_names (frequently used as plot labels by phyloseq) to be the same as the new unique informative names

taxa_names(all_data) <- tax_table(all_data)[,8]

Filter out contamination

all_data = subset_taxa(all_data, Rank1 != "Unclassified")
all_data = subset_taxa(all_data, Rank2 != "p__Cyanobacteria")
all_data = subset_taxa(all_data, Rank4 != "o__Rhodobacterales")
all_data = subset_taxa(all_data, Rank4 != "o__Rhizobiales")
all_data = subset_taxa(all_data, Rank5 != "f__Oxalobacteraceae")
all_data = subset_taxa(all_data, Rank6 != "g__Deviia")
all_data = subset_taxa(all_data, Rank6 != "g__Rhodococcus")
all_data = subset_taxa(all_data, Rank6 != "g__Vibrio")

Subset samples into groups

Need to remove Run 7 (as it is redundant)

sample_data(all_data)$Run <- as.factor(sample_data(all_data)$Run)
sample_data(all_data)$Visit <- as.factor(sample_data(all_data)$Visit)
sample_data(all_data)$Rx_step <- as.factor(sample_data(all_data)$Rx_step)

Sputum and Throat swab samples as one group (called Actual_samples)

Actual_samples <- subset_samples(all_data, Sample != "NA")
Actual_samples <- subset_samples(Actual_samples, Sample != "Negative")
Actual_samples <- subset_samples(Actual_samples, Sample != "Mock")
Actual_samples <- subset_samples(Actual_samples, Sample != "Reagent")
Actual_samples <- subset_samples(Actual_samples, Sample != "Saline")
Actual_samples <- subset_samples(Actual_samples, Sample != "TS_CNT")
Actual_samples <- subset_samples(Actual_samples, Status != "excluded")

Sputum samples

Subset samples to just sputum and prune out the 'excluded' samples

sputum <- subset_samples(Actual_samples, Sample == "Sputum")

Throat swab samples

Subset samples to just throat swab and prune out the 'excluded' samples

throat_swab <- subset_samples(Actual_samples, Sample == "TS")

Mock community

mock <- subset_samples(all_data, Sample == "Mock")

Negative control

negative_control <- subset_samples(all_data, Sample == "Negative")

Reagents

reagents <- subset_samples(all_data, Sample == "Reagent")

Saline controls

saline_control <- subset_samples(all_data, Sample == "Saline")

Throat swab controls (i.e. blank throat swabs)

throat_swab_control <- subset_samples(all_data, Sample == "TS_CNT")

Assessment of control samples (i.e. negative, reagent, TS_CNT (blank throat swab))

Sort taxon abundance in negative controls and highlight most abundant and prevalent

negative_abund <- sort(taxa_sums(negative_control))
reagents_abund <- sort(taxa_sums(reagents))
saline_control_abund <- sort(taxa_sums(saline_control))
throat_swab_control_abund <- sort(taxa_sums(throat_swab_control))
Negative controls

negative_abund = as.data.frame(negative_abund)
negative_abund$negative_abund = as.data.frame(cbind(rownames(negative_abund), negative_abund$negative_abund))
negative_abund$V2 = as.numeric(as.character(negative_abund$V2))
negative_topbugs = subset(negative_abund, V2 > 1000)  # V2 > x means filtering to OTUs with number greater than x

Reagents

reagents_abund = as.data.frame(reagents_abund)
reagents_abund$reagents_abund = as.data.frame(cbind(rownames(reagents_abund), reagents_abund$reagents_abund))
reagents_abund$V2 = as.numeric(as.character(reagents_abund$V2))
oreagents_topbugs = subset(reagents_abund, V2 > 1000)

Saline controls

saline_control_abund = as.data.frame(saline_control_abund)
saline_control_abund$saline_control_abund = as.data.frame(cbind(rownames(saline_control_abund), saline_control_abund$saline_control_abund))
saline_control_abund$V2 = as.numeric(as.character(saline_control_abund$V2))
saline_control_topbugs = subset(saline_control_abund, V2 > 1000)

Throat swab control

throat_swab_control_abund = as.data.frame(throat_swab_control_abund)
throat_swab_control_abund$throat_swab_control_abund = as.data.frame(cbind(rownames(throat_swab_control_abund), throat_swab_control_abund$throat_swab_control_abund))
throat_swab_control_abund$V2 = as.numeric(as.character(throat_swab_control_abund$V2))
throat_swab_control_topbugs = subset(throat_swab_control_abund, V2 > 1000)

Plot chart of negative control contamination

library(ggplot2)
library(grid)

Negative control

p1 <- ggplot(negative_topbugs, aes(x = V2, y = reorder(V1, V2)))
p1 = p1 + geom_segment(aes(yend=V1), xend = 0, colour = "grey50")
p1 = p1 + geom_point(size = 3, colour = "deepskyblue")
p1 = p1 + theme_bw()
p1 = p1 + theme(axis.text.x = element_text(angle = 60, hjust = 1), panel.grid.major.x = element_blank(),


panel.grid.minor.y = element_blank(),
panel.grid.major.y = element_blank(),
axis.title.y = element_blank())
p1 = p1 + xlab("Number of Reads")
p1 = p1 + ggtitle("Contaminant OTUs Negative PCR Controls")
p1

Reagents

p2 <- ggplot(reagents_topbugs, aes(x = V2, y = reorder(V1, V2))) +
geom_segment(aes(yend=V1), xend = 0, colour = "grey50") + geom_point(size = 3, colour =
"deepskyblue") + theme_bw() + theme(axis.text.x = element_text(angle = 60, hjust = 1),
panel.grid.major.x = element_blank(),
panel.grid.minor.y = element_blank(),
panel.grid.major.y = element_blank(),
axis.title.y = element_blank()) + xlab("Number of Reads") + ggtitle("Contaminant
OTUs Reagents")
p2

Saline controls

p3 <- ggplot(saline_control_topbugs, aes(x = V2, y = reorder(V1, V2))) +
geom_segment(aes(yend=V1), xend = 0, colour = "grey50") + geom_point(size = 3, colour =
"deepskyblue") + theme_bw() + theme(axis.text.x = element_text(angle = 60, hjust = 1),
panel.grid.major.x = element_blank(),
panel.grid.minor.y = element_blank(),
panel.grid.major.y = element_blank(),
axis.title.y = element_blank()) + xlab("Number of Reads") + ggtitle("Contaminant
OTUs Saline Flush Pre-bronchoscopy")
p3

Throat swab control

p4 <- ggplot(throat_swab_control_topbugs, aes(x = V2, y = reorder(V1, V2))) +
geom_segment(aes(yend=V1), xend = 0, colour = "grey50") + geom_point(size = 3, colour =
"deepskyblue") + theme_bw() + theme(axis.text.x = element_text(angle = 60, hjust = 1),
panel.grid.major.x = element_blank(),
panel.grid.minor.y = element_blank(),
panel.grid.major.y = element_blank(),
axis.title.y = element_blank()) + xlab("Number of Reads") + ggtitle("Contaminant
OTUs Blank Throat Swabs")
p4

Assess mock community

set.seed(220886) # Setting seed allows the 'same' randomisation to take place again. i.e.
allow someone else to reproduce your data
pos_glom <- tax_glom(mock, "Genus") # merge together all otus of the same genus into one group.
pos_glom_40 <- names(sort(taxa_sums(pos_glom), TRUE)[1:27])
pos_glom_40 <- prune_taxa(pos_glom_40, pos_glom)

pos_glom_40_rare <- rarefy_even_depth(pos_glom_40, 10000) # Rarefy to even depth. In this case to 1E5 reads

Plot mock community

library(plyr)
plot_ordered_bar(pos_glom_40, fill = "Genus", leg_size = 0.5) +
scale_fill_manual(values=pixar_up_27$hexCode) + ggtitle("Mock Communities")

plot_ordered_bar(pos_glom_40_rare, fill = "Genus", leg_size = 0.5) +
scale_fill_manual(values=pixar_up_27$hexCode) + ggtitle("Mock Communities Rarefied to 1E5")

Assessing for differences/ordination between runs

First, plot ordination NMDS of all runs. As 'Run' column is numerical (i.e. 1-6, now that Run 7 has been excluded), the NMDS plot will be colour graded, which makes it difficult to interpret. To make it easier to see, convert the 'Run' column as factor

sample_data(all_data)$Run <- as.factor(sample_data(all_data)$Run)
Plotting ordination of all runs

all_data_2 <- as.data.frame(all_data)
ord_all <- ordinate(all_data, "NMDS", "bray")
plot_ordination(all_data, ord_all, color = "Sample")+geom_point( size=5)

res <- simprof(data=all_data,method.distance="braycurtis")

Plot barplot using heirarchical clustering
All sputum samples

d = distance(sputum, "bray")

hc <- hclust(d, method="complete")
plot(hc, cex= 0.4,main = "", hang = -2, xlab="", ylab="")

names(hc)
o <- hcs$order
l <- hcs$labels

order <- l[o]
order # these are the samples arranged in order of their h.cluster!
write.csv(order, file = "sputum_HC.csv")

c
dh = as.dendrogram(hc)
plot(hcd)

Colour Dendrogram

load code of A2R function
source("http://addictedtor.free.fr/packages/A2R/lastVersion/R/code.R")
#

colored dendrogram

op = par(bg = "white")
A2Rplot(hc, k = 8, boxes = FALSE, col.up = "gray50", col.down = c("#53868B", 
"#FF4040", 
"#556270", 
"#EDC951", 
"#C44D58", 
"#458B74", 
"#00688B", 
"#228B22"), show.labels = FALSE)

Subset to Top 25 Sputum taxa
Top25Taxa_sputum = names(sort(taxa_sums(sputum), decreasing = TRUE)[1:25])
Top25_sputum = prune_taxa(Top25Taxa_sputum, sputum)

Phylum level

phylum_pal <- merge(master_phylum_pallet, unique(tax_table(Top25_sputum)[,2]))

plot_ordered_bar_x_order(Top25_sputum, 
    fill = "Phylum", 
    leg_size = 1, order.var = "Bar_order_sputum", 
    rarefaction_level = 1000 
) + scale_fill_manual(values = as.vector(phylum_pal$hexCode), na.value = "white")

At Genus level

genus_pal <- merge(master_genera_pallet, unique(tax_table(Top25_sputum)[,6]))

plot_ordered_bar_x_order(Top25_sputum, 
    fill = "Genus", 
    leg_size = 1, order.var = "Bar_order_sputum", 
    rarefaction_level = 1000 
) + scale_fill_manual(values = as.vector(genus_pal$hexCode), na.value = "white")

Indicator species analysis

library("indic Species", lib.loc="~/Library/R/3.1/library")

a = as.matrix(sample_data(sputum))
a <- as.data.frame(a)
str(a)
a$Sputum_group

otu_table_sputum <- t(otu_table(sputum))
```r
o <- as.matrix(otu_table_sputum)
o <- as.data.frame(o)

indval = multipatt(o, a$Sputum_group, control = how(nperm=999), duleg = TRUE)
summary(indval, indvalcomp=TRUE, alpha = 0.03)

Adonis test
# Assessing difference between asthma and healthy at day 8 post RV

```
```
JD_pos_v3 <- subset_samples(JD_pos, Stage == "C")
JD_pos_v3
df = as(sample_data(JD_pos_v3), "data.frame")
d = distance(JD_pos_v3, "bray")
D8_adonis = adonis(d ~ Disease, df)
D8_adonis

Procrustes test
ord_all3 <- ordinate(JD3, "NMDS", "bray") #JD3 – samples rarefied at 500 reads

ord_all2 <- ordinate(JD2, "NMDS", "bray") #JD2 – samples rarefied at 1000 reads

pro <- protest(ord_all3, ord_all2)
pro
plot(pro)
```
<table>
<thead>
<tr>
<th>Sample_ID</th>
<th>Sample_Name</th>
<th>Sample_Well</th>
<th>I7_Index_ID</th>
<th>Index2</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1</td>
<td>NT01</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a2</td>
<td>NT02</td>
<td>ECXATGAG M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a3</td>
<td>NT03</td>
<td>AGGAGGAG M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b1</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b2</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b3</td>
<td>NT11</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b4</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b5</td>
<td>NT11</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b6</td>
<td>NT01</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b7</td>
<td>NT02</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a1</td>
<td>NT08</td>
<td>AGGAGGAG M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a2</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a3</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a4</td>
<td>NT11</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a5</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a6</td>
<td>NT01</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a7</td>
<td>NT02</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a8</td>
<td>NT08</td>
<td>AGGAGGAG M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a9</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a10</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a11</td>
<td>NT11</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a12</td>
<td>NT11</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>a13</td>
<td>NT12</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b1</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b2</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b3</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b4</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b5</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b6</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b7</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b8</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b9</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b10</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b11</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
<tr>
<td>b12</td>
<td>NT10</td>
<td>TAYAGGCA M5OS</td>
<td>TAGATGC</td>
<td>Bpm_Myeny_20_percent</td>
<td>phi</td>
</tr>
</tbody>
</table>

10 Appendix 2 – 16S rRNA gene sequencing barcodes
11 Appendix 3 – Copyright clearance

OXFORD UNIVERSITY PRESS LICENSE
ERMS AND CONDITIONS
in 25, 2017

This Agreement between Ernie Hoi Cheung Wong ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number: 3955881115340
License date: Sep 25, 2016
Licensed content publisher: Oxford University Press
Licensed content publication: Human Molecular Genetics
Licensed content title: Sequencing the human microbiome in health and disease:
Licensed content author: Michael J. Cox, William O.C.M. Cookson, Miriam F. Moffatt
Licensed content date: 10/15/2013
Type of Use: Thesis/Dissertation
Institution name: n/a
Title of your work: The lung microbiome in virus-induced asthma exacerbations
Publisher of your work: n/a
Expected publication date: Jan 2017
Permissions cost: 0.00 GBP
Value added tax: 0.00 GBP
Total: 0.00 GBP

Requestor: Ernie Hoi Cheung Wong
Location: Flat 12 Willow Court
Corney Reach Way
London, W42TW
United Kingdom
Attn: Ernie Hoi Cheung Wong

Publisher Tax ID: GB125506730
Billing Type: Invoice
Billing Address: Ernie Hoi Cheung Wong
Flat 12 Willow Court
Corney Reach Way
London, United Kingdom W42TW
Attn: Ernie Hoi Cheung Wong
Total: 0.00 GBP
his Agreement between Ernie Hoi Cheung Wong ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

<table>
<thead>
<tr>
<th>License Number</th>
<th>3955860243211</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Sep 25, 2016</td>
</tr>
<tr>
<td>Licensed Content Publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed Content Publication</td>
<td>Medicine</td>
</tr>
<tr>
<td>Licensed Content Title</td>
<td>Asthma: diagnosis and management in adults</td>
</tr>
<tr>
<td>Licensed Content Author</td>
<td>Ernie H.C. Wong, Hugo A. Farne, David J. Jackson</td>
</tr>
<tr>
<td>Licensed Content Date</td>
<td>May 2016</td>
</tr>
<tr>
<td>Licensed Content Volume Number</td>
<td>44</td>
</tr>
<tr>
<td>Licensed Content Issue Number</td>
<td>5</td>
</tr>
<tr>
<td>Licensed Content Pages</td>
<td>10</td>
</tr>
<tr>
<td>Start Page</td>
<td>287</td>
</tr>
<tr>
<td>End Page</td>
<td>296</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>Format</td>
<td>electronic</td>
</tr>
<tr>
<td>Are you the author of this Elsevier article?</td>
<td>Yes</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Order reference number</td>
<td></td>
</tr>
<tr>
<td>Original figure numbers</td>
<td>Figure 1</td>
</tr>
<tr>
<td>Title of your thesis/dissertation</td>
<td>The lung microbiome in virus-induced asthma exacerbations</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Jan 2017</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>350</td>
</tr>
<tr>
<td>Elsevier VAT number</td>
<td>GB 494 6272 12</td>
</tr>
<tr>
<td>Requestor Location</td>
<td>Ernie Hoi Cheung Wong</td>
</tr>
<tr>
<td></td>
<td>Flat 12 Willow Court</td>
</tr>
<tr>
<td></td>
<td>London, W42TW</td>
</tr>
</tbody>
</table>
United Kingdom
Attn: Ernie Hoi Cheung Wong

Total
0.00 GBP