Clinical, immunological and microbiological phenotypes of chronic obstructive pulmonary disease (COPD) in ethnic Chinese populations in South-East Asia

Tiew, Pei Yee

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**CLINICAL, IMMUNOLOGICAL AND MICROBIOLOGICAL
PHENOTYPES OF CHRONIC OBSTRUCTIVE PULMONARY
DISEASE (COPD) IN ETHNIC CHINESE POPULATIONS IN
SOUTH-EAST ASIA**

TIEW PEI YEE

LEE KONG CHIAN SCHOOL OF MEDICINE

2020

**CLINICAL, IMMUNOLOGICAL AND MICROBIOLOGICAL PHENOTYPES
OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) IN ETHNIC
CHINESE POPULATIONS IN SOUTH-EAST ASIA**

TIEW PEI YEE

Lee Kong Chian School of Medicine

A thesis submitted to the Nanyang Technological University in partial fulfilment of
the requirement for the degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

26th July 2020

Date

Tiew Pei Yee

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from papers published in the following peer-reviewed journals where I was the first and/or corresponding author

Published manuscripts

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 - My contribution to this manuscript includes study conception, study protocol design, ethics application, COPD patient recruitment in Singapore, data collation and venous sample collection, all laboratory works presented in the manuscript including all statistical analysis (except validation clustering), interpretation of data and writing of the final manuscript.

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5. Anthony CA Yii, Sean CH Loh, **Tiew PY**, Huiying Xu, Aza Taha, Jansen Koh, Jessica Tan HY, Therese Lapperre, Antonio Anzueto, Augustine KH Tee. A clinical prediction model for hospitalized COPD exacerbations based on “treatable traits”. *Int J Chron Obstruct Pulmon Dis*. 2019 Mar 27; 14:719-728. (JIF:3.157)
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Conferences Abstracts:

Chapter 3:

1. **Tiew PY**, Ko FWS, Xu H, Poh ME, Narayana JK, Jiong GHS, Neo HY, Loh LC, Ong CK, Tan JHY, Lapperre TS, C, Abisheganaden JA, Tee A, Chotirmall SH. Chronic Obstructive Pulmonary Disease (COPD) in patients of Chinese ethnicity reveal clinically relevant phenotypes. European Respiratory Society (ERS) International Congress Madrid, 2019. (Poster discussion).
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Chapter 4:

1. **Tiew PY**, Fanny Wai San Ko, Mau Ern Poh, Sze Lei Pang, Sri Anusha Matta, Yang Yie Sio, Huiying Xu, Micheál Mac Aogáin, J X Kenny Lau, Tavleen Kaur Jaggi, Augustine Tee, John Arputhan Abisheganaden, Mariko Siyue Koh, Fook Tim Chew, Sanjay Haresh Chotirmall. Fungal sensitization and its association with frequent exacerbations in COPD. American Thoracic Society (ATS) Conference, 2020. (Poster presentation).
2. **Tiew PY**, Tavleen Kaur Jaggi, Micheál Mac Aogáin, J X Kenny Lau, Fransiskus Xavierius Ivan, Nicolas E. Gaultier, Mariko Siyue Koh, Ong Thun How, Fook Tim Chew, Stephan C. Schuster³, Sanjay Haresh Chotirmall. Indoor and outdoor home allergen load relates to clinical outcomes in COPD: A metagenomics approach. European Respiratory Society (ERS) International Congress, Virtual 2020. (Poster presentation).

Chapter 5:

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TABLE OF CONTENTS

TABLE OF CONTENTS	x
LIST OF FIGURES	xv
LIST OF TABLES	xx
ABBREVIATIONS	xxii
SUMMARY	xxvii
CHAPTER 1: INTRODUCTION	1
1.1 Background	1
1.3 Objectives of this thesis	5
CHAPTER 2: LITERATURE REVIEW	6
2.1 Chronic Obstructive Pulmonary Disease (COPD)	6
2.1.1 Epidemiology	7
2.1.3 The diagnosis and clinical assessment of COPD	8
2.1.4 Pathogenesis	11
2.1.5 Pathophysiology	15
2.1.6 COPD Treatment: The stable and acute exacerbation states	16
2.1.6.1 Non-pharmacological treatment	16
2.1.6.2 Pharmacological treatment of COPD	19
2.1.6.3 Treating acute exacerbations of COPD (AECOPD)	21
2.2 COPD in the Asian sub-continent	22
2.2.1 Prevalence of COPD	23
2.2.2 Are there clinical phenotypes of COPD in Asia?	24
2.2.3 COPD Exacerbations	28
2.3 COPD endophenotypes	31
2.3.1 Comorbidities	31
2.3.2 Frequent COPD exacerbators	32
2.3.3 The allergic (eosinophilic) COPD phenotype	34
2.3.4 Allergic sensitisation in COPD	34
2.3.5 COPD-Overlap Syndromes	38
2.3.5.1 Asthma-COPD Overlap (ACO)	38
2.3.5.2 Bronchiectasis-COPD overlap (BCO)	40
2.4 Infection in COPD	41
2.4.1 Acute infection in COPD	41
2.4.2 Chronic infection in COPD	43
2.4.2.1 Bacteria in COPD	43
2.4.2.2 Viruses in COPD	44

2.4.2.3 Fungi in COPD.....	45
2.5 The microbiome in COPD	47
2.5.1 The bacterial microbiome (bacteriome) in COPD	47
2.5.2 The fungal microbiome (mycobiome) in COPD.....	49
2.5.3 The virome in COPD	50
2.5.4 The interaction between bacteria, viruses and fungi in COPD	51
2.5.5 The Asian microbiome.....	52
2.5.6 Overall aim and hypothesis.....	53
 CHAPTER 3: CLINICAL AND INFLAMMATORY PHENOTYPES OF COPD IN SOUTH- EAST ASIA	 54
3.1 Introduction.....	54
3.2 Methods.....	57
3.2.1 Ethics statement	57
3.2.2 COPD patient recruitment.....	57
3.2.3 Derivation and validation cohorts	58
3.2.4 Clinical data collation.	59
3.2.5 Definitions of recorded co-morbidities.	60
3.2.6 Non-diseased (healthy individual) recruitment.	62
3.2.7 Blood sample collection.....	62
3.2.8 Cytokine assay	63
3.2.9 Statistical analysis	64
3.2.9.2 Survival analysis	67
3.2.9.3 Data visualisation.....	67
3.2.9.4 Cytokine (Inflammatory) analysis.....	67
3.3 Results.....	68
3.3.1 Demographics, co-morbidities and airway microbiology profiles are comparable between the derivation and validation cohorts of Chinese patients with COPD.....	68
3.3.2 Unsupervised hierarchical clustering reveals five clinical clusters with variable prognostic outcomes.	73
3.3.3 Clusters from the derivation cohort were validated using an independent (validation) cohort with comparable clinical and mortality outcomes.....	81
3.3.4 Decision tree for Chinese COPD cluster classification.....	88
3.3.5 The identified Chinese COPD clusters each demonstrate systemic and complex inflammatory networks that associate with mortality outcomes.	90
3.3.6 The five Chinese COPD clusters provide additional and incremental value to established GOLD classification by identifying ‘high-risk’ Chinese COPD patients.	98
3.4 Discussion	102

CHAPTER 4: IMMUNOLOGICAL (ALLERGIC) PHENOTYPES OF COPD IN SOUTH-EAST ASIA	108
4.1 Introduction.....	108
4.2 Methods.....	111
4.2.1 Ethics statement	111
4.2.2 Patient recruitment	111
4.2.3 Blood sample collection.....	112
4.2.4 Measurement of total Immunoglobulin (Ig)-E.....	112
4.2.5 Allergen panel	113
4.2.6 Outdoor air sampling for metagenomic sequencing	115
4.2.7 Fungal isolation from outdoor air	116
4.2.8 Sanger sequencing identification of cultured fungal species from outdoor air	117
4.2.9 Crude allergen preparation.....	117
4.2.10 Recombinant <i>Aspergillus fumigatus</i> proteins	118
4.2.11 Immuno dot-blot assays to determine specific Ig-E concentration	119
4.2.12 Indoor air and surface sampling for metagenomic sequencing	121
4.2.13 Metagenomic sequencing and data processing	122
4.2.14 Statistical analysis and data visualisation	123
4.2.14.1 Cluster analysis	123
4.2.14.2 Indoor Allergen mapping	124
4.3 Results.....	125
4.3.1 South-East Asians with COPD demonstrate high sensitisation rates to a wide range of allergens.....	125
4.3.2 Fungal sensitisation is associated with very frequent COPD exacerbator (vFE) status	128
4.3.3 Unsupervised clustering reveals three groups of ‘sensitised’ South-East Asians with COPD that demonstrate variable clinical outcomes.....	136
4.3.4 Sensitisation to outdoor air fungi is associated with exacerbations in South-East Asians with COPD.	141
4.3.5 Sensitisation to “air-fungi” from outdoor air in Singapore is not related to COPD exacerbations in patients from Malaysia and Hong Kong.	145
4.3.6 Indoor allergens are an important and under-recognised source of sensitisation in COPD.	147
4.4 Discussion.....	151
CHAPTER 5: MICROBIOLOGICAL (FUNGAL) PHENOTYPES OF COPD IN SOUTH-EAST ASIA	156

5.1 Introduction.....	156
5.2 Methods.....	159
5.2.1 Ethics Statement.....	159
5.2.2 Patient recruitment	159
5.2.3 COPD specimen collection and processing (sputum and serum).....	161
5.2.4 Sputum DNA extraction.....	161
5.2.5 ITS Primer optimisation and selection	162
5.2.6 Fungal mock community preparation	164
5.2.7 PCR amplification.....	166
5.2.8 Library preparation, mycobiome sequencing and data processing	167
5.2.9 Immuno-dotblot assays to measure specific-IgE responses against a range of detected fungi.....	168
5.2.10 Statistical analysis	168
5.2.10.1 Cluster analysis	171
5.2.10.2 Co-occurrence network analysis	171
5.3 Results.....	172
5.3.1 Primers targeting the ITS-2 region accurately identifies fungi within a mock community.	172
5.3.2 Distinct mycobiome profiles demonstrating geographic variation are observed in stable COPD.....	174
5.3.3 Altered airway mycobiome profiles in very frequent COPD exacerbators (vFE). 186	
5.3.4 Airway mycobiome profiles remain unchanged between the stable, exacerbation and post- exacerbation state in COPD despite antibiotic and steroid treatment.	192
5.3.5 Hierarchical clustering reveals two COPD patient groups (clusters) demonstrating variable clinical outcomes based on airway mycobiome profiling.	195
5.3.6 The COPD airway mycobiome associates with measurable systemic immune responses to its constituents.	200
5.3.7 Lower alpha diversity in the COPD airway mycobiome identified during acute exacerbations of COPD (AECOPD) is associated with poorer two-year survival.....	202
5.3.8 COPD frequent exacerbator is associates with decrease bacteria diversity.	206
5.4 Discussion	208
CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS	216
6.1 Novelty and Significance	216
6.1.1 The cardiovascular and ex-tuberculosis clinical phenotypes in South-East Asians with COPD demonstrate poorest survival and complex inflammatory networks	217

6.1.2 Allergic sensitisation is prevalent in South-East Asian COPD with important contributions from the indoor and outdoor environment	218
6.1.3. The airway fungal mycobiome is related to exacerbation frequency and mortality in South-East Asian COPD	219
6.2 Future directions	222
6.3 Summary of skills acquired and lessons learnt	224
6.4 Conclusion	225
REFERENCES:	226
APPENDIX: PUBLICATIONS AND AWARD	275

LIST OF FIGURES

Figure 2.1: GOLD grouping of COPD (A-D) based on patient symptoms and exacerbation history.

Figure 2.2: Pathogenesis of COPD.

Figure 2.3: Mean body mass index (BMI): comparisons between non-Asian (Western) (yellow) and Asian (blue) COPD studies.

Figure 2.4: Percentage of male COPD patients comparing non-Asian (Western) and Asian studies.

Figure 2.5: A summary of characteristics observed in Asian COPD patients.

Figure 3.1: Contour plot to determine optimal model parameter selection for the Regularised Discriminant Analysis (RDA) model used for determination of validation cluster membership.

Figure 3.2: Co-morbidities are comparable between the derivation (D) and validation (V) Chinese COPD cohorts.

Figure 3.3: Bubble plot illustrating the proportion of Chinese COPD patients with a positive sputum microbiology between the derivation and validation cohorts.

Figure 3.4: Dendrogram illustrating the five clinical clusters derived from the derivation cohort using a non-metric multidimensional scaling followed by hierarchical clustering.

Figure 3.5: Two-year survival between the five clinical clusters in the derivation cohort.

Figure 3.6: Bar chart demonstrating the proportion of COPD patients within each derived cluster with at least one severe (hospitalised) exacerbation at one-year following recruitment into the study.

Figure 3.7: Mortality and exacerbation outcomes within each respective cluster from the derivation cohort is unaltered by smoking status.

Figure 3.8: Co-morbidities are comparable between the derivation and validation COPD cohorts for each respective cluster.

Figure 3.9: Two-year survival between the five clinical clusters derived from the validation cohorts.

Figure 3.10: Mortality and exacerbations in each of the five clusters from the validation cohort is unaltered by smoking status.

Figure 3.11: Clinical decision tree generated by Classification and Regression trees (CART) for classification of a Chinese COPD patient into one of the five derived clusters.

Figure 3.12: A semi-quantitative expression of 120 cytokines between the five clusters used in the initial screening assay.

Figure 3.13: TNF-R2 is significantly increased in Chinese COPD patients demonstrating higher symptom scores (CAT scores) and greater frequency of severe (hospitalised) exacerbations (SE).

Figure 3.14: Inflammatory profiles and networks of the five derived Chinese COPD clusters.

Figure 3.15: An increased mortality in the cardiovascular and ex-TB clusters irrespective of GOLD grouping.

Figure 3.16: Reduced two-year survival in the cardiovascular and ex-TB Chinese COPD clusters irrespective of GOLD grading.

Figure 3.17: A forest plot illustrating the odds ratios of mortality in each of the derived Chinese COPD clinical clusters (compared to the LCLR cluster) after adjustment for age, sex, BMI, smoking pack year exposure, lung function (by FEV₁) and GOLD group.

Figure 4.1: The extensive allergen panels designed for used in this study.

Figure 4.2: Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentrations and temperatures used for the optimisation of *A. fumigatus* recombinant protein expression.

Figure 4.3: Outdoor fungi and recombinant *Aspergillus fumigatus* allergens preparation steps with overall process that includes the immuno-dot blot assays.

Figure 4.4: Increased sensitisation to a panel of allergens in South-East Asian patients with chronic obstructive pulmonary disease (COPD).

Figure 4.5: Very frequent exacerbator (≥ 3 exacerbations/year) status in South-East Asians with COPD associates with a significantly increased occurrence of fungal sensitisation.

Figure 4.6: No difference in sensitisation to (a) pollens, (b) house dust mite, cockroaches, (c) crude fungal, (d) major and (e) minor recombinant *Aspergillus fumigatus* allergens were detected between the GOLD lung function stages 1 ($FEV_1 > 80\%$ predicted), 2 ($FEV_1 50-79\%$ predicted), 3 ($FEV_1 30-49\%$ predicted) and 4 ($FEV_1 < 30\%$ predicted).

Figure 4.7: No difference in sensitisation to (a) pollens, (b) house dust mite, cockroaches, (c) crude fungal, (d) major and (e) minor recombinant *Aspergillus fumigatus* allergens were detected between the GOLD A (CAT ≤ 10 and < 2 exacerbations and no hospitalised exacerbations per year), B (CAT > 10 and < 2 exacerbations and no hospitalised exacerbations per year) C (CAT ≤ 10 and > 2 exacerbations/ ≥ 1 hospitalised exacerbations) and D (CAT > 10 and > 2 exacerbations/ ≥ 1 hospitalised exacerbations) groups.

Figure 4.8: Unsupervised clustering reveals three ‘sensitised’ South-East Asian COPD patient groups (clusters) that demonstrate varying clinical outcomes with the poorest outcomes in the highly sensitised (HS) fungal predominant group (cluster).

Figure 4.9: Sensitisation to outdoor air fungi are associated with exacerbations in Singaporean patients with COPD.

Figure 4.10: Sensitisation to outdoor “air-fungi” from Singapore assessed in COPD patients recruited from Hong Kong and Malaysia, respectively.

Figure 4.11: An increased number of detectable indoor allergens correlates with COPD symptoms and poorer lung function.

Figure 5.1: A schematic overview of the two different mock community preparation strategies used.

Figure 5.2: Bar charts illustrating the relative abundance of each individual fungal isolate using the 4 different primer sets.

Figure 5.3: Bar charts illustrating the comparisons between each individual tested primer set (P1-P4) in replicating the theoretical (T) composition of the fungal mock community using two separate methods: fungal amplicon (FA) and fungal DNA (FD) mock community preparations.

Figure 5.4: Airway mycobiome profiles remain unchanged at baseline and at 6-month follow up in a group of n=5 stable COPD patients. Stacked bar charts illustrating the relative abundance of the various fungal genera detected between baseline and 6- month interval sputum samples in stable COPD. a: baseline, b: 6-month-follow up.

Figure 5.5: The airway mycobiome differs between COPD and non-diseased (healthy) controls and demonstrates geographic variation.

Figure 5.6: Airway mycobiome profiles are not related to GOLD ABCD grouping in patients with stable COPD.

Figure 5.7: Airway mycobiome profiles are not affected by GOLD (lung function) (FEV₁) grade in patients with stable COPD.

Figure 5.8: A comparison of airway mycobiome profiles between very frequent (vFE) and non-frequent COPD exacerbators (non-FE).

Figure 5.9: The COPD airway mycobiome is unaffected by inhaled corticosteroid use in stable COPD patients.

Figure 5.10: The COPD airway mycobiome remains unchanged across an exacerbation event when assessed longitudinally at baseline, during an exacerbation and at post-exacerbation.

Figure 5.11: Unsupervised hierarchical clustering of fungal genera from airway mycobiome profiling in COPD reveals two clusters each demonstrating variable clinical outcomes.

Figure 5.12: Detectable associations between the COPD airway mycobiome and a measurable systemic immune response to specific fungal constituents.

Figure 5.13: COPD airway mycobiome that demonstrate lower fungal diversity at exacerbation associate with poorer two-year survival.

Figure 5.14: The airway bacterial microbiome profile in South-East Asians with COPD.

Figure 6.1: A summary of the identified clinical (chapter 3), immune (allergic) (chapter 4) and microbiological (fungal) (chapter 5) phenotypes of Asian COPD studied in this thesis and their association with relevant COPD outcomes.

LIST OF TABLES

Table 2.1: GOLD grading of COPD based on measured lung function.

Table 2.2: Diagnostic criteria for Asthma-COPD overlap (ACO).

Table 3.1 Definitions for recorded co-morbidities.

Table 3.2: Confusion matrix showing the predicted and actual clusters identities (expressed as number of patients, n and percentages; %) generated from the derivation cohort (n= 911) using Regularised Discriminant Analysis (RDA).

Table 3.3: Demographic table illustrating the derivation and validation cohorts of Chinese COPD patients recruited across six sites and three countries in South-East Asia.

Table 3.4: Baseline characteristics of the five derived Chinese COPD clusters (from the derivation cohort).

Table 3.5: Baseline characteristics of the five derived COPD clusters (from the validation cohort).

Table 3.6: Demographic table illustrating baseline characteristics and inflammatory (cytokine) assessments for the non-diseased (healthy) and COPD cohorts.

Table 3.7a: Demographic table illustrating the baseline characteristics and inflammatory (cytokine) assessment for the five derived COPD clusters.

Table 3.7b: Table illustrating significance (p-values) for each of the assessed cytokines between the clusters (including post-hoc analysis).

Table 4.1: Participant characteristics for the non-diseased (healthy) control and COPD cohorts.

Table 4.2: Systemic specific IgE (sIgE) binding (expressed as OD intensity) for each of the tested allergens across the within ‘sensitised’ South-East Asian COPD patient groups (clusters).

Table 4.3: Demographics comparing the three ‘sensitised’ South-East Asian patient groups

(clusters).

Table 4.4: Demographics of the COPD patient cohort where home visits were conducted including outdoor air, indoor air and surface sampling (n=11).

Table 5.1: Summary of the selected primer sets used to examine the efficiency of amplification of a fungal mock community.

Table 5.2: The fungal isolates employed to make up a ‘fungal mock community’ to examine and optimize the PCR amplification process and to choose the optimal primers for use in mycobiome sequencing experiments.

Table 5.3: Summary of (a) fungal and (b) bacteria genera identified as contaminants using the “Decontam” R package.

Table 5.4: Demographic table summarising the non-diseased (healthy) and COPD cohorts from Singapore / Kuala Lumpur (SG/KL) and Dundee (DD).

Table 5.5: Tables summarizing network analyses based on fungal genera comparing very frequent (vFE) with non-frequent (non-FE) COPD exacerbators.

Table 5.6: Demographic table of COPD patients in cluster 1 (n=178) and cluster 2 (n=159) respectively based on airway mycobiome profiling.

Table 5.7: Demographic table comparing COPD survivors (alive at 2-year follow up) (n=51) versus non-survivors (dead at 2-year follow up) (n=15) from an acute exacerbation of COPD (AECOPD) cohort.

ABBREVIATIONS

16S rRNA:	16S ribosomal RNA
AAT :	Alpha-1 antitrypsin
ABPA :	Allergic bronchopulmonary aspergillosis
ABPM :	Allergic bronchopulmonary mycoses
ACO :	Asthma-COPD overlap
AECOPD:	Acute exacerbation of COPD
BAL :	Bronchoalveolar lavage
BCO :	Bronchiectasis-COPD overlap
BDR :	Bronchodilator response
<i>Bla g</i> :	<i>Blattella germanica</i>
<i>Blo t</i> :	<i>Blomia tropicalis</i>
BMI :	Body mass index
BSA :	Bovine Serum Albumin
CART :	Classification and Regression trees
CAT :	COPD Assessment Test
CF :	Cystic fibrosis
CI :	Confidence interval
cm :	Centimetre
COPD :	Chronic Obstructive Pulmonary Disease
COTE :	COPD specific co-morbidity test
CRP :	C-reactive protein

CT	:	Computed tomography
CVS	:	Cardiovascular
CXCL	:	CXC-chemokine ligand
CXCR	:	CXC-chemokine receptor
DD	:	Dundee
<i>Der f</i>	:	<i>Dermatophagoides farinae</i>
<i>Der p</i>	:	<i>Dermatophagoides pteronyssinus</i>
DNA	:	Deoxyribonucleic acid
DRP1	:	Dynamic-1- like protein
DSM-V:		The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
ECM	:	Extracellular matrix
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Epidermal growth factor
EGFR	:	Epidermal growth factor receptor
eGFR	:	Estimated glomerular filtration rate
FE	:	Frequent exacerbator
FEV ₁	:	Forced expiratory volume in the first second
FVC	:	Forced vital capacity
GERD	:	Gastro-esophageal reflux disease
GINA	:	Global Initiative of Asthma
GOLD	:	Global initiative for chronic obstructive lung disease
HbA1c	:	Haemoglobin A1c
HDM	:	House dust mite

HIV	:	Human immunodeficiency virus
HR	:	Hazard ratio
HRCT	:	High-resolution computed tomography
ICAM-1:		Intercellular Adhesion Molecule 1
ICS	:	Inhaled corticosteroid
Ig-E	:	Immunoglobulin-E
IL-	:	Interleukin
LC3B-II:		Microtubule-associated protein 1 light chain 3B phosphatidylethanolamine conjugate
IPA	:	Invasive pulmonary aspergillosis
IPTG	:	Isopropyl β -D-1-thiogalactopyranoside
IQR	:	Interquartile range
ITS	:	Internal Transcribed Spacer
KDE	:	Kernel density estimation
LABA	:	Long-acting beta agonist
LAMA	:	Long-acting muscarinic antagonist
LCHR	:	Low co-morbidity high risk
LCLR	:	Low co-morbidity low risk
LDA	:	Linear Discriminant Analysis
LEfSe	:	Linear discriminant analysis effect size
LLN	:	Lower limit of normal
LOOCV:		Leave one out cross validation
MAPK	:	Mitogen-activated protein kinase

MCP	:	Monocyte chemoattractant protein
MMP	:	Matrix metalloproteinase
mMRC	:	Modified British Medical Research Council questionnaire
MPO	:	myeloperoxidase
NE	:	Neutrophil elastase
NF-KB	:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMDS	:	Non-metric multidimensional scaling
Nrf-2	:	Nuclear factor erythroid 2-related factor -2
ns	:	Not significant
OR	:	Odds ratio
PaO ₂	:	Partial pressure of oxygen
PBS	:	Phosphate-buffered saline
PCoA	:	Principle Coordinate Analysis
PCR	:	Polymerase chain reaction
PDE-4	:	Phosphodiesterase-4- inhibitor
PDGF	:	Platelet derived growth factor
PERMANOVA	:	Permutational multivariate ANOVA
PINK1	:	Phosphatase and tensin homolog-induced putative kinase-1
PPM	:	Potential pathogenic microorganism
PTBLD	:	Post-tuberculosis lung disease
rAsp f	:	Recombinant <i>Aspergillus fumigatus</i>
RDA	:	Regularised Discriminant Analysis
RIPK3	:	Regulator receptor- interacting protein kinase 3

ROS	:	Reactive oxygen species
RSV	:	Respiratory syncytial virus
SABA	:	Short-acting beta agonist
SAMA	:	Short acting muscarinic antagonist
SEA	:	South-East Asian
SDS-PAGE:		Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SG/KL	:	Singapore/Kuala Lumpur
TB	:	Tuberculosis
TGF	:	Transforming growth factor
Th-	:	T-helper cell
TLR-	:	Toll-like receptor-
TMB	:	Tetramethyl benzidine
TNF	:	Tumour necrosis factor
Treg	:	Regulatory T cells
VEGF	:	Vascular endothelial growth factor
vFE	:	Very frequent exacerbator
WHO	:	World health organization

SUMMARY

Chronic obstructive pulmonary disease (COPD) is a common chronic respiratory disease with increasing prevalence in Asia. It is associated with significant morbidity, socioeconomic burden and mortality. In Asia, the variation in risk factor exposure and ethnogenetic background results in a diverse disease presentation and response to treatment. Therefore, a need exists to better understand disease phenotypes in Asia for better future targeted COPD therapy and precision care across the continent. The overall aim of my thesis is to define the clinical, immunological and microbiological phenotypes of COPD in South-East Asian patients.

Using clinical parameters including comorbidities, I first define novel clusters of COPD with differing outcomes that associate with systemic inflammation in Chinese COPD patients recruited from Singapore, Hong Kong and Malaysia (Chapter 3: Tiew *et al*, Chest 2020). Five distinct clinical clusters (using derivation and validation cohorts) were identified with poorest (two-year) survival in the cardiovascular and ex-tuberculosis clusters. These clusters were accompanied by complex and unique inflammatory networks. Our identified clusters complement existing COPD assessment tools (GOLD), identifying those at risk of highest mortality despite being classified as ‘low risk’ by traditional COPD assessment.

Next, I illustrate an association of clinical outcomes with immunological phenotypes (allergic sensitisation) in Chinese COPD patients recruited from the same countries (Chapter 4; Tiew *et al*, European Respiratory Journal 2020). Sensitisation was further assessed in relation to both indoor and outdoor environments. High frequencies of sensitisation are observed in COPD compared to non-diseased controls, and very frequent exacerbators demonstrate fungal sensitisation, an important Asian phenotype. Cluster analysis reveals three sensitisation patient groups, where the ‘highly sensitised fungal predominant’ group demonstrates poorest clinical outcomes including low lung function, higher symptoms and more exacerbations. When sensitisation was assessed in relation to outdoor air fungi, increased exacerbations were associated with a demonstrable sensitisation to fungi detected in outdoor air. In a subset of

COPD patients, indoor air (at their homes) was collected and analysed through metagenomic sequencing. Interestingly, increased indoor air allergens (predominantly fungal allergens) were also correlated with COPD symptoms and poorer lung function. Collectively these suggest the importance of allergy and in particular fungal sensitisation in South-East Asian (Singapore, Malaysia and Hong Kong) COPD where sensitisation is closely linked to environmental exposures in this region.

Having identified that fungal sensitisation in Asian COPD is related to poor clinical outcomes, I next investigated the airway mycobiome (fungal microbiome) and its clinical associations by comparing cohorts of COPD patients recruited in Singapore, Malaysia and the United Kingdom (Chapter 5; Tiew *et al* European Respiratory Journal 2020). I performed the largest multicentre evaluation of the COPD airway mycobiome to date, including longitudinal assessment during the stable state, at exacerbation and during recovery (post exacerbation). Distinct mycobiome profiles were observed in COPD compared to non-diseased (healthy) controls and varied between very frequent and non-frequent exacerbators. Further differences were observed in airway mycobiome profiles based on country of origin (Singapore/Malaysia: Asian versus United Kingdom: non-Asian). No differences in the airway mycobiome were identified by longitudinal analysis during the stable state, at exacerbation and at post exacerbation despite oral antibiotic and/or corticosteroid treatment. Unsupervised clustering resulted in two clinically distinct patient groups, one with increased symptoms and *Saccharomyces* dominance and a second with frequent exacerbations and higher mortality characterized by *Aspergillus*, *Penicillium* and *Curvularia* with a concomitant increase in serum specific IgE levels against these very same fungi. During acute exacerbations of COPD, changes in mycobiome composition with increased α -diversity was associated with higher two-year mortality, illustrating the prognostic value of assessing the mycobiome particularly in South-East Asian (Singapore and Malaysia) COPD. The COPD mycobiome is therefore characterized by specific fungal genera associated with exacerbations, poor survival and sensitisation responses in South-East Asian (Singapore and Malaysia) patients. In addition, the airway mycobiome during

AECOPD reveals signatures with prognostic implication. As for bacterial microbiome, decreased diversity was detected in frequent COPD exacerbators, suggesting a potential role in COPD progression.

In summary, South-East Asian patients with COPD exhibit distinct clinical, immunological and microbiological phenotypes with all demonstrate association with poor clinical outcomes. Importantly, these phenotypes represent ‘treatable traits’ in South-East Asian COPD with a strong potential to inform clinical decision making, attract new clinical trials and potentially alter clinical practice guideline recommendations in the future with the inclusion of greater numbers and focused Asian COPD studies and patient groups.

CHAPTER 1: INTRODUCTION

1.1 Background

COPD is a heterogeneous disease with varying patient responses to guideline directed treatment. Despite treatment, the disease progresses with no available cure. The natural course of COPD fluctuates with high inter-individual variability, which in turn translates into variable treatment responses between patients despite adherence to “standardized” treatment derived from the best available evidence-based guidelines. Of note, such guidelines are derived from evidence collated mainly from non-Asian populations and most clinical trials in COPD do not include Asian patients. This leaves a significant knowledge gap in our understanding of COPD in Asia and particularly in the South-East Asian (SEA) region. In Asia, the variations in risk factor exposure and ethnogenetic background results in varying disease phenotypes that are currently overlooked by existing COPD guidelines. COPD-associated risk factors and clinical care in Asia also differs between countries affected by local resources, healthcare policies, cultural belief and practices (1).

The awareness of COPD remains low in Asia. Studies conducted in Japan and Korea found that less than 25% of the population had ever heard of COPD, and concerning a proportion of these patients were smokers with COPD related symptoms (2, 3). In rural areas of China, > 95% of COPD patients have never heard of the term COPD, and the majority were not on guideline directed treatment such as vaccinations, inhaled therapy and/or pulmonary rehabilitation (4). Cultural beliefs and socioeconomic status play a significant role in the health seeking behaviours in Asian patients: they perceive their symptoms as a result of ageing, the preferential of alternative medicines or home and traditional remedies, financial concern, fear of side effects and dependence on medication (5, 6). These factors also contribute to a lower number of inhaled therapies used, under- and delayed diagnosis of COPD, resulting in an overall increased mortality of COPD in the Asian sub-continent (7).

Indoor and outdoor pollution are important risk factors for COPD in Asia. The use of biomass fuel, an important energy source particularly in rural Asia, predisposes to COPD, and remains a major cause of COPD in non-smokers (8). Rapid urbanization in the Asia-Pacific region has resulted in poor air quality, increased allergic sensitisation and a higher risk of exacerbations and hospitalizations for COPD (9-11). In addition, the effect of incense burning, a popular religious practice across Asia, and its association with COPD is less well recognized and studied. It has been shown that incense burning releases high amounts of pollutants including particulate matter, carbon monoxide, sulphur dioxide, volatile organic compounds and nitrogen dioxide resulting in increased respiratory symptoms (12-14). Urbanization with poor air quality also further contributes to increased allergic sensitisation across Asia, with modulation of the immune system towards high susceptibility of allergic disease (15). Allergic sensitisation is reported in COPD, however, its role on disease progression and COPD outcomes remain undetermined (16, 17). Furthermore, a significant lack of data on allergic sensitisation in SEA COPD exists. Other common risk factors for COPD in Asia includes the high endemic prevalence of tuberculosis in several regions, occurrence of bronchiectasis and a range of occupational exposures (18-20).

The clinical characteristics of Asian COPD include low body mass index (BMI), a higher prevalence of male-related COPD and lower reported symptoms compared to non-Asian cohorts (18, 21-23). Despite the known differences exhibit in Asian COPD, the current GOLD guidelines do not provide any additional recommendation for Asian COPD patients. Higher adverse effects with COPD treatments, such as a high risk of pneumonia with inhaled corticosteroid (ICS) use and an increased risk of adverse effects and drug discontinuation with roflumilast due to the low BMI in Asian COPD warrants dedicated attention (22, 24-26). Dose adjustment should be considered in Asian COPD patients. In addition, ICS recommended for eosinophilic COPD should only be initiated after excluding parasitic infections which are more common in Asia and in themselves associate with eosinophilia (18).

Considering such unique factors and features of disease, it is likely that different COPD phenotypes exist across the region. Given such variations, there is a clear need to better characterise and phenotype COPD in Asia and uncover ‘treatable traits’ to impact care delivery. Therefore, the central aim of my thesis is to define clinical, immunological and microbiological phenotypes of COPD in the South-East Asian region, all of which have clear relevance for clinical translation and improvement to patient care.

1.2 Research gaps and Challenges

There remains lack of data focused on COPD phenotypes in South-East Asian populations, largely contributed by the low awareness and resource allocation to this disease. Multiple different COPD clinical phenotypes are identified in non-Asian settings, however, their applicability in the SEA context remains unknown. While some COPD phenotypes maybe similar and unrelated to country of origin or ethnicity, it remains uncertain whether differences do in fact exist in SEA COPD considering the variations described above.

Allergic sensitisation is shown to differ across geographic regions. Recently, our group has illustrated high sensitisation rates in non-cystic fibrosis bronchiectasis with variation in sensitisation patterns between SEA and European cohorts (27). This study clearly illustrates the importance of geographical differences in sensitisation profiles in bronchiectasis, however, it remains unknown if such comparable variation and difference occurs in SEA COPD. In addition, the influence of environmental allergen exposure on SEA COPD patients remains unexplored. Understanding sensitisation profiles and their potential influence on clinical COPD outcomes may uncover important SEA COPD phenotypes that may be amenable to a targeted treatment approach.

Infection remains an important cause of COPD exacerbations. Recent advances in sequencing technologies has enabled the identification of unculturable airway microorganisms with high precision. Most current airway microbiome studies have focused on bacterial communities, which have been used to phenotype COPD exacerbations, with increasing data supporting the importance of the microbiome in COPD pathogenesis and its related clinical outcomes including exacerbations. Critically, however, these studies are mainly derived from non-Asian cohorts with limited bacterial microbiome studies, performed on SEA COPD patients. Indeed, bacterial profiles are different using conventional culture methods during AECOPD with increased detection of *Pseudomonas* and *Klebsiella* in Asian patients, while *Haemophilus*, *Streptococcus* and *Moraxella* were more common in non-Asian COPD populations (28-31). Importantly, the role of fungal microbiome (the mycobiome) remains largely unexplored in COPD with no available data in SEA COPD patients. The tropical climate across most parts of SEA further predisposes to fungal persistence in the surrounding environment which can potentially impact the airway mycobiome and fungal sensitisation patterns in SEA patients with COPD, and therefore warrants investigation.

1.3 Objectives of this thesis

The objectives of this thesis are:

Chapter 3: To define clinical and inflammatory phenotypes in South-East Asian COPD patients, and determine if such phenotypes associate with mortality and clinical outcomes including their relation to existing COPD (GOLD) guidelines.

Chapter 4: To determine the immunological (allergic sensitisation) phenotype in South-East Asian COPD patients and further associate this to environmental allergens and clinical COPD outcomes.

Chapter 5: To elucidate the microbiological (airway microbiome and mycobiome) phenotype in South-East Asian COPD patients by evaluating geographic variation with non-Asian COPD cohorts and assess any clinical impact of such phenotypes including association with mortality.

CHAPTER 2: LITERATURE REVIEW

2.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a common chronic respiratory condition in adults and results in significant morbidity, mortality and socioeconomic burden (32, 33). COPD is characterized by “persistent airflow limitation that is progressive and associated with an enhanced chronic inflammatory response in the airways to noxious particles and gases” (34). Although smoking is a major risk factor for developing COPD, only 15-20% of smokers develop COPD illustrating the importance of other factors in its development (35). Moreover, despite smoking cessation, the disease progresses with no reliable or curative treatment. The precise mechanisms conferring susceptibility and/or leading to COPD and its subsequent progression remain to be fully understood. Several factors play important roles in disease progression, including persistent airway and systemic inflammation, sensitisation, chronic microbial infection and alterations to the airway microbiome (29, 36, 37).

Emphysema and chronic bronchitis are frequently used to describe COPD in its old classification (38). It is now clear that they only represent a subgroup of COPD and are not necessarily present in all COPD patients. Emphysema is a pathological diagnosis defined by a permanent enlargement of the distal airspace secondary to alveolar wall destruction which results in a loss of elastic recoil (38). Emphysema can occur without airflow obstruction. Chronic bronchitis is a clinical term used to describe the presence of a chronic cough with sputum production for at least 3 months each year in 2 consecutive years (38). Chronic bronchitis can occur without or preceding the development of COPD. The presence of chronic bronchitis in COPD has been associated with an increased risk of and severity of exacerbations (39).

2.1.1 Epidemiology

The World Health Organisation (WHO) estimates that 65 million people worldwide have moderate to severe COPD with a prevalence of 251 million cases reported in 2016 (7). COPD accounts for 5% (approximately 3.17 million) of all deaths globally with the highest mortality in low to middle income countries (7). The reported prevalence of COPD varies across countries with 8.5-18% in USA, Australia and Canada, while prevalence in Asia is between 3.5-13.7% (40). The lower reported prevalence in Asia is largely attributed to the paucity of available data rather than the true occurrence of COPD.

COPD results in a significant socioeconomic burden to both patients and their healthcare system (34). The total medical cost of COPD in the United States was estimated to be \$32.1 billion in 2010 and is projected to increase to \$49 billion by 2020 (41, 42). In Asia, the medical cost per patient is reported to be between \$2,803 and \$3,008, as reported in Korea and Taiwan (43, 44).

In Singapore, COPD was the 9th leading cause of death in 2018 and accounts for more than 3,000 annual hospital admissions, with an average length of stay of 3 days (45, 46). It is also the 9th leading cause of disability adjusted life years lost for males in Singapore aged 44-65 (45, 47). Hospitalisation is the major contributor to the financial burden of COPD, accounting for 70-90% of total disease health care costs (48). In a study of 2 out of the 3 health care clusters in Singapore, the mean total cost of COPD stood at S\$9.9 million per year to the health care system (48). At the level of the individual, a typical uncomplicated COPD admission in a public hospital will cost the patient approximately SGD\$2,000 (45).

Considering the ageing demographic and urbanization in Asia, the prevalence of COPD is expected to increase. This will have a significant impact on health care systems with a greater disease burden and health care utilisation.

Globally there are approximately 1.1 billion current smokers (the leading risk factor for COPD), of which each year 6 million deaths alone are attributed to the effects of tobacco (49). It is

estimated that by 2030, the numbers will increase to 2 billion despite overall decreases in smoking prevalence (50). This is contributed by the overall increase in the world's population (51-53). The national health survey in Singapore found that smoking rates among Singaporeans in 2017 were 12%, with the highest rates in males (54). A greater proportion of smokers were of the Malay ethnic group, concerning of younger age and with lowest levels of education (55). This reflects contrasting patterns even within our Singaporean multi-ethnic population where smoking habits, and therefore, COPD prevalence itself varies.

While smoking remains the leading risk factor for the occurrence of COPD, up to 40% of COPD patients are non-smokers (56). Other risk factors include occupational exposure, indoor air pollution, recurrent childhood respiratory tract infections, pulmonary tuberculosis, intrauterine growth restriction, and poorer socioeconomic status (38). These factors are prevalent across the Asian subcontinent and warrant attention.

2.1.3 The diagnosis and clinical assessment of COPD

The diagnosis of COPD requires the presence of persistent airflow limitation as evidenced by spirometry showing a post bronchodilator $FEV_1/FVC < 0.7$ in patients with respiratory symptoms and/or risk factor exposure (38). Following diagnosis, the clinical assessment of COPD includes determining the severity of airflow obstruction, the impact on patient health and the risk of future events including exacerbations. Severity of airflow obstruction is determined by lung function (FEV_1 % predicted), graded based on GOLD 1 through 4 (Table 2.1) (38). Lung function severity may be used for COPD prognostication and to determine use of interventional procedures such as bronchoscopy and/or surgical lung volume reduction as described in detail in Chapter 2.1.6.1. An alternate classification for COPD is GOLD ABCD. This assesses health status and future exacerbation events, based on reported patient symptoms using the COPD assessment test (CAT) and/or Modified British Medical Research Council (mMRC) dyspnoea questionnaire, and exacerbation history. GOLD ABCD is used in clinical practice to guide

pharmacotherapy (38) (Figure 2.1). This is in contrast to GOLD grade which focus on FEV₁ % predicted alone to determine disease severity for prognostication.

Table 2.1: GOLD grading of COPD based on measured lung function.

GOLD Grade: classification of airflow limitation in COPD “In a patient with FEV₁/FVC<0.7”:	
“GOLD 1: Mild”	“FEV ₁ ≥ 80%predicted”
“GOLD 2: Moderate”	“FEV ₁ 50-79% predicted”
“GOLD 3: Severe”	“FEV ₁ 30-49% predicted”
“GOLD 4: Very Severe”	“FEV ₁ <30% predicted”

FEV₁: Forced expiratory volume in the first second, FVC: Forced vital capacity. GOLD: Global Initiative for Chronic Obstructive Lung Disease. Adapted from GOLD 2018 (38)

Figure 2.1: GOLD grouping of COPD (A-D) based on patient symptoms and exacerbation history. mMRC: Modified British Medical Research Council questionnaire, CAT: COPD Assessment Test. Adapted from GOLD 2018 (38)

COPD is often diagnosed when patients are symptomatic, with excessive loss of lung function. At this stage, the condition is irreversible, and treatment focused on disease control, preventing further exacerbations, maintaining quality of life, and delaying disability associated with the disease.

It was previously thought that lung function decline rapidly leads to symptoms and progression of COPD in susceptible individuals exposed to noxious particles and/or smoking. Consequently, interventions focused on preventing such declines in lung function were employed in hope to halt disease progression, but, with disappointing results on overall prognosis (57-59). It is now clear that varying trajectories of lung function decline exist for each individual COPD patient with ensuing differing extents and speeds of lung function decline (60). This highlights the inherent heterogeneity in COPD, and a need to better define COPD phenotypes, endotypes and

endophenotypes that allow the identification of ‘treatable traits’ that may be amenable to therapeutic intervention in individual patients (61).

2.1.4 Pathogenesis

The precise mechanisms leading to COPD remain largely unknown. Smoking is the main risk factor for the development of COPD, however, despite smoking cessation, airway inflammation persists suggesting alternate mechanisms involved in perpetuating airway inflammation, including chronic infection and altering the airway microbiome (37, 62-64). Nevertheless, several factors have been linked to COPD pathogenesis, including an abnormal airway and systemic inflammatory response, protease-anti-protease imbalances, increased oxidative stress, mitochondrial dysfunction and alterations in lipid metabolism (Figure 2.2) (38).

Airway inflammation in COPD. Airway inflammation in COPD involves the recruitment of neutrophils, macrophages, and lymphocytes. Stimulation of airway epithelial cells and macrophages results in the release of neutrophil chemoattractants including CXCL1 and CXCL8, with subsequent recruitment of neutrophils and their release of proteinases, leading to a vicious cycle of ECM destruction and further inflammation (65). Macrophage activation induces ROS production, chemokine, and cytokine release including MCP-1, MMP-9 and MMP-12, which results in additional monocyte recruitment and lung destruction (65, 66). In addition, T-cells play an important role in COPD. Activated cytotoxic CD-8 T-cells release perforin and granzyme, resulting in cell apoptosis, which is increased in COPD airways compared to non-smokers (67). Furthermore, increased expression of CXCR3 and its ligand CXCL10 are observed in type-1 lymphocytes in COPD compared to non-smoking controls. This induces production of MMP-12 by macrophages and potentiates further tissue destruction (68, 69). The increase in airway inflammation observed in the COPD induces structural change with airway remodelling, that importantly includes small airways disease and obstruction in COPD (70).

The protease-antiprotease imbalance. Exposure to noxious particles particularly cigarette smoking activates the host immune response with a consequential influx of inflammatory and immune cells including neutrophils and macrophages (71). The activated neutrophil (and macrophage) releases proteases including neutrophil elastase (NE), proteinase 3, cathepsins and matrix metalloproteinases (MMPs) causing extracellular matrix (ECM) destruction and resulting in the development of emphysema (71). This particular mechanism is best evidenced by a genetic cause of COPD: alpha 1-antitrypsin (AAT) deficiency. AAT deficiency is an autosomal dominant disorder and key genetic cause of COPD, where increased neutrophilic inflammation and NE activity in the lung coupled with decreased AAT levels (an anti-protease) leads to emphysema of early onset in young sufferers, particularly if they smoke (72).

Increased oxidative stress. Exposure to cigarette smoking triggers lipid peroxidation and the production of reactive oxygen species (ROS). ROS then activates various transcription factors including nuclear factor kappa B (NF- κ B), c-Jun N terminal kinases and p38-mitogen-activated protein kinase (MAPK), leading to transcription of pro-inflammatory genes, which in turn, amplify airway inflammation (66). Nuclear factor erythroid 2-related factor 2 (Nrf-2) is a further key transcription factor that activates an antioxidant gene response, and critically regulates anti-oxidant defences. Increased apoptosis and oxidative stress induced cell death are also observed in the pneumocytes of Nrf-2 knock out mice, leading to a susceptibility towards the development of emphysema and associated lung inflammation, particularly with cigarette smoke exposure (73, 74). In COPD, expression of Nrf-2 on bronchial epithelial cells are lower than control subjects, suggestive of impaired anti-oxidant defence systems (75). Collectively, these suggest importance of the oxidative pathway and Nrf-2 in COPD pathogenesis.

Mitochondrial dysfunction. Mitochondria are vital organelles involved in cellular metabolism, homeostasis, intracellular signalling, energy generation and cell death. Mitochondrial dysfunction is associated with increased ROS production, apoptosis and autophagy, decreased ATP and an alteration in the airway morphology detected following cigarette smoke exposure (76, 77). Dose related responses to cigarette smoke extracts are observed in human epithelial

cell culture models, which demonstrate increased apoptosis at low concentrations. High concentrations result in necrosis and impaired apoptosis (76, 77). Autophagy, a programmed cellular-protein degradation process is activated by chronic cigarette smoke exposure, evidenced by the increased detection of the autophagosome marker microtubule-associated protein-1 light chain 3B phosphatidylethanolamine conjugate (LC3B-II) in pulmonary epithelial cells. Mitophagy is a form of autophagy to facilitate turnover of mitochondria (76). Dose dependent increases in mitophagy proteins, including phosphatase and tensin homolog-induced putative kinase-1 (PINK1), mitochondrial fission regulator, dynamic-1-like protein (DRP1) and the necroptosis regulator, receptor- interacting protein kinase 3 (RIPK3) are observed following cigarette smoke exposure (76, 78, 79). Mice with PINK1 deficiency are protected against mitochondrial dysfunction and emphysema associated with cigarette smoke exposure, suggestive of the involvement of cigarettes smoke induce mitophagy in COPD pathogenesis (79).

Lipid metabolism. Alterations to lipid metabolism has recently been shown to be involved in COPD pathogenesis. Cigarette smoke exposure enhances the activation of fatty acid oxidation pathways, resulting in ROS release and increased cell death. In addition, lipid mediators such as prostanoids, leukotrienes and resolvins, including oxidized cholesterol are all shown to induce airway inflammation in COPD (80-82). Ceramide is a basic unit of sphingolipids present in cell membranes and is involved in multiple cellular functions including signalling. High lung ceramide levels are found in smokers and patients with COPD, with a resulting increase in oxidative stress and cell apoptosis (83-85). In a mouse model, instillation of intratracheal ceramide resulted in emphysematous destruction, while an inhibition of ceramide biosynthesis prevented such damage (86). Overall, taken together, these observations suggest the potential role of lipid metabolism in the development of COPD.

Figure 2.2: COPD pathogenesis.

Exposure to cigarette smoke and noxious particles activates immune cells with an influx of macrophages and neutrophils leading to the release of proteases (neutrophil elastase (NE), matrix metalloproteinases (MMPs)) and proinflammatory cytokines (TNF- α , IL-1, IL-6, IL-8). In addition, lipid peroxidation and a release of reactive oxygen species (ROS) occurs which induces transcription of pro-inflammatory genes amplifying airway inflammation. Activated cytotoxic CD-8 T-cells release perforin and granzyme resulting in cell apoptosis, and, airway epithelial cells release growth factors (TGF- β , VEGF, EGF) leading to fibroblast proliferation. Collectively these mechanisms result in airway inflammation, alveolar destruction, and airway remodelling in the COPD lung. TNF- α : Tumour necrosis factor alpha, IL: interleukin, TGF: Transforming growth factor, VEGF: vascular endothelial growth factor, EGF: Epidermal growth factor.

2.1.5 Pathophysiology

The main clinical characteristic of COPD is the expiratory airflow obstruction. Airflow obstruction occurs because of chronic airway inflammation leading to mucus hypersecretion, small airways disease and parenchymal destruction.

Mucus hypersecretion is defined as chronic cough and sputum production. In COPD, exposure to noxious particles causes epithelial cell destruction, airway inflammation and oxidative stress, leading to the activation of epidermal growth factor receptors (EGFRs) and a cascade of signalling pathways resulting in goblet cell hyperplasia, mucosal gland hypertrophy and mucus hypersecretion (87, 88). The accumulation of excessive airway mucus causes luminal obstruction and airflow limitation (87). The presence of chronic mucus hypersecretion in COPD is associated with increased exacerbations, lung function decline and poorer quality of life (87-89).

Small airways disease. The concept of small airways disease (i.e. those <2mm diameter) was first introduced in 1968. Hogg and colleague found an increase in small airways resistance of 4-40 times in the emphysematous lung by wedging a small catheter into the peripheral airway of an explanted healthy and emphysematous lung. In addition, with increased mucus plugging, a narrowing and destruction of the small airway is seen in an emphysematous lung (90). This group further demonstrated an increase in the number of inflammatory cells and lymphoid follicles in the small airways with COPD progression (91). Increased small airway inflammation, mucus plugging with airway narrowing, obliteration and remodelling, all contribute to the airflow obstruction and gas trapping observed in COPD (38). Collectively this is termed small airways disease (92). Using micro-CT imaging, McDonough *et al.* (2011) further strengthened these findings by demonstrating a decrease in the absolute number of small airways, and narrowing with wall thickening of the terminal bronchioles in COPD patients (93). In addition, this phenomenon occurs before the onset of emphysematous destruction, and therefore, is suggested to be the precursor to the development of emphysema (92, 93).

2.1.6 COPD Treatment: The stable and acute exacerbation states

Treatment of COPD has advanced over the past decade. Previously, it was solely based on grading lung function before the introduction of GOLD ABCD in 2011, which incorporates symptoms and exacerbation risk in treatment decisions (94). Furthermore, there is increasing evidence supporting targeted therapy, treating individual patients based on disease phenotype. For instance, eosinophilic inflammation and frequent exacerbators with inhaled corticosteroids or phosphodiesterase-4 (PDE-4) inhibitors for those with chronic bronchitis and exacerbations. Those with emphysema predominant disease may be considered for surgical intervention (38, 95, 96). Current treatment guidelines in COPD do not distinguish between Asian and non-Asian populations, despite known clinical differences and potential variations in both treatment response and associated adverse effects.

2.1.6.1 Non-pharmacological treatment

The treatment of COPD can be divided into non-pharmacological and pharmacological. Nonpharmacological treatments include smoking cessation, vaccination, pulmonary rehabilitation, oxygen, and non-invasive ventilation, and where relevant, endoscopic intervention and even surgery.

As smoking remains the leading risk factor for COPD, smoking cessation is a key intervention and has the greatest influence on the natural course of COPD, resulting in the reduced rate of lung function decline and improved survival (97, 98). While greater benefit is seen in individuals who quit smoking at an earlier age, those quitting when they are older still benefit from smoking cessation with lower mortality recorded compared to older adults that continue to smoke (98). In addition, lung function declines in the ‘sustained quitter’ are comparable to those who have never smoked (99). Despite its benefits, the long-term success rate of smoking cessation is about 25%, with highest success in those with intensive smoking cessation programs that are combined

with behavioural support and pharmacotherapy (38, 100). Variation in smoking cessation rates is also observed across Asia, with <10% in China and India, while more than 20% achieved in Korea, Singapore and Taiwan (101). The differences in smoking cessation campaigns and policy may also have accounted for the observed variation. The availability, accessibility and affordability of smoking cessation services vary across countries (102). For instance, smoking cessation clinics are mainly available in secondary and tertiary hospitals, and treatment is not integrated into primary care in China (103). In Singapore, the smoking cessation counselling and medications are self-funded, without government subsidies. This may contribute to high attrition and low utilisation of such programmes (104). In addition, each country has its own regulations on smoking in public places, age limits related to smoking and taxes implemented on cigarettes purchased (102).

Influenza and pneumococcal vaccinations are recommended for all COPD patients with current evidence supporting a decrease in exacerbations and occurrence of pneumonia (105, 106). The benefit of pneumococcal vaccination is particularly relevant for COPD patients <65 years of age and who have severe airflow limitation (107). In Asia, the uptake of vaccination remains low, with variation in the perception of vaccination, including its efficacy, side effect profile, perceived vulnerability and lack of recommendations by physicians (108).

Pulmonary rehabilitation is a tailored intervention including education, exercise and self-management to enhance the adherence to medication and promote physical and psychological patient well-being for those with chronic respiratory diseases including COPD (109). The program is tailored to an individual's need, with optimum effect observed if performed for at least 6-8 weeks (38). Pulmonary rehabilitation improves overall health quality, exercise capacity, reduces symptoms, systemic inflammation and readmission rates for exacerbations (110-113). Despite the known benefits associated with pulmonary rehabilitation, uptake remains generally low globally, and particularly in Asia (114-116). The lack of knowledge on programs itself, their beneficial effect, sustainability of the benefits, compliance, accessibility of the rehabilitation centre, reproducibility with web based program and financial cost are all commonly cited as

limiting factors (117).

Long term oxygen therapy is prescribed to COPD patients with severe hypoxemia for at least 15 hours a day to confer a mortality benefit (38, 118). Severe resting hypoxemia is defined as a $\text{PaO}_2 < 55 \text{ mmHg}$ or $< 60 \text{ mmHg}$ with polycythaemia and/or pulmonary hypertension. In this group of patients, oxygen therapy when used appropriately can improve mortality outcomes (38, 118, 119).

Non- invasive ventilation is reserved for use in patients with concomitant obstructive sleep apnoea and/or those with persistent hypercapnia ($\text{PaCO}_2 > 52 \text{ mmHg}$) following recent hospitalisation (120-122). It has been shown to reduce readmission rates and improve COPD survival (121-123).

Other non-pharmacological interventions for COPD include surgical intervention with lung volume reduction surgery, bullectomy, lung transplant and/or bronchoscopic intervention with endobronchial valves, coils and vapor ablation. These therapies are shown to improve exercise capacity and health status, with some demonstrated improvement in lung function. However, each intervention has specific indications and must be considered carefully based on an individual patient need, disease severity and risk-benefit profile (38, 59, 124-126).

Despite optimal treatment, COPD patients with advanced disease often suffer persistent symptoms, with a decreased in quality of life. Palliative care aimed at symptom relief, nutritional support, advanced care planning including hospice use during end-of life care are all critically important components in the overall holistic management of the end-stage COPD patient.

2.1.6.2 Pharmacological treatment of COPD

Pharmacotherapy in COPD includes various groups of bronchodilators and anti-inflammatory therapies. To date, pharmacotherapy in COPD has failed to show a direct impact on survival but does importantly reduce symptoms, exacerbation frequency and improves overall health status, with relatively weak evidence for lung function improvements (26, 58, 127-130). Therefore, current guidelines advocate treatment based on a patients symptoms and exacerbation risk (38).

Inhaled bronchodilators are a key treatment for COPD where it decreases symptoms and exacerbations. Combination therapy with different groups of bronchodilators are more effective than any single therapy used alone (monotherapy) (26, 131, 132). Combinations of inhaled long acting muscarinic antagonists (LAMAs) and long acting beta-2 agonists (LABAs) are superior to single LABA or placebo, with improvements in lung function recorded. These medications are well tolerated by COPD patients including Asians (133). In the Asia FLAME study, which involved 7 Asian countries, LABA/LAMA combinations were found to be superior in reducing moderate to severe exacerbation, improved lung function and overall health status when compared with the LABA/inhaled corticosteroid (ICS) group (22). Interestingly, when compared to the western COPD cohort, a higher risk of pneumonia was observed in the Asia FLAME cohort which received LABA/ICS (4.8% in western versus 7.7% in Asian cohort) (22, 26). This is likely explained by a lower overall BMI observed in the Asian cohort that predisposes to an increased pneumonia risk.

Anti-inflammatory therapies include ICS, macrolides, phosphodiesterase-4- inhibitors (PDE4) and antioxidants. Anti-inflammatories are generally used in combination with bronchodilators in COPD to decrease exacerbations (38). ICS are usually added to a treatment regimen in patients with a history of frequent exacerbations, concomitant asthma and/or increased blood eosinophil counts (>300 cells/ul), however, ICS use has been associated with an increased risk of pneumonia (95, 134-136). Macrolides are recommended for patients with recurrent exacerbations for up to a one-year duration as prolonged use is associated with increased hearing

impairment, bacterial resistance and prolonged QTC intervals. Their efficacy beyond a year remains uncertain (137, 138). PDE-4 inhibitor therapy is recommended in patients with chronic bronchitis and severe COPD with exacerbations. It is often however, associated with gastrointestinal side effects and weight loss resulting in high withdrawal rates in clinical trials (96). The inherent low BMI in Asian COPD patients with a resultant high risk of adverse events with roflumilast largely explains its low utilization across Asia (24, 25). The role of anti-oxidant agents are less well-established in COPD; however, benefits on exacerbation rates have been observed in select patient groups (139, 140).

In Asia, there is an overall preferential use of oral over inhaled medications for the management of COPD (18). A COPD based population-based survey conducted in 9 different countries in South-East Asia (SEA) (n=4,289) illustrated that more than 50% of COPD patients were on oral medications and only 26% on inhaled medications (141). Dong *et al.* (2015) found excessive prescription of oral xanthine or beta-2 agonists (62.6%) when compared to inhaled bronchodilators (47.5%) in COPD patients (n=4,387) using the Taiwan National Health insurance database (142). The preferential use of oral medications in Asian COPD patients is likely contributed by the ease of oral medications compared to inhalers that require additional education and steps, financial concerns due to the high cost of bronchodilators/inhaler therapies, lack of education and underlying cultural belief that oral medications are somehow superior to inhaled medications. Suboptimal COPD treatment is a major concern across Asia and will necessitate better health care policies coupled with physician and patient education programmes to improve COPD outcomes in the region.

2.1.6.3 Treating acute exacerbations of COPD (AECOPD)

The goal of treating COPD exacerbations is to reduce adverse outcomes and prevent future events. Short acting bronchodilators, oral glucocorticoids and antibiotics are the mainstay of AECOPD treatment (38). In addition, respiratory support may be required in severe exacerbations which includes oxygen and ventilatory (invasive or non-invasive mechanical ventilation) therapy (38). Short-courses of glucocorticoid therapy (for up to 5-day duration) has been shown to improve lung function, oxygenation and promote earlier exacerbation recovery (143, 144). Antibiotics are recommended for bacterial-driven or severe exacerbations with systemic reviews illustrating a mortality benefit (145). It must however, be noted that the how and when to use antibiotics in bacterial COPD exacerbations remains controversial. Prior studies demonstrate that a reduction of symptoms are a good predictor, while others suggest the use of biomarker directed therapy with procalcitonin and/or C-reactive protein (CRP) (146-148). Current recommendations, however do suggest that oral antibiotics are used based on the presence of dyspnoea, increased sputum purulence and volume and/or a requirement for mechanical ventilation (38, 148).

2.2 COPD in the Asian sub-continent

Asia is the largest and one of the most populated continents in the world. With such large and diverse population groups, a wide range of cultural practices and ethnicities exist, potentially influencing the presentation of COPD and its associated phenotypes. For instance, (1) incense burning, practiced by the Buddhist and Hindu religions predominantly, may contribute to poorer indoor air quality and increased respiratory symptoms, (2) an overall lack of health seeking behavior among certain populations with preference for traditional and/or natural remedies which in turn may delay the diagnosis of COPD and its associated comorbidities, (3) dietary variation that influences the gut and respiratory microbiomes and potentially susceptibility to allergic disease, (4) skepticism toward western-based pharmacological medicines including a fear of side effects, leading to non-compliance and overall poorer prognostic outcomes and (5) societal pressures and associated stigmatization of female smokers which in turn contributes to a lower overall prevalence of COPD in females (5, 6, 149-156). In this thesis, my aim is to evaluate the existence of Asian COPD phenotypes with particular focus on the East and South-East Asian (SEA) region where data is more lacking, therefore throughout the thesis, I have used the term “Asian COPD” to refer to the East and South-East Asian geographic region which is the primary focus of the presented work.

2.2.1 Prevalence of COPD

COPD is a major global issue; with burden of disease estimated to be highest in Asian countries, contributed by ageing populations, increased cigarette smoking rates and consumption and poor air quality, the latter secondary to urbanization in the continent's developing regions (157, 158). Prior data has reported the global prevalence of COPD at 10.7% with the highest rates in the Americas and lowest in Asia. (159). In East and Southeast Asia, the prevalence of COPD was estimated to be 6.3% in 2003 which is a likely underestimate of true prevalence (160). This is illustrated by large population studies across Asia, where only 9.4%- 35.0% of patients with COPD received a formal diagnosis (161, 162). This is contributed to by a lack of awareness and underutilisation of spirometry, especially in rural areas, leading to an underestimation of actual COPD prevalence (40). Recent studies have affirmed that and shown significant increases in COPD prevalence across Asia, even far exceeding that observed in western countries. In a large cross-sectional study conducted in China in 57,779 individuals, COPD prevalence across all age groups was 8.6%, accounting for approximately 100 million people. In those above 40 years of age, prevalence rates were even higher at 13.7%, which is significantly higher than previously reported statistics (163). In Korea, the national prevalence of COPD is 13.4%, while in Japan, reported prevalence is at 8.4% (164, 165). Despite an overall dearth of updated studies focused on reporting COPD prevalence across Asia including SEA, the current available data does strongly suggest a trend toward increasing COPD prevalence in the Asian sub-continent.

2.2.2 Are there clinical phenotypes of COPD in Asia?

COPD is a clinically heterogeneous disease with high inter-individual variability in clinical presentation, disease progression and treatment response. In Asia, diverse cultural, genetic profiles and risk factor exposures together likely result in varying COPD phenotypes, which are not considered in existing COPD treatment guidelines. Indeed, existing studies have reported several unique factors associated to COPD in Asian patients but these have lacked focused study.

Smoking remains a key risk factor for COPD across Asia. While the overall rate of smoking is decreasing in higher income countries, its prevalence remains high across Asia particularly in the low-to middle income regions (166). In a recent large prospective study including 20 birth cohorts with >1 million participants from different parts of Asia, smoking related mortality is reported to be increasing among Asian men with a decreased mean age of starting to smoke and an increased number of cigarettes smoked per day (101). With such high smoking prevalence, the numbers of patients with COPD is estimated to further increase across the region.

While smoking remains the major risk factor for the development of COPD, 25-45% of COPD patients are interestingly never smokers (8). Exposure to biomass fuels, outdoor air pollution and occupational exposures are other important risk factors contributing to COPD in the Asian sub-continent (20, 167, 168). In Singapore for instance, it is estimated that 34.4% of COPD patients are non-smokers, a figure higher to that observed in other countries such as the USA (25%) and UK (22.9%) (49, 160). These figures are particularly significant as smokers and non-smokers with COPD have different clinical characteristics and disease outcomes (49, 56). Biomass fuel is the main source of domestic energy in most rural parts of Asia (167). These substances have low combustion efficiency with high indoor air concentration and therefore exposure. A study performed by the Asian Network for Obstructive Lung Disease (ANOLD) included data from 7 Asian cities and 922 patients with COPD. It reported prior exposure to biomass fuel in a significant number (32.1%) of the cohort. Women are at greater risk of such exposures as they are often responsible for daily cooking at home, however, interestingly the

majority of COPD across Asia (>90%) occurs in males. Occupational exposures are another important cause of COPD. In Asia, up to 40% of patients report exposure to toxic gases, dust or fumes at the workplace, which in turn associates with more symptoms and higher COPD severity (20).

A further important characteristic of COPD across Asia is the higher prevalence of pulmonary tuberculosis (18, 169, 170). A history of previous pulmonary tuberculosis is shown to associate with airflow obstruction and accelerated lung function decline especially if recurrent (19, 171, 172). Furthermore, a diagnosis of COPD tends to occur earlier in those with a previous history of tuberculosis (173). Established complications of tuberculosis include bronchiectasis and structural lung change, both of which contribute to an increased susceptibility to non-tuberculous mycobacteria (NTM) and have each in turn been associated to the development of COPD (19, 29, 174). Greater declines in pulmonary function, increased risks of exacerbation and higher mortality are associated with NTM, tuberculosis and bronchiectasis in COPD (175, 176).

An under- or delayed diagnosis of COPD remains a further major challenge to disease management and addressing the poor COPD outcomes across Asia (158, 162, 177). The diagnosis of COPD is often based on clinical symptoms with underutilization of spirometry in most Asian settings. The absence of spirometry availability coupled with a lack of knowledge and skill in primary care settings for performing and interpreting spirometry hinders a proper and formal COPD diagnosis (158). Poor public awareness of COPD is a further factor requiring attention. Poor socioeconomic status across many Asian regions may further contribute to delays in seeking medical attention and therefore a diagnosis of COPD (18). In a large, national, cross sectional study performed in China, spirometry was performed in only 12% of those with documented COPD (162). An epidemiological study in Japan illustrates that up to 90% of those with spirometry confirmed COPD did not receive any prior diagnosis (177). Differences in respiratory symptoms are also observed in COPD across various regions in the Asian sub-continent, with Asians generally reporting less chronic bronchitis like-symptoms of cough and

sputum production (169, 178, 179). This again contributes to additional delays in diagnosis and therefore treatment of COPD in the Asian region.

While there are no direct comparisons between COPD in Asia and Western countries, prior clinical trials clearly illustrate different demographics between global regions. Lower body mass index (BMI) and higher prevalence of male COPD is observed in Asia compared to Western cohorts (Figure 2.3, 2.4). For instance, in the FLAME study, the average BMI for the western cohorts were 27 with 76% of individuals being male, while Asia FLAME had an average BMI of 22.4 with >95% being male (22, 26). Such observations are also noted in other large COPD studies including ECLIPSE, TIOSPIR and TORCH trials in comparison to Asian studies such as ANOLD, HOKKAIDO, Asia TIOSPIR and Asia FLAME (20, 26, 58, 180-183). The higher COPD male prevalence observed in Asia is possibly contributed to the existing social prohibitions and stigmatisation of smoking in females (156). However, with rapid changes to socio-economic status, gender equality and more westernised cultural influences across the Asian region, smoking prevalence and therefore COPD in females is expected to rise in the coming decade (184). BMI is an important prognostic indicator in COPD, and increased mortality is observed in those with the lowest BMI. BMI is also an important component of the composite score used in the BODE index for COPD prognostication (185, 186). It is yet unknown whether the low BMI in Asian COPD patients translates to higher mortality when compared to Western cohorts.

Figure 2.3: Mean body mass index (BMI), comparisons across COPD studies between non-Asians (Western cohorts) (yellow) and Asians (blue). Source: Rhee *et al.* (2019) (18)

Figure 2.4: Percentage of male COPD patients comparing non-Asian (Western cohorts) and Asian studies. Colouration denotes gender: Male (blue), Female (pink)

A further interesting feature across the Asian sub-continent is the high rate of parasitic infection resulting in blood eosinophilia. The eosinophilic phenotype is now well described in COPD and used as a predictor for expected inhaled corticosteroid (ICS) response (95). Current COPD guidelines recommend the use of ICS with blood eosinophil counts of >300 cells/ μ l; however, in Asia, parasitic infections must be out-ruled prior to ICS initiation (38).

Taken together, the clear variation in risk factor exposure, diverse cultural backgrounds and genetic differences across the Asian region likely contributes to specific phenotypes of COPD (Figure 2.5). Given such variation, a clear knowledge gap and need exists to better characterize Asian COPD and its associated phenotypes to improve identification, outcomes and overall patient care.

2.2.3 COPD Exacerbations

Acute exacerbations of COPD (AECOPD) represent acute events with worsening of underlying respiratory symptoms (34). They are associated with increased mortality, risks of hospitalisation, lung function decline, poorer quality of life and increased healthcare utilisation and cost for the patient and healthcare system (187, 188). Existing guidelines recommend that all AECOPD to be treated with corticosteroids and potentially antibiotics based on the presence of clinical symptoms of bacterial infection (34). Critically, AECOPD are not all equal and themselves appear clinically heterogeneous. Their aetiology (viral and/or bacterial or non-infective), clinical presentation (with or without symptoms of bacterial infection) and patterns of airway inflammation (eosinophilic, neutrophilic, pauci-inflammatory) can all vary between patients and even AECOPD events. More recently, the microbiome is proposed as a tool to assist in COPD exacerbation endotyping, with variation detected in diversity between bacterial, eosinophilic and viral exacerbations (189). Variable responses to guideline-directed treatment are also observed. The prescription of antibiotics to treat AECOPD remains controversial with inconsistent results of benefit reported (145). Treatment failure following antibiotics remains

between 12-26% in AECOPD (30). High prevalence rates of antimicrobial resistance further complicates antimicrobial usage and results in a significant rise in multi-drug resistance organisms in the respiratory tract, another major issue in Asian settings (190). Therefore, an appropriate and evidence-based use of antibiotics in clinical practice is paramount to prevent multi-drug resistance and its associated higher health-care costs. There is a clear need to better understand COPD pathogenesis, particularly AECOPD to better identify patients who are most likely to benefit from antibiotic therapy.

Approximately 50% of COPD exacerbations are secondary to bacterial infection (*Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*) (29, 30). These organisms however, are also associated with the stable COPD state and perpetuate deleterious immune responses in stable patients. Gram-negative bacilli, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are more common among Asian COPD patients experiencing AECOPD when compared to European cohorts (31). This suggests that understanding local COPD phenotypic profiles (including microbes) is a key step toward greater personalisation of COPD management, with the potential to improve clinical care and patient outcomes as well as achieving cost savings for under-resourced healthcare systems.

Figure 2.5: A summary of characteristics observed in Asian COPD patients. ↑: Increased, BMI: Body mass index.

2.3 COPD endophenotypes

2.3.1 Comorbidities

The occurrence of comorbidities in COPD complicates the disease resulting in increased mortality, health care utilisation and poorer quality of life. Comorbidities can co-exist in patients with COPD due to the relatively older age of patients with concurrent high exposure to cigarette smoke, which predisposes to increased risks of cardiovascular, metabolic disorder and malignancies that are independent of COPD. Conversely, these co-morbidities may also occur because of COPD. Up to 98% of all COPD patients have at least one comorbid disease, with the average number of comorbidities ranging between 1.2 and 4 per individual (191). Systemic inflammation is a crucial mechanism linking COPD and its various co-existing comorbidities, with increased systemic inflammatory markers such as CRP, TNF- α , IL-6 and fibrinogen observed in COPD when compared to controls. GOLD guidelines acknowledge the importance of comorbidities in COPD, and, recommend their assessment and treatment based on individual and relevant comorbid disease guidelines (38). The presence of COPD in itself also complicates the disease process associated with a co-existing co-morbidity, for instance co-existing cardiovascular disease results in higher morbidity and mortality when compared to those without COPD (192). Despite the importance of comorbidities in COPD prognosis, current available tools used in the assessment and stratification of COPD importantly do not account for such underlying comorbidities. Furthermore, clinical trials in COPD often exclude patients with significant comorbidities, due to the inherent complexity and potential interaction with trial medications. Numerous studies have attempted to incorporate comorbidities into a clustering analysis of COPD patients, and, a cardiovascular group is consistently associated with higher systemic inflammation, symptoms and mortality (193-195). In the Asian setting, differences in genetic, environmental, cultural, and behavioural background may result in differing COPD manifestations and accompanying comorbidities when compared to Western cohorts. Lee HY *et al.* (2018) for instance, demonstrates significant racial differences in comorbidities between cohorts from the United States and Korea with a lower prevalence in Koreans (196). Higher

numbers across Asia also illustrate a prior history of pulmonary tuberculosis and/or asthma, particularly in the most severe COPD patients when compared to Polish or American cohorts (169). Most reported data to date however, only involves a single Asian country or region and importantly fails to account for vast heterogeneity that exists across Asia: the studied Korean cohort for example is homogenous and therefore the effects of ethnicity on COPD manifestations has not been adequately expounded in the wider Asian setting.

2.3.2 Frequent COPD exacerbators

Exacerbations are significant COPD events that associate with increased mortality, poorer quality of life and increased health care utilisation including cost. In any COPD cohort, patient subgroups with frequent exacerbator (FE) status (≥ 2 exacerbations/year) are recognised and importantly it is a state not influenced by lung function severity (180, 197). These COPD FEs are more likely to have impaired quality of life scores and associated systemic inflammation, comorbidities and higher risk of mortality (198, 199). The identification of such patients has resulted in changes to COPD guidelines in 2009, with the addition of the “ABCD” grouping to account for those with FEs. This contrasts traditional COPD assessment that was based solely on lung function (GOLD stage) (200). The heterogeneity of COPD exacerbations in themselves pose a further challenge, with multiple exacerbation phenotypes now recognised. This suggests that the susceptibility to exacerbation in the first place is likely multifactorial. The interaction between airway and systemic inflammation, host immune response, microbial exposures, and exacerbation susceptibility, likely together contribute to pathogenesis and the occurrence of FEs in COPD patients. Higher levels of airway and systemic inflammation is demonstrated in FEs with an increased sputum IL-6, IL-8, and plasma fibrinogen. Despite recovery from exacerbations, FEs demonstrate a persistent heightened inflammatory state and higher risk of future exacerbations (199). Therefore, current guidelines recommend the addition of anti-inflammatory treatment in COPD patients with FEs (38).

Systemic inflammation in COPD is associated with an increased risk of comorbidities, including cardiovascular, gastro-oesophageal reflux disease (GERD), depression and osteoporosis. Increased systemic inflammation results in a prothrombotic state and increases the risk of cardiac events following COPD exacerbations. GERD is also frequently associated with FEs, and while treatment with proton-pump inhibitors are shown to reduce COPD exacerbations, mechanisms to explain this remain uncertain (201). Therefore, it is critical that in COPD FEs, optimisation of the underlying treatment of comorbidities are undertaken to prevent future events.

FE represent an important COPD phenotype with clear prognostic implications. Greater declines in lung function are observed, with up to a 25% additional decline in pulmonary function when compared to non-FEs (187). Moreover, patients with history of exacerbations are at risk of recurrent future exacerbations and all-cause mortality (180, 202).

Exacerbation frequency differs between Asian and Western COPD cohorts. In UPLIFT, Japanese COPD patients reported a lower overall exacerbation frequency compared to the western recruits but with a higher number of 'severe exacerbations' requiring hospitalisation (203). The perception of disease severity and exacerbations are also likely different across various regions, ethnic groups and even religions, evidenced by better health quality scores despite comparable lung function between Asian and Western cohorts (203). Other contributing factors that must be considered include the accessibility to health care and potential genetic influences on exacerbation susceptibility (204). Hence, it is of importance to understand and characterise Asian COPD and attempt to validate findings from non-Asian studies to assess their true relevance in the Asian context.

2.3.3 The allergic (eosinophilic) COPD phenotype

Eosinophilia is a hallmark finding in some forms of asthma but is now increasingly being recognised in COPD. Eosinophilic inflammation is observed in stable and even during exacerbations of COPD and can be used to predict responses to corticosteroid treatment. Blood eosinophil counts correlate with sputum eosinophils and a cut off of 2% or $>300\text{cells}/\mu\text{l}$ identifies patients who demonstrate corticosteroid responsiveness (95, 205, 206). During AECOPD, the use of blood eosinophils to guide treatment with corticosteroids is associated with lower rates of treatment failure and an overall reduced corticosteroid prescription (207). In stable COPD, blood eosinophils $\geq 2\%$ are associated with a response to ICS with a resulting decreased exacerbation frequency (95, 206, 208). Available data on the direct association between blood eosinophilia and COPD exacerbations however, remains inconsistent, with some studies suggesting no difference to exacerbation frequency, while others report associations with moderate to severe exacerbations, predominantly in ex-smokers (209-211). Due to such discrepancies, current recommendations for the use of eosinophilia in COPD remains only to predict corticosteroid responsiveness.

2.3.4 Allergic sensitisation in COPD

Increasing evidence supports the ‘treatable trait’ concept in chronic airways diseases such as COPD, with a targeted treatment approach used to improve disease outcomes (61). One such trait is allergy and its related sensitisation but this importantly lacks dedicated investigation in Asian patients with COPD. With rapid urbanisation and global climate change, allergic disease is being increasingly reported across Asian countries. Increased exposure to air pollution secondary to urbanisation can ‘prime’ the host immune system towards a pro-allergic response, and facilitate the entry of allergens through mucosal damage and inflammation resulting in an increased risk of allergic sensitisation (212, 213). In addition, the hygiene hypothesis has been advanced in relation to allergy and allergic disease where early childhood endotoxin exposure

is thought to be protective against future allergic disease, however direct causality is yet to be established (214, 215). The susceptibility to allergic disease is complex, and involves multiple immunoregulatory pathways including alterations of the human microbiome (skin and gut), which in turn may be influenced by genetic factors, diet and lifestyle (216). Geographic variation, climate and air quality are all-important factors determining the prevalence, type and the allergenic potential of a particular region or group of allergens. In Asia, house dust mite (HDM) remains the most common allergen, and is strongly associated with allergic airway disease. This is in contrast to pollens and animal dander which are found at lower frequencies and with regional differences across the continent (217, 218). Indeed, prior studies have compared sensitisation patterns across Japan with >10 million serum-IgE results assessed over a 10-year period. This demonstrates variation in sensitisation profiles toward environmental allergens likely secondary to climatic differences (219). Changes in climate from the subtropical (Okinawa) to subarctic (Hokkaido) can further leads to differing sensitisation profiles, with higher insect sensitisation in subtropical areas and pollens in temperate regions attributed to surrounding habitats (219). Environmental exposure plays a significant role in determining sensitisation profiles. Andiappan *et al.* (2014) have shown that migrants from China into Singapore acquired similar sensitisation frequencies to HDM compared to native Singaporeans after residing in Singapore for approximately 8 years (220). When taken together, these data illustrate the critical importance of considering geographic location in evaluating allergic sensitisation in COPD.

Allergic sensitisation is the outcome of a complex interaction between host immune response and exposure to allergenic stimuli. Allergens may enter epithelial cells through disruption of the epithelial tight junction barrier, protease-activator receptors and/or pathogen recognition receptors. Exposure to cigarette smoke and noxious particles can further induce epithelial disruption and promote recruitment of immune cells to the airway with their associated inflammatory consequence. This in turn facilitates allergen entry and susceptibility to sensitisation (221). In allergic asthma for instance, HDM binds TLR-4 at the epithelial cell

surface, and, results in the release of inflammatory mediators which then activates dendritic cells that initiate Th-2 inflammation (222, 223). Activation of Th-2 inflammation with release of IL-4 and IL-13 induced class-switching in B-cells, eventually results in the production of immunoglobulin (Ig)-E (223). Binding of allergen to the IgE receptor on mast cells or basophils triggers their degranulation and the release of inflammatory mediators including histamine, leukotrienes and prostaglandins resulting in allergic symptoms (224). Following the initial acute response, the recruitment of other immune cells ensues including neutrophils, eosinophils, monocytes and T-cells perpetuating late-phase inflammation (224, 225). While Th2 inflammation is well recognised in relation to allergic sensitisation, Th17 and Treg's are also shown to have involvement in allergic-related inflammation (226). The differentiation of CD-4 T-cells into its various subtypes are mediated by signals received from antigen presenting cells (APCs) and cytokines (223). Persistent and recurrent allergic inflammation eventually leads to airway remodelling and hyperresponsiveness which contributes to the development of allergic airways disease such as the co-existence of asthma with COPD (225). Sensitisation has been reported in COPD, without the presence of co-existing asthma, however, its precise role and contribution to disease pathogenesis remains uncertain and necessitates study. This may be achieved through prospective follow up studies of COPD patients who demonstrate variable sensitisation patterns (e.g. fungal, house dust mite or pollen sensitisation). These can be compared to non-sensitised COPD patients and clinical outcomes closely monitored in a longitudinal design including exacerbation rate, lung function, quality of life and survival.

In an European multicentre study involving mild to moderate COPD patients, atopy was associated with increased respiratory symptoms, particularly in younger age groups, males and obese individuals (17). Similarly, a study from the USA that included 77 COPD patients illustrated that 30% had atopy and suffered with an increased exacerbation frequency and greater respiratory symptom burden compared to those that did not (16). Likewise, Bafadhel *et al* (2014) detected atopy in one third of COPD patients with 13% sensitised to *Aspergillus fumigatus*, leading to worse lung function (227). This contrasted a Japanese study (n=265) where no

association between atopy (25%) and lung function decline or exacerbations was observed (183). The presence of asthma-like features (defined as a positive bronchodilator response, blood eosinophilia (≥ 300 cells/ μ l) and atopy) also associates with better COPD outcomes with appropriate treatment. In agreement with the Japanese data, Queiroz *et al.* (2016) and Neves *et al.* (2013) reported no association with clinical outcomes or lung function decline in atopic COPD patients (228, 229). Such contrasting findings between studies are likely explained by geographic variation, recruited patient cohorts, methodologies used, and the wide range of allergens studied. Furthermore, prior studies often report outcomes based on ‘overall’ sensitisation rather than at the individual allergen level. Sensitisation to specific allergens such as *Aspergillus fumigatus* have been associated with frequent exacerbations in asthma and lung function declines in COPD. However, its precise role of sensitisation in COPD and the contribution of the environment as an exposure source remains uncertain and require study (230, 231). It remains unknown whether the exposure to environmental allergens lead to the development of sensitisation or result in worsening clinical outcomes in sensitised COPD patients. Future study collecting the outdoor and indoor environmental allergens data of COPD and non-COPD cohort through metagenomic sequencing, and its relations to clinical outcomes with longitudinal follow up may provide a greater understanding of the role of sensitisation in COPD.

2.3.5 COPD-Overlap Syndromes

The existence of overlap syndromes in airways disease is a subject of ongoing debate, in particular because of a lack of clear definitions to describe these entities. Overlap represents a combination of disease phenotypes rather than a single disease entity (232). As airways diseases themselves are heterogenous, some share common risk factors and therefore patients may present with features of more than a single disease entity at any one time. The conceptual advance therefore of ‘treatable traits’ emerged as an alternate to disease focused management and allows a “label-free” precision approach, to aid both the diagnosis and management of airways disease based on the recognition of endophenotypic patterns in individual patients (61). This approach allows for the identification of common underlying mechanisms leading to the appearance of features from different airways disease, allowing for a more holistic and precision treatment approach for individual patients.

2.3.5.1 Asthma-COPD Overlap (ACO)

While the presence of overlap syndromes are controversial, GINA guidelines have proposed a stepwise approach to assist clinicians in recognising patients with asthma and COPD overlap (232). Importantly, it does acknowledge the limited existing evidence-base, as its suggestions are made largely based upon consensus expert opinions rather than primary research to provide interim advice to clinicians until more data becomes available (232). The lack of clear definitions of ACO results in its variable reported prevalence and outcomes. Patients with ACO are commonly excluded from large clinical trials. Despite the uncertainty, ACO is associated with increased healthcare utilisation, poorer quality of life, greater lung function declines and higher mortality, particularly when associated with late-onset asthma (36, 233-236). Its operational definition, developed through panel discussions, consists of 3 major and 3 minor criteria that can be considered (Table 2.2). The presence of 3 major and one minor criteria in a particular individual may be used to define ACO (237). Hopefully, these proposed definitions

will facilitate future research in a more uniform manner to better understand the disease and its optimal management strategies.

Table 2.2: Diagnostic criteria for Asthma-COPD overlap (ACO). “Three major and one minor criteria” define the presence of ACO.

Major criteria	Minor criteria
“Persistent airflow limitation (post-bronchodilator FEV ₁ /FVC <0.70 or LLN) in individuals 40 years of age or older” *LLN is preferred	“Documented history of atopy or allergic rhinitis”
“At least 10 pack-years of tobacco smoking OR Equivalent indoor or outdoor air pollution exposure”	“BDR of FEV ₁ ≥200 mL and 12% from baseline values on 2 or more visits”
“Documented history of asthma before 40 years of age OR BDR of >400 mL in FEV ₁ ”	“Peripheral blood eosinophil count of ≥300 cells/μl”

LLN: lower limit of normal, BDR: bronchodilator response #Adapted from Don D. Sin *et al.* (2016). (237)

2.3.5.2 Bronchiectasis-COPD overlap (BCO)

Bronchiectasis is defined as the presence of permanent and irreversible airway dilatation on high-resolution computed tomography (HRCT), while the presence of COPD is based on physiological measures along with the presence of risk factor exposure and airflow limitation (38, 238, 239). Up to 30% of COPD patients have bronchiectasis, and COPD may develop in patients with a primary diagnosis of bronchiectasis. Both diseases therefore commonly co-exist, and the presence of airflow limitation is a criterion for disease severity in bronchiectasis. In COPD, bacterial colonisation and chronic infection results in thickening of airway walls, another common feature of bronchiectasis (240, 241). Moreover, chronic cough and sputum production are common symptoms in both disease states, which further complicates diagnosis. CT scanning is also being increasingly used in COPD which inadvertently led to a surge in the identification of BCO. Radiological bronchiectasis is defined by a bronchial-arterial ratio >1 , lack of bronchial tapering and visualisation of bronchi within 1cm of the pleural surface (242). Bronchial-arterial ratios >1 without airway wall thickening should be evaluated with caution, particularly in the elderly and patients with cardiopulmonary disease (243). The presence of hypoxic vasoconstriction or pulmonary hypertension may also result in a missed or over-diagnosis of bronchiectasis. Tan *et al.* (2016), report that approximately 1 in 5 healthy individuals have radiological features of bronchiectasis without overt clinical symptoms (244). The absence of a clear definition for BCO has led to large discrepancies in its reported prevalence that ranges from 4-69% (243, 245, 246). Nevertheless, the presence of BCO is most likely to involve the lower lobes with an increased airway inflammation, symptomatology, exacerbations and all-cause mortality (175, 246, 247). In Asia, the reported prevalence of bronchiectasis in COPD is up to 50%, likely explained by the high prevalence rate of post-infection and tuberculosis-related bronchiectasis (36, 238).

2.4 Infection in COPD

We have made great progress in understanding the role of infection in COPD (29). First described in the 1950s, recurrent infection and mucus hypersecretion was in fact proposed as the root cause of COPD. However, subsequent studies did not find any association between infection and lung function decline (248). Exposure to noxious particles and gases were subsequently identified as the predominant cause of COPD, while infection and chronic mucus hypersecretion were considered epiphenomena. Over the past decade, however, emerging evidence supports the important role of infection in the development and pathogenesis of COPD. Microorganisms are often detected in stable disease or during acute exacerbations (29). From initial insult due to noxious particle exposure followed by an impairment of innate immune defences, microbial colonisation ensues leading to epithelial injury and inflammation that perpetuates an ongoing cycle of disease progression in COPD (249).

2.4.1 Acute infection in COPD

AECOPD are important clinical events with increased morbidity and mortality. Up to 80% of exacerbations are attributed to bacterial and/or viral infection (29). Seasonal variation in exacerbation frequency is reported with increased exacerbations occurring in lower temperature zones (250).

The potential pathogenic microorganisms (PPMs) frequently isolated in COPD include *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (251). Impaired phagocytosis by alveolar macrophages with decreased TLR-2 expression and increased ROS results in the susceptibility of the COPD lung to bacterial colonisation and subsequent exacerbations (252-254).

As PPMs have been isolated in the stable COPD state, it remains difficult to predict whether episodes of exacerbation are due to the same pathogen. Employing molecular typing in

longitudinal analyses with repeated sampling during stable and exacerbation states, an acquisition of new bacterial strains appears to be associated with AECOPD (255). Following antibiotic treatment, airway inflammation decreases and bacteria are suppressed and/or eradicated (256). Taken together, these data support the causative role of bacteria in AECOPD.

Viral induced exacerbations are common in COPD and often result in a prolonged recovery with overall higher levels of airway inflammation (257, 258). Rhinovirus, which causes the common cold, is regularly implicated in AECOPD (259). It adheres to airway epithelial cells by binding the surface protein intracellular adhesion molecule 1 (ICAM-1) and increased airway inflammation further induces its expression. During AECOPD, rhinovirus can be found in induced sputum, accompanied by increases in sputum IL-6 suggestive of its involvement in the detectable lower airway inflammation that results in AECOPD (260). Since the introduction of influenza vaccination, the number of influenza-related AECOPD has substantially decreased, particularly in Western countries (259). Influenza vaccination is associated with decreased outpatient visits, hospitalisations and mortality especially during influenza seasons (261). In Asia however, the low uptake of influenza vaccination is problematic, and therefore influenza remains the commonest cause of viral-induced AECOPD in this region (108, 262).

Co-infection or secondary infection by bacteria following a virus is possible in COPD, a disruption of the airway epithelial cell barrier occurs initially caused by the virus which then results in an increased susceptibility to bacterial invasion (263). Likewise, bacterial infection can exaggerate airway inflammation conferring a subsequent susceptibility to viral infection (264). Co-infection during AECOPD is associated with greater hospital lengths of stay and a higher risk of readmission (265, 266).

While bacteria and viruses remain the commonest causes of AECOPD, geographic variation does exist particularly in Asia and among the isolated PPMs during AECOPD events. A higher prevalence of *Klebsiella*, *Pseudomonas aeruginosa* and Influenza is reported in Asia compared

to Western countries and often associates with drug resistance (267). This is likely attributed to climatic variation, ease of antibiotics acquisition, and variable antibiotic resistance profiles.

2.4.2 Chronic infection in COPD

In COPD, defective phagocytosis by alveolar macrophages and monocyte-derived-macrophages (MDM) contributes to the persistence of bacteria in the airway, even during times of clinical stability (253, 268). Bacterial colonisation associates with a heightened airway inflammation and release of pro-inflammatory cytokines and chemokines including IL-6, IL-8, myeloperoxidase (MPO), NE and MMP (269-271). Collectively these result in chronic infection in COPD which is complicated by increased symptoms, exacerbations and higher mortality (272, 273).

2.4.2.1 Bacteria in COPD

Haemophilus influenzae remains the most frequently isolated organism in COPD either in the stable state or during exacerbations. Its unique characteristics include its outer membrane proteins P2 and P5 which adhere to the epithelium and ECM, and P6 that induces inflammation, allowing evasion of the host immune response. Intracellular survival leads to its airway persistence and therefore chronic infection (274-277). In mouse models exposed to *Haemophilus influenzae*, changes likened to COPD occur including airway fibrosis and inflammation (278, 279). In COPD, chronic infection with *Haemophilus influenzae* results in poorer health and lower lung function (280, 281).

Increased airway inflammation in COPD is also reported with *Moraxella* and *Streptococcus*, albeit with a lower frequency when compared to *Haemophilus Influenzae* (282-284). Short term

acquisition followed by clearance and immune protection are seen in particular with *Moraxella* (285).

While mucoid strains of *Pseudomonas aeruginosa* are often reported in relation to cystic fibrosis (CF), they can be associated with COPD and lead to disease progression (286, 287). In COPD, such strains associate with severe disease, and occur at higher frequencies in patients with co-existing bronchiectasis and in those with recurrent exacerbations necessitating antibiotic and steroid use. While *Pseudomonas* infection in COPD is associated with poorer prognosis (increased BODE index), the benefits of *Pseudomonas* clearance in COPD remain uncertain, with only a single centre study reporting no survival benefit that contrasts with that of *Pseudomonas* infection in CF (288, 289).

2.4.2.2 Viruses in COPD

Viruses are commonly implicated in AECOPD, however, their role in stable COPD is less appreciated. Adenovirus and respiratory syncytial virus (RSV) are identified in stable COPD and associate with activation of the host inflammatory response (290-294). Adenovirus preferentially targets epithelial cells, and following cell entry, its DNA can persist in the host epithelial cell genome, remaining latent (295). Therefore, lower levels of adenovirus are detected in sputum. In animal models, latent adenovirus infection amplified the effect of cigarette smoke on alveolar surfaces resulting in an increased emphysematous destruction (296). Increased adenoviral transactivating protein E1A has been detected in the lung tissues of smokers with COPD, with 5 to 40-fold higher prevalence in patients with emphysema. This is coupled to an increased abundance of airway immune cells including lymphocytes, B cells, eosinophils and polymorphonuclear cells (291, 295). In addition, an increased expression of IL-8 and ICAM-1 are found in human epithelial cells transfected with adenovirus, suggesting its involvement in persistent airway inflammation and COPD progression (290, 293, 294).

RSV is an RNA virus of the family Paramyxoviridae. Once within a cell, it replicates on the luminal surface of the airway epithelium with a preference for ciliated epithelial cells (297, 298). The major infection site is the small airways, with increased inflammation, mucus secretion and small airway obstruction described (299). RSV is commonly associated with acute infection; however, low-grade RSV is also detected in stable COPD, associated with raised plasma fibrinogen, serum IL-6 and an accelerated lung function decline (292, 294). The persistence of low-grade RSV infection may contribute to disease progression in the subgroup of affected COPD patients.

Other viruses detected in the stable COPD state include rhinovirus, coronavirus, influenza, and parainfluenza, however, at lower numbers compared to RSV. Detection of these viruses in stable COPD is associated with a history of frequent exacerbations and small airway inflammation, suggesting a potential role for chronic viral infection in COPD (257, 300).

2.4.2.3 Fungi in COPD

The significance of a positive fungal culture in COPD remains indeterminate. The isolation of fungi in the stable state may represent transition to more severe disease, a benign coloniser or be an early sign of invasive disease including pulmonary aspergillosis (IPA). Positive fungal culture is reported in up to half of COPD patients, of which 75% are *Aspergillus* and are associated with ICS dosage but no differences in COPD severity (227). In other work, 22% of COPD patients with a positive *Aspergillus* culture have IPA (301). Therefore, isolation of fungus, particularly *Aspergillus* in COPD should be treated with caution and carry a high index of suspicion for clinical relevance and potential deterioration.

Aspergillus sensitisation is associated with severe disease in asthma, bronchiectasis and CF (27, 230, 302), however, its role in COPD remains uncertain, with inconsistency in reported study

outcomes (174, 227). The association between positive sputum culture and hypersensitivity, although reported in asthma, does not appear to have any association in COPD (227, 303).

Pneumocystic jirovecii is an opportunistic fungus, associated with life-threatening pneumonia in the immunocompromised host, particularly in human immunodeficiency virus (HIV) infection. Interestingly, colonisation is reported in 8-55% of stable COPD patients and is linked to disease severity. Furthermore, upregulation of the CXCR3 receptor ligand in Th1 lymphocytes is observed in COPD complicated by *Pneumocystic jirovecii* colonisation suggestive of a potential contribution to disease progression (304).

2.5 The microbiome in COPD

Advances in sequencing technology has enabled access to genomic information with high accuracy and allowed a characterisation of the unculturable airway microbial community (305). The microbial community (or microbiome) comprises of bacteria, fungi, viruses, and archaea. Most microbiome studies in COPD have focused on the airway bacterial microbiome, with a lack of study on the fungal mycobiome, virome and archaea. The airway microbiota plays a crucial role in the development and function of host immunity, in endo-phenotyping efforts and can indicate through change disease progression. It can be influenced by age, infection, genetics, the environment and associated exposures, medications and even diurnal rhythms (189, 306-308). Lim *et al.* (2016) examined the airway bacterial microbiome profile in 257 sputum samples from Korean twin families, and found close association between the microbiome with host genetics and lifestyle factors (309).

2.5.1 The bacterial microbiome (bacteriome) in COPD

Bacterial composition of the COPD airway has been examined with sequencing of the 16S rRNA gene, which represents a highly conserved region for each respective bacterial grouping. 16s rRNA sequencing reveals a bronchial tree that contains over 2000 bacterial genomes per cm² in a healthy airway (310). Changes to microbiome composition is increasingly associated with the development of airways disease. Indeed, the characteristic airway microbiome detected in controls is perturbed in patients with stable COPD. This has been demonstrated in the upper and lower respiratory tracts, although differences in specific microbiota are observed across both compartments (310-312). A decreased sputum microbiome diversity with increased prevalence of *Proteobacteria* is reported in COPD, while *Bacteroides* dominates healthy individuals (310). This diversity is further reduced with increasing disease severity and during exacerbations (313-317). During AECOPD, an increased *Proteobacteria* (*Haemophilus*, *Moraxella*) with decreased Firmicutes (*Veillonella*) and Bacteroides (*Prevotella*) are detected (313, 314, 318-320).

Antibiotic use reduces Proteobacteria, whereas steroids alone can lead to its enrichment (307). Exacerbations may be further characterised as bacterial, eosinophilic or virus predominant, with lower diversity, higher load of Proteobacteria and symptoms in the bacterial exacerbation group (189, 307). In contrast, eosinophilic exacerbations are associated with higher diversity, increased Firmicutes and Bacteroidetes with lesser symptoms (189, 307, 318). This suggests that the airway bacterial microbiome is intimately linked to COPD pathogenesis and its exacerbation states and importantly may potentially be modulated by existing therapies to influence COPD trajectory. The stability of the bacterial microbiome through longitudinal assessment remains inconsistent, with high variability in the AERIS study while COPDMap found lower overall temporal variability of beta diversity in FE (314, 318). During AECOPD, a decreased alpha diversity is associated with an increased one-year mortality. A higher abundance of *Staphylococcus* and the absence of *Veillonella* remains a poor prognostic indicator (321). Similarly, during stable COPD, a lower diversity and increased Proteobacteria with predominant *Haemophilus* predicts poorer survival (317). These studies together advocate for the importance and prognostic of airway bacterial microbiome in COPD.

Similar evidence evaluating the bacterial microbiome in Asian COPD settings remain lacking. The Asian COPD microbiome studies performed to date are mainly from Korea and China, with small sample sizes and largely single centred (322-325). In addition, no data assessing mortality or exacerbations in relation to airway bacterial microbiomes from Asian COPD cohorts have been published. Available data simply suggests the similarity of the dominant taxa between Asian and non-Asian studies. Studies focusing on the microbiome's relation to clinical outcome therefore remains unknown in larger Asian COPD cohorts.

2.5.2 The fungal microbiome (mycobiome) in COPD

The number of bacterial microbiome studies continues to rise; however, the role of fungal microbiome (the mycobiome) has lagged. This is largely because of a lack of standardised methodologies in mycobiome sequencing with challenge from sample acquisition to bioinformatic analyses (326).

Despite such challenges, the emerging evidence does support the involvement of the mycobiome in disease pathogenesis and severity. In respiratory disease, dysbiosis of the airway mycobiome has been reported in asthma, CF and bronchiectasis when compared with non-diseased (healthy) individuals (326). Changes in mycobiome diversity associate with poorer lung function in CF (326). In bronchiectasis, the airway mycobiome relates to *Aspergillus* sensitisation and unfavourable disease outcomes (327). In COPD, only two studies to date assess the airway mycobiome. A study of HIV infected individuals with or without COPD found an overrepresentation of *Pneumocystis spp.* in those with co-existing HIV with COPD, potentially suggestive of involvement in disease pathogenesis in this very specific clinical state (328). A small single centre study including six AECOPD patients report a predominance of the phyla Ascomycota and genus *Candida*, *Phialosimplex*, *Aspergillus*, *Penicillium*, *Cladosporium*, and *Eutypella* in COPD sputum (322). The role and stability of the pulmonary mycobiome and its interaction with the host immune system, COPD treatment and clinical outcomes therefore remains largely unknown and requires study. Prospective longitudinal studies that include repeated sampling of the airway mycobiome at different stages and severities of COPD may help address this. This includes assessment of the stable, exacerbation, and post-exacerbation state, including its correlations with airway and systemic inflammation and clinical outcome. Such work will provide clearer insights into the specific importance of the pulmonary mycobiome to clinical COPD outcomes. A better understanding of the airway mycobiome and its interaction with other microbial communities in COPD may have important implications in treatment related decisions and clinical COPD trajectories. This may be of particular importance in Asia where hot and humid climates favours fungal abundance and persistence.

2.5.3 The virome in COPD

While evidence for a respiratory virome is emerging, current available data is mainly focused on acute respiratory tract infections and in particular paediatric cohorts (329). Studies comparing sputa of healthy individuals and those with cystic fibrosis (CF) report a discrete set of bacteriophages in the healthy lung which differs from that in CF (330). The role of respiratory virome in COPD remains poorly studied with limited data available to date. Using metagenomic sequencing, Van Rijn *et al.* (2019) recruited 63 patients with AECOPD, and compared the virome in patients with positive versus negative viral polymerase chain reaction (PCR) results. A total of 0.1% of the whole sequence reads were assigned to the virome with high sensitivity and specificity (96% and 100%) when compared to PCR (331). Positive viral PCR was associated with reduced bacteriophages and diversity when compared to patients with viral negative PCRs. No significant differences between viromes were detectable based on disease severity, lung function, duration and number of exacerbations (331). In the Human virome protein cluster database study, a predominance of Myoviridae, Siphoviridae, Phycodnaviridae and Podoviridae viral families were detected in bronchoalveolar lavage (BAL) samples from healthy, moderate and severe COPD patients (332). Increased bacteriophage burdens were observed in COPD with a decreased diversity in severe COPD (332). These studies clearly uncover the important prevalence of bacteriophages and the presence of a COPD virome, however, larger studies complete with longitudinal analysis is now required to better understand its precise role and contribution to COPD pathogenesis.

2.5.4 The interaction between bacteria, viruses and fungi in COPD

Bacteria, viruses and fungi have all been isolated in stability and during acute exacerbations of COPD, however, studies integrating the bacteriome, virome and mycobiome are currently lacking. Culture-based and molecular microbiological techniques however demonstrate important interactions between such microbial kingdoms that may affect disease outcomes. Co-culture models illustrate antagonistic effects between *Aspergillus* and *Pseudomonas*; where gliotoxin (secreted by *Aspergillus*) has anti-*Pseudomonas* activity while supernatant from *Pseudomonas* cultures inhibits *Aspergillus* biofilm formation (333-335). In mouse models, co-infection by *Aspergillus* and *Pseudomonas* surprisingly demonstrates improved survival compared to *Aspergillus* infected mice alone, further evidence of the antifungal effects of *Pseudomonas* (336). Importantly, however, in patients with cystic fibrosis, co-colonisation is associated with poorer clinical outcomes including increased exacerbations and reduced lung function (337). Conversely in AECOPD, positive *Pseudomonas* sputum cultures are an important risk factor for *Aspergillus* colonisation, however the true clinical significance of this relationship remains unclear, with no demonstrable exacerbation and/or mortality risk (34). Some have proposed that the antagonistic effect between these microbes is not universal and contingent on *Pseudomonas* strain and underlying disease (338). Importantly, bacterial-fungal interactions have also been described in other respiratory conditions and include *Klebsiella*, *Acinetobacter*, *Staphylococcus aureus* and *Candida* (334).

Bacteria and virus are common causes of respiratory infection and co-infection is often also described. Respiratory viruses disrupt the airway epithelium and increase susceptibility to secondary bacterial infection, while the presence of bacteria enhances the inflammatory response to viruses (265, 339). In COPD, rhinovirus infection associates with an increased airway bacterial load, for example *Haemophilus influenzae* (340). Bacterial and viral co-infection also often further results in more severe exacerbations with greater hospital stays and a higher risk of readmission (265, 266).

Taken together, these data do suggest important and underappreciated roles for interactions between the various microbial kingdoms in the airway, and future studies integrating more holistically these different groups of microbes may provide improved insights into the pathogenesis and clinical consequences of infection in the COPD airway.

2.5.5 The Asian microbiome

Oral and gut microbiome studies illustrate key differences in microbiome composition between Western and Asian populations, which are influenced by ethnicity, diet and environmental factors (341, 342). However, data directly comparing the airway microbiome in Asian and non-Asian COPD populations remains lacking. Geographic differences in the airway mycobiome have been illustrated in non-cystic fibrosis bronchiectasis (327). Mac Aogain *et al.* (2019) report diverse mycobiome profiles between Asian and European bronchiectasis cohorts with an increased abundance of *Simplicillium*, *Trichosporon* and *Aspergillus* in the Asian group, while *Wickerhamomyces*, *Clavispora* and *Cryptococcus* were higher in matched European cohort (327). Differences in airway microbiomes may also be driven by the variable management practices between geographic regions. The high prevalence of antibiotic resistance combined with diverse cultural and genetic backgrounds across Asia likely results in distinct airway microbiomes which require further investigation in Asian COPD. This would involve the recruitment of COPD patients of different ethnic backgrounds in a number of countries across Asia, where a comparison of sputum microbiomes with age, treatment and disease severity can be performed against ‘matched’ European cohorts to better elucidate differences in microbial profiles and their associated clinical consequences.

2.5.6 Overall aim and hypothesis

With clear differences described in Asian COPD, there exists a need to better understand characteristics and/or phenotypes that associate with poorer clinical outcome. The aim of my thesis is to characterise the clinical, immunological, and microbiological phenotypes of COPD in three South-East Asian countries (Singapore, Malaysia, and Hong Kong). I hypothesise that distinct clinical, immunological, and microbiological phenotypes exist, and associate with clinical outcomes.

CHAPTER 3: CLINICAL AND INFLAMMATORY PHENOTYPES OF COPD IN SOUTH-EAST ASIA

3.1 Introduction

As outlined in Chapter 2, COPD is a progressive and heterogeneous disease with significant morbidity, mortality and socioeconomic burden (343, 344). Prevalence in the Asian sub-continent is likely grossly under-estimated due to a lack of awareness and delays in diagnosis (162). In South East Asia, diverse cultural backgrounds, genetics, variable health care policies and available resources all influence the diagnosis, clinical presentation and subsequent management of COPD which in turn are thought to contribute to disease phenotypes (1, 345). Of particular note is the higher prevalence of males, non-smokers and the occurrence of less severe symptoms reported in COPD among Asians when compared to non-Asian cohorts (21-23). Prior work assessing racial variation in COPD outlines important differences in COPD risk, susceptibility and severity in Asian cohorts, particularly those of Chinese descent when compared to non-Asian (western cohorts including Caucasians and African-Americans). Critically, however, these works have been performed predominantly outside Asia and only include small numbers of Asian COPD patients (346-349).

In parallel, varying clinical phenotypes of COPD have been described by cluster analysis which includes but is not restricted to the following: young, old, degree of airflow obstruction, emphysematous- versus airway predominant, symptomatic, exacerbator, low co-morbidity and cardiovascular further emphasizing the clinical heterogeneity inherent in COPD (194, 195, 350, 351). The aim of COPD phenotyping however remains to identify relatively homogenous patient groups for which targeted treatment can be applied.

Despite multiple studies focused on phenotyping COPD, available data particularly for the South-East Asian region remains lacking. Kim *et al.* (2018) identifies three clinical COPD clusters from patients recruited in 13 Asian countries: cluster 1 demonstrates poor lung function and few symptoms, cluster 2 poorer lung function with high symptomatic burden and cluster 3

with mild COPD and relatively preserved lung function (352). Unsurprisingly, cluster 2 associates with frequent exacerbations and greater declines in pulmonary function which likely relate to the measured higher symptom scores at 1-year follow up. While the studied cohort was large and included patients from multiple centres across the continent, variables used for clustering were limited and was restricted to BMI, Charlson comorbidity index, the St. George's Respiratory Questionnaire and FEV₁ (352). Most critically, follow up data was available in only ~15% of the total cohort at 1-year follow up as high rates of dropout were recorded. Furthermore, the study did not employ any validation cohort overall diminishing its strength, impact and translatability into clinical care.

As outlined in Chapter 2, the presence of co-morbidities are critically important in COPD as they complicate the disease process. Co-morbidities are often neglected in studies focused on COPD phenotyping, as composite scores including the Charlson co-morbidity index is frequently used as a surrogate. As a consequence, a collective approach as opposed to analysis of individual co-morbidities has been employed to date with relatively few studies selecting and including specific individual co-morbidities such as cardiovascular disease (195, 352, 353). Accounting for individual co-morbidities is therefore important, as a wide range exists, particularly in the Asian sub-continent. Specific co-morbidities may be relevant in individual COPD patients or groups because of their variation in susceptibility across differing regions, climates and even ethnic groups. Such co-morbidities can also be further influenced by the lifestyle, cultural and genetic factors, all of which exhibit are relevant when comparing between Asian and Western cohorts (196, 346). Studies that incorporate a comprehensive list of co-morbidities in COPD have importantly found differences in clustering outcomes (193, 354, 355). Importantly, however, such works lack longitudinal data and follow up, affecting their usefulness in terms of prognostication. Furthermore, like prior studies, no validation cohort was included and some remain focused on a single centre dataset. To date, no study has reported on COPD clustering using individual co-morbidities in the South East Asian region.

Recent work performed at the population level reports on 57,779 adults in China and illustrates a COPD prevalence of 8.6% across all age groups and 13.7% in those aged forty years of age and above (163). These results were validated in a second study that included 67,752 Chinese adults demonstrating a COPD prevalence of 13.6% (162). With such large numbers of COPD in ethnic Chinese populations, it is likely that different COPD phenotypes do indeed exist, which likely requires targeted and specific treatment interventions. This is further attested by the reported mortality differences in COPD patients of Chinese ethnicity, currently postulated to be due to variations in smoking prevalence, socioeconomic background, education, COPD awareness and critically geographic region (356, 357). It is however likely that, other factors also contribute to such varying mortality outcomes. A significant number of ethnic Chinese reside across South-East Asia; however, little is known about COPD and possible clinical phenotypes in this population. As COPD demonstrates a high reported burden and associated mortality in these populations, a need to better understand and phenotype COPD is now required to improve risk stratification and provide appropriate intervention (358). Here, in a large multicentre study including COPD patients of Chinese ethnicity recruited from three different countries across South-East Asia, I aim to describe associated clinical phenotypes and evaluate their clinical outcomes and accompanying inflammatory signatures.

3.2 Methods

3.2.1 Ethics statement

The work presented in this chapter was approved by the institutional (ethics) review boards of all participating hospitals as follows: DSRB 2012/01110, CIRB 2018/2186 (2013/184/C), CIRB 2016/2715, CIRB 2017/3010, CIRB 2017/2933 (all mutually recognized by DSRB, Singapore), NNMR-13-313-15138, UMMC 2018725-6524 (Malaysia), CREC 2011.146, CREC 2015.164 and CREC 2018.042 (Hong Kong). Non-diseased (healthy) recruitment was approved by the NTU institutional (ethics) review board under IRB-2017-12-010.

3.2.2 COPD patient recruitment

Chinese patients aged > 40 years of age with stable COPD were prospectively recruited through six tertiary hospitals across three different countries; Singapore General Hospital, Singapore, Changi General Hospital, Singapore, Tan Tock Seng hospital, Singapore, RCSI-UCD Malaysia Campus, Malaysia, University Malaya Hospital, Malaysia and Prince of Wales Hospital, Hong Kong. Chinese ethnicity was defined as documented evidence of both parents being of Chinese lineage. COPD was diagnosed according to global initiative for chronic obstructive lung disease (GOLD) criteria (200). Clinical stability was defined as the absence of any exacerbation in the six weeks preceding study recruitment. All patients received COPD therapy, in accordance to established guidelines and at the discretion of the treating clinician (200). GOLD criteria of $FEV_1/FVC < 0.7$ is used to diagnose COPD in the presented studies. This is because the management of COPD at all participating recruiting hospitals is based on GOLD recommendations. In addition, Bhatt SP *et al.* (2019) have recently illustrated that using $FEV_1/FVC < 0.7$ is better at discriminating COPD-related hospitalisation and mortality when compared to $FEV_1/FVC < \text{lower limit of normal (LLN)}$ (359). The FEV_1 predicted values were

obtained from Morris *et al.* 1971 (360) and an adjustment factor of 0.94 was applied for FEV₁ as recommended for Asian patients (361).

3.2.3 Derivation and validation cohorts

To establish if clinical phenotypes in South-East Asians with COPD of Chinese ethnicity residing outside China exist (i.e. Singapore, Malaysia, and Hong Kong), a total of 1,480 patients were recruited into a derivation and validation cohort respectively. The assignment to either the derivation or validation cohort was based on the year of initial recruitment. The derivation cohort was recruited from 2012 and the validation cohort from 2013. The derivation cohort comprised of 911 patients from four different sites over the study period from 2012: Singapore General Hospital (2012-2016) and Changi General Hospital (2012-2016) (both Singapore), RCSI-UCD Malaysia Campus (2012-2015) (Malaysia) and Prince of Wales Hospital (2012-2015) (Hong Kong). The validation cohort was recruited from five different sites from 2013 onwards: Tan Tock Seng Hospital (2013-2018), Singapore General Hospital (2016-2018) and Changi General Hospital (2017-2018) (Singapore), University Malaya Hospital (2018) (Malaysia) and Prince of Wales Hospital (2016-2018) (Hong Kong). Blood was collected from a subset of patients in the derivation (n=213) and validation cohort (n=123) respectively. The study ended in March 2019.

3.2.4 Clinical data collation.

Clinical data was collected at recruitment and included demographics, smoking history and pack years, lung function, COPD assessment test (CAT) scores, number of prior exacerbations requiring hospitalisation in the preceding year, sputum microbiology, comorbidities and COPD specific co-morbidity test (COTE) score (362). Sputum microbiology was obtained through spontaneous expectorated and representative sputum (<10 squamous epithelial cells and >25 leucocytes per low-power microscopic field) (363, 364). Comorbidities were collated by the recruiting clinician at study entry with verification from patient medical records and included the following: anxiety, atrial fibrillation/flutter, coronary artery disease, congestive heart failure, diabetes mellitus, liver cirrhosis, malignancy, gastric or duodenal ulcers (peptic ulcer disease), chronic kidney disease, cerebrovascular disease/stroke, peripheral arterial disease, asthma, prior documented history of confirmed pulmonary tuberculosis, chronic respiratory failure, pulmonary hypertension and osteoporosis. The number of acute exacerbations in the subsequent year following recruitment and two-year mortality were prospectively recorded. The cause of mortality was as recorded on the death certificate where a respiratory cause of death was defined as death secondary to pneumonia, chronic obstructive pulmonary disease or respiratory failure. COPD exacerbations were defined as an acute deterioration of respiratory symptoms requiring additional therapy such as antibiotics and/or corticosteroids (33, 200). Severe exacerbation was defined as exacerbations requiring hospitalisation (33, 200).

3.2.5 Definitions of recorded co-morbidities.

Co-morbidities were obtained from patient histories and verified through medical records, including medication lists and therapies received for specific disease states. Where data was unavailable or could not be verified for any one co-morbidity, the patient was classified as not demonstrating that respective co-morbidity. For standardisation of co-morbidities, definitions in Table 3.1 are used for the work presented in this chapter.

Table 3.1 Definitions for recorded co-morbidities.

Co-morbidities	Diagnosis
Anxiety	Based on DSM-V criteria and confirmed by a registered psychiatrist (365).
Atrial fibrillation/flutter	Absence of P waves, irregular R-R intervals and atrial activity on electrocardiogram (366).
Congestive heart failure	Based on clinical, physical, echocardiogram and echocardiography features and confirmed by a registered cardiologist (367).
Coronary artery disease	By functional testing, radiological imaging and/or coronary angiography (368).
Diabetes mellitus	Either fasting blood glucose levels of ≥ 7.0 mmol/l, blood glucose levels of ≥ 11.1 mmol/l two-hour post oral glucose tolerance test, random blood glucose level ≥ 11.1 mmol/l with hyperglycaemia symptoms, or HbA1c $\geq 6.5\%$ (369).
Liver cirrhosis	Presence of clinical, biochemical and radiological features of liver failure with or without liver biopsy (370).

Malignancies	Radiological imaging and/or histological confirmation of malignancy from any organ system.
Peptic ulcer disease	Diagnosed by endoscopy with features of ulcer disease in the gastric and/or duodenal region (371).
Chronic kidney disease	Estimated glomerular filtration rate (eGFR) <60 ml/min, according to the National Kidney Foundation, Kidney Disease Outcome Quality Initiative guidelines (372).
Cerebrovascular disease/stroke	Documented central nervous system infarction and/or haemorrhage (373).
Peripheral arterial disease	Ankle brachial index of ≤ 0.9 (374).
Asthma	Defined according to the Global Initiative of Asthma (GINA) guidelines based on past history, symptoms and the presence of variable expiratory airflow limitation (375).
Previous pulmonary tuberculosis	Prior positive sputum analysis for <i>Mycobacteria tuberculosis</i> including positive acid-fast bacilli smear, culture or nucleic acid amplification, radiological features (on chest radiography or computed tomography) and/or prior pharmacological treatment for pulmonary tuberculosis (376-378).
Osteoporosis	Defined by radiological imaging with bone mineral density of 2.5 standard deviations or more below the young adult mean values (379).

3.2.6 Non-diseased (healthy individual) recruitment.

To make appropriate comparisons, non-diseased (healthy) controls (n=24) was recruited for the purpose of inflammatory analysis. Non-diseased (healthy) controls with no prior history of COPD were recruited from Nanyang Technological University (NTU), Singapore. Spirometry was performed and subjects with no prior medical history, receiving no long-term medications and with an FEV₁/FVC >0.7 and FEV₁ ≥80% predicted were recruited.

3.2.7 Blood sample collection

For inflammatory analysis, venous blood was collected from COPD (n=336) and non-diseased (healthy) controls (n=24) upon study entry. Venous blood was centrifuged at 1300g for 10 minutes at 18°C. Plasma was isolated and maintained at -80°C till further processing.

3.2.8 Cytokine assay

Based on established clustering determined within the original derivation and validation cohorts to which the patients were recruited into, as described in chapter 3.2.9.1, I next assessed the inflammatory phenotypes from the identified clinical (phenotypic) clusters. First, I performed a semiquantitative assay using one hundred pooled plasma samples (20 from each respective cluster) as an initial screening step. In this step, the expression of 120 cytokines were screened semi-quantitatively using the Raybiotech human cytokine array C2000 kit according to the manufacturer's instructions. Briefly, diluted plasma (1:8) were incubated with membranes containing the antibody arrays at 4 degrees Celsius overnight. Subsequently the membranes were washed with wash buffer and incubated with a secondary antibody followed by horseradish peroxidase labelled streptavidin for detection. Data were analysed with Raybio[®] analysis software tool provided by the manufacturer.

Six cytokines (PDGF-AA, PDGF-AB, PDGF-BB, VEGF, TNF-R1, TNF-R2) were then selected for confirmatory assays based on 2.5-fold difference cut-off from at least one other cluster and using the established COPD literature. A customized ProcartaPlex multiplex immunoassay panel (Thermo Fisher Scientific) consisting of PDGF-BB, VEGF-A, TNF-R1 and TNF-R2 along-with human PDGF-AB and PDGF-AA ELISAs were used for confirmation. The panel was tested on a subset of the initially recruited cohort: non-diseased (n=24) and COPD patients (n=336). Magnetic beads with twenty-five microlitres of plasma (in duplicates) were incubated for 120 minutes at room temperature with shaking. Following the washing steps, detection antibody was added followed by Streptavidin-PE. Each respective step included an incubation for 30 minutes with shaking and washing (x2) in-between. Subsequently, beads were resuspended with reading buffer and the plate read with the Bio-Plex-200 system (Bio-Rad). The concentration of each cytokine was then determined by generating a standard curve using the Bio-Plex manager software 6.1.

3.2.9 Statistical analysis

Data was analysed using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria.). Shapiro-Wilks test was used to assess data distribution. Data is presented as median with interquartile ranges due to non-normal distributions. Continuous data was compared using Mann-Whitney U or Kruskal-Wallis testing, as appropriate. Benjamin-Hochberg correction for false discovery rate was performed for multiple comparisons. For categorical variables Chi-square and Fisher exact testing was used. A p value of ≤ 0.05 was defined as significant.

3.2.9.1 Clustering analysis

Continuous and categorical data including demographics, smoking history, lung function, CAT scores, comorbidities and sputum microbiology were used to perform clustering. Data was first transformed to a Gower dissimilarity matrix using the R “cluster” package followed by non-metric multidimensional scaling (NMDS) using $k=8$, determined by Scree plot. Subsequently, Ward's minimum-variance unsupervised hierarchical clustering method was applied on this transformed dataset using an agglomerative approach. The optimal number of cluster and cluster stability (Jaccard similarities index) was determined using the R “Nbclust” and “fpc” packages. The optimal number of clusters was 5 and a Jaccard similarity index of 0.79 indicated the stabilities of the derived clusters.

For the validation cohort, a Regularised Discriminant Analysis (RDA) model was implemented using the R “klaR” package. RDA was trained on the data-transformed derivation cohort and using optimally tuned model parameters (lambda and gamma). A nested approach with 100 iterations was implemented with optimal model parameters determined numerically to minimise misclassification rate, estimated by dividing the data into a 70% training and 30% testing group with bootstrapping over 100 iterations. The median of the optimal model parameters was determined by kernel density estimation (KDE) generated using the “seaborn” library in python.

The darkest region of the KDE density plot corresponded to the median optimal lambda and gamma, 0.012 and 0.130 respectively (Figure 3.1). The leave one out cross validation (LOOCV) was used to approximate the unbiased estimate of RDA accuracy. The LOOCV accuracy of the RDA model with optimal parameters was found to be 97.9%. To validate the derived cluster groups, the validation cohort was data-transformed and the trained RDA model on 70% of the derivation cohort (training dataset) was used to predict the class membership probabilities of the patients from the validation cohort (Table 3.2). Class labels were assigned based on the maximum probability of a patient being in that class.

Figure 3.1: Contour plot to determine optimal model parameter selection for the Regularised Discriminant Analysis (RDA) model used for determination of validation cluster membership. Kernel density estimation (KDE) is represented in a density plot with marginal distributions of gamma and lambda on the x-axis and y-axis respectively. Contour colours correspond to the probability where the optimal gamma and lambda lie (dark blue: highest probability, light blue: lowest probability). The optimal gamma and lambda of the Regularised Discriminant Analysis (RDA) model was chosen from the darkest region of the KDE plot and corresponds to gamma=0.012 and lambda=0.130.

Table 3.2: Confusion matrix showing the predicted and actual clusters identities (expressed as number of patients, n and percentages; %) generated from the derivation cohort (n= 911) using Regularised Discriminant Analysis (RDA). This model was used to predict cluster identity for each individual patient in the validation cohort based on the maximum probability of belonging to each derived cluster.

<div> <div>Predicted</div> <div>Actual</div> </div>	Ex-tuberculosis, n (%)	Diabetic, n (%)	Low co-morbidity: low-risk (%)	Low co-morbidity: high-risk, n (%)	Cardiovascular, n (%)	Total number of patients	Accuracy
Ex –tuberculosis, n (%)	156 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	156	99.9%
Diabetic, n (%)	0 (0.0%)	108 (99.1%)	0 (0.0%)	0 (0.0%)	1 (0.9%)	109	99.0%
Low co-morbidity: low-risk, n (%)	0 (0.0%)	0 (0.0%)	191 (99.5%)	1 (0.5%)	0 (0.0%)	192	99.7%
Low co-morbidity: high-risk, n (%)	0 (0.0%)	7 (2.0%)	2 (0.6%)	324 (95.6%)	6 (1.8%)	339	98.2%
Cardiovascular, n (%)	1 (0.9%)	1 (0.9%)	0 (0.0%)	0 (0.0%)	113 (98.2%)	115	99.0%

3.2.9.2 *Survival analysis*

Kaplan-Meier curves with log-rank testing was used to assess all-cause mortality. For multivariate analysis, cox proportional hazard regression and logistic regression models adjusted for age, sex, BMI, FEV₁ and smoking pack years were calculated using the R “survival” and “survminer” package and “glm” function. Competing risk analysis comparing between respiratory and non-respiratory causes of death was performed using the R “cr17” package.

3.2.9.3 *Data visualisation.*

Sankey plots were used to visualise the mortality and severe exacerbations in each cluster in relation to smoking status using R “ggalluvial” package.

3.2.9.4 *Cytokine (Inflammatory) analysis*

Inflammatory cytokines were corrected for batch effects with the R “MdimNormn” package and the median normalised values plotted on a radar chart using the R “ggiraphExtra” package (27). Network diagrams of the six cytokines for each respective clinical cluster was generated using “tidygraph” and the “ggraph” package in R. Nodes represent the percentage of patients positive for the respective cytokine, the edge (line) illustrates cytokine connectivity and edge attributes (line thickness) represent the proportion of patients demonstrating a positive cytokine connection. Positive cytokines were defined as a value above the 95th percentile of the non-diseased (healthy) control group (380).

3.3 Results

3.3.1 Demographics, co-morbidities and airway microbiology profiles are comparable between the derivation and validation cohorts of Chinese patients with COPD.

A total of 1,480 patients were recruited into derivation (n=911) and validation (n=569) cohorts respectively with comparable demographic profiles (Table 3.3). While a higher number of current smokers were recruited in the derivation compared to validation cohort, no significant differences were observed in the overall smoking pack years between the cohorts. When comorbidities were assessed, differences were identified between the cohorts and their respective country of origin (recruitment). In the derivation cohort, a higher proportion of prior pulmonary tuberculosis (18.2% versus 8.4%; $p<0.001$), osteoporosis (3.7% versus 1.4%; $p<0.01$) and asthma (7.1% versus 1.9%; $p<0.001$) was evident, whilst malignancy (8.0% versus 17.2%; $p<0.001$) was more common in validation cohort (Figure 3.2a). Coronary artery disease was significantly higher in Singapore ($p<0.01$) and prior pulmonary tuberculosis in Malaysia ($p<0.01$), reflective of the geographic tuberculosis prevalence (Figure 3.2b). Peptic ulcer disease was found at a greater proportion in the derivation cohort from Malaysia but was lowest in their validation cohort ($p<0.01$). For sputum microbiology, higher proportions of *Streptococcus pneumoniae* (3.6% versus 1.6%, $p<0.05$) and *Haemophilus influenzae* (8.9% versus 5.3%, $p<0.05$) were observed in the derivation cohort (Figure 3.3).

Table 3.3: Demographic table illustrating the derivation and validation cohorts of Chinese COPD patients recruited across six sites and three countries in South-East Asia.

Characteristics	Overall n=1,480	Derivation cohort n=911	Validation cohort n=569
Age (years), Median (IQR)	74 (68-79)	74 (68-79)	74 (69-81)
Country, n (%)			
Singapore	630 (42.6)	406 (44.5)	224 (39.4)
Hong Kong	720 (48.6)	425 (46.7)	292 (51.8)
Malaysia	130 (8.8)	80 (8.8)	50 (8.8)
Sex (Male), n (%)	1396 (94.6)	868 (95.3)	531 (93.3)
Smoking status, n (%)			
Current smoker	690 (46.6)	547 (60.0)	143 (25.1)
Ex- smoker	773 (52.2)	362 (39.8)	411 (72.2)
Non-smoker	17 (1.2)	2 (0.2)	15 (2.7)
Smoking pack years, Median (IQR)	50.0 (35.0-65.5)	50.0 (32.0-67.5)	50.0 (40.0-60.0)
BMI (kg/m ²), Median (IQR)	21.0 (18.0-23.6)	20.9 (18.0-23.5)	21.3 (18.1-23.9)
FEV ₁ (%predicted), Median (IQR)	45.0 (34.0-60.0)	47.3 (35.3-61.8)	42.0 (33.0-58.0)
FEV ₁ /FVC (%predicted), Median (IQR)	49.9 (40.5-59.0)	50.0 (41.3-60.0)	47.0 (39.9-56.1)
Total number of exacerbations per year, Median (IQR)	1 (0-2)	1 (0-3)	1 (0-2)
Hospitalized exacerbations, Median (IQR)	0 (0-2)	1 (0-2)	0 (0-1)
COTE Index, Median (IQR)	0 (0-2)	0 (0-2)	1 (0-2)

Data are presented as number of patients (n) (with percentage; %) or median (and interquartile range; IQR). BMI: body mass index; FEV₁: forced expiratory volume in the 1st second; FVC: forced vital capacity, COTE: chronic obstructive pulmonary disease-specific co-morbidity test

Figure 3.2: Co-morbidities are comparable between the derivation (D) and validation (V) Chinese COPD cohorts. (a) Bubble plots illustrate the proportion of patients with comorbidities between the derivation and validation cohorts. Colours correspond to patient groups: light green: derivation (overall), dark green: validation (overall), red: Singapore, blue: Hong Kong, purple: Malaysia. PUD: peptic ulcer disease, pTB: history of prior pulmonary tuberculosis, PAD: peripheral arterial disease, Other Ca: all malignancies excluding lung, oesophageal, pancreatic and/or breast carcinoma, DM: diabetes mellitus, CVA: cerebrovascular disease, CKD: chronic kidney disease, CHF: congestive heart failure, CAD: coronary artery disease, Ca: lung, oesophageal, pancreatic and/or breast carcinoma, AF: atrial fibrillation.

Figure 3.2: (b) Bubble plots illustrate the proportion of patients with comorbidities between country of origin. Colours correspond to patient groups: light green: derivation (overall), dark green: validation (overall), red: Singapore, blue: Hong Kong, purple: Malaysia. PUD: peptic ulcer disease, pTB: history of prior pulmonary tuberculosis, PAD: peripheral arterial disease, Other Ca: all malignancies excluding lung, oesophageal, pancreatic and/or breast carcinoma, DM: diabetes mellitus, CVA: cerebrovascular disease, CKD: chronic kidney disease, CHF: congestive heart failure, CAD: coronary artery disease, Ca: lung, oesophageal, pancreatic and/or breast carcinoma, AF: atrial fibrillation.

Figure 3.3: Bubble plot illustrating the proportion of Chinese COPD patients with a positive sputum microbiology between the derivation and validation cohorts. *H. influenzae*: *Haemophilus influenzae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. pneumoniae*: *Streptococcus pneumoniae*, *M. catarrhalis*: *Moraxella catarrhalis*, *K. pneumoniae*: *Klebsiella pneumoniae*.

3.3.2 Unsupervised hierarchical clustering reveals five clinical clusters with variable prognostic outcomes.

Five clinically distinct clusters were identified by unsupervised hierarchical clustering of the derivation cohort. The clusters were named based on the predominant clinical features and their mortality outcomes as follows: (1) prior pulmonary tuberculosis (Ex-TB; n=156); (2) co-existing diabetes (diabetic; n=109); (3) low co-morbidity: low risk (LCLR; n=192); (4) low-comorbidity: high risk (LCHR; n=339) and (5) co-existing cardiovascular disease (CVS; n=115) (Figure 3.4 and Table 3.4). While two of the five clusters have a low co-morbidity burden (LCLR and LCHR), the LCHR and CVS clusters closely resembled one another, separated only by dendrogram branching (Figure 3.4a). Two-year all-cause mortality was highest in the CVS (42.6%) followed by Ex-TB (34.0%), LCHR (25.7%), diabetic (23.1%) and LCLR (18.2%) groups respectively (log-rank $p < 0.05$) (Figure 3.5a). This remained significant after adjustment for age, gender, BMI, FEV₁ and smoking pack year exposure using cox hazard regression models (Figure 3.5b). The hazard ratio (HR) for each cluster compared to LCLR group was as follows: CVS (HR: 2.94; 95% CI 1.51-5.72; $p < 0.01$), Ex-TB (HR: 2.10; 95% CI 1.16-3.80; $p < 0.05$), LCHR (HR: 2.01; 95% CI 1.18-3.43; $p = 0.01$) and diabetic (HR: 1.67; 95% CI 0.82-3.40; $p = \text{ns}$). When causes of death were further classified as respiratory versus non-respiratory causes, interestingly, a similar trend was observed in all-cause mortality, with greater mortality in CVS, Ex-TB and LCLR clusters respectively ($P < 0.01$) (Figure 3.5c).

The proportion of patients with hospitalised exacerbations in the year following recruitment was also measured and compared between clusters. The CVS and Ex-TB clusters illustrated the highest proportions of hospitalised exacerbations when compared to either of the low co-morbidity clusters ($p < 0.01$) (Figure 3.6). Smoking status differed significantly between the two low co-morbidity clusters with predominantly current smokers in the LCHR and ex-smokers in the LCLR cluster (Table 3.4). In view of this, I next assessed if underlying smoking status within each respective cluster influenced mortality and/or exacerbation severity (Figure 3.7a-b).

Smoking status did not influence the mortality or exacerbations within each respective derived cluster (Figure 3.7a-b).

Table 3.4: Baseline characteristics of the five derived Chinese COPD clusters (from the derivation cohort).

Characteristics	Overall	Ex-tuberculosis	Diabetic	Low co-morbidity: low-risk	Low co-morbidity: high-risk	Cardio-vascular	p-value
n (%)	911	156 (17)	109 (12)	192 (21)	339 (37)	115 (13)	
Country, n (%)							<0.001
Singapore	406 (44.5)	73 (46.8)	45 (41.3)	107 (55.7)	101 (29.8)	80 (69.6)	
Hong Kong	425 (46.7)	53 (34.0)	54 (49.5)	50 (26.1)	234 (69.0)	34 (29.5)	
Malaysia	80 (8.8)	30 (19.2)	10 (9.2)	35 (18.2)	4 (1.2)	1 (0.9)	
Age (years), Median (IQR)	74 (68-79)	74 (67-79)	72 (67-79)	74.5 (68-78)	73 (67-79)	73 (68-78)	0.861
Sex (Male), n (%)	868 (95.3)	148 (94.9)	103 (94.5)	182 (94.8)	327 (96.6)	108 (93.9)	0.766
Smoking status, n (%)							<0.001
Current smoker	542 (59.5)	66 (42.3)	66 (60.6)	15 (7.8)	323 (95.3)	72 (62.6)	
Ex- smoker	362 (39.8)	86 (55.1)	43 (39.4)	176 (91.7)	14 (4.1)	43 (37.4)	
Non-smoker	7 (0.8)	4 (2.6)	0 (0.0)	1 (0.5)	2 (0.6)	0 (0.0)	
Smoking pack years, Median (IQR)	50.0 (32.0-67.5)	50.0 (33.9-77.3)	50.0 (29.5-80.0)	51.0 (40.0-79.9)	47.0 (35.0-61.0)	50.0 (30.0-60.0)	0.015
Body mass index (kg/m ²), Median (IQR)	20.9 (18.0-23.5)	19.5 (17.3-23.2)	21.4 (19.0-25.0)	21 (18.3-23.5)	20.9 (18.2-23.0)	21 (17.8-23.8)	0.049
FEV ₁ (% predicted), Median (IQR)	47.3 (35.3-61.8)	46.0 (33.0-61.8)	50.0 (35.8-62.5)	50.6 (38.7-69.0)	44.3 (32.9-57.0)	50.0 (40.6-63.0)	<0.001
FEV ₁ /FVC (% predicted), Median (IQR)	50.0 (41.3-60.0)	49.9 (40.0-58.0)	53.0 (42.8-60.0)	51.0 (45.1-60.0)	50.0 (40.0-58.6)	50.0 (42.0-60.0)	0.007
COPD Assessment test (CAT), Median (IQR)	14 (9-20)	13 (8-21)	13 (8-19)	13 (9-19)	15 (8-20)	16 (10-23)	0.271
Total number of exacerbations per year, Median (IQR)	1 (0-3)	1 (0-4)	1 (0-3)	1 (0-3)	1 (0-2)	2 (0-5)	0.005
Hospitalised exacerbations, Median (IQR)	1 (0-2)	1 (0-2)	1 (0-2)	0 (0-2)	0 (0-2)	1 (0-3)	0.003
COTE Index, Median (IQR)	1 (0-2)	0 (0-2)	2 (2-3)	0 (0-1)	0 (0-0)	1 (1-3)	<0.001
Sputum culture positive, n (%)	185 (20.3)	30 (19.2)	24 (22.0)	14 (7.3)	100 (29.5)	17 (14.8)	<0.001
<i>H.influenzae</i> , n (%)	81 (8.9)	14 (9)	9 (8.3)	0 (0.0)	56 (16.5)	2 (1.7)	<0.001
<i>P.aeruginosa</i> , n (%)	43 (4.7)	9 (5.8)	3 (2.8)	4 (2.1)	22 (6.5)	5 (4.3)	0.154
<i>S.pneumoniae</i> , n (%)	33 (3.6)	6 (3.8)	5 (4.6)	8 (4.2)	11 (3.2)	3 (2.6)	0.917
<i>M.catarrhalis</i> , n (%)	24 (2.6)	4 (2.6)	3 (2.8)	2 (1.0)	11 (3.2)	4 (3.5)	0.606
<i>K.pneumoniae</i> , n (%)	13 (1.4)	1 (0.6)	6 (5.5)	1 (0.5)	3 (0.9)	2 (1.7)	0.004
<i>Acinetobacter spp.</i> , n (%)	10 (1.1)	2 (1.3)	1 (0.9)	0 (0.0)	6 (1.8)	1 (0.9)	0.451
Fungi, n (%)	9 (1.0)	3 (1.9)	1 (0.9)	0 (0.0)	5 (1.5)	0 (0.0)	0.259
Other bacteria, n (%)	22 (2.4)	1 (0.6)	1 (0.9)	0 (0.0)	15 (4.4)	5 (4.3)	0.004

Data are presented as number of patients (n) (with percentage; %) or median (and interquartile range; IQR). FEV₁: forced expiratory volume in the 1st second; FVC: forced vital capacity, COPD: chronic obstructive pulmonary disease; COTE: COPD specific co-morbidity test. *H. influenzae*: *Haemophilus influenzae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. pneumoniae*: *Streptococcus pneumoniae*, *M. catarrhalis*: *Moraxella catarrhalis*, *K. pneumoniae*: *Klebsiella pneumoniae*.

Figure 3.4: Dendrogram illustrating the five clinical clusters derived from the derivation cohort using a non-metric multidimensional scaling followed by hierarchical clustering. Colours represents the cluster membership: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk. LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

Figure 3.5: Two-year survival between the five clinical clusters in the derivation cohort. (a) Kaplan-Meier curves illustrating the two-year survival between the five clusters (b) Survival probability after adjustment for age, gender, body mass index, forced expiratory volume in the 1st second (FEV₁) and smoking pack years. Colours represent cluster membership: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk, LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

Figure 3.5: (c) Cumulative incidence curves for respiratory causes of death between the five clusters from the derivation cohort. Colours represent cluster membership: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk, LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

Figure 3.6: Bar chart demonstrating the proportion of COPD patients within each derived cluster with at least one severe (hospitalised) exacerbation at one-year following recruitment into the study. Colour of each respective bar indicates the five clinical clusters as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk. LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

Figure 3.7: Mortality and exacerbation outcomes within each respective cluster from the derivation cohort is unaltered by smoking status. Sankey plots illustrating the (a) mortality and (b) exacerbation differences between the five-clusters divided according to smoking status. Horizontal flow colours indicate the five clinical clusters as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Width of flow indicates the proportion of patients within each group. SE: severe exacerbator (≥ 1 exacerbation requiring hospitalisation), Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk. LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

3.3.3 Clusters from the derivation cohort were validated using an independent (validation) cohort with comparable clinical and mortality outcomes.

Having identified five clinically distinct clusters with variable prognostic outcome, I next validated these findings in an independently recruited COPD cohort (n=569). The patients from the validation cohort were assigned into each derived cluster using RDA as follows: Ex-TB (n=102; 17.9%), diabetic (n=72; 12.7%), LCLR (n=88; 15.5%), LCHR (n=193; 33.9%) and CVS (n=114; 20.0%). The validation cohort illustrated comparable comorbidity profiles and proportions by number of patients to that detected in the derivation cohort (Tables 3.4 and Table 3.5, Figure 3.8). The leave one out cross validation (LOOCV) accuracy of the RDA model was 97.8%, illustrating the robustness of our model (Table 3.2 and Figure 3.1). Two-year all-cause mortality was also comparable with that found in the derivation cohort with poorest survival in the CVS (43.0%) and Ex-TB (26.0%) clusters (log rank test, $p=0.001$) (Figure 3.9a). The hazard ratios remain significant, even after adjustment for age, sex, BMI, FEV₁ and smoking pack year exposure (Figure 3.9b). The hazard ratio for each cluster compared to LCLR group was determined as follows: CVS (HR: 3.08; 95% CI 1.74-5.44; $p<0.0001$), Ex-TB (HR: 2.01; 95% CI 1.07-3.80; $p<0.05$), LCHR (HR: 1.59; 95% CI 0.89-2.85; $p=ns$) and diabetic (HR: 1.73; 95% CI 0.86-3.45; $p=ns$). Similar trends were also observed for respiratory causes of death with poorest survival in the CVS, Ex-TB and LCHR clusters, albeit not statistically significant ($p=ns$) (Figure 3.9c). In contrast to the derivation cohort, no significant differences in hospitalised exacerbations were observed ($p=ns$) however as found in with the derivation cohort, mortality and exacerbation frequency in each cluster was not influenced by smoking status (Figure 3.10 a-b).

Table 3.5: Baseline characteristics of the five derived COPD clusters (from the validation cohort).

Characteristics	Overall	Ex-tuberculosis	Diabetic	Low co-morbidity: low-risk	Low co-morbidity: high-risk	Cardio-vascular	p- value
n (%)	569	102 (17.9)	72 (12.7)	88 (15.5)	193 (33.9)	114 (20.0)	
Country, n (%)							0.281
Singapore	224 (39.4)	39 (38.2)	19 (26.4)	35 (39.8)	80 (41.5)	51 (44.7)	
Hong Kong	295 (51.8)	50 (50.0)	45 (62.5)	45 (51.1)	99 (51.3)	56 (49.1)	
Malaysia	50 (8.8)	13 (12.8)	8 (11.1)	8 (9.1)	14 (7.2)	7 (6.2)	
Age (years), Median (IQR)	74 (69-81)	77 (71-81)	74 (70-80)	75 (70-82)	72 (67-79)	74 (68-81)	0.019
Sex (Male), n (%)	531 (93.3)	90 (88.2)	66 (91.7)	82 (93.2)	185 (95.9)	108 (94.7)	0.147
Smoking status, n (%)							<0.001
Current smoker	143 (25.1)	7 (6.9)	12 (16.7)	3 (3.4)	88 (45.6)	33 (29.0)	
Ex- smoker	411 (72.2)	94 (92.1)	58 (80.5)	83 (94.3)	98 (50.8)	78 (68.4)	
Non-smoker	15 (2.7)	1 (1.0)	2 (2.8)	2 (2.3)	7 (3.6)	3 (2.6)	
Smoking pack years, Median (IQR)	50.0 (40.0-60.0)	50.0 (40.0-60.0)	60.0 (50.0-80.0)	50.0 (40.0-80.0)	50.0 (33.6-60.0)	50.0 (40.0-60.0)	0.006
Body mass index (kg/m ²), Median (IQR)	21.3 (18.1-23.9)	19.9 (18.0-25.4)	22.7 (19.7-25.9)	21.8 (19.0-24.2)	20.5 (17.2-23.6)	21.9 (18.6-23.6)	0.315
FEV ₁ (% predicted), Median (IQR)	42.0 (33.0-58.0)	47.4 (32.0-65.0)	40.0 (31.0-50.2)	39.7 (29.0-48.5)	43.0 (34.0-61.6)	43.0 (31.2-52.1)	0.001
FEV ₁ /FVC (% predicted), Median (IQR)	47.0 (39.9-56.1)	49.0 (40.8-61.5)	43.6 (38.2-54.8)	46.0 (36.1-52.6)	47.0 (40.0-58.1)	48.3 (40.7-56.1)	0.024
Total number of exacerbations per year, Median (IQR)	1 (0-2)	1 (0-2)	1 (0-1)	1 (0-2)	0 (0-1)	1 (0-2)	0.369
Hospitalised exacerbations, Median (IQR)	0 (0-1)	0 (0-1)	0 (0-1)	1 (0-2)	0 (0-1)	0 (0-2)	0.314
COTE Index, Median (IQR)	1 (0-2)	0 (0-2)	2 (2-3)	0 (0-2)	0 (0-2)	2 (1-3)	<0.001
Sputum culture positive, n (%)	89 (15.6)	30 (29.4)	8 (11.1)	7 (8.0)	28 (14.5)	16 (14.0)	<0.001
<i>H.influenzae</i> , n (%)	30 (5.3)	6 (5.9)	1 (1.4)	3 (3.4)	14 (7.3)	6 (5.3)	0.377
<i>P.aeruginosa</i> , n (%)	19 (3.3)	9 (8.8)	2 (2.8)	0 (0.0)	4 (2.1)	4 (3.5)	0.012
<i>S.pneumoniae</i> , n (%)	9 (1.6)	3 (2.9)	0 (0.0)	1 (1.1)	1 (0.5)	4 (3.5)	0.135
<i>M.catarrhalis</i> , n (%)	13 (2.3)	4 (3.9)	3 (4.2)	1 (1.1)	4 (2.1)	1 (0.9)	0.423
<i>K.pneumoniae</i> , n (%)	7 (1.2)	4 (3.9)	1 (1.4)	0 (0.0)	0 (0.0)	2 (1.8)	0.035
<i>Acinetobacter spp.</i> , n (%)	6 (1.1)	1 (1.0)	0 (0.0)	1 (1.1)	3 (1.6)	1 (0.9)	0.970
Fungi, n (%)	3 (0.5)	3 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.011
Other bacteria, n (%)	9 (1.6)	3 (3.0)	1 (1.4)	0 (0.0)	4 (2.1)	1 (0.9)	0.535

Data are presented as number of patients (n) (with percentage; %) or median (and interquartile range; IQR). FEV₁: forced expiratory volume in the 1st second; FVC: forced vital capacity, COPD: chronic obstructive pulmonary disease; COTE: COPD specific co-morbidity test. *H. influenzae*: *Haemophilus influenzae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. pneumoniae*: *Streptococcus pneumoniae*, *M. catarrhalis*: *Moraxella catarrhalis*, *K. pneumoniae*: *Klebsiella pneumoniae*.

Figure 3.8: Co-morbidities are comparable between the derivation and validation COPD cohorts for each respective cluster. Bubble plots illustrating co-morbidity profiles between the derivation and validation COPD cohorts by cluster. Colours represent cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk, LCHR: low co-morbidity: high-risk, CVS: cardiovascular. PUD: peptic ulcer disease, pTB: history of prior pulmonary tuberculosis, PAD: peripheral arterial disease, Other Ca: all malignancies excluding lung, esophageal, pancreatic and/or breast carcinoma, DM: diabetes mellitus, CVA: cerebrovascular disease, CKD: chronic kidney disease, CHF: congestive heart failure, CAD: coronary artery disease, Ca: lung, esophageal, pancreatic and/or breast carcinoma, AF: atrial fibrillation.

Figure 3.9: Two-year survival between the five clinical clusters derived from the validation cohorts. (a) Kaplan-Meier curves illustrating two-year survival between the five clusters (b) Survival probability after adjustment for age, gender, BMI, FEV₁ and smoking pack years. Colours represent cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink).

Figure 3.9: (c) Cumulative incidence curves for respiratory causes of death between the five clusters from the validation cohort. Colours represent cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink).

Figure 3.10: Mortality and exacerbations in each of the five clusters from the validation cohort is unaltered by smoking status. Sankey plots illustrating the (a) mortality and (b) exacerbation differences between the five clusters assessed by smoking status. Horizontal flow colours indicate the five clinical clusters as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Width of flow indicates the proportion of patients within each group illustrating a particular outcome. SE: severe exacerbator (≥ 1 exacerbation requiring hospitalisation), Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk. LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

3.3.4 Decision tree for Chinese COPD cluster classification.

To further validate the cluster algorithm with RDA, and for ease of clinical translation of our findings, I next sought to derive a clinical decision tree. A simplified model for a clinical decision tree (Figure 3.11) was generated using Classification and Regression trees (CART) to stratify patients into each respective cluster, with accuracy of 72.4%, slightly lower than that of the RDA model.

Figure 3.11: Clinical decision tree generated by Classification and Regression trees (CART) for classification of a Chinese COPD patient into one of the five derived clusters. Previous pulmonary tuberculosis was defined as a prior history of documented tuberculosis with positive sputum analysis for *Mycobacteria tuberculosis* including positive acid-fast bacilli smear, culture or nucleic acid amplification, radiological features (on chest radiography or computed tomography) and/or prior pharmacological treatment for pulmonary tuberculosis. Diabetes mellitus was defined as the presence of either fasting blood glucose levels of ≥ 7.0 mmol/l, blood glucose levels of ≥ 11.1 mmol/l two-hour post oral glucose tolerance test, random blood glucose level ≥ 11.1 mmol/l with hyperglycaemia symptoms, or HbA1c $\geq 6.5\%$. Coronary artery disease was defined based on functional testing, radiological imaging, and/or coronary angiography. Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk, LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

3.3.5 The identified Chinese COPD clusters each demonstrate systemic and complex inflammatory networks that associate with mortality outcomes.

After identifying and validating the five clinical clusters as outlined above, I next assessed the inflammatory signatures between these clusters by first employing a semiquantitative cytokine array that included 120 different cytokines as described above (chapter 3.2.8). Cytokines demonstrating at least a 2.5-fold difference between any two of the five clusters were selected for further confirmatory assessment (Figure 3.12). Of the 120 cytokines screened, 6 cytokines (PDGF-AA, PDGF-BB, PDGF-AB, TNF-R1, TNF-R2, VEGF) were selected for confirmatory assays based on the fold-change cut-off and also (Figure 3.12) from prior COPD studies (193). The six cytokines were next compared between the non-diseased (healthy) controls and the COPD clusters (Table 3.6). Overall TNF-R2 associated with increased symptoms (as measured by CAT scores) and exacerbations, independent of the patients underlying cluster membership (Figure 3.13). Cytokine expression by cluster was as follows: TNF-R2 and PDGF-AA in the CVS cluster (median: 83.8 pg/ml ($p=0.01$) and 2.4×10^5 pg/ml ($p<0.05$) respectively); VEGF in the ex-TB cluster (median: 398.8 pg/ml) trending toward significance ($p=0.077$) and PDGF-AB and PDGF-BB in the LCHR cluster (median: 4595.8 pg/ml ($p<0.05$) and 259.1 pg/ml ($p<0.05$) respectively) (Figure 3.14a; Table 3.7a-b). With the observed differences in cytokine expression between clusters, I next evaluated how these cytokines potentially interact within an inflammatory network for each cluster. This was achieved using network plots (Figure 3.14b). The CVS cluster demonstrates the most complex cytokine network of all the five clusters with the highest number of patients with positive cytokines (defined as a value above the 95th percentile of the non-diseased (healthy) control group) ($p<0.05$) and the highest proportion of patients demonstrating cytokine interaction. ($p<0.05$). This is followed by the Ex-TB, LCHR, diabetic and LCLR clusters. Interestingly, the inflammatory network complexity followed closely the observed mortality trend for both the derivation and validation COPD cohorts where increased mortality in CVS, followed by Ex-TB, LCHR, diabetic and LCLR.

Table 3.6: Demographic table illustrating baseline characteristics and inflammatory (cytokine) assessments for the non-diseased (healthy) and COPD cohorts.

Characteristics	Overall n=360	Non-diseased (Healthy) n=24	COPD n=336	p-value
Age (years), Median (IQR)	73 (67-79)	64 (59-68)	74 (67-79)	<0.0001
Sex (Male), n (%)	332 (92.2)	9 (38.0)	323 (96.1)	<0.001
Current smoker, n (%)	221 (61.4)	3 (12.5)	218 (64.9)	<0.0001
Smoking pack years, Median (IQR)	50 (40-68)	40 (37-47)	50 (40-70)	0.364
Body mass index (kg/m ²), Median (IQR)	21.4 (18.1-23.8)	22.8 (20.3-25.9)	21.4 (18.1-23.8)	0.070
FEV ₁ (% predicted), Median (IQR)	47.0 (34.1-61.8)	84.0 (83.0-90.5)	47.0 (34.0-61.0)	<0.0001
FEV ₁ /FVC (% predicted), Median (IQR)	50.0 (41.4-59.3)	78.0 (78.0-80.0)	49.7 (41.2-59.1)	<0.0001
COPD assessment test (CAT), Median (IQR)	14 (9-20)	NA	14 (9-20)	NA
Hospitalized exacerbations, Median (IQR)	0 (0-2)	NA	0 (0-2)	NA
COTE Index, Median (IQR)	1 (0-2)	NA	1 (0-2)	NA
TNF-R1 (pg/ml), Median (IQR)	1090 (597-1927)	279 (148-343)	1131 (722-2036)	<0.001
TNF-R2 (pg/ml), Median (IQR)	41 (16-89)	5 (2.5-8.4)	46 (19-92)	<0.001
VEGF (pg/ml), Median (IQR)	310 (40-625)	3.3 (0-33)	363 (90-690)	<0.001
PDGF-AB (pg/ml), Median (IQR)	3965 (2129-6497)	2017 (994-3074)	4054 (2353-7009)	<0.001
PDGF-AA (x 10 ⁵ pg/ml), Median (IQR)	2 (1- 5)	2 (1 - 3)	2 (1-5)	0.648
PDGF-BB (pg/ml), Median (IQR)	198 (71-455)	131 (43-240)	204 (72-460)	0.149

Data are presented as number of patients (n) (with percentage; %) or median (and interquartile range; IQR). FEV₁: forced expiratory volume in the 1st second; FVC: forced vital capacity, COPD: chronic obstructive pulmonary disease; COTE: COPD specific co-morbidity test; TNF-R1: Tumour Necrosis Factor receptor-1; TNF-R2: Tumour Necrosis Factor receptor-2; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet Derived Growth Factor.

Table 3.7a: Demographic table illustrating the baseline characteristics and inflammatory (cytokine) assessment for the five derived COPD clusters.

Characteristics	Overall	Ex-tuberculosis	Diabetic	Low co-morbidity: low-risk	Low co-morbidity: high-risk	Cardio-vascular	p-value
n, (%)	336	59 (17)	63 (19)	33 (10)	131 (39)	50 (15)	
Centre, n (%)							
Singapore	74 (22)	12 (20)	7 (11)	4 (12)	33 (25)	18 (36)	0.035
Hong Kong	213 (63)	35(60)	47 (75)	23 (70)	84 (64)	24 (48)	
Malaysia	49 (15)	12 (20)	9 (14)	6 (18)	14 (11)	8 (16)	
Age (years), Median (IQR)	74 (67-79)	74 (69-81)	73 (68-79)	75 (71-80)	73 (65-79)	74 (69-79)	0.728
Sex (Male), n (%)	323 (96.1)	56 (94.9)	59 (93.7)	32 (97.0)	128 (97.7)	48 (96.0)	0.633
Smoking status, n (%)							
Current smoker	218 (64.9)	28 (47.5)	45 (71.4)	10 (30.3)	104 (79.4)	31 (62.0)	<0.001
Ex-smoker	118 (35.1)	31 (52.5)	18 (28.6)	23 (69.7)	27 (20.6)	19 (38.0)	
Smoking pack years, Median (IQR)	50 (40-70)	50 (40-62)	50 (30-80)	60 (48-77)	50 (40-63)	49 (32-73)	0.437
Body mass index (kg/m ²), Median (IQR)	21.4 (18.1-23.8)	20.8 (17.7-24.5)	22.6 (19.6-25.6)	19.8 (17.3-21.9)	21.5 (18.0-23.5)	21.7 (17.9-23.6)	0.039
FEV ₁ (% predicted), Median (IQR)	47.0 (34.0-61.0)	42.0 (31.3-63.8)	47.1 (35.3-59.0)	47.0 (36.5-57.5)	48.0 (33.1-61.0)	42.6 (36.3-57.8)	0.997
FEV ₁ /FVC (% predicted), Median (IQR)	49.7 (41.2-59.0)	46.0 (40.0-60.9)	55.0 (44.0-61.5)	48.5 (42.0-57.7)	48.9 (40.7-58.3)	47.4 (41.0-57.5)	0.140
COPD assessment test (CAT), Median (IQR)	14 (9-20)	14 (9-21)	13 (8-18)	13 (11-22)	13 (8-19)	14 (10-22)	0.785
Total number of exacerbations per year, Median (IQR)	1 (0-2)	1 (0-2)	1 (0-3)	0 (0-2)	1 (0-2)	1 (0-3)	0.609
Hospitalized exacerbations, Median (IQR)	0 (0-2)	0 (0-2)	0 (0-1)	0 (0-1)	0 (0-2)	1 (0-3)	0.724
COTE Index, Median (IQR)	1 (0-2)	0 (0-2)	2 (2-3)	0 (0-1)	0 (0-0)	2 (1-4)	<0.001
TNF-R1 (pg/ml), Median (IQR)	1131 (722-2036)	1175 (793-2243)	1204 (709-2111)	994 (730-2043)	1093 (633-1828)	1379 (795-2138)	0.556
TNF-R2 (pg/ml), Median (IQR)	46 (19-92)	53.8 (16-110)	43.8 (21.9-90.8)	33 (17-122.8)	38 (15.7-70.0)	84 (34.3-151.3)	0.010
VEGF (pg/ml), Median (IQR)	363 (90-690)	550 (235-815)	294 (15-537)	343 (84-792)	272 (40-722)	399 (98-793)	0.077
PDGF-AB (pg/ml), Median (IQR)	4054 (2353-7009)	4472 (2833-8978)	3395 (1681-5465)	4110 (1314-9210)	4596 (2843- 7756)	3609 (1999-5462)	0.031
PDGF-AA (X10 ⁵ pg/ml), Median (IQR)	2 (1 - 5)	2 (1 – 6)	1 (0 - 5)	2 (1 - 5)	2 (1 – 4)	2 (1 -6)	0.037
PDGF-BB (pg/ml), Median (IQR)	204 (72-460)	158 (82-404)	218 (89-511)	107 (42-818)	259 (121-460)	86 (22-333)	0.026

Data are presented as number of patients (n) (with percentage; %) or median (and interquartile range; IQR). FEV₁:

forced expiratory volume in the 1st second; FVC: forced vital capacity, COPD: chronic obstructive pulmonary

disease; COTE: COPD specific co-morbidity test; TNF-R1: Tumour Necrosis Factor receptor-1; TNF-R2: Tumour Necrosis Factor receptor-2; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet Derived Growth Factor.

Table 3.7b: Table illustrating significance (p-values) for each of the assessed cytokines between the clusters (including post-hoc analysis).

Cluster/ Cytokines	TNF-R1	TNF-R2	VEGF	PDGF-AB	PDGF-AA	PDGF-BB
Ex-TB vs Diabetic	ns	ns	ns	ns	ns	ns
Ex-TB vs LCLR	ns	ns	ns	ns	ns	ns
Ex-TB vs LCHR	ns	ns	ns	ns	ns	ns
Ex-TB vs CVS	ns	ns	ns	ns	ns	ns
Diabetic vs LCLR	ns	ns	ns	ns	ns	ns
Diabetic vs LCHR	ns	ns	ns	ns	ns	ns
Diabetic vs CVS	ns	ns	ns	ns	0.03	ns
LCLR vs LCHR	ns	ns	ns	ns	ns	ns
LCLR vs CVS	ns	ns	ns	ns	ns	ns
LCHR vs CVS	ns	0.003	ns	ns	ns	0.02

ns: not significant, Ex-TB: ex-tuberculosis, LCLR: low co-morbidity low risk, LCHR: low co-morbidity high risk, CVS: cardiovascular, TNF-R1: Tumour Necrosis Factor receptor-1; TNF-R2: Tumour Necrosis Factor receptor-2; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet Derived Growth Factor.

Figure 3.12: A semi-quantitative expression of 120 cytokines between the five clusters used in the initial screening assay. Semi-quantitative assay with the Raybiotech Human cytokine C2000 antibody array using 20 specimens of pooled plasma from each respective cluster. The selected cytokines for confirmatory assay were those demonstrating at least a 2.5-fold difference from at least one other cluster and are highlighted by coloured boxes as follows: PDGF-BB (red), VEGF (blue), PDGF-AA (green), TNFR-2 (yellow), PDGF-AB (purple), TNF-R1 (orange).

Figure 3.13: TNF-R2 is significantly increased in Chinese COPD patients demonstrating higher symptom scores (CAT scores) and greater frequency of severe (hospitalised) exacerbations (SE). Colours represent cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). CAT: COPD assessment test, SE: severe exacerbator, TNF-R2: Tumor Necrosis Factor receptor-2. * $p \leq 0.05$. The groups were compared using the Mann-Whitney U Test.

Figure 3.14: Inflammatory profiles and networks of the five derived Chinese COPD clusters.

(a) Radar plots illustrating the median normalised value of the six selected cytokines compared between the five derived Chinese COPD clusters. Colours represent cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). TNF-R1: Tumour Necrosis Factor receptor-1, TNF-R2: Tumor Necrosis Factor receptor-2, VEGF: vascular endothelial growth factor, PDGF: platelet derived growth factor. * $p \leq 0.05$, # $p < 0.1$.

(b)

Figure 3.14: (b) Network plots illustrating the systemic inflammatory network between clusters arranged in descending order of network complexity. Cardiovascular followed by ex-TB demonstrated higher numbers of positive cytokines and an increased number of cytokine interactions. Each circle (node) represents a cytokine from the final assessed panel: circle size corresponds to percentage of patients within that cluster demonstrating a positive value. Lines connecting two nodes indicate positive detection of both cytokines and line thickness illustrates the proportion of patients with a positive result for both cytokines. Colours represent cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). TNF-R1: Tumour Necrosis Factor receptor-1, TNF-R2: Tumor Necrosis Factor receptor-2, VEGF: vascular endothelial growth factor, PDGF: platelet derived growth factor.

3.3.6 The five Chinese COPD clusters provide additional and incremental value to established GOLD classification by identifying 'high-risk' Chinese COPD patients.

Having identified five 'clusters' of Chinese patients with COPD each demonstrating prognostic relevance, distinct inflammatory signatures and cytokine network complexity, I next assessed the added value that these 'clusters' provide when compared to established COPD guideline-based classification: GOLD ABCD grouping and GOLD I-IV grading of lung function. All COPD patients (derivation and validation cohorts; n=1,480) were classified into their respective GOLD groups based on symptoms (CAT score) and exacerbation history at study enrolment as follow: A (17.0%; n=252), B (29.1%; n=430), C (12.9%; n=191) and D (41.0%; n=607). They were also assigned to their respective GOLD grade (based on FEV₁ criteria): I (6.4%; n=95), II (34.5%; n=510), III (43.9%; n=650) and IV (15.2%; n=225). Mortality at two-years were assessed based on cluster membership within each GOLD ABCD group and GOLD I-IV grade. The CVS and ex-TB clusters exhibited the highest mortality in GOLD A, C and D, while the CVS and LCHR clusters demonstrate the highest mortality in GOLD group B (Figure 3.15). Similarly, for GOLD grading, the CVS and ex-TB clusters demonstrate a significantly poorer survival from grades II through IV (Figure 3.16). By multivariate logistic regression analysis, the CVS (adjusted odd ratio (OR) 2.98 (95% CI 1.88-4.73; p<0.001)) and ex-TB clusters (adjusted OR 1.852 (95% CI 1.17-2.92; p<0.01)) demonstrate a significantly higher mortality, after adjustment for age, sex, BMI, FEV₁, smoking pack year exposure, and GOLD group compared to the LCLR cluster (Figure 3.17). Taken together, these data illustrate the additional and incremental benefits conferred by our derived clusters in the risk stratification (the identification of individuals at highest risk) of Chinese COPD patients.

Figure 3.15: An increased mortality in the cardiovascular and ex-TB clusters irrespective of GOLD grouping. Tree map illustrating the mortality for each respective Chinese COPD cluster within their GOLD ABCD group. Rectangles represent the proportion of mortality at 2-years within each group. Colour represents cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk, LCHR: low co-morbidity: high-risk, CVS: cardiovascular, CAT: Chronic obstructive pulmonary disease (COPD) assessment test.

Figure 3.16: Reduced two-year survival in the cardiovascular and ex-TB Chinese COPD clusters irrespective of GOLD grading. Kaplan-Meier curves illustrating two-year survival for each respective Chinese COPD cluster between their GOLD grades (based on FEV₁). * $p \leq 0.05$, ** $p < 0.001$ by log-rank test. Colours represents cluster membership as follows: Ex-tuberculosis (green), diabetic (blue), low co-morbidity: low-risk (violet red), low co-morbidity: high-risk (orange) and cardiovascular (pink). Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk. LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

Figure 3.17: A forest plot illustrating the odds ratios of mortality in each of the derived Chinese COPD clinical clusters (compared to the LCLR cluster) after adjustment for age, sex, BMI, smoking pack year exposure, lung function (by FEV₁) and GOLD group. Circles correspond to the odds ratio and colours denote significance levels: red ($p \leq 0.05$), grey ($p > 0.05$; ns). The error bar indicates the 95% confidence interval (CI). FEV₁: forced expiratory volume in the 1st second. Ex-TB: ex-tuberculosis, LCLR: low co-morbidity: low-risk, LCHR: low co-morbidity: high-risk, CVS: cardiovascular.

3.4 Discussion

To address my research question and determine if clinical phenotypes exist in COPD within South-East Asia, I focused on patients of Chinese ethnicity. I discovered five novel clinical clusters with prognostic significance in a large multi-centre study including 1,480 COPD patients of Chinese ethnicity across three South-East Asian countries (and six separate sites). The five derived clusters were: (1) ex-TB; (2) diabetic; (3) low co-morbidity: low risk (LCLR); (4) low co-morbidity: high risk (LCHR) and (5) cardiovascular (CVS). The cardiovascular and ex-tuberculosis clusters were identified as the “high risk” clusters with poorest two-year survival. This novel clinical ‘cluster’ classification provides added and incremental value to GOLD assessments alone. In addition, I describe accompanying inflammatory signatures within each cluster and assess their network complexity which in itself is shown to be associated with overall mortality.

The identified ex-TB cluster is particularly unique to Asia where TB is endemic. Despite having completed TB treatment with demonstrable clinical and microbiological cure, its long-term sequelae remain. The risk of pulmonary obstruction ranges from 15.0 to 86.8% post TB-treatment (19, 29, 381, 382). Precise mechanisms that drive this susceptibility to airflow obstruction post-TB however remains unclear. Structural damage, as a consequence of TB infection, with pulmonary cavitation, bronchiectasis and endobronchial disease are all associated with an increased risk of airflow obstruction. However, despite even minimal change on chest radiography, the risk of developing lung obstruction remains, indicating alternate mechanisms that may contribute to the development of COPD in the post-TB state (377). It is plausible that TB infection disrupts the host immune response with an increased susceptibility to noxious particle stimuli, its resultant inflammation and tissue remodelling (381, 383). Once COPD develops, the host immune response and genetic factors determine the precise disease trajectory that each patient will follow. A previous history of TB is associated with higher COPD morbidity and mortality (173, 384), consistent with our findings in the ex-TB cluster. In addition, our inflammatory results demonstrate complex inflammatory network interaction in

the ex-TB group, further highlighting the potential for an ongoing inflammatory response despite successful treatment of pulmonary TB. This in turn may contribute to poorer overall outcomes (385). Importantly, ethnicity has also been shown to influence TB induced inflammation and its disease manifestations (386, 387). This may have relevance in the context of our COPD patients of Chinese ethnicity belonging to the ex-TB cluster.

Cardiovascular disease and diabetes are both well-recognised comorbidities in COPD. Clusters demonstrating these have been consistently illustrated in prior COPD studies, suggesting a stability of such cluster across all COPD patients irrespective of geographic origin (193, 354, 355). In Asia, the increased urbanization with improved socioeconomic status has resulted in a modification of lifestyles and consequently has predisposed to a higher risk of developing CVS and metabolic disease. In COPD, CVS disease is often under-treated owing to poor recognition due to overlap symptoms and the concern of medication interaction. As a result, CVS disease often associates with poor COPD outcomes, consistent with our cluster findings. Prior COPD studies from Northeast and Southeast Asia illustrate that patients were less likely to receive optimal treatment for CVS disease in these settings, with the exception of Japan (388). Differences in resource availability and clinical practice also exists across the Asian subcontinent which potentially contributes to the higher mortality observed in our CVS ‘cluster’. A co-existence of diabetes in COPD is previously shown to confer a worse overall prognosis and increased mortality (389, 390). Interestingly, in our COPD cohort, the diabetic group associated with a better prognosis compared to those in the CVS and Ex-TB clusters. The differences in study cohorts may at least in part account for such discrepancies, with prior studies demonstrating ethnic differences in diabetes-related mortality with best survival in South Asia and in particular Chinese patients (391). The low co-morbidity clusters can be classified into those at low and high risk respectively based on their mortality outcomes. Despite having a low comorbidity burden, the mortality differences observed between these two clusters are likely attributed to underlying smoking history, with a predominance of current smokers in the LCHR and ex-smokers in LCLR clusters respectively. Smoking cessation is further known to attenuate

the decline of lung function in COPD and directly alters its natural course. This results in overall more favourable COPD outcomes after an individual stops smoking (392-394).

Low-grade systemic inflammation is often reported in COPD, but only a subgroup of COPD patients exhibits an increased persistent systemic inflammation which associates with high mortality (395). Systemic inflammation in COPD involves the interaction of multiple cytokines and pathways (395). Using network analyses, I illustrate the complexity of cytokine interaction and their associated mortality differences within each derived cluster. Systemic inflammation is also evident where CVS disease is present with increased pro-inflammatory cytokines, angiogenesis and growth factors, all promoting atherosclerotic plaque formation and disease progression (396, 397). The co-existence of both COPD and CVS disease therefore contributes to even greater increases in systemic inflammation, which in turn affects disease severity and outcome including higher mortality. Tumour necrosis factor (TNF), a pro-inflammatory cytokine, upon its stimulation causes tissue inflammation and injury. In CVS disease, TNF induces reactive oxygen species, increases endothelial permeability and vascular injury, which together results in deleterious outcomes, again consistent with my findings (397, 398). Vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis has a paradoxical role in COPD: decreased VEGF expression is observed in emphysema, whilst increased levels are reported with bronchitis. VEGF also plays an essential role in the pathogenesis of TB enhancing granulomatous inflammation and angiogenesis (399, 400). VEGF levels are further increased during active TB and correlate with disease severity and decrease following successful treatment (401-403). Importantly, however, its role in ex-TB COPD remains unknown. Higher levels of VEGF were observed in our ex-TB Chinese COPD group despite successful completion of TB treatment, suggesting a potential role for VEGF in the development of COPD in a susceptible individual post-TB subject. The role of platelets in COPD has also been of great recent interest, with associations between thrombocytosis and COPD mortality described (404, 405). Platelet derived growth factor (PDGF)-related cytokines play important roles in cell signalling and lung development, and when dysfunctional, can be associated to CVS disease (406). Increased PDGF

concentrations are reported in relation to cigarette smoke, which results in small airway remodelling (407). In addition, as a potent stimulator of smooth muscle cells, PDGF has been associated with fibrotic disease and atherosclerosis (408). The role PDGF in COPD however is less well described: the increased PDGF observed in our Chinese COPD cohort is likely contributed to by the significant underlying comorbidities and smoking status, as increased levels were detected in the CVS and LCHR clusters respectively.

The derived Chinese COPD clusters described in this chapter provide clear added value to existing GOLD classification used in COPD assessment. The CVS and ex-TB clusters illustrate higher two-year mortality and more complex cytokine networks irrespective of their underlying GOLD classification. This suggests a possible existence of an important subgroup within each GOLD classification that performs poorly and requires further attention. A comprehensive tool for risk stratification, that incorporates the presence of CVS disease and a history of ex-TB will likely be of value in COPD patients of Chinese ethnicity to improve disease prognostication and/or direct appropriate treatment. The CVS, diabetic and low comorbidity clusters identified here are consistent with prior COPD studies in non-Chinese cohorts (193, 354, 355). However, in contrast to prior studies, the number of patients with anxiety disorder was relatively low in our cohort, hence we could have missed this additional clinical group previously described in non-Asian COPD populations. The stigma associated with such a diagnosis coupled to the different diagnostic criteria employed likely explains its absence in my work. As in prior works, there was also no underweight COPD group identified in our cohort; however, our Ex-TB cluster did illustrate an overall low BMI, a potentially comparable cluster with high mortality relevant particularly in Asian countries where TB is endemic.

In this chapter, I have presented the largest multicentre study to date to cluster South-East Asian COPD patients of Chinese ethnicity. This work includes longitudinal follow up and separate derivation and validation cohorts with use of robust statistical modelling to derive these clinically relevant clusters. Multivariate modelling was employed for mortality analysis and to adjust for potential confounders to improve the accuracy and clinical relevance and

translatability of our cluster outcomes. A further strength to my work is the diagnostic criteria employed to define COPD (i.e. an FEV₁/FVC ratio of <0.7). This has recently been shown to be of high accuracy in identifying COPD with high mortality (409).

Despite its clear strengths, my presented work in this chapter does have limitations. While it is quite broadly representative, this study is restricted to COPD patients of Chinese ethnicity from three countries and six tertiary hospitals, therefore our results may not be generalisable to the milder COPD patient group. More than 90 percent of our COPD cohort was male, and although this is representative of the COPD population in Asia (21-23), it does potentially limit the applicability of our results to female COPD patients. The healthy controls in this study are younger compared to the COPD cohort (Median age: 64 versus 74), and cytokines are known to increase with age, therefore, the measured cytokines may be of higher values in the healthy age-matched controls. However, the median values of TNF-R1, TNF-R2 and VEGF, which is significantly different between the COPD and healthy controls (Table 3.6) is of at least 4-fold higher difference in COPD, and therefore it is likely that such differences suggest that values in COPD are higher compared to the age-matched (healthy) controls. All patients had a chest radiograph performed, and chest computed tomography (CT) was not available for all patients, therefore the potential sequelae of post-TB lung injury including bronchiectasis may not be completely excluded in all patients. A simplified decision tree was generated for the classification of patients into each derived cluster, but its accuracy was lower than the RDA model, therefore further study is required before implementing this into routine practice. I used a restricted panel of cytokines for inflammatory work, selected based on my initial semi-quantitative screen of 120 cytokines with 2.5-fold difference between clusters and those relevant from previous studies. By decreasing the fold difference cut-off, I could have increased the number of cytokines in the final panel for quantitative study. The comparisons of our clusters with other mortality prediction models including the BODE index was not assessed. This is because 6-minute walk test results were not performed or collected in the original study design. However, when compared to other mortality indices such as the ADO (410), no statistically

significant differences between the clusters were detectable. The five Chinese COPD clusters were determined based on clinical data collected at the onset of recruitment; and the stability of the clusters with time was not examined. Furthermore, the validity of our clusters in Chinese patients outside Asia and in other ethnic groups within Asia was also not examined: all these are important avenues for future study. Further studies could adopt our current study design and be applied to non-Chinese Asian COPD cohorts, or alternatively to ethnically Chinese COPD patients living outside the Asian sub-continent. Such work would be useful in validating my findings, and determining if the identified clusters in this thesis can be applied more widely and to all COPD patients of Asian background.

In summary, I have identified five novel clinical phenotypes of COPD in patients of Chinese ethnicity in three South-East Asian countries at six separate sites. Using cluster analysis and their accompanying unique systemic inflammatory signatures, I further demonstrate the incremental and prognostic value this clustering provides when compared with established GOLD grouping and staging of COPD. The ex-TB cluster is novel and of importance in South-East Asia where TB demonstrates high prevalence. Importantly, this phenotype demonstrates poorer survival that is not identifiable by conventional COPD assessment tools (GOLD ABCD group or grade). TB and COPD while associated, are independent key public health concerns, and a clear unmet need to address these particularly when they co-occur is required. Future research should therefore now focus on identifying pathways for immunomodulation, determining immuno-genetic correlates of hyper-inflammation and airway remodelling, or performing genome wide association studies (GWAS) focused on this 'ex-TB' Chinese COPD cluster. All of these approaches will assist in delineating mechanisms to better understand and treat this 'high-risk' COPD patient group.

CHAPTER 4: IMMUNOLOGICAL (ALLERGIC) PHENOTYPES OF COPD IN SOUTH-EAST ASIA

4.1 Introduction

Innate and adaptive immune responses play a vital role in the pathogenesis of COPD (411). Exposure to noxious stimuli such as cigarette smoke activates the host immune response and results in the recruitment of immune cells to the airway that in turn induce a cascade of inflammatory consequences that are further exaggerated in exacerbations (66). Several immunologic phenotypes are described in COPD, including neutrophilic, eosinophilic and pauci- granulocytic, each with association to clinical outcomes (205, 228, 412). Allergic sensitisation is an additional and key immunologic phenotype, less well studied compared to others and of importance in South East Asians with COPD.

Allergic sensitisation is an established risk factor for poorer clinical outcomes in asthma and an increasing prevalence is reported in other respiratory disease states including COPD. The prevalence of sensitisation in COPD is reported to be between 18 and 34%, statistics comparable to asthma (16, 17, 183, 227, 413). Sensitisation, particularly to fungi have been associated with worse clinical outcomes in asthma, however, their implications in other respiratory disease states remains indeterminate, with no current recommendations necessitating any screening nor evaluation of sensitisation in the management in COPD (230). In addition, prior studies on sensitisation in COPD remain inconsistent, with some stating important associations with COPD symptoms, exacerbations and lung function decline, while others illustrate a lack of any such association with clinical outcomes (16, 17, 183, 229, 413). Fattahi *et al.* (2013) illustrate an important association between respiratory symptoms and allergic sensitisation in COPD using serum specific IgE to common allergens, employing an ImmunoCAP panel (17). This contrasts the work of Neves *et al.* (2013) which report less severe COPD where sensitisation is present, while Queiroz *et al.* (2016) describe no association between COPD severity and allergic sensitisation using skin prick testing (229). Critically, heterogeneity between study populations,

the variable methodologies employed, wide range of allergens examined and geographic variation are all important factors that have likely accounted for such discrepancies in reported outcomes.

With such rapid urbanisation occurring across the Asian sub-continent, allergic disease is increasingly being recognized, however, its precise role on COPD in this region, and in particular in South-East Asia lacks dedicated study (217). Furthermore, whether the presence of sensitisation itself, allergen patterns and variable patient exposures across the region play a role in determining endophenotypes of COPD requires dedicated study.

Geographic variations to sensitisation patterns are also described. A high prevalence of sensitisation to house dust mites (HDM) are reported in up to 90% of atopic Asian individuals, while sensitisation to pollen and animal dander are less common (present in <10%) when compared to atopic western cohorts (217). Warm climates and humid environments across South-East Asia may further predispose to mould sensitisation, and associate with the development of allergic rhinitis and severe asthma (230, 414). Karvonen *et al.* (2015) and Oluwole *et al.* (2017) found that exposure to indoor mould allergens during early life represents a risk factor for the development of asthma (415, 416). Exposure to outdoor allergens such as pollen and fungi are important factors that precipitate respiratory events even in the healthy, however, these exposures can induce strong and deleterious outcomes in patients with chronic respiratory disease states such as asthma (417). Climate and air pollution affect the chemical and biological composition of outdoor allergens and can alter their allergenic potential, triggering for instance, exacerbations in asthma. One key example is that of thunderstorm-associated-asthma, where a release of allergenic particles from pollen and/or *Alternaria* by osmotic shock, causes outbreaks of asthma exacerbation during thunderstorm periods (213, 418). Environmental factors including climate and humidity are therefore of particular importance in the Asian sub-continent and influence the allergen type, time and state of an individual's exposure.

No prior work has to date addressed sensitisation in COPD in relation to environmental exposures and in particular separating indoor and outdoor allergens. In addition, data focused on sensitisation and its related clinical outcomes in COPD in the South-East Asian region remains lacking (27, 212, 217, 220).

In this chapter, I first aim to investigate if allergic sensitisation is clinically important in COPD. To achieve this, I recruited Chinese patients from three different regions across South-East Asia namely Singapore, Malaysia and Hong Kong. To further extend this and better understand the relevance of sensitisation in COPD within South-East Asia, I next evaluate if allergic (or immunological) phenotypes, each with clinical relevance, exists in the stable COPD state. Finally, and for the first time, I assess the separate influence of indoor and outdoor allergen exposure using a state-of-the-art metagenomic sequencing approach and relate these findings to clinical COPD outcomes.

4.2 Methods

4.2.1 Ethics statement

The work presented in this chapter has been approved by the Institutional Review Boards (IRBs) of all participating hospitals and institutions. Written informed consent was obtained from all participants. Reference numbers pertaining to ethical approvals at each site is as follows: CIRB 2016/2549 (2013/184/C), CIRB 2016/2715, CIRB 2017/2933, CIRB 2017/2109 (all mutually recognized by DSRB, Singapore), UMMC 2018725-6524 and U/SERC/03/2016 (Malaysia), CREC 2011.146, CREC 2015.164 and CREC 2018.042 (Hong Kong) and IRB-2017-12-010 (NTU Singapore).

4.2.2 Patient recruitment

Stable Chinese COPD patients aged ≥ 40 years old were recruited from respiratory outpatient departments of each participating hospital between 2014 and 2018 through five different sites (total COPD patients recruited, $n=446$); Singapore General hospital, Changi General Hospital and Tan Tock Seng hospital (Singapore); University Malaya Hospital (Kuala Lumpur, Malaysia) and Prince of Wales Hospital (Hong Kong). Patients with a documented prior diagnosis of asthma as defined by GINA guidelines (variable respiratory symptoms and airflow limitation), individuals on long-term oral corticosteroids and/or immunosuppressive agents were also excluded (232). COPD was defined as per GOLD guidelines as described in Chapter 3.2.2 (200). Frequent exacerbators were defined as individuals demonstrating ≥ 2 exacerbations per year and very frequent exacerbators (vFE) as those demonstrating ≥ 3 exacerbations per year.

Non-diseased (healthy) controls: Non-diseased (healthy) controls were recruited as a comparison group to obtain a baseline sensitisation profile to a range of allergens to compare with our COPD cohort. Non-diseased (healthy) controls ($n=51$) were recruited at two sites: Nanyang Technological University, Singapore and the University Tunku Abdul Rahman

(UTAR) Kampar, Malaysia. All participants had no prior history of any respiratory disease, were receiving no long-term medications and had normal spirometry measured at recruitment and defined by an $FEV_1/FVC > 0.7$ and $FEV_1 > 80\%$ predicted.

4.2.3 Blood sample collection

Venous blood was collected from each recruited participant for immunological assays as described. Blood was collected in vacutainer EDTA tubes (BD biosciences) and plasma isolated by centrifugation at 1300g for 10 minutes at 18°C and then stored at -80°C until further processing.

4.2.4 Measurement of total Immunoglobulin (Ig)-E

Twenty microlitres of plasma (in duplicate) were used for the measurement of total Ig-E (Abcam ab108650). Each plate consisted of six IgE standards and negative controls in duplicate. Participant and patient plasma and accompanying standards were incubated in appropriate wells with mixing for 30 minutes at room temperature according to the manufacturer's instructions. Washing steps were performed with deionised water in 5 repeat steps following which 150ul of enzyme conjugate reagent was added and incubated at room temperature for 30 minutes. This was followed by further washing steps and incubation with tetramethyl benzidine (TMB) solution for 15 minutes. Plates were read after adding stop solution at an optical density of 450nm with an appropriate microplate reader.

4.2.5 Allergen panel

To examine the sensitisation profile in healthy and COPD participants, a customised allergen panel comprising of the most common allergens implicated in airways disease across Asia was designed and included the top eight culturable fungi isolated from the outdoor air in Singapore based on published metagenomics data (218, 419, 420). This included *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Blomia tropicalis*, *Elaeis guineensis*, Panicoids (Johnson grass (*Sorghum halepense*)), Pooids (Timothy grass (*Phleum pratense*)), Meadow fescue (*Festuca pratensis*), Perennial ryegrass (*Lolium perenne*)), Chloroids (Bermuda grass (*Cynodon dactylon*)), Weeds (*Brassica spp*, *Ambrosia artemisiifolia*, *Helianthus annuus*), *Blattella germanica*, *Periplaneta Americana* and the fungi: *Curvularia* (*C.lunata*, *C.spicifera*, *C.inequalis*), *Penicillium* (*P. citrinum*, *P.chrysogenum*, *P.notatum*, *P.digitatum*), *Aspergillus fumigatus* and recombinant *Aspergillus fumigatus* (rAsp f) 1, 2, 6, 8, 15, 17. The top 8 culturable air fungi are *Trametes* (*T.sanguinea*), *Schizophyllum* (*S.commune*), *Fusarium* (*F.solani*, *F.moniliforme*), *Aspergillus* (*A. terreus*, *A. sydowii*), *Penicillium* (*P.herquei*, *P.griseofulvum*), *Neurospora spp.*, *Byssoschlamys* (*B.spectabilis*) and *Cladosporium* (*C. tenuissimum*, *C. xanthochromaticum*). (Figure 4.1).

Figure 4.1: The extensive allergen panel designed for use in this study. HDM: house-dust mite, *Der f*: *Dermatophagoides farinae*, *Der p*: *Dermatophagoides pteronyssinus*, *Blo t*: *Blomia tropicalis*, *Bla g*: *Blattella germanica*, and rAsp f: recombinant *Aspergillus fumigatus*.

4.2.6 Outdoor air sampling for metagenomic sequencing

To create a comprehensive allergen panel that incorporates the common outdoor air fungi, we first determined the top outdoor air fungi in Singapore using a state-of-the art metagenomic sequencing protocol. Outdoor air samples were collected on a rooftop at Nanyang Technological University, Singapore, 20 metres above the ground for five consecutive days using methods previously described (420). Air was collected using filter-based air samplers: SASS3100 (Research International, USA) mounted on SASS bioaerosol electret filters (Research International). Air sampling was performed at 300 L/minute airflow rate at a height of 1.5 metres above the ground at the rooftop balcony. The SASS filter following sampling was then immediately transferred to the lab for processing or stored at -20°C prior to processing. For processing, the SASS filter was transferred to a sterile tube after being washed with PBS-Triton X-100 three times. Resultant washed solutions were then filtered through a 0.02µm Anodisc filter (Whatman) and DNA extracted using the Dneasy PowerWater Kit (Qiagen). The extracted DNA (including an extraction blank and sterile filter) were sequenced using the HiSeq 2500 Illumina platform (Illumina, USA). All sequence data from the outdoor air study has been uploaded to the National Center for Biotechnology Information (NCBI) Sequence read archives (SRA) under project accession PRJNA436039.

4.2.7 Fungal isolation from outdoor air

The top 8 culturable fungi from outdoor air were then selected based on the metagenomic sequencing profiles from outdoor air sampling as described above (420). *Schizophyllum commune* was collected from National Institute of Education Carpark 3, *Trametes sanguinea* from Kent Ridge Park (Permission for fungi specimen collection was filed under NParks Permit NP/RP19-078). *Aspergillus spp.* (*A. terreus*, *A. sydowii*), *Cladosporium spp.* (*C. tenuissimum*, *C. xanthochromaticum*), *Fusarium spp.* (*F. solani*, *F. moniliforme*), *Neurospora spp.*, *Byssosclamyces spectabilis* and *Penicillium spp.* were isolated directly from air samples collected at the rooftop balcony at Nanyang Technological University, Singapore using the Air agar impactor (IUL S.A., Barcelona, Spain) at a sampling height of 156cm. Air was impacted onto nutrient media with a flow rate of 100L/min for 1 minute in 90mm Petri dishes followed by incubation at 25°C for 3-5 days as described by Sánchez-Muñoz, Muñoz-Vicente (421). The media used included Tryptic Soy Agar (Becton-Dickinson, USA), Potato Dextrose Agar (Sigma-Aldrich, USA), cellulose agar (Sigma-Aldrich, USA), Malt Extract Agar (MEA) (Sigma-Aldrich, USA) and Reasoner's 2A Agar (Becton-Dickinson, USA). Single colonies were selected and re-streaked onto fresh plates, and the process repeated until a single morphology of fungal species was isolated and free of contamination. The identity of the isolated fungi was then confirmed by Sanger sequencing as described below.

4.2.8 Sanger sequencing identification of cultured fungal species from outdoor air

Fungal DNA was extracted using the Qiagen PowerWater DNA extraction kit (Qiagen, Germany) and the ITS-4 and ITS-5 regions amplified by polymerase chain reaction (PCR). The primers used were as follows: ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). PCR was performed using a total of 25µl reaction mix consisting of 12.5µl of 2x KAPA HiFi Hotstart Ready Mix (Kapa Biosystems, USA), 10µM of forward and reverse primers (0.75µl each), 1µl of 5 ng template DNA and 10µl sterile H₂O. PCR cycles for ITS-4 and ITS-5 were performed as follows: 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds with a final elongation step of 72°C for 5 minutes. The PCR products were sent for sanger sequencing to AITbiotech Singapore. Reads were filtered and trimmed using a Phred score cut-off of <20. Forward and reverse reads were merged using BioEdit v7.2.6.1 and final species identification obtained using blastn (422, 423).

4.2.9 Crude allergen preparation

To prepare crude allergens, whole fungi were homogenised with PBS and incubated at 4 °C for 4 hours. The supernatant was then filtered using glycerol and final protein concentrations determined by Bradford assay and stored at -20°C until further processing.

4.2.10 Recombinant *Aspergillus fumigatus* proteins

In addition to crude allergens, recombinant *Aspergillus fumigatus* proteins were also included in our allergen panel due to the established antigenic potential of *Aspergillus fumigatus*. Recombinant *Aspergillus fumigatus* proteins were expressed using the pET system (Novagen, Germany) containing the kanamycin resistance gene using an *Escherichia coli* strain Rosetta-gami (DE3) and purified with the Histrap HP column (5ml). The transformed bacterial cells were then grown in Luria Bertani (LB) medium containing kanamycin (500ug/ml) overnight at 37°C until OD600 reached 0.6. The recombinant protein expression was then optimised with different Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentrations and temperatures as shown (Figure 4.2). After 4 hours, pellets (achieved by centrifugation at 6000g for 15 minutes at 4°C) were harvested and re-suspended in binding buffer. Cells were then lysed by sonication at amplitude 30% for 3 minutes (1 second pulse on and 0.1 second pulse off). The pellets and supernatants were then assessed by SDS PAGE to determine optimal conditions. Large-scale production commenced after determination of the optimal conditions of IPTG (37° with 1mM IPTG) and purified with the Histrap HP column using 50, 100, 250 and 500mM concentration of imidazole for elution. Protein concentrations and purity were determined by Bradford assays and using SDS page.

Figure 4.2: Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentrations and temperatures used for the optimisation of *A. fumigatus* recombinant protein expression

4.2.11 Immuno dot-blot assays to determine specific Ig-E concentration

The specific IgE (sIgE) response to both the crude and recombinant protein extracts were then assessed using immuno-dot blot assays by previously published methods (Figure 4.3) (424-427). The crude proteins included *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Blomia tropicalis*, *Elaeis guineensis*, Panicoids (*Sorghum halepense*), Pooids (*Phleum pratense*, *Festuca pratensis*, *Lolium perenne*), Chloroids (*Cynodon dactylon*), Weeds (*Brassica spp*, *Ambrosia artemisiifolia*, *Helianthus annuus*), *Blattella germanica*, *Periplaneta Americana*, *Curvularia* (*C.lunata*, *C.spicifera*, *C.inequalis*), *Penicillium* (*P. citrinum*, *P.chrysogenum*, *P.notatum*, *P.digitatum*), *Aspergillus fumigatus* and the top 8 culturable air fungi (as described in section 3.2.7). The recombinant proteins used for analysis included Asp f 1 (M83781), Asp f 2 (U56938), Asp f 6 (U53561), Asp f 8 (AJ224333), Asp f 15 (AJ002026) and Asp f 17 (AJ224865). Each allergen was blotted onto nitrocellulose membranes in duplicate together with Bovine Serum Albumin (BSA) as a protein control, and PBS as a negative control. The membranes were first blocked with 0.1% PBS - Tween-20 for 40 minutes followed by overnight (16 hours) incubation with diluted plasma (1:8 in PBS) at 4°C. Washing steps were performed as follows: 0.05% PBS-Tween-20 for 15 minutes followed by 7 minutes in repeat. Subsequently, the membranes were incubated with anti-human IgE antibodies (1:1000) conjugated with alkaline phosphatase for two hours. Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3'-indolylphosphate (BCIP) solution (Thermo Fisher Scientific) was then added for the detection of alkaline phosphatase activity (10 minutes). The data were analysed using Syngene imaging software and inter- and intra-assay reproducibility was found to be 90% and 95% respectively.

Figure 4.3: Outdoor fungi and recombinant *Aspergillus fumigatus* allergen preparation steps with overall process that includes the immuno-dot blot assays. IPTG: Isopropyl β -D-1-thiogalactopyranoside. SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

4.2.12 Indoor air and surface sampling for metagenomic sequencing

To determine the association between indoor allergens and clinical outcomes, eleven COPD patients were recruited for indoor air sampling. Air samples were collected from the patient's bedroom and balcony using filter-based air samplers SASS3100 (Research International, USA) at a flow rate of 100 L/min for 8 hours. In addition, the surfaces (either an air-conditioner or fan) were swabbed using 4N6Floq (Copan, USA) swabs pre-moistened with 0.1% PBS-Triton-X100. DNA was then extracted from the swabs using the DNeasy PowerWater kit. Extraction blanks and sterile swabs were processed in parallel as controls. Sequencing was performed using the Illumina HiSeq 2500 platform (Illumina, USA) and library preparation including sequencing methodologies were performed using published protocols (420). All sequence data from the indoor air study has been uploaded to the National Center for Biotechnology Information (NCBI) Sequence read archives (SRA) under project accession PRJNA608611.

4.2.13 Metagenomic sequencing and data processing

Extracted DNA was quantified using the Qubit fluorometer 2.0 double-stranded DNA (dsDNA) high sensitivity assay (Invitrogen, USA) and Promega Quantifluor fluorometer with Invitrogen's Picogreen assay (Invitrogen, USA). DNA concentrations of $<0.25\text{ng}/\mu\text{l}$ was deemed insufficient and not used in any further processing. Sequencing libraries was prepared using the Swift Biosciences Accel-NGS 2S Plus DNA Kit with DNA sheared to $\sim 450\text{bp}$. Bioanalyzer DNA 7500 chip (Agilent) was used to determine the average library size. Pooled libraries of 10 to 11pM were then loaded onto the Illumina HiSeq 2500 platform (2x 251bp reads) (Illumina, USA) for sequencing. Following sequencing, the raw metagenomic sequences with Phred scores >20 were subjected to adaptor removal and quality trimmed with Cutadapt (version 1.8.1) (428). The metagenomic reads were then aligned to the NCBI nonredundant protein database using RAPSearch version 2.15 and imported into MEGAN for assignment of taxon ID (based on NCBI taxonomy with the lowest common ancestry (LCA) algorithm with a minimum score of 100 and support of ≥ 25) (429).

4.2.14 Statistical analysis and data visualisation

Statistical analysis was performed using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria). Data distribution was assessed with the Shapiro-Wilk test. For non-normally distributed data, the continuous variables are presented as median with interquartile range (IQR), or as mean $\pm$ standard deviation (SD) for normally distributed data. Kruskal-Wallis or Mann-Witney U testing was used for group comparisons in non-normally distributed data. Categorical data were compared by Chi-squared or Fisher-exact testing as appropriate. Multiple comparisons were corrected for false discovery rate (FDR) using Benjamin Hochberg corrections. Logistic regression corrected for age, gender, BMI and smoking pack years was performed using “glm” function in R. Incidence rate ratio (IRR) was performed using the “glm.nb” function of the “MASS” package. Indoor allergen abundance was visualized using the “pheatmap” package with row z-score and regression plots plotted with *ggscatter* function using the R “ggpubr” package with Pearson correlations. Bubble charts were plotted using MEGAN (429). Sensitisation was defined as a specific-IgE (sIgE) binding intensity above the 95th percentile of that measured from the non-diseased (healthy) control cohort. Air-fungi sensitised individuals are defined as an individual illustrating sensitisation to one or more of the 8 outdoor air fungi assessed. Significance is defined as a $p \leq 0.05$.

4.2.14.1 Cluster analysis

Systemic specific-IgE (sIgE) with patient demographics and treatments were used for cluster analysis using the R “cluster” package. The detailed cluster analysis methodologies are described in Chapter 3 (section 3.2.9.1). Mean Jaccard values for each detected cluster in this chapter was 0.998, 1.0 and 0.999 respectively, suggesting that the identified clusters were highly stable.

4.2.14.2 Indoor Allergen mapping

The established allergens obtained from sampling of indoor air (n=977) were extracted using the R “rvest” package from the established Allergen Nomenclature (<http://www.allergen.org/>) (430). Nucleotide sequences of the 786 allergens whose GenBank Accessions were available were retrieved using the R “ape” package (431). The raw paired-end Illumina HiSeq sequencing reads with a minimum Phred score of 20 and >30bp length were selected and adapter-trimmed using Cutadapt (version 1.8.1) (428). The trimmed reads were then mapped against the GRCh38 human reference genome using bowtie2 (version 2.3.3.1) (432). The unmapped non-human reads were extracted using samtools (version 1.3.1) and bedtools2 (version 2.8.0), and, aligned to the 786 allergens nucleotide sequences using bowtie2 in its local mode (433, 434). Samtools was next used to calculate the number of alignments with mapping quality >30 (raw hits). The number of raw hits (H) to a specific allergen’s nucleotide sequence was then normalized using similar methodology to ShortBRED (435), whereby read length (R), allergen nucleotide sequence length (L), and sequencing depth were adjusted after removal of human reads (N) according to the following formula:

$$C = \frac{H}{\left(\frac{|L - R|}{10^3}\right)\left(\frac{N}{10^6}\right)} = \frac{H}{|L - R| \cdot N} \times 10^9$$

The normalised count (relative abundance) is presented as units of reads per kilobase of reference sequence per million sample reads (RKPM).

4.3 Results

4.3.1 South-East Asians with COPD demonstrate high sensitisation rates to a wide range of allergens

For work presented in this chapter, a total of n=446 South-East Asians Chinese with COPD and n=51 healthy (non-diseased) controls were recruited from Singapore, Malaysia and Hong Kong (Table 4.1). In comparison to the non-diseased (healthy) controls, COPD patients illustrate high specific IgE (sIgE) binding across a range of allergens including pollens, house-dust mite, cockroach, and fungi (Figure 4.4). Of all tested allergens, house dust mites (HDM) (*Blot*, *Der p* and *Der f*) and grass pollens (Pooids and Panicoids) demonstrate the highest median specific IgE binding intensities. When sensitisation is classified as either positive or negative (based on a sIgE binding intensity >95th percentile of the non-diseased (healthy) controls), a high number of COPD patients demonstrate sensitisation to fungal allergens (n=249; 55.8%) followed by HDM (229; 51.3%).

Table 4.1: Participant characteristics for the non-diseased (healthy) control and COPD cohorts

Demographic	Non-diseased control (≤40 Years old)	Non-diseased control (>40 Years old)	COPD
n (%)	26	25	446
Age (years), Median (IQR)	22 (20-23)	63 (61-64)	75 (68-80)
Sex (Male), n (%)	10 (38.5)	12 (48.0)	426 (96.2)
BMI (kg/m ²), Median (IQR)	22.0 (21.0-24.0)	24.0 (20.6-26.0)	21.5 (18.7-23.8)
Smoking status, n (%)			
Current	0 (0.0)	4 (16.0)	303 (67.9)
Ex-smoker	1 (3.8)	3 (12.0)	139 (31.2)
Never	25 (96.2)	18 (72.0)	4 (0.9)
Smoking pack years, Median (IQR)	0 (0-0)	0 (0-20)	50 (38-65)
FEV1 % predicted, Median (IQR)	96.5 (85.3-107.8)	93.5 (84.0-97.8)	45.0 (33.6-59.0)
FEV1/FVC (% predicted), Median (IQR)	86.6 (82.3-93.2)	78.6 (75.0-84.7)	50.4 (41.2-59.5)
No of Exacerbations 1-year preceding, n (%)			
0-1	NA	NA	293 (65.7)
>1	NA	NA	153 (34.3)
Hospitalization 1-year preceding, n (%)			
Yes			208 (46.6)
No			238 (53.4)
COPD assessment test (CAT), Median (IQR)	NA	NA	14 (9-19)
Blood eosinophil count (x10 ⁹ /L)	NA	NA	0.12 (0.00-0.29)
Total IgE (IU/ml)	NA	NA	24.5 (5.0-119.3)
Treatment, n (%)			
SAMA/SABA			125 (28.0)
LAMA			29 (6.5)
LAMA/LABA	NA	NA	43 (9.7)
LABA/ICS			115 (25.8)
LAMA/ICS			18 (4.0)
LAMA/LABA/ICS			116 (26.0)

n: number of participants; IQR: interquartile range; COPD: Chronic Obstructive Pulmonary Disease, BMI: body mass index, FEV₁: forced expiratory volume in the 1st second, FVC: forced vital capacity, IQR: interquartile range, NA: non-applicable. SAMA: short acting muscarinic antagonist, SABA: short-acting beta-2 agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta-2 agonist, ICS: inhaled corticosteroid.

Figure 4.4: Increased sensitisation to a panel of allergens in South-East Asian patients with chronic obstructive pulmonary disease (COPD). Scattered boxplots illustrating the systemic specific Ig-E binding to (a) pollens, (b) house dust mite, cockroaches and (c) fungi between COPD and non-diseased (healthy) controls (expressed as log₁₀ optical density (OD) intensity). Benjamin-Hochberg adjusted p-values are shown and dot coloration indicates the respective patient cohort: non-diseased ≤40 years (blue), non-diseased >40 years (orange) and COPD (red). COPD: chronic obstructive pulmonary disease, HDM: House Dust Mite, ns: not significant, *p≤0.05, **p≤0.01, ***p≤0.001

4.3.2 Fungal sensitisation is associated with very frequent COPD exacerbator (vFE) status

Sensitisation profiles were next assessed in relation to exacerbation status and pulmonary function in COPD. Interestingly, very frequent COPD exacerbators (vFEs) illustrate a significantly increased systemic specific IgE binding to crude fungal allergens including *Curvularia* (p=0.028), *Penicillium* (p=0.028) and *Aspergillus fumigatus* (p=0.041) (Figure 4.5c). The cockroach allergen *Blattella germanica* is also significantly increased in this patient group (p=0.046) (Figure 4.5b). No differences in sensitisation to pollens (Figure 4.5a), HDM and the cockroach allergen *Periplaneta americana* were detected (Figure 4.5b). In view of the detectable and significantly increased sensitisation to crude *Aspergillus fumigatus* allergen in vFEs with COPD, I next investigated this association further using selected recombinant *Aspergillus fumigatus* allergens to determine the specific binding protein of *Aspergillus fumigatus* which has been shown to be of higher specificity compared to crude allergen (27, 436). Importantly, I only detected a significant association for the minor and not major recombinant *Aspergillus fumigatus* (rAsp f) allergens (rAsp f6 (p=0.046), f8 (p=0.028), f15 (p=0.035) and f17 (p=0.028)) (Figure 4.5d-e). On further multivariate analysis, which was adjusted for age, gender, body mass index (BMI) and smoking pack year history, the *Penicillium* and *Aspergillus fumigatus* crude fungal allergens and the specific recombinant *Aspergillus fumigatus* allergens rAsp f 1, 15 and 17 were significantly associated with vFE status (Figure 4.5f). Of note, no association between sensitisation, GOLD ABCD group (Figure 4.6) or GOLD stage (lung function as FEV₁) was detected (Figure 4.7).

Figure 4.5: Very frequent exacerbator (≥ 3 exacerbations/year) status in South-East Asians with COPD associates with a significantly increased occurrence of fungal sensitisation. Scattered boxplots illustrate the specific Ig-E binding to (a) pollens, (b) house dust mite, cockroaches, (c) crude fungal allergens between non-frequent (Non-FE) and very frequent COPD exacerbators (vFEs) expressed as log₁₀ optical density (OD) intensity. Benjamin-Hochberg adjusted p-values are shown. Coloration denotes the exacerbator group: non-frequent exacerbator (grey), frequent exacerbator (dark green). Error bars indicate the 95% confidence interval (CI) and dots represent the odds ratios (ORs) with colour indicating significance level: red ($p < 0.05$), grey ($p > 0.05$). FE: frequent exacerbator, HDM: house-dust mite, Der f: *Dermatophagoides farinae*, Der p: *Dermatophagoides pteronyssinus*, Blo t: *Blomia tropicalis*, Bla g: *Blattella germanica*, ns: not significant. * $p \leq 0.05$.

Figure 4.5: Scattered boxplots illustrate the specific Ig-E binding to (d) major and (e) minor recombinant *Aspergillus fumigatus* allergens between non-frequent (Non-FE) and very frequent COPD exacerbators (vFEs) expressed as log₁₀ optical density (OD) intensity. Benjamin-Hochberg adjusted p-values are shown. Coloration denotes the exacerbator group: non-frequent exacerbator (grey), frequent exacerbator (dark green). Error bars indicate the 95% confidence interval (CI) and dots represent the odds ratios (ORs) with colour indicating significance level: red (p<0.05), grey (p>0.05). FE: frequent exacerbator, rAsp f: recombinant *Aspergillus fumigatus*, ns: not significant. *p≤0.05.

(f)

Figure 4.5: (f) Forest plot illustrating the adjusted odd ratio of each fungal allergen for very frequent COPD exacerbators after adjustment for age, gender, body mass index and smoking pack years. Error bars indicate the 95% confidence interval (CI) and dots represent the odds ratios (ORs) with colour indicating significance level: red ($p < 0.05$), grey ($p > 0.05$). FE: frequent exacerbator. *rAsp f*: recombinant *Aspergillus fumigatus*, *Der f*: *Dermatophagoides farinae*, *Der p*: *Dermatophagoides pteronyssinus*, *Blo t*: *Blomia tropicalis*, *Bla g*: *Blattella germanica*, CI: confidence interval, ns: not significant.

Figure 4.6: No difference in sensitisation to (a) pollens, (b) house dust mite and cockroaches allergens were detected between the GOLD lung function stages 1 ($FEV_1 > 80\%$ predicted), 2 ($FEV_1 50-79\%$ predicted), 3 ($FEV_1 30-49\%$ predicted) and 4 ($FEV_1 < 30\%$ predicted). Systemic specific IgE (sIgE) binding is expressed as $\log_{10}$ OD intensity. Boxplot colouration represents the GOLD (lung function; FEV_1) stage: GOLD stage 1 (light grey), GOLD stage 2 (medium grey), GOLD stage 3 (grey), GOLD stage 4 (dark grey). ns: not significant, *Blo t*: *Blomia tropicalis*, *Der f*: *Dermatophagoides farinae*, *Der p*: *Dermatophagoides pteronyssinus* and *Blat g*: *Blattella germanica*.

Figure 4.6: No difference in sensitisation to (c) crude fungal, (d) major and (e) minor recombinant *Aspergillus fumigatus* allergens were detected between the GOLD lung function stages 1 (FEV₁ >80% predicted), 2 (FEV₁ 50-79% predicted), 3 (FEV₁ 30-49% predicted) and 4 (FEV₁ <30% predicted). Systemic specific IgE (sIgE) binding is expressed as log₁₀ OD intensity. Boxplot colouration represents the GOLD (lung function; FEV₁) stage: GOLD stage 1 (light grey), GOLD stage 2 (medium grey), GOLD stage 3 (grey), GOLD stage 4 (dark grey). ns: not significant, rAsp f: recombinant *Aspergillus fumigatus*.

Figure 4.7: No difference in sensitisation to (a) pollens, (b) house dust mite and cockroaches, allergens were detected between the GOLD A (CAT ≤ 10 and < 2 exacerbations and no hospitalised exacerbations per year), B (CAT > 10 and < 2 exacerbations and no hospitalised exacerbations per year) C (CAT ≤ 10 and > 2 exacerbations/ ≥ 1 hospitalised exacerbations) and D (CAT > 10 and > 2 exacerbations/ ≥ 1 hospitalised exacerbations) groups. Systemic specific IgE (sIgE) binding is expressed as log₁₀ OD intensity. Boxplot colouration corresponds to GOLD ABCD group: GOLD A (light grey), GOLD B (medium grey), GOLD C (grey), GOLD D (dark grey). ns: not significant, *Blo t*: *Blomia tropicalis*, *Der f*: *Dermatophagoides farinae*, *Der p*: *Dermatophagoides pteronyssinus* and *Bla g*: *Blattella germanica*.

Figure 4.7: No difference in sensitisation to (c) crude fungal, (d) major and (e) minor recombinant *Aspergillus fumigatus* allergens were detected between the GOLD A (CAT ≤ 10 and < 2 exacerbations and no hospitalised exacerbations per year), B (CAT > 10 and < 2 exacerbations and no hospitalised exacerbations per year) C (CAT ≤ 10 and > 2 exacerbations/ ≥ 1 hospitalised exacerbations) and D (CAT > 10 and > 2 exacerbations/ ≥ 1 hospitalised exacerbations) groups. Systemic specific IgE (sIgE) binding is expressed as log₁₀ OD intensity. Boxplot colouration corresponds to GOLD ABCD group: GOLD A (light grey), GOLD B (medium grey), GOLD C (grey), GOLD D (dark grey). ns: not significant, rAsp f: recombinant *Aspergillus fumigatus*.

4.3.3 Unsupervised clustering reveals three groups of 'sensitised' South-East Asians with COPD that demonstrate variable clinical outcomes

To assess if different groups of 'sensitised' South-East Asians (Singapore, Malaysia and Hong Kong) Chinese with COPD exist, I next employed an unsupervised clustering approach which included patient demographics, COPD treatment and sIgE results from the comprehensive panel screening. Three separate patient groups (clusters) were identified as follows, each defined by their sensitisation (specific IgE binding pattern) to specific allergens within the tested panel (Table 4.2) (Figure 4.8a-b): (1) A highly-sensitised (HS) fungal predominant group (n=115), (2) A low sensitisation group (LS) (n=114) and (3) A moderately sensitised (MS) *Blomia* predominant group (n=217) (Table 4.3). Most importantly, the HS fungal predominant patient group exhibits the poorest clinical outcomes with increased symptoms (measured as CAT score), lowest lung function and increased exacerbations (Table 4.3) (Figure 4.8c-e). Interestingly, however, no differences in total blood eosinophil count between the clusters was detected (median absolute eosinophil count :0.11; IQR 0-0.18 versus 0.11; IQR 0-0.21 versus 0.13 IQR 0-0.21 for each cluster respectively; p=0.535) (Figure 4.8f). The cluster stability was determined using Jaccard similarities index with the mean value of 0.998, 1.0 and 0.999 respectively, suggesting that the identified clusters were highly stable.

Table 4.2: Systemic specific IgE (sIgE) binding (expressed as OD intensity) for each of the tested allergens across the within ‘sensitised’ South-East Asian COPD patient groups (clusters).

Allergens	Total, OD, Median (IQR)	HS fungal predominant, OD Median (IQR)	LS, OD Median (IQR)	MS <i>Blomia</i> predominant, OD Median (IQR)	p-value
<i>Elaeis guineensis</i>	2.59 (1.55-6.51)	3.31 (1.68-9.44)	2.55 (1.55-7.5)	2.47 (1.48-5.55)	0.011
Panicoids	5.20 (2.01-16.92)	7.98 (2.49-22.24)	4.60 (1.86-16.57)	4.76 (1.95-15.52)	0.078
Pooids	5.56 (2.35-17.94)	7.49 (3.02-23.24)	4.93 (2.16-15.88)	5.22 (2.26-15.86)	0.064
Chloroids	4.09 (1.85-12.78)	5.22 (2.1-18.11)	3.40 (1.66-10.47)	3.75 (1.84-11.77)	0.057
Weeds	2.51 (1.48-6.06)	3.39 (1.77-6.49)	1.93 (1.31-6.15)	2.35 (1.5-5.66)	0.041
<i>Blomia tropicalis</i>	4.88 (2.25-15.6)	5.34 (2.21-16.8)	3.68 (1.86-9.55)	5.89 (2.67-17.52)	0.050
<i>Dermatophagoides farina</i>	4.34 (1.96-12.71)	4.56 (2.27-12.9)	4.27 (1.65-11.79)	4.17 (1.89-13.32)	0.321
<i>Dermatophagoides pteronyssinus</i>	4.20 (1.92-9.53)	4.92 (2.21-11.32)	3.43 (1.66-8.56)	4.23 (1.84-9.62)	0.159
<i>Blattella germanica</i>	2.63 (1.57-6.06)	2.91 (1.87-6.93)	2.31 (1.4-6.06)	2.62 (1.57-5.65)	0.134
<i>Periplaneta americana</i>	3.37 (1.65-7.56)	3.83 (2.03-8.59)	3.29 (1.48-6.18)	3.24 (1.59-8.44)	0.130
<i>Curvularia spp.</i>	2.35 (1.43-5.13)	2.68 (1.63-5.44)	2.05 (1.26-5.77)	2.32 (1.45-4.65)	0.147
<i>Penicillium spp.</i>	2.58 (1.48-5.98)	3.19 (1.82-6.92)	2.30 (1.33-6.16)	2.51 (1.45-5.53)	0.050
<i>Aspergillus fumigatus</i>	2.34 (1.44-5.26)	3.39 (1.64-6.38)	2.04 (1.42-5.05)	2.31 (1.44-4.75)	0.016
rAsp f 1	2.66 (1.26-7.51)	3.23 (1.15-8.98)	2.90 (1.47-7.81)	2.31 (1.14-6.78)	0.217
rAsp f 2	0.13 (0.02-1.02)	0.19 (0.02-1.69)	0.09 (0.03-0.93)	0.12 (0.02-0.86)	0.326
rAsp f 6	2.40 (1.48-6.26)	2.87 (1.54-7.33)	2.11 (1.49-6.18)	2.34 (1.4-5.81)	0.203
rAsp f 8	2.41 (1.42-6.03)	3.00 (1.58-7.3)	2.29 (1.39-5.83)	2.25 (1.31-5.69)	0.034
rAsp f 15	2.83 (1.48-6.63)	3.50 (1.59-7.81)	2.56 (1.49-6.04)	2.57 (1.43-6.31)	0.085
rAsp f 17	2.44 (1.41-5.71)	3.10 (1.51-6.88)	2.11 (1.32-4.82)	2.39 (1.43-5.08)	0.043

HS: highly-sensitised, MS: moderately sensitised, IQR: interquartile range, ns: not significant, OD: optical density.

Table 4.3: Demographics comparing the three ‘sensitised’ South-East Asian patient groups (clusters).

Demographics	Cluster 1 Highly-sensitised (HS)fungal predominant	Cluster 2 Low sensitised	Cluster 3 Moderately- sensitised (MS) <i>Blomia</i> predominant	p-value
n	112	114	217	
Age (years), Median (IQR)	72 (67-78)	77 (71-82)	74 (68-80)	0.001
Sex (Male), n (%)	108 (96.4)	110 (96.5)	208 (95.9)	0.946
BMI (kg/m ²), Median (IQR)	21.6 (18.2-23.6)	21.2 (19.0-23.9)	21.6 (18.8-23.7)	0.938
Smoking status, n (%)				0.012
Current	72 (64.3)	92 (80.7)	138 (63.6)	
Ex-smoker	40 (35.7)	21 (18.4)	76 (35.0)	
Never	0 (0.0)	1 (0.9)	3 (1.4)	
Smoking pack years, Median (IQR)	50 (40-66)	50 (35-67)	50 (36-63)	0.300
FEV1 % predicted, Median (IQR)	41.1 (32.5-57.0)	41.0 (31.7-56.3)	49.0 (36.7-62.2)	0.002
FEV1/FVC (% predicted), Median (IQR)	47.3 (40.0-57.0)	50.0 (40.5-59.5)	51.5 (42.3-60.9)	0.100
No. of Hospitalisations/year, Median (IQR)	1 (0-3)	1 (0-2)	0 (0-1)	<0.001
No. of Exacerbations/year, Median (IQR)	1 (0-3)	1 (0-2)	0 (0-2)	<0.001
COPD assessment test (CAT), Median (IQR)	16 (10-22)	15 (10-19)	12 (8-17)	0.002

n: number of patients; IQR: interquartile range, BMI: body mass index, FEV₁: forced expiratory volume in the 1st second, FVC: forced vital capacity.

(a)

(b)

Figure 4.8: Unsupervised clustering reveals three ‘sensitised’ South-East Asian COPD patient groups (clusters) that demonstrate varying clinical outcomes with the poorest outcomes in the highly sensitised (HS) fungal predominant group (cluster). (a) non-metric multidimensional scaling plot and (b) dendrogram illustrating the 3 sensitised COPD patient groups (clusters).

Figure 4.8: Clinical outcomes (c) symptoms (CAT score) (d) lung function (FEV₁% predicted) (e) exacerbation frequency and (f) total eosinophil counts compared between the 3 identified patient groups (clusters). Colours correspond to each respective patient group (cluster): (1) Highly-sensitised (HS) fungal predominant (turquoise) (cluster 1); (2) Low sensitisation (LS) (purple) (cluster 2) and (3) Moderately-sensitised *Blomia* predominant (violet-red). Error bar indicates the 95% confidence interval (CI) and dots each respective patient or the incidence rate ratio for exacerbation respectively. HS: Highly-sensitised, MS: Moderately-sensitised, LS: Low-sensitisation, CI: confidence interval, ns: not significant. Dotted line color indicates eosinophil cut offs: standard (0.45 x 10⁹/L) (grey) and COPD GOLD cut off of eosinophil for treatment with inhaled corticosteroid (0.30 x 10⁹/L) (red) (38). *p≤0.05, **p≤0.01, ***p≤0.001, ns: not significant.

4.3.4 Sensitisation to outdoor air fungi is associated with exacerbations in South-East Asians with COPD.

Having demonstrated poor outcomes in association to fungal sensitisation in South-East Asians (Singapore, Malaysia and Hong Kong) Chinese with COPD, I next evaluated if culturable fungi found in the outdoor air may represent an important and under-recognised source of sensitisation in the South-East Asian region. In addition, I evaluated any detectable sensitisation response to outdoor air fungi with clinical outcomes in COPD. I selected the top 8 culturable fungi from outdoor air for further assessment. This was based on available and published metagenomic sequencing data obtained prospectively from outdoor air in Singapore (Sg) on five consecutive days (420) (Figure 4.9a). The dot-blot specific Ig-Es assay against the selected outdoor fungi were prepared and then used to evaluate specifically any air-fungi sensitisation in COPD patients recruited from Sg (n=82). Compared to non-diseased (healthy) controls (also recruited in Sg), a significantly increased systemic specific IgE binding to the outdoor air fungi: *Schizophyllum* (p=0.001), *Aspergillus* (p<0.001), *Penicillium* (p<0.001), *Byssoschlamys* (p<0.001) and *Cladosporium* (p=0.001) were observed in COPD patients (Figure 4.9b). This is a novel finding in that it represents the first time that a measurable systemic immune response to any outdoor air microbe is detected in any respiratory state including COPD.

To better appreciate the clinical relevance of these findings and in particular the relevance if any of the ‘sensitised’ state to outdoor air fungi, I next assessed for its relevance to clinical COPD outcomes. To do this, a presence of sensitisation to one or more outdoor air fungi in Sg was defined as “air-fungi sensitised” (n=61) and was compared to the non-sensitised (n=21) group from the Sg COPD cohort. Interestingly, increased exacerbation rates (median: 1, IQR 0-3 versus 0, IQR 0-1; p=0.049) (Figure 4.9c) were observed in the “air-fungi sensitised” group of patients which remained significant after adjustment for age, gender, BMI, lung function and smoking pack years (Figure 4.9d) (Incidence rate ratio (IRR) 2.29, 95% CI 1.12-4.68, p=0.02). Importantly, no such relationship to symptoms (CAT score) (Figure 4.9e), lung function

(FEV₁% predicted) (Figure 4.9f), eosinophil count (Figure 4.9g) or total IgE (Figure 4.9h) was detected between the “air fungi sensitised” and the non-sensitised COPD groups.

Figure 4.9: Sensitisation to outdoor air fungi are associated with exacerbations in Singaporean patients with COPD. (a) Bubble chart illustrating the metagenomic read count for the top eight culturable air fungi from outdoor air in Singapore prospectively assessed over five consecutive days.

Figure 4.9: (b) Scattered boxplots illustrating specific IgE binding expressed as $\log_{10}$ optical density (OD) intensity to the top 8 outdoor air fungi in Singapore with comparisons between Singaporean COPD patients and non-diseased (healthy) controls, (c) the exacerbation frequency between the “air fungi sensitised” and “non-sensitised” COPD patient groups, (d) forest plot illustrating a multivariate analysis of incidence rate ratio (IRR) for exacerbations, adjusted for age, body mass index (BMI), smoking pack years and lung function (post bronchodilator (BD) FEV₁% predicted). Scattered plot colours correspond to the respective cohort: non-diseased (healthy) <40 years old (blue), non-diseased (healthy) >40 years old (orange) and COPD (red), non-sensitised (black), air-fungi sensitised (dark pink). BMI: body mass index, Post BD FEV₁: post bronchodilator forced expiratory volume in the 1st second, CI: confidence interval, CAT: COPD assessment test, FEV₁: forced expiratory volume in the 1st second, IgE: immunoglobulin-E, ns: not significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 4.9: (e) scattered box plot for symptoms (CAT score), (f) lung function (FEV₁ % predicted), (g) total blood eosinophil count (as 10⁹/L) and (h) total serum IgE between the “air fungi sensitised” and “non-sensitised” COPD patient groups. Scattered plot colours correspond to the respective cohort: non-sensitised (black), air-fungi sensitised (dark pink). CAT: COPD assessment test, FEV₁: forced expiratory volume in the 1st second, IgE: immunoglobulin-E, ns: not significant.

4.3.5 Sensitisation to “air-fungi” from outdoor air in Singapore is not related to COPD exacerbations in patients from Malaysia and Hong Kong.

In view of the relationship detected between sensitisation to outdoor “air-fungi” and exacerbations in Singaporean COPD patients, I next evaluated similar relationships in COPD patients recruited from Malaysia and Hong Kong. As air sampling was only conducted in Singapore, all assessment was made in relation to outdoor “air-fungi” detected in Singapore. Interestingly, despite having large number of COPD patients recruited from Malaysia (n=25, 53%) and Hong Kong (n=194, 61%) respectively which demonstrate clear sensitisation to outdoor “air-fungi” from Singapore (Figure 4.10a), no significant relationship with exacerbation frequency was detectable between the sensitised and non-sensitised patient groups (p=0.266 Hong Kong; p=0.453 Malaysia) (Figure 4.10b-c). This is in contrast to Singaporean COPD cohort which demonstrated a significant association between sensitisation to outdoor “air-fungi” and COPD exacerbations (Figure 4.9c).

Figure 4.10: Sensitisation to outdoor “air-fungi” from Singapore assessed in COPD patients recruited from Hong Kong and Malaysia, respectively. No association between “air-fungi” sensitisation and COPD exacerbations was detected. (a) Bar plot illustrating the proportion of COPD patients in Singapore, Hong Kong and Malaysia respectively that demonstrate at least a single sensitisation response to the top 8 outdoor air fungi detected in Singapore. (b) Scattered box plots demonstrating no significant differences in exacerbation frequency in COPD patients recruited from Hong Kong and Malaysia with and without detectable sensitisation to outdoor “air-fungi” from Singapore.

4.3.6 Indoor allergens are an important and under-recognised source of sensitisation in COPD.

Having detected a significant association between sensitisation to outdoor “air-fungi” and exacerbations in Singaporean patients with COPD, I next investigated the potential role of indoor allergens on clinical COPD outcomes. To achieve this, home visits were conducted in a subgroup of Singaporean COPD patients (n=11) with demographics of the cohort indicated below (Table 4.4). Home visits included a detailed clinical and environmental assessment including the prospective collection of air samples from indoor air (patient’s bedroom), outdoor air (patient’s balcony), and also indoor surfaces by an optimised swabbing technique (air-conditioner or fan). All these samples were then subjected to our state-of-the-art metagenomic sequencing protocols as described in the methods. Allergens were identified by aligning the metagenomic reads against the established WHO/IUIS Allergen Nomenclature database. A total of 43 allergens were mapped from the collected specimens, including fungi, house-dust mites, and plant allergens (Figure 4.11a). Most of the mapped allergens were interestingly fungi (n=34), with a particularly high abundance detected on indoor surfaces (Figure 3.11a). Of particular interest, the number of indoor (bedroom) and surface but not outdoor (balcony) allergens were positively correlated with COPD symptoms (CAT score) ($r=0.75$, $p=0.0076$ versus $r=0.5$, $p=0.12$) (Figure 4.11b-d), and negatively correlated with lung function (FEV_1 %predicted) ($r=-0.61$, $p=0.047$ versus $r=-0.28$; $p=0.41$) (Figure 4.11c-e). Taken together, and for the first time using metagenomic sequencing in the home environment, I have identified indoor air (in the patient’s bedroom) and indoor surfaces (such as air-conditioners and fans) as a potential source of allergen exposure with clinical implications in COPD. These findings may have importance particularly in hot and humid climates observed across many Asian settings.

Table 4.4: Demographics of the COPD patient cohort where home visits were conducted including outdoor air, indoor air and surface sampling (n=11).

Characteristics	
Number of patient homes sampled	11
Age (years), Mean $\pm$ SD	69 $\pm$ 3
Sex (Male), n (%)	11 (100)
BMI (kg/m ²), Mean $\pm$ SD	22.5 $\pm$ 3.7
Smoking status, n (%)	
Current	5 (45.5)
Ex-smoker	6 (54.5)
Never	0 (0.0)
Smoking pack years, Median (IQR)	50.0 (47.5-65.0)
FEV ₁ % predicted, Mean $\pm$ SD	57.7 $\pm$ 21.2
FEV ₁ /FVC (% predicted), Mean $\pm$ SD	56.7 $\pm$ 12.5
No. of Hospitalisations/year, Median (IQR)	0 (0-1)
No. of Exacerbations/year, Median (IQR)	0 (0-1)
COPD assessment test (CAT), Mean $\pm$ SD	12.3 $\pm$ 11.1
Blood Eosinophils (x10 ⁹ /L)	0.21 (0.09-0.42)
Total IgE (IU/ml)	26.2 (5.0-251.6)
Treatment, n (%)	
LAMA	4 (36)
LAMA/LABA	4 (36)
LAMA/LABA/ICS	3 (28)

SD: standard deviation; IQR: interquartile range; BMI: body mass index; FEV₁: forced expiratory volume in the 1st second; FVC: forced vital capacity.

Figure 4.11: An increased number of detectable indoor allergens correlates with COPD symptoms and poorer lung function. (a) Heatmap illustrating the relative abundance of indoor allergens detected through metagenomic sequencing of indoor air (bedroom), outdoor air (balcony) and indoor surfaces by swabbing (air-conditioners and/or fans) in 11 separate homes of COPD patients (ID 1-11). The highest abundance is indicated in purple and side colour bars denote allergen source: fungi (pink), house dust mite (grey) and plants (green) (y-axis). HDM: house-dust mite.

Figure 4.11: (b) Scatterplot illustrating the correlation between COPD symptoms (as CAT scores) and lung function (as FEV₁% predicted) against the number of indoor (b-c) and outdoor allergens (d-e). Blue dotted lines correspond to the Pearson regression and the grey shaded areas represent the 95% confidence interval. Each black dot represents a single patient. CAT: COPD assessment test; FEV₁: forced expiratory volume in the 1st second.

4.4 Discussion

This chapter aimed to identify if allergic sensitisation has importance in South-East Asians (Singapore, Malaysia and Hong Kong) of Chinese ethnicity with COPD and further elucidate if clinical phenotypes based on sensitisation patterns exist in relation to clinical COPD outcomes, assessing for the first time the outdoor and indoor environment. The presented work is novel, advances our understanding of South-East Asians of Chinese ethnicity with COPD in Singapore, Malaysia and Hong Kong respectively and represents the first prospective dataset obtained through metagenomic sequencing linking the outdoor and indoor environment to sensitisation responses in COPD. This dataset is also the largest study to date focusing on sensitisation and COPD outcomes in Chinese patients from South-East Asia (Singapore, Malaysia and Hong Kong).

I describe a high frequency of sensitisation across a range of common allergens including fungi in COPD when compared to non-diseased (healthy) individuals. Sensitisation to fungi importantly associate with very frequent exacerbations in COPD. Furthermore, and of clinical relevance, unsupervised clustering reveals a “highly-sensitised fungal predominant” patient group, who demonstrate poor clinical outcomes. Notably, for the first time in COPD, I demonstrate that the environment serves as a key reservoir of fungal allergens and that measurable sensitisation responses to fungi in the air has a relationship with COPD exacerbation frequency. Taking this concept further, a number of allergens are identifiable within the indoor environment, including air and surfaces in the patient’s home and this in turn correlates with COPD symptoms and poorer lung function.

An ‘allergic’ disease phenotype, particularly that related to fungal sensitisation is increasingly reported in several chronic airway diseases (27, 230, 326, 327). In asthma, it represents a poor prognostic indicator that is associated with disease severity and exacerbations. Recent work in non-cystic fibrosis bronchiectasis also reports a high frequency of sensitisation, and in particular fungal sensitisation is associated with increased inflammation (pro-inflammatory cytokines)

and poorer clinical outcome when compared to other forms of sensitisation such as house-dust mites (HDM) (27). In COPD, however, and particular in the Asian setting, studies assessing the relevance of sensitisation responses in association with clinical outcomes such as lung function have been inconsistent, with some studies reporting poorer lung function with *Aspergillus* sensitisation, while others did not find such a relationship (227, 437). We too did not find any direct association between *A. fumigatus* sensitisation and lung function, however, our highly-sensitised fungal predominant patient group demonstrate significantly worse clinical outcomes including poorer lung function, higher symptoms and increased exacerbations when compared to the low sensitisation and *Blomia* predominant sensitisation groups. The variable outcomes observed between studies is most likely attributable to differences in the recruited study groups, sample size, and methodologies used to define a sensitisation response. My work has focused on South-East Asians, recruited across several jurisdictions (Singapore, Malaysia and Hong Kong), represents the largest such study in the region to date and used a comprehensive, novel and metagenomics approach to assess for sensitisation.

While fungi are ubiquitously found in nature with high abundance in outdoor air, direct exposure rarely results in overt disease, particularly if an individual is immunocompetent (420). Prior work has reported on associations between outdoor fungal spore concentration and adverse outcomes in asthma, with increases in exacerbations, symptoms, inhaler used and decreased evening peak flow readings all described (438, 439). In addition, heightened exposures during the *Alternaria* season have been associated with life threatening asthma in sensitised individuals (440). Importantly, however, no study has thus far evaluated the association between outdoor air fungi and sensitisation responses in COPD. Similar to prior studies in asthma (438, 439), the work presented in this chapter suggests that an important and previously unappreciated association exists between outdoor “air-fungal” sensitisation and COPD exacerbations. Notably *Schizophyllum*, *Penicillium*, *Aspergillus*, *Byssosclamyces* and *Cladosporium* are all allergenic air fungi that appear to associate with systemic specific-IgE responses in our recruited COPD cohort. My findings importantly suggest the exposure to fungi in the outdoor air environment

may play a role in precipitating exacerbation events especially in a 'sensitised' COPD patient, but this can only apply to COPD patients living in the same region where such outdoor air fungi are isolated.

The indoor environment also importantly harbours a significant amount of fungus with overlapping species detected between the outdoor and indoor environments, likely due to air exchange, for instance by opening windows (326). In asthma, an increased concentration of the indoor fungi, *Aspergillus*, *Alternaria*, *Cladosporium* and *Penicillium* have been associated with increased symptoms and more exacerbations (441). Interestingly, I detected similar allergens in the homes of COPD patients and identified that, where an increased number of allergens was present in an individual home, this correlated with greater symptoms and poorer lung function. The allergens identified indoors are similar to those identified outdoors (from outdoor air), further supporting a concept of microorganism interchange between these two environments. Importantly, fungal composition differs between individual homes, attributed to the contrasting built environment, resident behaviour and daily activities. My results, for the first time in COPD, importantly suggest that the indoor environment represents a critical source of allergen exposure; and because of differences between individual homes and fluctuations with time and activity, it does represent a 'modifiable' risk factor in sensitised COPD patients that needs to be addressed at the individual level.

As mentioned previously, prior studies evaluating sensitisation responses in COPD have reported contrasting results, for instance the presence of asthma like features associate with better outcomes in a Japanese cohort and in single centre work from Brazil, atopy was associated with less severe COPD (183, 229). This contrasts larger multicentre work involving nine European countries which found increased respiratory symptoms in atopic COPD patients (17). In agreement with the European work and using two national cohorts from the United States, the 'allergic' phenotype is associated to both COPD symptoms and the occurrence of exacerbations (16). Sensitisation importantly also exhibits geographic variation, which in turn is influenced by climate, air quality, genetic, social and cultural differences in any particular

region. This again may have a role in the conflicting outcomes of currently available studies (27, 220). Such geographic variability is also important across the Asian sub-continent as some have dramatically contrasting environmental and climatic conditions to that observed in Europe or North America. In addition, the outcomes reported in existing work often represent and therefore reports sensitisation to all tested allergens holistically rather than comparing between different groups of allergens, as I have presented in this chapter.

My work is the largest multicentre study focused on sensitisation in South-East Asians (Singapore, Malaysian and Hong Kong) Chinese with COPD. Our unsupervised clustering approach confirms the important presence and clinical relevance of ‘sensitised’ subgroups of COPD patients with the worst clinical outcomes observed in the “highly-sensitised fungal predominant” group. My work is also the first to assess the role of sensitisation in COPD in relation to environmental allergens and is the largest to date to be performed in South-East Asia. In addition, novel home air and surface sampling was performed and allergens identified through a metagenomics approach and then correlated with features of COPD including symptoms and lung function.

Despite the clear novelty and strength of my work presented here, the study does have some limitations. First, as a cross-sectional study, the stability of a sensitisation response with time and measured longitudinally was not assessed. Second, chest computed tomography (CT) was not available in all patients, therefore, I was unable to infer any association with bronchiectasis as previously reported by *Everaerts et al* (2017) (174). Third, patients with physician diagnosed asthma were excluded at study entry, however, it is difficult to ascertain for the absence of asthma-COPD overlap in the recruited cohort, as currently a lack of consensus exists in defining asthma-COPD overlap. Fourth, data on outdoor air fungi that exist in Malaysia and Hong Kong were not available as outdoor air sampling was only performed in Singapore, therefore, the outdoor “air-fungal” sensitisation referred to in this chapter strictly can only apply to the Singaporean COPD cohort (where an association with exacerbations was established). While an increased sIgE to outdoor air fungi was detectable in COPD patients, the specific exposure

duration, timing and quantity that results in such sensitisation was not assessed and was beyond the scope of this chapter but should be considered in future studies. Specific IgE were measured by immuno-dot blot rather than ImmunoCAP assays, but this was necessary, as the outdoor air fungal allergens from Singapore necessitated cultivation directly from air and are not readily available by standard ImmunoCAP approaches. The non-diseased controls are importantly of younger age compared to the COPD cohort, and therefore the differences in allergen sensitisation observed in this work between the controls and COPD patients may not be generalisable to other non-diseased groups, in view of the established change to sensitization responses observed with advancing age (442). While sensitisation is known to decrease with age, our older non-diseased controls (>40) do show slightly higher sIgE levels compared to younger controls (<40), likely attributed to differences in smoking status (443, 444). Finally, this study was performed in COPD patients of Chinese ethnicity from Singapore, Malaysia and Hong Kong respectively, and therefore the presented results may not be generalisable to all countries in the South-East Asian region.

In conclusion, sensitisation and in particular fungal sensitisation is prevalent in South-East Asians with COPD from Singapore, Malaysia and Hong Kong respectively, where it associates with poor clinical outcomes including frequent exacerbations. A highly-sensitised fungal predominant patient sub-group exists in COPD, which associates with the poorest clinical outcomes, and therefore represent a high-risk sub-group of COPD in these countries. Fungal sensitisation is therefore another important and potentially 'treatable trait' in COPD. Outdoor and indoor environments including air and surfaces are important sources of allergen exposure that can lead to measurable sensitisation responses in COPD with clear relevance in South-East Asians from Singapore, Malaysia and Hong Kong.

CHAPTER 5: MICROBIOLOGICAL (FUNGAL) PHENOTYPES OF COPD IN SOUTH-EAST ASIA

5.1 Introduction

The significance of detecting fungi in the airway of COPD patients remains poorly defined. Limited studies, however, do suggest some important associations with disease severity and clinical outcomes which is further attested by my work presented in chapter 4 of this thesis (227, 437, 445). Impaired host immunity coupled with long term inhaled corticosteroid use with frequent bursts of oral steroid prescription during exacerbations can all predispose to fungal-associated disease including allergic, chronic and even invasive manifestations, the latter with high mortality (446, 447).

In the previous chapter of this thesis (Chapter 4), I demonstrated high frequencies of fungal sensitisation in association with poor clinical outcomes in COPD across South-East Asia. Despite a clear association between the presence of fungi in the airway and the occurrence of various fungal disease states, the significance of fungal isolates, in particular *Aspergillus fumigatus*, in the stable COPD state remains unclear and often disregarded by treating clinicians (445). Positive airway fungal cultures have been reported in up to 43% of COPD patients with documented association to inhaled corticosteroid dose and increased sputum neutrophil counts, suggestive of the involvement of host immunity in the presence of airway fungi (227).

While prior work on the bacterial microbiome in COPD illustrates a clear relationship with disease severity, exacerbations and mortality, the role of the underappreciated and understudied fungal microbiome (the mycobiome) lacks dedicated study (37, 306, 307, 321, 341). The mycobiome field has several inherent challenges precluding its widespread study. This includes the absence of standardised methodologies in sequencing protocols including the optimal primers to use for amplification of the Internal Transcriber Spacer (ITS) regions and poorly populated reference databases to support bioinformatic pipelines, leading to large number of unidentified reads, and inaccurate classification to the species level (326, 448).

Although present in an overall lower abundance when compared to bacterial microbiomes, a healthy airway mycobiome does exist with a predominance of *Candida*, *Saccharomyces* and *Grammothele* (327). Moreover, dysbiosis of the mycobiome is linked to disease severity in asthma and cystic fibrosis but studies in COPD are lacking (449, 450). In non-cystic fibrosis bronchiectasis, pathogenic fungal taxa are identified within the airway and have been described in association to various *Aspergillus*-associated disease states. In COPD, very limited data exists focused on elucidating the role of the mycobiome. In HIV patients with COPD, *Pneumocystis jirovecii* has been found as the dominant airway taxa (328). In a small single centre study, *Candida*, *Phialosimplex* and *Aspergillus* have been identified in COPD sputum during acute exacerbations. Critically, no study to date has reported on the potential role of the mycobiome in South-East Asian patients with COPD and related this to clinical outcomes, COPD treatments nor assessed dynamic changes in longitudinal analysis.

Current available data, predominantly bacteriome focused, has illustrated key differences in COPD when compared with healthy controls (310, 313, 323, 451, 452). Most importantly, work on COPD (bacterial) microbiomes demonstrate its prognostic value and link specific features with clinical outcomes including frequent exacerbators, mortality and even COPD symptoms as described in Chapter 2.5.1 (189, 317, 321). Decreased diversity with increased Proteobacteria with the predominant of *Hemophilus*, associated with poor survivor, while increased abundance of *Hemophilus*, *Moraxella* and *Pseudomonas* are seen in frequent exacerbator (314, 317). Furthermore, during AECOPD, increased relative abundance of *Staphylococcus* and decreased *Veillonella* are associated with higher mortality (321). However, this in itself has been poorly studied in the Asian context where a constellation of unique factors including climate, terrain, environment and cultural and dietary habits can all influence the microbiome. A small Chinese study evaluated 40 patients with stable COPD and interestingly found associations between sputum bacterial microbiomes and COPD symptoms including an increased in *Neisseria spp.* in the most symptomatic COPD patients (325). Several other small and descriptive studies, all including <50 patients have been performed in Korea and China and, report differences in

microbial composition between COPD patients and healthy controls but importantly associations with clinical outcomes were not formally assessed (322-324). Here, in this chapter, my aim is to elucidate the presence of clinically relevant microbiological (fungal) phenotypes (using mycobiome sequencing) in South-East Asians with COPD by characterising the airway mycobiome, for the first time, in a large cohort of COPD patients recruited from Singapore, Malaysia and as a comparator, the United Kingdom (Dundee, Scotland). In addition, I assessed the COPD airway mycobiome during and following acute COPD exacerbations and related these findings to important clinical COPD outcomes including exacerbations, mortality and systemic specific IgE responses to the detected fungi. Bacterial microbiome profiles were also evaluated in a smaller group of South-East Asians with COPD predominantly recruited from Singapore and Malaysia.

5.2 Methods

5.2.1 Ethics Statement

The work presented in this chapter was approved by the Institutional Review Boards (IRBs) of all participating hospitals and institutions. Written informed consent was obtained from all participants. Reference numbers pertaining to ethical approvals at each site were as follows: CIRB 2016/2549, CIRB: 2016/2715, CIRB 2017/2933, CIRB 2017/2109 (all mutually recognized by DSRB, Singapore), IRB-2017-12-010 (NTU, Singapore), UMMC 2018725-6524 (Malaysia) and 13/ES/0030 (Dundee, United Kingdom).

5.2.2 Patient recruitment

Patients aged ≥ 40 years old with COPD were recruited through five different sites: Singapore General hospital, Changi General Hospital, and Tan Tock Seng hospital (Singapore), University Malaya Hospital (Kuala Lumpur, Malaysia) and Ninewells Hospital (Dundee, UK). COPD patients were recruited into three separate cohorts between 2014 and 2019 as follows: stable COPD (n=337), acute exacerbation of COPD (AECOPD) (n=66) and a longitudinal COPD cohort (n=34) (Table 5.2). COPD was defined by the presence of respiratory symptoms with appropriate risk factor exposure and a post bronchodilator FEV₁/FVC ratio of <0.7 as per GOLD guidelines as detailed in Chapter 3.2.2 (200). Patients recruited into the longitudinal study arm had samples collected during clinical stability, within 24 hours of a documented acute exacerbation (AECOPD) and the 2 weeks following recovery and treatment (post exacerbation). All patients in the longitudinal study arm received oral antibiotics (co-amoxiclav or doxycycline for 1 week) and a burst of oral corticosteroids (prednisolone 30-40mg for 5 consecutive days) during their AECOPD. Stable COPD was defined as absence of an exacerbation in the six-week period preceding study recruitment and an acute exacerbation defined as an acute worsening of respiratory symptoms (defined as increased dyspnea, cough, sputum purulence and volume

and/or wheeze) necessitating treatment with antibiotics and/or corticosteroids based on GOLD guidelines (33, 200). The AECOPD cohort was followed up over a two-year period prospectively and individual mortality outcome was recorded. Patients with previous diagnosis of asthma (defined by GINA guidelines) or with variable respiratory symptoms and airflow limitation, on long-term oral corticosteroids and/or immunosuppressive agents, active mycobacterial infection and malignancy (on active treatment) were excluded (232). Frequent COPD exacerbators were defined as ≥ 2 exacerbations/year and very frequent exacerbator (vFE) defined as ≥ 3 exacerbations per year (180). Use of inhaled corticosteroids was defined as use of a daily maintenance dose of at least 100 μ g fluticasone propionate or an equivalent inhaled corticosteroid.

Non-diseased (healthy) controls: An independent group of non-COPD (healthy) controls (n=47) was recruited from Nanyang Technological University, Singapore, to serve as a comparison group for the COPD cohort. The non-COPD control (healthy) cohort participants all had no prior history of respiratory disease, were lifelong non-smokers and had normal measured spirometry with an $FEV_1/FVC > 0.7$ and $FEV_1 \geq 80\%$ predicted.

5.2.3 COPD specimen collection and processing (sputum and serum)

Sputum: Spontaneously expectorated and/or induced sputum was obtained and collected in sterile containers (453). Sputum collected was homogenised with the same volume of Sputasol (Oxoid Limited, UK) at 37°C with shaking. This was followed by adding two-parts of RNAlater (Life Technologies, New Zealand) and stored at -80°C for subsequent DNA extraction and mycobiome analysis (454).

Serum: Venous blood was collected for specific IgE assays as described in Chapter 4.2.11. Serum was used to determine if any correlations exist between the measured airway mycobiome and a systemic sensitisation response to these fungi. Venous blood was collected in serum vacutainer tubes (BD biosciences), centrifuged at 1300g for 10 minutes at 18°C to separate serum which was used for subsequent immunological studies.

5.2.4 Sputum DNA extraction

Sputum DNA was extracted using the Zymo DNA isolation kit with appropriate blank controls (Zymo Research, Irvine, CA, USA) included as per manufacturer's instructions. Briefly, sputum was centrifuged at 13,000g for 10 minutes and resultant pellets were resuspended with elution buffer. Next, the homogenised samples were incubated at 55 °C for 10 minutes with storage buffer and proteinase K. Subsequently the samples with genomic binding buffer was transferred to a Zymo-spin column and centrifuged for 1 minute at 12,000g. The columns were then washed with wash buffers and the final DNA was eluted in 50ul of elution buffer. All DNA was quantified using the Qubit. fluorometer 2.0 double-stranded DNA (dsDNA) assay (Invitrogen, Carlsbad, CA, USA).

5.2.5 ITS Primer optimisation and selection

Prior to initiation of mycobiome sequencing, the Internal Transcribed Spacer (ITS) primers were optimised using fungal mock communities. Four different primer sets used regularly in prior published mycobiome studies and in human samples targeting the ITS-1 or ITS-2 region (Table 5.1) were selected and compared for efficacy. Each primer set was employed to profile the fungal mock community (Table 5.2) using a targeted amplicon sequencing approach.

Table 5.1: Summary of the selected primer sets used to examine the efficiency of amplification of a fungal mock community.

Primer set	Target	Forward primer	Reverse primer	References
P1	ITS1	ITS1F: CTTGGTCATTTAGAGGAAGTAA	ITS2: GCTGCGTTCTTCATCGATGC	(455)
P2	ITS1	ITS1-30F: GTCCCTGCCCTTTGTACACA	ITS1-217R: TTTCGCTGCGTTCTTCATCG	(456)
P3	ITS2	gITS7ngs: GTGARTCATCRARTYTTTG	ITS4ngs: TCCTSCGCTTATTGATATGC	(457, 458)
P4	ITS2	Fseq: ATGCCTGTTTGAGCGTC	Rseq: CCTACCTGATTTGAGGTC	(459, 460)

Table 5.2: The fungal isolates employed to make up a ‘fungal mock community’ to examine and optimize the PCR amplification process and to choose the optimal primers for use in mycobiome sequencing experiments.

Phylum	Species
Ascomycota	<i>Candida tropicalis</i>
	<i>Curvularia lunata</i>
	<i>Aspergillus fumigatus</i>
	<i>Aspergillus terreus</i>
Basidiomycota	<i>Schizophyllum commune</i>

5.2.6 Fungal mock community preparation

Mock fungal communities were prepared using two different methods: fungal amplicons (FA) and fungal DNA (FD) (Figure 5.1). Extracted DNA from individual fungal isolates was amplified using 4 different primer sets and the resultant amplicon from each fungal isolate normalized and pooled as a fungal amplicon (FA) derived mock community. For the fungal DNA (FD) mock community, DNA from each fungal isolate was extracted and pooled prior to amplification. The pooled DNA was then subjected to amplification using the 4 primer sets (P1-4).

Figure 5.1: A schematic overview of the two different mock community preparation strategies used. (i) The fungal amplicon (FA) method where DNA was extracted from each fungal isolate and amplified using 4 different primer sets (P1-4) prior to pooling as the final mock community. (ii) The fungal DNA (FD) method which pools extracted DNA from each fungal isolate prior to amplification with the 4 primer sets. Source: Ali et al.(461)

5.2.7 PCR amplification

Primer set 4 targeting the fungal ITS-2 region was selected for final mycobiome sequencing due to its superior fungal coverage, accuracy in identification of mock community and high sensitivity in amplification of ITS region in sputum samples based on optimisation experiments as details in Chapter 5.3.1. For the bacterial microbiome, the V3-V4 regions were amplified using 16S amplicon PCR primers: forward sequence: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, reverse sequence: 5'-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. Forward and reverse adaptors were added as per Illumina Fungal and 16S metagenomics protocols to the ITS2 and 16S primer pairs (Integrated DNA technologies, IDT, USA).

PCR conditions and cycling for fungal mycobiome experiments were optimised as follows: 10ul KAPA HiFi HotStart ReadyMix polymerase (Kapa Biosystems, South Africa), 4ul (2uM) of forward and reverse primers (IDT, USA) and 6ul of sputum DNA. Initial denaturation at 95°C for 3 minutes, 35 cycles at 95°C for 15 seconds followed by 50°C for 15 seconds, 72°C for 30 seconds and then a final extension step at 72°C for 7 minutes using the Verti Thermal cycler (Invitrogen, USA).

For the bacterial microbiome analysis, the PCR conditions and cycling were as follows: 10ul KAPA HiFi HotStart ReadyMix polymerase (Kapa Biosystems, South Africa), 8ul (1uM) of 16S forward and reverse primers (IDT, USA) and 2ul of sputum DNA. Initial denaturation at 95°C for 3 minutes, 25 cycles at 95°C for 30 seconds followed by 55°C for 30 seconds, 72°C for 30 seconds and then a final extension step at 72°C for 5 minutes using the Verti Thermal cycler (Invitrogen, USA).

5.2.8 Library preparation, mycobiome sequencing and data processing

After PCR amplification, PCR products were visualized using 1.5% agarose gels to ensure amplification with the presence of an appropriately sized band prior to further purification. Purification was then performed using Ampure XP beads (Beckman Coulter Genomics, part #A63881) with a modified two-step clean up, first with an 0.6 ratio volume of the Ampure XP bead to PCR product following which supernatant was collected for a second clean up using an 0.8 ratio with final elution in 20ul of elution buffer.

Second stage index PCR and clean up was performed to include the index sequences and flow-cell attachment regions. Agarose gel electrophoresis (1.5%) was again performed to ensure successful purification and the absence of primer dimers prior to pooling. Final libraries were denatured and quantified with the Kappa library quantification kit (Kapa Biosystems, South Africa). Pooled libraries of 8.5pM with 20% phiX was loaded onto the Illumina MiSeq platform (2x 101bp reads) for sequencing (Illumina, USA). Extraction blanks and negative controls for all PCR steps were included in the mycobiome sequencing runs.

Data processing: Analysis was performed using the Illumina Miseq platform with validated wet-lab (illumine) and cloud-based analytic workflows (Basespaces Labs) (461). ITS sequences were mapped to the UNITE database v7.2 described by Kõljalg *et al.* using the Illumina ITS Metagenomics pipeline (version 1.0.1; Basespace labs, San Diego, CA, USA). 16S rRNA sequences were mapped to the illumine-curated version of greengenes 16S rRNA database (16S Meatgenomics, v1.0.1; Basespace labs, San Diego, CA, USA) with high-performance implementation of the Ribosomal Database project (RDP) classifier (462, 463). All sequence data from this chapter has been uploaded to the National Center for Biotechnology Information (NCBI) Sequence read archives (SRA) under the project accession PRJNA609892.

5.2.9 Immuno-dotblot assays to measure specific-IgE responses against a range of detected fungi

To assess for any correlation between the presence of specific fungi within the airway mycobiome and a systemic specific-IgE (sIgE) immune response against selected fungi identified within the COPD airway mycobiome serum from a subset of n=42 COPD patients were screened by immuno-dotblot assays against the following fungi: *Aspergillus*, *Curvularia*, *Penicillium*, *Cladosporium*, *Schizophyllum*, *Fusarium* and *Trametes* using methodologies as described in Chapter 4.2.11.

5.2.10 Statistical analysis

Data was analysed using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria.) and python (version 3.8). Shapiro-Wilks testing was used to assess data distribution. All continuous variables was not normally distributed; hence all continuous data are presented as medians with interquartile ranges. For between group comparisons, Wilcoxon's, Mann-Whitney-U and/or Kruskal-Wallis testing with Benjamin-Hochberg correction for false discovery was performed as appropriate. Categorical data was analysed using Chi-squared or Fisher-exact tests as appropriate. Contamination was identified with the R "Decontam" package with a prevalence threshold of 0.5 (464). The fungal genera identified as contaminants were removed from downstream analysis (Table 5.3). Shannon and Simpson diversity indices were calculated using the "diversity" function of the R package "vegan". Bray-Curtis dissimilarity index was generated with the "vegdist" function of the R package "vegan". Beta diversity was visualised using principal coordinate analysis (PCoA) plots with Bray-Curtis dissimilarity. The distance to centroids were calculated using the "betadisper" function of the R package "vegan". Between group differences were derived by permutation-based testing of multivariate homogeneity of group dispersion (PERMDISP2). For multivariate analysis adjusted for age, gender, body mass index, smoking status (as pack year exposure), post bronchodilator FEV₁,

inhaled corticosteroid use and country of origin, permutational multivariate analysis of variance using distance matrices (PERMANOVA) was computed with 10,000 permutations using the “*adonis*” function of the R package “*vegan*”. Linear discriminant analysis effect size (LEfSe) was performed using the webtool: <http://huttenhower.sph.harvard.edu/galaxy/> using a cut-off Linear Discriminant Analysis (LDA) score of >3.5. Graphs were generated using the R package “*ggplot2*” and significance defined as $p < 0.05$.

Table 5.3: Summary of (a) fungal and (b) bacteria genera identified as contaminants using the “Decontam” R package. Probability: true probability of a genera being present in an examined sample. Cut off <0.5 in prevalence was identified as a contaminant.

(a)

Genus	Probability
<i>Dichomitus</i>	0.028
<i>Trichosporon</i>	0.057
<i>Dekkera</i>	0.085
<i>Hortaea</i>	0.127
<i>Puccinia</i>	0.127
<i>Polyporus</i>	0.141
<i>Pichia</i>	0.258
<i>Tinctoporellus</i>	0.276
<i>Wickerhamia</i>	0.327
<i>Hyphodontia</i>	0.456

(b)

Genus	Probability
<i>Ruminococcus</i>	0.051
<i>Gluconacetobacter</i>	0.066
<i>Pelomonas</i>	0.103
<i>Yersinia</i>	0.103
<i>Escherichia</i>	0.118
<i>Serratia</i>	0.213
<i>Enterobacter</i>	0.301
<i>Pseudidiomarina</i>	0.316
<i>Luteimonas</i>	0.324
<i>Methylobacterium</i>	0.331
<i>Glaciecola</i>	0.338
<i>Microbulbifer</i>	0.368
<i>Roseospira</i>	0.368
<i>Marinomonas</i>	0.375
<i>Arsenophonus</i>	0.397
<i>Thiomonas</i>	0.397
<i>Tolomonas</i>	0.397
<i>Marinobacter</i>	0.412
<i>Vibrio</i>	0.412
<i>Vogesella</i>	0.419
<i>Alcanivorax</i>	0.426
<i>Gluconobacter</i>	0.426
<i>Lachnospira</i>	0.426
<i>Paucibacter</i>	0.426
<i>Roseomonas</i>	0.441
<i>Lentibacillus</i>	0.449
<i>Sporotomaculum</i>	0.449
<i>Amaricoccus</i>	0.456
<i>Candidatus.Regiiella</i>	0.456
<i>Hyphomonas</i>	0.456
<i>Maricaulis</i>	0.456
<i>Candidatus.Phytoplasma</i>	0.463
<i>Atopobium</i>	0.471

Genus	Probability
<i>Anaerobacillus</i>	0.478
<i>Caloramator</i>	0.478
<i>Candidatus.Amoebophilus</i>	0.478
<i>Gallibacterium</i>	0.478
<i>Geobacillus</i>	0.478
<i>Halomonas</i>	0.478
<i>Kushneria</i>	0.478
<i>Marinitoga</i>	0.478
<i>Providencia</i>	0.478
<i>Thalassospira</i>	0.478
<i>Desulfotomaculum</i>	0.485
<i>Hydrocarboniphaga</i>	0.485
<i>Litoricola</i>	0.485
<i>Micromonospora</i>	0.485
<i>Pasteurella</i>	0.485
<i>Peptococcus</i>	0.485
<i>Peptoniphilus</i>	0.485
<i>Sedimentibacter</i>	0.485
<i>Sneathia</i>	0.485
<i>Sphingomonas</i>	0.485
<i>Thermodesulfovibrio</i>	0.485
<i>Chromobacterium</i>	0.493
<i>Exiguobacterium</i>	0.493
<i>Hydrogenophilus</i>	0.493
<i>Propionigenium</i>	0.493
<i>Rhodothermus</i>	0.493
<i>Salinicoccus</i>	0.493
<i>Sphingobacterium</i>	0.493
<i>Sphingobium</i>	0.493
<i>Staphylococcus</i>	0.493
<i>Stenoxybacter</i>	0.493
<i>Virgibacillus</i>	0.493
<i>Weissella</i>	0.493

5.2.10.1 Cluster analysis

As *Candida* was overwhelmingly the predominant fungal genera detected in the COPD mycobiome ($\geq 65\%$ relative abundance), it was excluded from clustering analyses to allow for the identification, if relevant, of other differentiating fungi in relation to COPD outcomes. Unsupervised hierarchical clustering with Ward's minimum variance was performed with the “*hclust*” function of the R “Cluster” package and visualised with *heatmap.2* using the R “*gplot*” package. Optimal clusters were assessed by the R “Nbclust” package. Cluster stability was assessed using Jaccard similarities index with bootstrapping over 100 iterations. The mean Jaccard similarities index for the reported clusters 1 and 2 in this chapter respectively were 0.91 and 0.92 suggesting that the identified clusters were highly stable (Jaccard index ≥ 0.75 suggests valid and stable clusters (465)).

5.2.10.2 Co-occurrence network analysis

Co-occurrence network analyses was generated using methodologies described by *Fraust et al*, with slight modification (466). Pearson's correlation, Spearman's correlation, Bray-Curtis index, Generalized Boosted Linear Models (GBLMs) and mutual information were all ensembled with a weighted Sime's test and visualised using Cytoscape (version 3.7.2) (467). Fungal genera present in the airway mycobiome in at least 5% of the total COPD patient population at an abundance of $>1\%$ were included for co-occurrence analyses.

5.3 Results

5.3.1 Primers targeting the ITS-2 region accurately identifies fungi within a mock community.

Four different primer sets targeting the ITS-1 or ITS-2 regions were selected for comparison in their accuracy of identifying a fungal mock community as described in the methods. These primer sets were chosen based on prior studies that illustrate good taxonomic coverage and/or were previously used in studies of human specimens.

The four ITS primer sets each identified the individual fungal isolates included in the mock community accurately with the exception of *Curvularia lunata* which was misclassified as *Exserohilum spp.* using primer set 1 (P1) and primer set 2 (P2) (Figure 5.2). The fungal mock communities were reproducible with all tested primer sets, except for *Curvularia lunata*, which again was misclassified using P1 and P2 as *Exserohilum spp.* (Figure 5.3). The fungal mock community composition detected varied between the two methods employed as described above: FA and FD-derived mock communities with a higher *Candida* and lower *Curvularia* detected with an FD derived fungal mock community (Figure 5.3). Primer sets 3 and 4 targeting the ITS-2 region were associated with higher accuracy in taxonomic classification of the fungal mock community. The sensitivity of the four primer sets in amplifying the ITS region of clinical samples were assessed with a subset of COPD sputum (n=15). Primer set 4 (P4) has the highest yield for ITS amplification (80%), compared to the other 3 primer sets (P3:33%, P2: 60% and P1: 53%). In view of these results, primer set 4 was selected for further mycobiome sequencing of sputum samples.

Figure 5.2: Bar charts illustrating the relative abundance of each individual fungal isolate using the 4 different primer sets. Colored bars indicate the individual fungal taxa.

Figure 5.3: Bar charts illustrating the comparisons between each individual tested primer set (P1-P4) in replicating the theoretical (T) composition of the fungal mock community using two separate methods: fungal amplicon (FA) and fungal DNA (FD) mock community preparations.

5.3.2 Distinct mycobiome profiles demonstrating geographic variation are observed in stable COPD

COPD patients were recruited into 3 separate cohorts as described in the methods: stable COPD (n=337), acute exacerbation of COPD (AECOPD) (n=66) and a longitudinal COPD arm (n=34) which includes repeated sampling at stability, within 24 hours of an exacerbation and then 2 weeks following treatment and recovery (post exacerbation). The clinical characteristic of each cohort is summarised in Table 5.4.

The average sequence read counts for control blanks and clinical (sputum) samples were 406 and 20,908, respectively. The intra-individual differences in airway mycobiome profiles were compared in a small number of stable COPD patients with sputum collected at baseline and after 6-months. No significant differences were observed in the mycobiome profiles between the baseline and 6-month sample follow-up (Figure 5.4).

The airway mycobiome in stable COPD (n=337) illustrates a higher α -diversity as measured by the Shannon index when compared to non-diseased (healthy) controls which are either young (<40 years of age (n=24)) or older (>40 years of age (n=23) (p=0.003) (Figure 5.5a). *Saccharomyces*, *Hanseniaspora*, *Ganoderma*, *Schizophyllum*, *Fusarium*, *Phlebiopsis* and *Grammothele* were the predominant fungal genera identified in the non-diseased (healthy) airway, while *Alternaria*, *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Mycosphaerella*, *Wickerhamomyces*, *Trametes*, *Penicillium*, *Resinicium*, *Itersonilia*, *Aureobasidium*, *Botrytis* and *Wallemia* were specific to the COPD airway (Figure 5.5b-c).

Twelve discriminants fungal taxa were evident when comparing between Asian (recruited in Singapore and Kuala Lumpur; SG/KL) and non-Asian COPD patients (recruited in Dundee; DD). *Saccharomyces*, *Curvularia*, *Aspergillus*, *Schizophyllum*, *Penicillium*, and *Grammothele* were the predominant fungi in the SG/KL cohort while *Cladosporium*, *Debaryomyces*, *Hanseniaspora*, *Trametes* and *Wickerhamomyces* were predominant in the DD cohort respectively, all demonstrating a linear discriminant analysis (LDA) score >3.5 (all p<0.05)

(Figure 5.5d -e). Critically, in stable COPD, airway mycobiome composition remains unaltered by either GOLD ABCD group or GOLD (lung function) grade (Figure 5.6 and 5.7).

Figure 5.4: Airway mycobiome profiles remain unchanged at baseline and at 6-month follow up in a group of n=5 stable COPD patients. Stacked bar charts illustrating the relative abundance of the various fungal genera detected between baseline and 6- month interval sputum samples in stable COPD. a: baseline, b: 6-month-follow up.

Table 5.4: Demographic table summarising the non-diseased (healthy) and COPD cohorts from Singapore / Kuala Lumpur (SG/KL) and Dundee (DD).

Characteristics	Non-diseased (<40 years old)	Non-diseased (>40 years old)	Stable COPD	SG/KL	Dundee (DD)	AECOPD	SG/KL	Dundee (DD)	Longitudinal
n	24	23	337	175	162	66	29	37	34
Age (years), Median (IQR)	28 (25-33)	63 (61-64)	72(67-77)	72 (67-77)	72 (66-77)	69 (64-76)	68 (63-72)	70 (65-77)	70 (65-77)
BMI (Kg/m²), Median (IQR)	21.17 (19.14-23.0)	23.86 (20.94-26.23)	24.7 (21.9-29.0)	22.5 (20.2-26.3)	27.0 (24.0-31.0)	25.0 (21.0-30.6)	21.7 (17.5-27.9)	28.0 (24.3-31.8)	25.0 (23.0-29.9)
Gender (Male), n (%)	11 (45.8)	6 (26.1)	273 (81.0)	167 (95.4)	106 (65.4)	53 (80.3)	29 (100)	24 (64.9)	26 (76.5)
Ethnicity									
Chinese	22 (91.7)	21 (91.3)	117 (34.7)	117 (66.9)		19 (28.8)	19 (65.5)		5 (14.7)
Malay	2 (8)	2 (8.7)	23 (6.8)	23 (13.1)		8 (12.1)	8 (27.6)		
Indian			35 (10.4)	35 (20.0)		2 (3.0)	2 (6.9)		
Caucasian			162 (48.1)		162 (100)	27 (56.1)		37 (100)	29 (85.3)
Current smoker, n (%)	0 (0.0)	0 (0.0)	186 (55.2)	61 (34.9)	125 (77.2)	44 (66.7)	11 (38.0)	33 (89.2)	27 (79.4)
Ex-smoker, n (%)	0 (0.0)	0 (0.0)	151 (44.8)	114 (65.1)	37 (22.8)	22 (33.3)	18 (62.0)	4 (10.8)	7 (20.6)
Smoking pack years, Median (IQR)	0 (0.0)	0 (0.0)	50.0 (33.1-64.5)	50.0 (40.0-80.0)	40.0 (30.0-54.5)	41.5 (37.8-67.3)	49.0 (40.0-75.0)	40.0 (30.0-60.0)	40.0 (26.3-69.5)
CAT Score, Median (IQR)	NA	NA	20.0 (12.0-25.0)	20.0 (11.3-25.0)	19.0 (13.0-25.0)	23.0 (18.0-27.0)	22.0 (17.5-27.0)	23.0 (18.8-27.3)	23.0 (17.0-27.3)
FEV₁ (% predicted), Median (IQR)	96.5 (85.8-107.3)	93.5 (83.3-96.8)	59.0 (42.0- 74.5)	52.0 (37.0-66.0)	67.5 (51.8-79.3)	51.7 (41.0-73.0)	49.0 (41.0- 59.0)	61.8 (43.4-79.9)	61.8 (50.7-76.9)
FEV₁/FVC (% predicted), Median (IQR)	86.6 (81.5-93.1)	79.0 (77.5-84.1)	53.0 (44.0-63.0)	55.0 (44.0-65.0)	52.0 (43.1-60.9)	50.7 (42.3-59.8)	49.0 (43.0-59.0)	51.0 (42.0-60.0)	49.0 (41.0-56.0)
No. of Exac/year, Median (IQR)	NA	NA	1 (0-3)	0 (0-2)	2 (1-4)	3 (2-4)	2 (1-3)	3 (2-4)	3 (2-4)
LABA, n (%)	NA	NA	8 (2.4)	3 (1.7)	5 (3.1)	1 (1.5)	0 (0.0)	1 (2.7)	1 (2.9)
LAMA, n (%)	NA	NA	34 (10.1)	20 (11.4)	14 (8.6)	5 (7.6)	4(13.8)	1(2.7)	2 (5.9)
LABA/LAMA, n (%)	NA	NA	112 (33.2)	70 (40.0)	42(25.9)	13(19.7)	6 (20.7)	7 (18.9)	6 (17.6)
LABA/ICS, n (%)	NA	NA	41 (12.1)	14 (8.0)	27 (16.7)	5 (7.6)	1 (3.4)	4 (10.8)	4 (11.8)
Triple Therapy, n(%)	NA	NA	130(38.6)	63 (36.0)	67 (41.4)	38 (57.5)	17 (58.7)	21 (56.8)	19 (55.9)
SAMA/SABA, n (%)	NA	NA	12 (3.6)	5 (2.9)	7 (4.3)	4 (6.1)	1 (3.4)	3 (8.1)	2 (5.9)
Macrolide, n (%)	NA	NA	34 (10.1)	10 (5.7)	24 (14.8)	8 (12.1)	1 (3.4)	7 (18.9)	5 (14.7)

Characteristics	Non-diseased (<40 years old)	Non-diseased (>40 years old)	Stable COPD	SG/KL	Dundee (DD)	AECOPD	SG/KL	Dundee (DD)	Longitudinal
GOLD Group, n (%)									
A	NA	NA	81 (24.0)	45 (25.7)	36 (22.2)	3 (4.5)	0 (0.0)	3 (8.1)	1 (2.9)
B			104 (30.9)	76 (43.4)	28 (17.3)	1 (1.5)	7 (24.1)	1 (2.7)	5 (14.7)
C			41 (12.2)	8 (4.6)	33 (20.4)	10 (15.2)	0 (0.0)	10 (27.0)	0 (0.0)
D			111 (32.9)	46 (26.3)	65 (40.1)	52 (78.8)	22 (75.9)	23 (62.2)	28 (82.4)
GOLD Grade									
1	NA	NA	57 (16.9)	20 (11.4)	37 (22.8)	12 (18.2)	3 (10.4)	9 (24.3)	8 (23.5)
2			163 (48.4)	71 (40.6)	92 (56.8)	29 (43.9)	11 (37.9)	18 (48.7)	19 (55.9)
3			92 (27.3)	66 (37.7)	26 (16.1)	20 (30.3)	13 (44.8)	7 (18.9)	4 (11.8)
4			25 (7.4)	18 (10.3)	7 (4.3)	5 (7.6)	2 (6.9)	3 (8.1)	3 (8.8)

n: number of patients; IQR: interquartile range, BMI: body mass index, CAT: COPD assessment test, FEV₁: forced expiratory volume in the 1st second, FVC: forced vital capacity, IQR: interquartile range, NA: not applicable, GOLD: global initiative for COPD, SAMA: short acting muscarinic antagonist, SABA: short-acting beta-2 agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta-2 agonist, ICS: inhaled corticosteroid.

Figure 5.5: The airway mycobiome differs between COPD and non-diseased (healthy) controls and demonstrates geographic variation. (a) Boxplot illustrating the α -diversity (Shannon index) of the non-diseased (healthy) controls <40 and ≥ 40 years of age compared to the COPD cohort. (b) Bar chart illustrating the relative abundance and Respective colouration denotes group membership: non-diseased (healthy) controls <40 years of age (orange), non-diseased (healthy) controls ≥ 40 years of age (brown). COPD: Chronic obstructive pulmonary disease. * $p < 0.05$.

Figure 5.5: (c) Prevalence of the fungal genera (with relative abundance of $>1\%$) between non-diseased (healthy) individuals <40 ($n=24$) and ≥ 40 years of age ($n=23$) compared to COPD ($n=337$). Respective colouration denotes group membership: non-diseased (healthy) controls <40 years of age (orange), non-diseased (healthy) controls ≥ 40 years of age (brown). COPD: Chronic obstructive pulmonary disease.

Figure 5.5: (d) Airway mycobiome composition between South-East Asian i.e. Singapore/Kuala Lumpur (SG/KL) and non-Asian patients i.e. Dundee (DD) with COPD illustrating the top 25 fungal genera. COPD: Chronic obstructive pulmonary disease.

(e)

Figure 5.5: (e) Linear discriminant analysis effect size (LEfSe) illustrating the discriminant mycobiome genera between the South-East Asian and non-Asian groups. Respective colouration denotes group membership: SG/KL (dark turquoise) and DD (purple) COPD cohorts. SG/KL: Singapore/Kuala Lumpur, DD: Dundee.

Figure 5.6: Airway mycobiome profiles are not related to GOLD ABCD grouping in patients with stable COPD. (a) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera between COPD patients in GOLD A (n=81), GOLD B (n=104), GOLD C (n=41) and GOLD D (n=111) groups. GOLD: global initiative for COPD. ns: not significant.

Figure 5.6: No significant changes in α -diversity as measured by the (b) Shannon and (c) Simpson indexes and (d) Principle Coordinate Analysis (PCoA) plot between GOLD groups. GOLD: global initiative for COPD. ns: not significant.

Figure 5.7: Airway mycobiome profiles are not affected by GOLD (lung function) (FEV_1) grade in patients with stable COPD. (a) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera between patients in GOLD stage 1 ($n=57$), stage 2 ($n=163$), stage 3 ($n=92$) and stage 4 ($n=25$). GOLD: global initiative for COPD. ns: not significant.

Figure 5.7: No significant change in α -diversity measured by the (b) Shannon and (c) Simpson indexes and (d) a Principle Coordinate Analysis (PCoA) plot between the grades. GOLD: global initiative for COPD. ns: not significant.

5.3.3 Altered airway mycobiome profiles in very frequent COPD exacerbators (vFE).

No significant differences in mycobiome composition and/or diversity between non-frequent and frequent exacerbators (≥ 2 exacerbations/year) with COPD was detected. Importantly, however, differences were observed when exacerbation frequency was increased to ≥ 3 exacerbations/year (a clinical state described in this thesis of very frequent exacerbators).

Wickerhamomyces is the only identified discriminant taxa that differentiates very frequent from non-frequent exacerbators with an LDA score > 3.5 ($p=0.036$) (Figure 5.8a). β -diversity with Bray Curtis index reveals significant differences in mycobiome composition between very frequent ($n=92$) and non-frequent COPD exacerbators ($n=245$) (median distance to centroid: 0.61 Interquartile range (IQR) 0.49-0.67 versus 0.53 IQR 0.39-0.69; $p=0.007$) (Figure 5.8 b-c), which remains significant on multivariate analysis with permutational multivariate ANOVA (PERMANOVA) adjusted for age, gender, smoking pack years, body mass index, country of origin and inhaled corticosteroid use ($p=0.0022$) (Figure 5.8b).

Having detected differences in mycobiome composition between COPD exacerbator groups, I next assessed, using network analysis, interactions within the airway mycobiome itself in the exacerbator group. Interactions between fungi were assessed using network analysis and 15 fungal genera demonstrating $>1\%$ abundance in at least $>5\%$ (>17) of the study population. A higher number of co-occurrence interactions (positive associations between taxa) were observed in the non-FE COPD group while vFE exhibit a higher number of co-exclusive interactions (negative associations between taxa) (Figure 5.8d). This pattern suggests the presence of fungal dysbiosis where co-exclusive interactions occur, potentially resulting in the observed clinical state of frequent exacerbations. In the vFE COPD group, the key taxa responsible in maintaining network integrity include *Alternaria*, *Aspergillus*, *Cryptococcus*, *Curvularia*, *Lodderomyces*, *Malassezia*, *Penicillium* and *Saccharomyces* with the highest number of edge (interaction) counts, stress centrality and betweenness centrality values (indicating the critical and/or influential microbes respectively within the network). In the non-FE COPD group, the key taxa

identified include *Candida* and *Saccharomyces* (Table 5.5). Interestingly, when the airway mycobiome profiles are compared between COPD groups based on use of inhaled corticosteroids alone, no differences were observed in the underlying mycobiome profiles (Figure 5.9a-c) suggesting alternate mechanisms to the influence of treatment (particularly corticosteroids) in mediating the detectable changes to COPD airway mycobiome profiles.

Figure 5.8: A comparison of airway mycobiome profiles between very frequent (vFE) and non-frequent COPD exacerbators (non-FE). (a) Stacked bar charts illustrating the top 25 fungal genera between very frequent and non-frequent exacerbators. vFE: very frequent exacerbator.

Figure 5.8: (b) Principle coordinate analysis (PCoA) plot illustrating the β -diversity between groups using the Bray-Curtis index. (c) Box plot of the average distance to centroid between groups in the PCoA further illustrating group related differences. (d) Co-occurrence analysis reveals more complex and increased co-exclusive interactions within the mycobiome in very frequent COPD exacerbators (vFE) compared to non-frequent exacerbators (Non-FE). Fungal genera demonstrating >1% relative abundance in at least 5% of the studied COPD population are shown. vFE: very frequent exacerbator. Coloration denotes the interaction: positive/co-occurrence (blue), negative/ co-exclusive (red). * $p < 0.05$.

Table 5.5: Tables summarizing network analyses based on fungal genera comparing very frequent (vFE) with non-frequent (non-FE) COPD exacerbators. The number of interactions (edge counts), critical (stress centrality) and influential (betweenness centrality) measures are illustrated for between group comparisons. The top taxa with highest number of interactions, and the most influential and critical general within the network are highlighted.

Non- FE			
Microbes	No. of interactions (Edges)	Critical (Stress Centrality)	Influential (Betweenness Centrality)
<i>Candida</i>	28	79	0.131846
<i>Saccharomyces</i>	24	51	0.068446
<i>Alternaria</i>	22	36	0.048775
<i>Penicillium</i>	22	29	0.03046
<i>Cryptococcus</i>	20	28	0.044562
<i>Malassezia</i>	18	19	0.019183
<i>Curvularia</i>	18	14	0.011748
<i>Aspergillus</i>	18	14	0.013139
<i>Lodderomyces</i>	17	10	0.008272
<i>Cladosporium</i>	13	12	0.019885
<i>Mycosphaerella</i>	14	10	0.010178
<i>Debaryomyces</i>	8	6	0.008425
<i>Hanseniaspora</i>	8	4	0.00641
<i>Wickerhamomyces</i>	18	14	0.011517
<i>Schizophyllum</i>	14	2	0.001221

vFE			
Microbes	No. of interactions (Edges)	Critical (Stress Centrality)	Influential (Betweenness Centrality)
<i>Alternaria</i>	28	11	0.006207
<i>Aspergillus</i>	28	11	0.006207
<i>Cryptococcus</i>	28	11	0.006207
<i>Curvularia</i>	28	11	0.006207
<i>Lodderomyces</i>	28	11	0.006207
<i>Malassezia</i>	28	11	0.006207
<i>Penicillium</i>	28	11	0.006207
<i>Saccharomyces</i>	28	11	0.006207
<i>Debaryomyces</i>	26	8	0.00464
<i>Cladosporium</i>	25	4	0.002198
<i>Candida</i>	26	2	0.000881
<i>Hanseniaspora</i>	26	2	0.000881
<i>Mycosphaerella</i>	26	2	0.000881
<i>Schizophyllum</i>	26	2	0.000881
<i>Wickerhamomyces</i>	19	1	0.000423

Figure 5.9: The COPD airway mycobiome is unaffected by inhaled corticosteroid use in stable COPD patients. (a) Stacked bar charts of the top 25 fungal genera compared between COPD patients receiving ICS and those not on ICS respectively. ns: not significant. ICS: inhaled corticosteroid.

Figure 5.9: Alpha diversity measured by the (b) Shannon and (c) Simpson indexes between the respective groups. (d) A Principle Coordinate Analysis (PCoA) plot between patient groups receiving ICS and those not on ICS respectively. ns: not significant. ICS: inhaled corticosteroid.

5.3.4 Airway mycobiome profiles remain unchanged between the stable, exacerbation and post- exacerbation state in COPD despite antibiotic and steroid treatment.

Longitudinal analysis of airway mycobiome profiles in n=34 patients with COPD was undertaken to assess for any change between the stable, exacerbation and post-exacerbation state. Respiratory specimens (sputum) was collected at baseline (during clinical stability), within 24 hours of a documented exacerbation (exacerbation) and then 2 weeks post-exacerbation on recovery and completion of treatment (post-exacerbation). All patients received an oral antibiotic (either doxycycline or co-amoxiclav) for one week and a burst of oral corticosteroids (prednisolone) for 5 consecutive days during treatment of their exacerbation. Airway mycobiome profiles importantly remain unchanged across all three timepoints suggesting that the exacerbation event itself and its associated treatment does not alter the airway mycobiome (Figure 5.10a). There were no significant differences observed in the α - (Shannon and Simpson indices) ($p=0.939$, $p=0.9144$) (Figure 5.10b-c) or in β -diversity (Figure 5.10d) across the three times points in COPD patients within the longitudinal cohort. Taken together, these data suggest that changes in the COPD airway mycobiome are not influenced by exacerbation events or associated treatment with oral antibiotics and corticosteroids.

(a)

Figure 5.10: The COPD airway mycobiome remains unchanged across an exacerbation event when assessed longitudinally at baseline, during an exacerbation and at post-exacerbation. (a) Stacked bar chart illustrating the relative abundance of the top 25 fungal genera at baseline (B), within 24 hours of a documented exacerbation (E) and at 2-weeks post-exacerbation following treatment and on recovery (PE).

Figure 5.10: (b) Shannon, (c) Simpson index and (d) Principle coordinate analysis plots reveal no significant differences in airway mycobiome profiles in longitudinal samples obtained at baseline (B), within 24 hours of a documented exacerbation (E) and at 2-weeks post-exacerbation following treatment and on recovery (PE). Dotted lines connect each individual patient at the different assessed timepoints. ns: not significant.

5.3.5 Hierarchical clustering reveals two COPD patient groups (clusters) demonstrating variable clinical outcomes based on airway mycobiome profiling.

Two COPD patients' groups (clusters) based on airway mycobiome profiling were identified using unsupervised hierarchical clustering (excluding *Candida*) (Figure 5.11a) (Table 5.6). *Candida* was excluded from clustering analyses to allow identification of other differentiating fungi as described in Chapter 5.2.10.1. Cluster one (n=178) is dominated by *Saccharomyces* (p<0.001) (Figure 5.11b) while cluster two (n=159) demonstrates a significant increase in *Aspergillus* (p<0.001), *Curvularia* (p=0.029) and *Penicillium* (p<0.001) (Figure 5.11c-e). On univariate analysis, cluster 1 associates with an increased CAT score (median: 20, interquartile range (IQR) 14.3-26.8) versus cluster 2 (median 18, IQR 20-25; p=0.02), while cluster 2 demonstrates higher exacerbation frequency (median: 2, IQR 0-3) versus cluster 1 (median 1, IQR 0-2; p=0.002) and also greater mortality (n=21 (13.2%) mortality compared to cluster 1 that demonstrates n=10 (5.6%) mortality, p=0.027) (Table 5.6). These differences remain significant even after adjustment for age, gender, BMI, smoking pack years and lung function (FEV₁% predicted) on multivariate logistic regression. Multivariate logistic regression reveals an increased burden of symptoms in cluster 1 (CAT>10) (odds ratio (OR): 0.45, 95% CI 0.24-0.85, p=0.0135), while cluster 2 was associated with increased exacerbations (OR 1.12, 95% CI 1.01-1.24, p=0.393) and higher mortality (OR: 2.43, 95% CI 1.04-5.69, p=0.041) (Figure 5.11f).

Table 5.6: Demographic table of COPD patients in cluster 1 (n=178) and cluster 2 (n=159) respectively based on airway mycobiome profiling.

Characteristics	Cluster 1	Cluster 2	p-value
n	178	159	
Age (years), Median (IQR)	73 (67-77)	72 (66-77)	0.361
BMI (Kg/m ²), Median (IQR)	25.0 (21.5-29.0)	24.3 (22.0-29.0)	0.920
Gender (Male), n (%)	151 (84.8)	122 (76.7)	0.079
Current smoker, n (%)	80 (44.9)	106 (66.7)	<0.001
Ex-smoker, n(%)	98 (55.1)	53 (33.3)	
Smoking pack years, Median (IQR)	50.0 (39.5-71.3)	45.0 0-60.0)	0.075
CAT Score, Median (IQR)	20.0 (14.3-26.8)	18.0 (10.0-25.0)	0.020
FEV ₁ (% predicted), Median (IQR)	57.4 (41.7-75.0)	61.8 (43.7-74.0)	0.451
FEV₁/FVC (% predicted), Median (IQR)	56.4 (45.6-65.0)	50.6 (41.0-60.3)	0.007
No. of Exacerbations/year, Median (IQR)	1 (0-2)	2 (0-3)	0.002
Mortality, n (%)	10 (5.6)	21 (13.2)	0.027
LABA, n (%)	1 (0.6)	7 (4.4)	0.054
LAMA, n (%)	17 (9.5)	17 (10.7)	0.868
LABA/LAMA, n (%)	65 (36.5)	47 (29.6)	0.216
LABA/ICS, n (%)	24 (13.5)	17 (10.7)	0.538
LABA/LAMA/ICS, n (%)	64 (36.0)	66 (41.5)	0.351
SAMA/SABA, n (%)	7 (3.9)	5 (3.1)	1.000
Macrolide, n (%)	18 (10.1)	16 (10.1)	1.000

n: number of patients; IQR: interquartile range; BMI: body mass index, CAT: COPD assessment test, FEV₁: forced expiratory volume in the 1st second, FVC: forced vital capacity, IQR: interquartile range, GOLD: global initiative for COPD, SAMA: short acting muscarinic antagonist, SABA: short-acting beta-2 agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta-2 agonist, ICS: inhaled corticosteroid, ns: not significant.

Figure 5.11: Unsupervised hierarchical clustering of fungal genera from airway mycobiome profiling in COPD reveals two clusters each demonstrating variable clinical outcomes. (a) Heatmap illustrating the relative abundance of the various fungal genera within the airway mycobiome.

Figure 5.11: Bar plot based on the two distinct clusters differentiated based on abundance of (b) *Saccharomyces* (c) *Aspergillus* (d) *Curvularia* and (e) *Penicillium*. Coloration denotes cluster membership: Cluster 1 (brown) and Cluster 2 (green). * $p < 0.05$.

Figure 5.11: (f) Forest plot with adjusted odds ratio (OR) and 95% confidence interval (CI) illustrating the clinical differences between the clusters. Cluster 1 demonstrates significantly higher symptoms (CAT score>10) ($p=0.014$) while Cluster 2 demonstrates significantly more exacerbations ($p=0.039$) and higher mortality ($p=0.041$). Circles represent ORs, circle coloration indicates the significance level: $p<0.05$ (red), non-significant (grey) with corresponding error bars and 95% confidence intervals.

5.3.6 The COPD airway mycobiome associates with measurable systemic immune responses to its constituents.

As one of the two derived clusters (cluster 2) demonstrates poor clinical outcomes with increased exacerbations (median: 2, interquartile range (IQR) 0-3 versus cluster 1 with median 1, IQR 0-2; $p=0.002$) and higher mortality ($n=21$ (13.2%) versus cluster 1 with $n=10$ (5.6%), $p=0.027$), I next assessed the association of the mycobiome's fungal constituents in this "high-risk" cluster with any measurable systemic immune response in a subset of the recruited COPD cohort ($n=42$).

Systemic specific IgE response against the fungal general detected at an increased relative abundance in cluster 2 (i.e. *Aspergillus*, *Curvularia* and *Penicillium*) and control fungi which are not elevated in the airway mycobiome of either cluster 1 or 2 (i.e. *Cladosporium*, *Fusarium*, *Schizophyllum* and *Trametes*) were assessed in a subset of patients from our recruited COPD cohort. I also compared such responses between the clusters. When compared to cluster 1, COPD patients in cluster 2 (the 'high-risk' cluster) demonstrate a significantly increased systemic and specific IgE response to *Aspergillus* ($p=0.033$), *Curvularia* ($p=0.027$) and *Penicillium* ($p=0.013$) (Figure 5.12 a-c) which corresponds to the increased relative abundance of the same taxa from airway mycobiome assessments. Importantly, however, no significant differences in systemic sIgE responses between the clusters to the control fungi including *Cladosporium* ($p=0.259$), *Fusarium* ($p=0.062$), *Schizophyllum* ($p=0.295$) and *Trametes* ($p=0.06$) were detected (Figure 5.12 d-g).

Figure 5.12: Detectable associations between the COPD airway mycobiome and a measurable systemic immune response to specific fungal constituents. (a-g) Bar plots illustrating the systemic specific Immunoglobulin (Ig)-E binding to selected fungi between the two identified COPD clusters based on mycobiome profiles and clinical outcomes. ns: not significant. * $p < 0.05$.

5.3.7 Lower alpha diversity in the COPD airway mycobiome identified during acute exacerbations of COPD (AECOPD) is associated with poorer two-year survival.

While no change to COPD airway mycobiome profiles were observed between the stable state, during exacerbations and on recovery (post exacerbation) in my longitudinal analyses, I next evaluated mycobiome profiles during AECOPD and associated the findings with COPD mortality. Respiratory specimens (sputum) were obtained during AECOPD in a subset of COPD patients (n=66) (Table 5.7) and these patients were closely followed prospectively over a two-year period and survival outcomes compared. The composition of the COPD airway mycobiome differed between the survival groups, with a decreased α -diversity (Shannon and Simpson index) observed in COPD patients who had poorer survival (n=16) (p=0.021 and p=0.031 respectively). Calculated hazard ratio (HR) remained significant for α -diversity in the mycobiome profiles even after adjustment for age, gender, BMI, and smoking pack year status (Shannon index HR: 0.31, 95% CI 0.11-0.89, p=0.029; Simpson index HR 0.11 95% CI 0.01-0.92, p=0.042) (Figure 5.13a-e). This suggests that assessment of the airway mycobiome profile during an AECOPD may have important prognostic implications and assist in identifying COPD patients at higher risks of death.

Table 5.7: Demographic table comparing COPD survivors (alive at 2-year follow up) (n=51) versus non-survivors (dead at 2-year follow up) (n=15) from an acute exacerbation of COPD (AECOPD) cohort.

Patient Characteristics	Alive	Dead	p-value
n	51	15	
Age (years), Median (IQR)	67 (63-73)	72 (69-78)	0.022
BMI (Kg/m ²), Median (IQR)	26.0 (22.0-30.8)	22.3 (17.8-26.0)	0.013
Gender (Male), n (%)	42 (82.4)	11 (73.3)	0.733
Current smoker, n (%)	36 (70.6)	8 (53.3)	0.318
Ex-smoker, n (%)	15 (29.4)	7 (46.7)	
Smoking pack years, Median (IQR)	40.0 (30.0-67.0)	50.0 (40.0-65.0)	0.354
CAT Score, Median (IQR)	22.5 (18.0-27.0)	24.0 (19.0-27.0)	0.570
FEV ₁ (% predicted), Median (IQR)	51.9 (41.5-72.6)	50.0 (38.6-69.9)	0.204
FEV ₁ /FVC (% predicted), Median (IQR)	51.0 (42.5-59.5)	50.0 (41.5-59.0)	0.589
No. of Exacerbations/year, Median (IQR)	3 (2-4)	2 (2-4)	0.896
GOLD Group, n (%)			
A	2 (3.9)	1 (6.7)	0.434
B	7 (13.7)	1 (6.7)	
C	9 (17.7)	1 (6.7)	
D	33 (64.7)	12 (80.0)	
GOLD Grade, n (%)			
1	10 (19.6)	2 (13.3)	0.281
2	23 (45.1)	6 (40.0)	
3	15 (29.4)	5 (33.4)	
4	3 (5.9)	2 (13.3)	
LABA, n (%)	1 (2.0)	0 (0.0)	1.000
LAMA, n (%)	3 (5.9)	2 (13.3)	0.406
LABA/LAMA, n (%)	9 (17.7)	4 (26.7)	0.316
LABA/ICS, n (%)	4 (7.8)	1 (6.7)	0.108
LABA/LAMA/ICS, n (%)	32 (62.7)	6 (40.0)	0.900
SAMA/SABA, n (%)	2 (3.9)	2 (13.3)	0.445
Macrolide, n (%)	6 (11.8)	2 (13.3)	1.000

n: number of patients; IQR: interquartile range; BMI: body mass index, CAT: COPD assessment test, FEV₁: forced expiratory volume in the 1st second, FVC: forced vital capacity, IQR: interquartile range, NA: non- applicable, GOLD: global initiative for COPD, SAMA: short acting muscarinic antagonist, SABA: short-acting beta-2 agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta-2 agonist, ICS: inhaled corticosteroid.

Figure 5.13: COPD airway mycobiome that demonstrate lower fungal diversity at exacerbation associate with poorer two-year survival (a) Bar plot illustrating the prevalence of the fungal genera (with relative abundance of >1%) between the survival groups (n=51 alive, n=15 dead) during acute exacerbations of COPD (AECOPD). The text coloration represents the predominant taxa between the groups: dead (blue), alive (grey). The earlier cluster taxa are highlighted: cluster 1 (purple), cluster 2 (light green).

Figure 5.13: (b) Significantly lower α -diversity is observed in the group with poorer survival as measured by the Shannon and (c) Simpson index. (d) Forest plot illustrating the adjusted hazard ratio for the α -diversity index, with inverse association between α -diversity and survival. (e) Principle coordinate analysis demonstrating β -diversity between the groups. Circles represent the adjusted hazard ratio and error bars represent the 95% confidence interval. Hazard ratio was adjusted for age, gender, smoking pack years and body mass index. CI: confidence interval. * $p < 0.05$.

5.3.8 COPD frequent exacerbator is associates with decrease bacteria diversity.

As the COPD bacteriome has been studied extensively in prior works, bacterial microbiome sequencing was performed in our cohort in only a small subset of patients (n=76) to ensure comparability and representativeness to the wider literature and identify, if any, any potential differences in South-East Asians with COPD (314, 317, 318). Average sequence read count of the samples were 182,664 and the top five bacterial genera were as reported in the wider COPD literature as follows: *Streptococcus*, *Prevotella*, *Haemophilus*, *Fusobacterium* and *Neisseria* (Figure 5.14a). This confirmed the representativeness of our COPD cohort to others reported in the literature (314, 317, 318, 325). The dominant microbiome taxa remain similar compared to non-Asian COPD cohort as previously described (314, 323). Importantly, frequent COPD exacerbators (FE; ≥ 2 exacerbations/year) demonstrate a decreased α -diversity when compared to non-frequent exacerbators (non-FE) ($p=0.005$ Shannon index, $p=0.009$ Simpson index) (Figure 5.14b-c). *Klebsiella* was the single bacterial genera that allowed a differentiation between FE and non-FE in South-East Asians with COPD in our cohort (LDA >3.5 , $p=0.04$). No significant differences observed in bacterial microbiome composition and diversity with lung function severity and COPD treatments.

Figure 5.14: The airway bacterial microbiome profile in South-East Asians with COPD. (a) Bar plot illustrating the relative abundance of the top 25 bacterial genera. (b) Boxplot of Shannon and (c) Simpson indices between non-frequent (Non-FE) and frequent COPD exacerbators (FE) in South-East Asia. ** $p < 0.01$.

5.4 Discussion

This chapter focused on determining if the presence of microbiological, and more specifically fungal phenotypes of COPD, which relate to clinical outcomes were detectable in South-East Asians (Singapore and Malaysia) with COPD. A focus on the fungal microbiome (the mycobiome) was selected because it has been underrepresented and understudied in the COPD literature, is highly relevant to the climatic and environmental factors in South-East Asia and have illustrated clinical relevance in other chronic inflammatory respiratory disease states including asthma, severe asthma, bronchiectasis and cystic fibrosis. I detected important fungal (microbiological) phenotypes of COPD in patients recruited from South-East Asia (Singapore and Malaysia) which strongly associated to adverse clinical outcomes. Airway mycobiomes in COPD varied by geographic region from which a patient was recruited and associates with a variety of clinical symptoms and outcomes in COPD. Very frequent COPD exacerbators (≥ 3 exacerbations/year) illustrate increased inter-fungal interactions, while frequent COPD exacerbators (≥ 2 exacerbations/year) demonstrate a decreased bacterial microbial diversity. Of particular interest, a *Saccharomyces* predominant COPD cluster demonstrates increases symptoms (by higher CAT score) and a second ‘high-risk’ *Aspergillus*, *Penicillium* and *Curvularia* predominant cluster associates with COPD exacerbations, poorer survival and measurable systemic sIgE responses to these fungi. Furthermore, when the airway mycobiome was assessed during COPD exacerbations, a decreased α -diversity was linked to increased mortality. Interestingly, the airway mycobiome remains stable across longitudinal sampling during and following exacerbations, despite the patients receiving treatment with oral corticosteroids and antibiotics, suggestive that the observed mycobiome perturbation in the airway is likely secondary to factors other than treatments received and/or the exacerbation state.

Primer selection for targeted amplicon sequencing of fungi is crucial in determining the taxonomic resolution and coverage. The Internal Transcribed Spacer (ITS) region is known as the ‘universal fungal barcode’ and (similar to its correlate 16S rRNA in bacteria) remains highly conserved (457, 468). Primers targeting the two subregions of the ITS (ITS1 and ITS2) are most

commonly used in mycobiome sequencing experiments. While the ITS1 region is frequently selected, it is shown to suffer from amplification bias and fails to accurately identify fungal mock communities (469-471). As a consequence, the ITS2 region is proposed to be of superior coverage with lower length variation (457, 460, 461). Several primers targeting different ITS regions have therefore been used for more accurate mycobiome sequencing, with some documented variation in fungal coverage based on the sample profile and sample source employed (470). I selected 4 different primer sets targeting ITS-1 and ITS-2 region for comparisons and optimisation prior to conducting my COPD focused work. These primers were selected based on prior studies illustrating good taxonomic coverage and/or have been used in experimentation with human specimens in the existing literature (455-460). Consistent with prior studies, our results suggest that primers covering the ITS-2 region perform well, with good fungal coverage and better profiling of the tested mock communities as compared to ITS-1 primers (458, 472).

In agreement with prior work focused on non-cystic fibrosis bronchiectasis, differences in the COPD mycobiome profiles were observed between patients recruited in South-East Asian when compared to the United Kingdom (UK) (327). Geographic variation, and in particular differences in climate, humidity and air quality coupled to a range of host factors (genetics, lifestyle and dietary variation) may all have contributed to such observations. In addition, the respiratory tract is in constant and direct communication with the external environment, and it is likely that microbes dynamically traffic between the airway and wider environment, illustrating the importance of environmental surroundings when considering the airway mycobiome and its composition. Rather than a static resident airway mycobiome, the dynamic interchange with the environment should be considered and this is of particular relevance in the Asian sub-continent where climate can play a significant role to the type, form, range, concentration and exposure to fungi, which in turn may influence clinical outcomes in patients with chronic lung diseases such as COPD.

Prior studies in COPD have demonstrated alterations to its bacterial microbiome composition in the airway particularly in frequent COPD exacerbators (≥ 2 exacerbations per year) (318, 473). This is in line with my observations in a small subset COPD patient recruited in South-East Asia (Singapore and Malaysia), which demonstrate a decreased overall bacterial microbiome diversity when compared to non-FE. In terms of the airway fungal mycobiome, no significant differences were observed based in frequent COPD exacerbators (≥ 2 exacerbations per year), however, changes became evident when very frequent COPD exacerbators (vFE) demonstrating ≥ 3 exacerbations per year were evaluated. Of particular note, a more complex and increased number of negative inter-fungal interactions were identified in the mycobiome of vFE COPD exacerbators. It is therefore possible perhaps that the initial exacerbation event contributes to a dysbiosis of the bacterial community, and disruption of fungal communities only occurs as the frequency of exacerbations increase with a demonstrable heightened inter-fungal (and co-exclusive) interaction. Changes to the dynamics of the COPD airway mycobiome occurs in vFEs which demonstrate an increased number of negative (co-exclusive) inter-fungal interactions, which result in an overgrowth of specific fungal taxa that in turn drive exacerbation frequency. While vFE COPD exacerbators receive more overall courses of oral antibiotics and/or corticosteroids, which in turn may contribute to the differences in interactions observed between vFE and non-FE COPD exacerbators, our longitudinal analysis reveals an overall stability of the airway mycobiome despite receipt of antibiotics and/or corticosteroids during an exacerbation event. This suggests the presence of alternate mechanisms rather than COPD treatment alone that drives this remodelling of the airway mycobiome community that I have detected in this chapter, which, at least in part may result in further exacerbations and explain the poorer clinical outcomes observed.

Despite demonstrating important differences in the COPD airway mycobiome between non-FE and vFEs, the stability of the airway mycobiome over the longitudinal analysis performed, reveals that its composition remains stable over the period of stability, exacerbation and even when assessed post-exacerbation following treatment. This is regardless of the treatments (i.e.

antibiotics and steroids) received during the exacerbation. These findings are consistent with prior exacerbation studies of the mycobiome in other respiratory disease states, such as cystic fibrosis (CF) (474, 475). Similarly, inhaled corticosteroid use demonstrated no impact on the COPD airway mycobiome which is in stark contrast with bacterial COPD microbiome studies. In assessments of the bacterial microbiome in COPD, alterations were observed between the stable, exacerbation and post exacerbation states, and critically, were also influenced by the treatment received (307). Such observed variations between bacteriome and mycobiome study outcomes in COPD suggests that bacteria and fungi likely have different roles in relation to COPD pathogenesis, progression, exacerbations and clinical outcomes including its treatment.

While our current knowledge of the airway mycobiome in COPD remains limited, its contribution to disease pathogenesis is an area of great interest. The work presented in this chapter clearly illustrates an important influence on exacerbation frequency and mortality, a strong starting point to better understand the role and importance of fungi in the COPD lung. Available work strongly indicates that a distinct mycobiome profile exists across a range of different chronic respiratory disease states with an increased burden of *Candida* and *Aspergillus* noted in CF, *Psathyrella*, *Malassezia*, *Termitomyces* and *Grifola* in asthma, and, *Aspergillus*, *Cryptococcus* and *Clavispora* in non-CF bronchiectasis (327, 449, 476-478). In stable COPD, very limited mycobiome work has been performed and was largely restricted to that in HIV infected individuals with COPD. Alterations to the airway mycobiome with an increased *Pneumocystis jirovecii* abundance is reported in HIV-infected COPD patients (328). Importantly, I did not detect *Pneumocystis* in our COPD cohort, however, other COPD specific fungal taxa were identified and include *Alternaria*, *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Mycosphaerella*, *Wickerhamomyces*, *Trametes* and *Penicillium*. During AECOPD, a prior and very small single centre study that evaluated n=6 COPD patients illustrate that a predominance of *Candida*, *Phialosimplex*, *Aspergillus*, *Penicillium* and *Cladosporium* existed with clear inter-patient differences making interpretation of such work challenging. When compared to our much larger AECOPD cohort, we found some similarities in airway mycobiome profiles, except

for *Phialosimplex*, however, we describe important associations with clinical outcomes in COPD that prior studies did not assess (322).

Unsupervised clustering analysis revealed two clinically distinct COPD patient groups based on their airway mycobiomes: one with increased symptoms (measured by CAT score) and with *Saccharomyces* dominance and a second with increased exacerbations and higher mortality, and, characterised by the presence of *Aspergillus*, *Penicillium* and *Curvularia*. Of particular interest and in this latter patient group, an increased airway *Aspergillus*, *Penicillium* and *Curvularia* associated with a measurable and significant systemic sIgE response to the same fungi.

Aspergillus, *Penicillium* and *Curvularia* are thermophilic fungi capable of surviving and replicating within the human body and possess pathogenic potential. In addition, these fungi are commonly associated with allergic bronchopulmonary mycosis (ABPM). Isolation of *Aspergillus* and *Penicillium* in sputum culture is associated with sensitisation responses and reduced lung function in asthma (303). Fungal sensitisation, is less well described in COPD, however, is associated with disease severity and exacerbations in asthma (230). Therefore, when these data are taken together with my results from chapter 4, it remains plausible that increased abundance of these fungi in the airway, as part of the resident mycobiome, can activate host immune responses with a resulting ‘sensitised state’ and poorer clinical COPD outcomes, as I have observed in this chapter.

When the airway mycobiome was evaluated during acute exacerbations of COPD (AECOPD), a reduced α -diversity clearly associated with an increased two-year mortality. This remains consistent with findings from prior equivalent bacterial microbiome studies in COPD, where survival was assessed one year following a documented exacerbation. A decreased alpha diversity in the bacterial COPD microbiome was detected in those with poorest survival (321). The fungal genera with highest prevalence in the poorest COPD survivors include *Penicillium*, *Cladosporium*, *Trametes* and *Lodderomyces*. Interestingly, *Penicillium* also was characteristic of the “high risk” cluster of stable COPD patients identified. While these fungi individually can

associate with invasive fungal infection and/or sensitisation responses in humans, their specific roles in COPD remain uncertain and warrant further research. Nevertheless, my data presented in this chapter does suggests that, airway mycobiome composition during COPD exacerbations may have prognostic value and early identification of patients with poorer survival outcomes.

Despite only studying a limited number of COPD patients, the composition of the bacterial microbiome in COPD in my work was strongly comparable to prior studies confirming the applicability and representativeness of the recruited COPD group. I detected a predominance of *Streptococcus*, *Prevotella*, *Hemophilus* and *Neisseria* and an overall decreased diversity in frequent COPD exacerbators (310, 315-317, 319). While the dominant taxa identified are similar to prior COPD studies, I did not find any association between *Hemophilus* and frequent exacerbator state or disease severity as reported by Mayhew *et al.* (2018) and Dicker *et al.* (2020) (314, 317). *Klebsiella* is the only taxa which discriminate between frequent and non-frequent exacerbator. This suggest the potential differences in exacerbations pathogens between Asian and Non-Asian COPD cohort, in consistent with prior Asian studies using sputum culture (31). Bacterial microbiome studies focusing on Asian COPD populations and its specific relations with clinical outcomes remain scarce. Diao *et al.* (2018) reported an increased relative abundance of *Neisseria* in COPD patients who demonstrate severe symptoms using the St. George's Respiratory Questionnaire (SGRQ) (325). In our cohort, no such association with symptoms was identified, likely explained by the contrasting methodologies and symptom measures used. For instance, I used CAT scoring (as recommended in COPD guidelines) to define symptom severity.

In this chapter, I present the largest and first multicenter study evaluating the mycobiome in COPD which includes longitudinal sampling. Despite its clear strengths, novelty and added value to the COPD field, the approach does have limitations. The included healthy cohort was small and all individuals in this group were recruited in Singapore, while the COPD patients were recruited from Singapore, Kuala Lumpur (Malaysia) and Dundee (UK), hence the described “healthy” and COPD mycobiome profiles may have limited generalisability outside

these countries. Although the non-diseased controls were of relatively younger age compared to the recruited COPD patients, recent airway mycobiome studies in healthy cohorts have shown little difference to the alpha and beta- mycobiome diversity with age (479). While both bacteriome and mycobiomes were assessed in South-East Asians (Singapore and Malaysia) with COPD, any potential role and/or interaction with the virome was not assessed. This has particular importance especially in the context of COPD exacerbations. Future studies integrating the various microbial kingdoms and the specific and individual host responses will be important to better understanding their roles in COPD pathogenesis. Despite optimising the ITS fungal sequencing protocol, there exists inherent limitations in the field at large which include weak fungal reference databases that preclude confident resolution to the species level. In addition, targeted amplicon sequencing lacks the functional annotation afforded by metagenomics and therefore, my work in this chapter can only forward potential associations rather than determine causation. The direct pathogenic role for any of the detected bacterial and fungal taxa cannot be determined from this study and future work focused on mechanistic confirmation should be pursued using germ-free mice inoculated with potential pathogenic fungal species including *Aspergillus*, *Penicillium* and *Curvularia*, which in this study are associated with poorer COPD outcomes. The actual abundance of the identified taxa cannot be accurately determined by targeted amplicon sequencing and therefore the results presented in this chapter must be interpreted accordingly. The work presented here provides a first insight into the COPD lung mycobiome and further work should evaluate the specific relevance of the predominant genera. Actual abundance of specific fungi may have more relevance in determining their pathogenicity within the lung's microbiome, however, this would necessitate additional experimental approaches including metagenomics supplemented with qPCR before further assessments can be made. Finally, I only performed the systemic specific IgE assays in a subset of COPD patients and with selected fungi. Validation against a more comprehensive fungal panel (which includes their accompanying allergens) will be a logical next step and this work should incorporate comparisons between various ethnic populations that exist within Asia and internationally.

In summary, distinct microbiological, and specifically fungal profiles were observed in South-East Asians (Singapore and Malaysia) with COPD which importantly associate with clinical outcomes including exacerbations and mortality. The airway mycobiome in COPD is important and represents an avenue of untapped potential in understanding COPD pathogenesis. The airway mycobiome in COPD demonstrates geographic variation and is characterised by specific fungal genera, where frequent exacerbators exhibit mycobiome profiles that remain unaltered by antibiotic and/or corticosteroid treatment. In addition, changes to mycobiome composition identifiable at exacerbation by a decreased α -diversity demonstrates prognostic implication and predicts two-year survival. Increased airway *Aspergillus*, *Penicillium* and *Curvularia* in the stable COPD state associates with exacerbations, poorer survival and systemic specific-IgE responses against these fungi. While future research is clearly necessary to better understand and evaluate the importance of the COPD mycobiome, the work presented in this chapter is novel and represents the important first steps for necessary future work across Asia and internationally. Such future work may include mechanistic studies employing germ-free mouse models and also smoke-induced COPD mice with inoculation of various ‘biome’ constituents as presented in this chapter. Clinical and inflammatory outcomes can be measured including the potential benefit of anti-fungal treatment in a more controlled experimental setting in contrast to my observational human studies.

CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS

6.1 Novelty and Significance

The available studies on COPD endo-phenotyping in South-East Asia remain limited, despite a reported increasing prevalence in this region. A variety of unique risk factors predispose to COPD in the South-East Asian region including environmental exposures, genetic background, cultural beliefs, air quality, climate and the availability of health care resources to recognise, educate and address the diverse COPD phenotypes that occur across the region. Asia represents the largest and most populated continent globally, therefore even within Asia, a diverse range of cultural backgrounds, genetics and even climates exist. In the context of my PhD thesis, I focused predominantly on East and South-East Asia where little data in regards to COPD and its associated phenotypes have been reported to date. I recruited a large cohort of patients with COPD across three different countries in South-East Asia (Singapore, Malaysia and Hong Kong) and identified novel key clinical, immunological (allergic) and microbiological (fungal) phenotypes, and related each in turn to important clinical outcomes in COPD including exacerbations and mortality affirming their translational importance (Figure 6.1). Of particular importance, is that the recognition of these phenotypes may assist clinicians across the region in identifying ‘high-risk’ COPD patients and that some of the identified features characterising these phenotypes represent potential ‘treatable traits’ in South-East Asians with COPD.

6.1.1 The cardiovascular and ex-tuberculosis clinical phenotypes in South-East Asians with COPD demonstrate poorest survival and complex inflammatory networks

Using clinical clustering, in chapter 3, I identified five important clinical COPD phenotypes in individuals of Chinese descent. The cardiovascular and ex-tuberculosis phenotypes in particular, associated with highest two-year mortality and represent COPD patients in the region that are ‘high-risk’. While the cardiovascular COPD phenotype has been established in non-Asian cohorts, the ex-TB phenotype is novel and described for the first time in the COPD literature with great relevance to the Asian subcontinent where TB is endemic. Such clinical COPD phenotyping is distinct from and further complements the existing GOLD COPD assessment strategies. The presence of subgroups based on my work within each GOLD ABCD classification and GOLD FEV₁ stages can now be identified, as these patient sub-groups illustrate poor clinical outcomes and requires close attention, management and follow up. While the cardiovascular COPD group expectedly associates with poor survival, consistent with prior studies, our ex-TB phenotype is particularly important, relevant and of clinical value to identify early due to the high prevalence rates of TB across the South-East Asian region. Despite making a full clinical and microbiological recovery from a prior episode of pulmonary TB, interestingly, this clinical COPD phenotype associated with higher mortality, increased systemic inflammation and demonstrated more complex inflammatory networks. A further important and interesting observation was the increased VEGF concentrations detected in the ex-TB phenotype. VEGF is known to play essential roles in pathogenesis of active TB and levels decrease with treatment. Its specific role in the ex-TB COPD setting remains unknown and further exploration including longitudinal study should be undertaken.

6.1.2 Allergic sensitisation is prevalent in South-East Asian COPD with important contributions from the indoor and outdoor environment

The prevalence and potential consequences of sensitisation in COPD, particularly in the South-East Asian region remains undetermined. In chapter 4, I have illustrated the high prevalence of sensitisation and in particular fungal sensitisation in COPD, which represents an important and novel allergic phenotype in South-East Asians (Singapore, Malaysia and Hong Kong). Fungal sensitisation is associated with COPD exacerbations illustrating the clinical relevance and importance of this state.

Unsupervised clustering revealed three novel COPD patient groups based on sensitisation responses demonstrating that subgroups of patient with differing sensitisation patterns exist with the overall poorest outcomes observed in those demonstrating fungal sensitisation. The three identified groups were as follows: a highly-sensitised fungal predominant group which illustrates the poorest clinical outcomes, a low sensitisation group, and a moderately sensitised *Blomia* predominant group which demonstrates the best clinical outcomes among the three identified groups. These groups, for the first time illustrate important and varying clinical outcomes in COPD that are associated with differing sensitisation patterns in South-East Asians with COPD, and, in particular the importance of fungal sensitisation.

The novelty of my work was further when fungal sensitisation was assessed in relation to the outdoor and indoor environment using a state-of-the-art metagenomics approach. Sensitisation to outdoor air fungi are demonstrated in South-East Asians (Singapore) with COPD for the first time and associated to exacerbations, illustrating a direct association between the outdoor environment and associated exposures with COPD exacerbation. In addition, I identified the indoor (home) environment as another key source of allergen exposure in COPD. The indoor (home) environment was shown to harbor large numbers of allergens (including fungal), which in turn was correlated to increased COPD symptoms (by CAT score) and poorer lung function. My results clearly illustrate, for the first time, the association between fungal sensitisation and

exacerbations in ‘sensitised’ COPD patients, and, further demonstrates the importance of the indoor and outdoor environment as a potential source of allergen (and in particular fungal) exposure and its related sensitisation response.

6.1.3. The airway fungal mycobiome is related to exacerbation frequency and mortality in South-East Asian COPD

Having shown a novel association between fungal sensitisation and poorer clinical outcomes in South-East Asians (Singapore, Malaysia and Hong Kong) with COPD in chapter 4, I next examined, for the first time and in the largest multicenter study to date in chapter 5, the airway fungal mycobiome in COPD. I related the airway mycobiome profiles detected to clinical COPD outcomes and sensitisation status.

Using a large multicenter cohort of COPD patients recruited from Singapore, Malaysia and Dundee, distinct mycobiome profiles were observed in COPD when compared to healthy controls with demonstrable geographic differences. Lower alpha diversity in the COPD mycobiome during acute exacerbations associated significantly with an increased two-year mortality. Importantly, neither treatment with antibiotics or steroids (oral and inhaled) influenced the airway mycobiome profile. During longitudinal analysis, again performed for the first time for the mycobiome in COPD at three timepoints: stability, during and post exacerbation revealed no effect on mycobiome profiles, which contrasts equivalent bacterial microbiome studies in COPD.

Bacteria and viruses are established causes of AECOPD; however, the role of fungi remains unknown. Although no significant differences in the airway mycobiome with longitudinal sampling was observed in my work, critically, poorer survival is seen in patients with lower alpha diversity during AECOPD. Importantly, both bacterial microbiome and fungal mycobiome profiles are further altered in frequent and very frequent COPD exacerbators with a decreased diversity detected in my work.

Interestingly, I illustrate that the presence of some fungi in the COPD airway mycobiome are associated with a measurable systemic immune response to these fungi. An increased relative abundance of airway *Aspergillus*, *Curvularia* and *Penicillium* in stable COPD is associated with sIgE responses to these fungi, which in turn is linked to increased exacerbations and poorer overall survival. It is plausible that a constant exposure to allergenic fungi from the environment, particularly those demonstrating thermotolerance, when coupled to poor mucociliary clearance in the COPD lung leads to their persistence within the terminal bronchioles resulting in an activation of host immunity and a sensitisation response. Such heightened immune and inflammatory responses from fungal exposures then eventually result in increased symptoms and exacerbations, reflected by the data presented in chapters 4 and 5.

In the South-East Asian region, the tropical environment, characterised by high humidity promotes the growth of thermophilic fungi including *Aspergillus*, *Penicillium* and *Curvularia*, which demonstrate survival and infective capabilities. The detected geographic variation in our COPD mycobiome profiles are also likely influenced by the variable climatic and differing abundance of environmental fungi across jurisdictions, which in turn leads to the varying sensitisation profiles in different countries.

The reported fungal sensitisation in chapters 4 and 5 of this thesis, and its association with the COPD airway mycobiome represent an important and novel COPD phenotype, and potential ‘treatable trait’ that has the ability to improve clinical outcomes in South-East Asians (Singapore, Malaysia and Hong Kong) with COPD. While our work cannot establish causation and associated mechanisms at this stage, our mycobiome studies do represent the first of its kind in COPD, and, illustrate important key association with disease status and clinical outcomes. This represents a strong platform from which future COPD phenotyping work across Asia and internationally can be built in relation to understanding the role of fungi and fungal sensitisation in COPD.

Figure 6.1: A summary of the identified clinical (chapter 3), immune (allergic) (chapter 4) and microbiological (fungal) (chapter 5) phenotypes of Asian COPD studied in this thesis and their association with relevant COPD outcomes. COPD: chronic obstructive pulmonary disease, TB: tuberculosis, ↑: increased, ↓: decreased, AECOPD: acute exacerbation of COPD.

6.2 Future directions

In this thesis, I identify important and novel clinical, immunologic (allergic) and microbiologic (fungal) phenotypes in South-East Asians (Singapore, Malaysia, Hong Kong) with COPD, each with a demonstrable relevance to clinical outcomes. The phenotypes identified are descriptive and focus on South-East Asians from Singapore, Malaysia, Hong Kong and future studies that build on my current data, and that exploring underlying mechanisms, therapeutic trials, and validation of my results in larger longitudinal cohorts outside these countries are warranted.

In chapter 3, a prior history of TB is associated with an increased detection of VEGF and mortality in South-East Asians (Singapore, Malaysia, Hong Kong) with COPD. The precise mechanisms leading to COPD susceptibility following TB remains unknown and prospective follow-up studies of patients from first onset of pulmonary TB including at least yearly spirometry, inflammatory markers (e.g. VEGF) and chest CT is warranted to better appreciate the natural history and development of lung destruction (including co-existing bronchiectasis) post-recovery. Such work will better identify particular individuals and/or groups of individuals who are highest risks of developing COPD and further elucidate potential associated mechanisms that may be targeted for treatment.

Post-TB lung disease (PTBLD) is another unique clinical entity in countries demonstrating high prevalence rates of TB. The progression from post pulmonary TB to PTBLD, COPD and/or bronchiectasis is an important area in need of future research. It would be important to identify individuals at high-risk of progression and associated mechanisms to allow for therapeutic intervention. Different trajectories and/or mechanisms may relate to the development of different disease entities, best revealed with prospective longitudinal studies. In addition, a validation of our identified clinical clusters in chapter 3 in countries outside the Asian sub-continent which demonstrate high TB prevalence rates such as Africa and South America should be pursued.

Using a customised outdoor air fungal panel described in chapter 4, I demonstrate an association between sensitisation to outdoor air fungi and COPD exacerbations in South-East Asian (Singapore, Malaysia, Hong Kong) patients. Future work focused on validating a more universally applicable sIgE panel for outdoor and indoor air fungi and other related allergens using for instance ImmunoCAP assays may assist in identifying COPD patients at highest risk of poor clinical outcomes. In addition, work focused on “precision” COPD risk stratification based on sensitisation, perhaps, by evaluating individual risk exposure and measurable sensitisation responses that incorporate metagenomic approaches to indoor and outdoor air, correlation with air quality, temperature and humidity, may lead to a more targeted treatment approach. For instance, and based on risk stratification, anti-fungal and/or anti-IgE therapy may be used, and environmental and/or lifestyle changes advised. Validation of my findings by prospective longitudinal studies focused on clinical outcomes and sensitisation in COPD, assessing outdoor and indoor air-fungi outside Singapore is a further key avenue of investigation and where data currently lacks. This should be followed by prospective studies and clinical trials addressing the treatment of ‘sensitised’ COPD patients with for instance anti-fungal and or anti-IgE therapy. Those at highest risk of recurrent exacerbations would be an appropriate initial target group for such studies. In addition, larger studies focused on indoor allergen and in particular fungal exposure with correlation to airway mycobiome profiles and sIgE assessment will provide potential avenues for targeted and potentially ‘precision’ approaches to COPD risk stratification and therapy. Lastly, evaluation of fungal sensitisation in COPD overlap diseases (such as BCO and ACO), and how this relates to clinical outcomes is important and an area in need of data. How sensitisation responses in themselves may also influence the development of COPD overlap diseases, particularly in the context of ACO.

In chapter 5, I describe, for the first time, distinct mycobiome profiles associated with COPD exacerbations, mortality and a systemic immune response using targeted ITS sequencing. Future work using metagenomic sequencing and germ-free mice for instance, may provide greater mechanistic and functional assessments in relations to clinical outcomes in COPD. Furthermore,

a holistic approach, integrating various microbes (bacterial, viral and fungal) within the lung with further extension across multiple organ systems (e.g. lung-gut relationships) in tandem with environmental microbial assessment will likely provide an improved understanding of their individual and specific disease contribution to COPD pathogenesis. An assessment of underlying systemic and airway inflammatory responses associated to the COPD airway mycobiome coupled to extensive fungal immunoassays including individuals from multiple ethnic backgrounds should be pursued in future work. This has the potential to lead to ‘precision medicine’ in COPD combining clinical, microbial, immunological and geographic assessment to inform therapeutic intervention that goes beyond pharmacology, one that may include manipulation of the airway and environmental microbiome.

6.3 Summary of skills acquired and lessons learnt

My PhD has provided me opportunities to acquire a wide range of different skill sets as exemplified by the primary research work presented in Chapters 3 through 5. I was primarily responsible for clinical data and biological sample collation from all participating national and international hospitals, and performed direct patient recruitment myself at the Singapore General Hospital, where my clinical practice is based. I performed all presented laboratory experiments including the accompanying analysis (with the sole exception of the regularised discriminant analysis, classification and regression trees and cluster stability for the validation cohort presented in Chapter 3 and metagenomic data in Chapter 4). This was performed in collaboration with an expert statistician. Throughout my PhD experience, I have acquired the principles and skills associated with handling patient specimens for scientific investigation, in particular blood and sputum.

In Chapter 3, I have performed ELISA assays and their accompanying statistical analysis including survival analysis, clustering methodologies and included a wide variety of data presentation approaches in this thesis.

In Chapter 4 specifically, I designed the presented study from conception to experiments and learned to generate crude and recombinant protein allergens. Using these generated allergens, I performed immuno-dot-blot assays for all recruited patients. In addition, I recruited all 11 COPD patients and visited their homes for indoor air sampling and was subsequently involved in sample processing and data analysis. I also completed the statistical except metagenomics analysis presented in Chapter 4.

For Chapter 5, I designed the study, executed all experimental work independently, and analysed all the targeted amplicon sequencing data including bioinformatics. From this study, I have acquired skills and knowledge related to sputum collection, processing, DNA extraction, polymerase chain reaction (PCR), sequencing library preparation and targeted sequencing including the bioinformatics skills necessary for microbiome and mycobiome analysis. This work involved a significant amount of optimization. Overall, the skills acquired throughout my PhD experience has significantly improved my understanding of basic science and ‘wet lab’ research. Coupled to my clinical knowledge base, this experience serves to equip me to better integrate translational medicine into patient care in my future role as a clinician-scientist.

6.4 Conclusion

Clinical, allergic and fungal phenotypes have been identified in South-East Asians (Singapore, Malaysia, Hong Kong) with COPD using a multicentered, multi-country approach. These COPD phenotypes associate with increased symptoms, poorer survival, and greater exacerbations (Figure 6.1). Taken together, the novel findings arising from my thesis will hopefully represent the first important and key steps in improving risk stratification and targeted treatment approaches to COPD in the South-East Asian region. A better understanding of the unique features of COPD across Asia is critical to ensure optimal education, improved diagnosis, the success of clinical trials and an ability to fully realise the potential for targeted and ‘precision’ COPD therapy for all.

REFERENCES:

1. Tan WC, Ng TP. COPD in Asia: where East meets West. *Chest*. 2008;133(2):517-27.
2. Seo JY, Hwang YI, Mun SY, Kim JH, Kim JH, Park SH, et al. Awareness of COPD in a high risk Korean population. *Yonsei Med J*. 2015;56(2):362-7.
3. Asai M, Tanaka T, Kozu R, Kitagawa C, Tabusadani M, Senjyu H. Effect of a chronic obstructive pulmonary disease (COPD) intervention on COPD awareness in a regional city in Japan. *Intern Med*. 2015;54(2):163-9.
4. Lou P, Zhu Y, Chen P, Zhang P, Yu J, Zhang N, et al. Vulnerability, beliefs, treatments and economic burden of chronic obstructive pulmonary disease in rural areas in China: a cross-sectional study. *BMC Public Health*. 2012;12:287.
5. Chan CQH, Lee KH, Low LL. A systematic review of health status, health seeking behaviour and healthcare utilisation of low socioeconomic status populations in urban Singapore. *Int J Equity Health*. 2018;17(1):39.
6. Widayanti AW, Green JA, Heydon S, Norris P. Health-Seeking Behavior of People in Indonesia: A Narrative Review. *J Epidemiol Glob Health*. 2020;10(1):6-15.
7. World Health organisation. Chronic Obstructive Pulmonary Disease. [https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-\(copd\)](https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd)).
8. Salvi SS, Barnes PJ. Chronic obstructive pulmonary disease in non-smokers. *Lancet*. 2009;374(9691):733-43.
9. Nicolaou N, Siddique N, Custovic A. Allergic disease in urban and rural populations: increasing prevalence with increasing urbanization. *Allergy*. 2005;60(11):1357-60.
10. Eguiluz-Gracia I, Mathioudakis AG, Bartel S, Vijverberg SJH, Fuertes E, Comberiati P, et al. The need for clean air: The way air pollution and climate change affect allergic rhinitis and asthma. *Allergy*. 2020.
11. Jiang XQ, Mei XD, Feng D. Air pollution and chronic airway diseases: what should people know and do? *J Thorac Dis*. 2016;8(1):E31-40.

12. Cohen R, Sexton KG, Yeatts KB. Hazard assessment of United Arab Emirates (UAE) incense smoke. *The Science of the total environment*. 2013;458-460:176-86.
13. Kurmi OP, Lam KB, Ayres JG. Indoor air pollution and the lung in low- and medium-income countries. *Eur Respir J*. 2012;40(1):239-54.
14. Lin TC, Krishnaswamy G, Chi DS. Incense smoke: clinical, structural and molecular effects on airway disease. *Clin Mol Allergy*. 2008;6:3.
15. Wong GW, Leung TF, Ko FW. Changing prevalence of allergic diseases in the Asia-pacific region. *Allergy Asthma Immunol Res*. 2013;5(5):251-7.
16. Jamieson DB, Matsui EC, Belli A, McCormack MC, Peng E, Pierre-Louis S, et al. Effects of allergic phenotype on respiratory symptoms and exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2013;188(2):187-92.
17. Fattahi F, ten Hacken NH, Lofdahl CG, Hylkema MN, Timens W, Postma DS, et al. Atopy is a risk factor for respiratory symptoms in COPD patients: results from the EUROSCOP study. *Respir Res*. 2013;14:10.
18. Rhee CK, Chau NQ, Yunus F, Matsunaga K, Perng DW, on behalf the CAotA. Management of COPD in Asia: A position statement of the Asian Pacific Society of Respiriology. *Respirology*. 2019;24(10):1018-25.
19. Menezes AM, Hallal PC, Perez-Padilla R, Jardim JR, Muino A, Lopez MV, et al. Tuberculosis and airflow obstruction: evidence from the PLATINO study in Latin America. *Eur Respir J*. 2007;30(6):1180-5.
20. Oh YM, Bhome AB, Boonsawat W, Gunasekera KD, Madegedara D, Idolor L, et al. Characteristics of stable chronic obstructive pulmonary disease patients in the pulmonology clinics of seven Asian cities. *Int J Chron Obstruct Pulmon Dis*. 2013;8:31-9.
21. Martin A, Badrick E, Mathur R, Hull S. Effect of ethnicity on the prevalence, severity, and management of COPD in general practice. *Br J Gen Pract*. 2012;62(595):e76-81.
22. Wedzicha JA, Zhong N, Ichinose M, Humphries M, Fogel R, Thach C, et al. Indacaterol/glycopyrronium versus salmeterol/fluticasone in Asian patients with COPD at a

high risk of exacerbations: results from the FLAME study. *Int J Chron Obstruct Pulmon Dis*. 2017;12:339-49.

23. Tan WC, Seale P, Ip M, Shim YS, Chiang CH, Ng TP, et al. Trends in COPD mortality and hospitalizations in countries and regions of Asia-Pacific. *Respirology*. 2009;14(1):90-7.

24. Kim TO, Shin HJ, Kim YI, Rhee CK, Lee WY, Lim SY, et al. Adherence to the GOLD Guideline in COPD Management of South Korea: Findings from KOCOSS Study 2011-2018. *Chonnam Med J*. 2019;55(1):47-53.

25. Kim KH, Kang HS, Kim JS, Yoon HK, Kim SK, Rhee CK. Risk factors for the discontinuation of roflumilast in patients with chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. 2017;12:3449-56.

26. Wedzicha JA, Banerji D, Chapman KR, Vestbo J, Roche N, Ayers RT, et al. Indacaterol-Glycopyrronium versus Salmeterol-Fluticasone for COPD. *N Engl J Med*. 2016;374(23):2222-34.

27. Mac Aogain M, Tiew PY, Lim AYH, Low TB, Tan GL, Hassan T, et al. Distinct "Immunoallertypes" of Disease and High Frequencies of Sensitization in Non-Cystic Fibrosis Bronchiectasis. *Am J Respir Crit Care Med*. 2019;199(7):842-53.

28. Tiew PY, Koh MS, Tan JHY, Soh RY, Ong TH, Loo CM, et al. P148 Bacteria profile of acute exacerbations of chronic obstructive pulmonary disease in Singapore. *Chest*. 2017;151(5):A45.

29. Leung JM, Tiew PY, Mac Aogain M, Budden KF, Yong VF, Thomas SS, et al. The role of acute and chronic respiratory colonization and infections in the pathogenesis of COPD. *Respirology*. 2017;22(4):634-50.

30. Miravittles M. Exacerbations of chronic obstructive pulmonary disease: when are bacteria important? *Eur Respir J Suppl*. 2002;36:9s-19s.

31. Hui DS, Ip M, Ling T, Chang SC, Liao CH, Yoo CG, et al. A multicentre surveillance study on the characteristics, bacterial aetiologies and in vitro antibiotic susceptibilities in patients with acute exacerbations of chronic bronchitis. *Respirology*. 2011;16(3):532-9.

32. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med.* 2006;3(11):e442.
33. Vogelmeier CF, Criner GJ, Martinez FJ, Anzueto A, Barnes PJ, Bourbeau J, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report. GOLD Executive Summary. *Am J Respir Crit Care Med.* 2017;195(5):557-82.
34. Huerta A, Soler N, Esperatti M, Guerrero M, Menendez R, Gimeno A, et al. Importance of *Aspergillus* spp. isolation in Acute exacerbations of severe COPD: prevalence, factors and follow-up: the FUNGI-COPD study. *Respiratory research.* 2014;15:17.
35. Terzikhan N, Verhamme KM, Hofman A, Stricker BH, Brusselle GG, Lahousse L. Prevalence and incidence of COPD in smokers and non-smokers: the Rotterdam Study. *Eur J Epidemiol.* 2016;31(8):785-92.
36. Poh TY, Mac Aogain M, Chan AK, Yip AC, Yong VF, Tiew PY, et al. Understanding COPD-overlap syndromes. *Expert Rev Respir Med.* 2017;11(4):285-98.
37. Sze MA, Dimitriu PA, Hayashi S, Elliott WM, McDonough JE, Gosselink JV, et al. The lung tissue microbiome in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2012;185(10):1073-80.
38. GOLD. Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2018. (<http://goldcopd.org/>); 2018.
39. Kim V, Han MK, Vance GB, Make BJ, Newell JD, Hokanson JE, et al. The chronic bronchitic phenotype of COPD: an analysis of the COPDGene Study. *Chest.* 2011;140(3):626-33.
40. Ho T, Cusack RP, Chaudhary N, Satia I, Kurmi OP. Under- and over-diagnosis of COPD: a global perspective. *Breathe (Sheff).* 2019;15(1):24-35.
41. Guarascio AJ, Ray SM, Finch CK, Self TH. The clinical and economic burden of chronic obstructive pulmonary disease in the USA. *Clinicoecon Outcomes Res.* 2013;5:235-45.

42. Ford ES, Murphy LB, Khavjou O, Giles WH, Holt JB, Croft JB. Total and state-specific medical and absenteeism costs of COPD among adults aged ≥ 18 years in the United States for 2010 and projections through 2020. *Chest*. 2015;147(1):31-45.
43. Kim C, Yoo KH, Rhee CK, Yoon HK, Kim YS, Lee SW, et al. Health care use and economic burden of patients with diagnosed chronic obstructive pulmonary disease in Korea. *Int J Tuberc Lung Dis*. 2014;18(6):737-43.
44. Tsai YH, Yang TM, Lin CM, Huang SY, Wen YW. Trends in health care resource utilization and pharmacological management of COPD in Taiwan from 2004 to 2010. *Int J Chron Obstruct Pulmon Dis*. 2017;12:2787-93.
45. MOH: Total Hospital Bills - By condition/procedure | Ministry of Health. Available on: https://www.moh.gov.sg/content/moh_web/home/costs_and_financing/hospital-charges/Total-Hospital-Bills-By-condition-procedure.html.
46. Principal Causes of Death. Ministry of Health Singapore 2016-2018. Available from <https://www.moh.gov.sg/resources-statistics/singapore-health-facts/principal-causes-of-death>.
47. MOH Singapore Burden of Disease Study 2010 Report. Available on: https://www.moh.gov.sg/content/moh_web/home/statistics/Health_Facts_Singapore/Disease_Burden.html.
48. Teo WS, Tan WS, Chong WF, Abisheganaden J, Lew YJ, Lim TK, et al. Economic burden of chronic obstructive pulmonary disease. *Respirology*. 2012;17(1):120-6.
49. Sundeep S Salvi PJB. Chronic obstructive pulmonary disease in non-smokers. *Lancet* 2009 Aug 29;374(9691):733-43.
50. A Publication of the National Registry of Diseases Office & Substance Abuse Department of Health Promotion Board, Singapore 2015. Available from: <https://www.nrdo.gov.sg/docs/librariesprovider3/default-document-library/nrdo-wntd-info-paper-2015.pdf?sfvrsn=0>.
51. Ng M, Freeman MK, Fleming TD, Robinson M, Dwyer-Lindgren L, Thomson B, et al. Smoking prevalence and cigarette consumption in 187 countries, 1980-2012. *JAMA*. 2014;311(2):183-92.

52. Waatevik M, Skorge TD, Omenaas E, Bakke PS, Gulsvik A, Johannessen A. Increased prevalence of chronic obstructive pulmonary disease in a general population. *Respir Med.* 2013;107(7):1037-45.
53. Xie M, Liu X, Cao X, Guo M, Li X. Trends in prevalence and incidence of chronic respiratory diseases from 1990 to 2017. *Respir Res.* 2020;21(1):49.
54. Eller J, Ede A, Schaberg T, Niederman MS, Mauch H, Lode H. Infective Exacerbations of Chronic Bronchitis. *Chest.* 1998;113(6):1542-8.
55. Picco L, Subramaniam M, Abdin E, Vaingankar JA, Chong SA. Smoking and nicotine dependence in Singapore: findings from a cross-sectional epidemiological study. *Ann Acad Med Singapore.* 2012;41(8):325-34.
56. Zeng G, Sun B, Zhong N. Non-smoking-related chronic obstructive pulmonary disease: a neglected entity? *Respirology.* 2012;17(6):908-12.
57. Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S, et al. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *N Engl J Med.* 2008;359(15):1543-54.
58. Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, et al. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med.* 2007;356(8):775-89.
59. Fishman A, Martinez F, Naunheim K, Piantadosi S, Wise R, Ries A, et al. A randomized trial comparing lung-volume-reduction surgery with medical therapy for severe emphysema. *N Engl J Med.* 2003;348(21):2059-73.
60. Lange P, Celli B, Agusti A, Boje Jensen G, Divo M, Faner R, et al. Lung-Function Trajectories Leading to Chronic Obstructive Pulmonary Disease. *N Engl J Med.* 2015;373(2):111-22.
61. Agusti A, Bel E, Thomas M, Vogelmeier C, Brusselle G, Holgate S, et al. Treatable traits: toward precision medicine of chronic airway diseases. *Eur Respir J.* 2016;47(2):410-9.
62. Pei Yee Tiew, Jessica Han Ying Tan, Ruiya Soh, Karen Li Leng Tan, Thun How Ong, Therese Sophie Lapperre, Sanjay Haresh Chotirmall, Mariko Siyue Koh. Prior Pulmonary Tuberculosis In Chronic Obstructive Pulmonary Disease (COPD) Is Associated with Mortality

and Occurrence of Radiological Bronchiectasis. *Am J Respir Crit Care Med* 2018;197:A3250 (Abstract).

63. Lapperre TS, Postma DS, Gosman MM, Snoeck-Stroband JB, ten Hacken NH, Hiemstra PS, et al. Relation between duration of smoking cessation and bronchial inflammation in COPD. *Thorax*. 2006;61(2):115-21.

64. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, et al. Anti-elastin autoimmunity in tobacco smoking-induced emphysema. *Nat Med*. 2007;13(5):567-9.

65. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest*. 2008;118(11):3546-56.

66. Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J*. 2008;31(6):1334-56.

67. Chrysafakis G, Tzanakis N, Kyriakoy D, Tsoumakidou M, Tsiligianni I, Klimathianaki M, et al. Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest*. 2004;125(1):71-6.

68. Saetta M, Mariani M, Panina-Bordignon P, Turato G, Buonsanti C, Baraldo S, et al. Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2002;165(10):1404-9.

69. Grumelli S, Corry DB, Song LZ, Song L, Green L, Huh J, et al. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS Med*. 2004;1(1):e8.

70. Wang Y, Xu J, Meng Y, Adcock IM, Yao X. Role of inflammatory cells in airway remodeling in COPD. *Int J Chron Obstruct Pulmon Dis*. 2018;13:3341-8.

71. Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation. *Int J Chron Obstruct Pulmon Dis*. 2011;6:413-21.

72. McCarthy C, Reeves EP, McElvaney NG. The Role of Neutrophils in Alpha-1 Antitrypsin Deficiency. *Ann Am Thorac Soc*. 2016;13 Suppl 4:S297-304.

73. Reddy NM, Kleeberger SR, Cho HY, Yamamoto M, Kensler TW, Biswal S, et al. Deficiency in Nrf2-GSH signaling impairs type II cell growth and enhances sensitivity to oxidants. *Am J Respir Cell Mol Biol*. 2007;37(1):3-8.
74. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest*. 2004;114(9):1248-59.
75. Yamada K, Asai K, Nagayasu F, Sato K, Ijiri N, Yoshii N, et al. Impaired nuclear factor erythroid 2-related factor 2 expression increases apoptosis of airway epithelial cells in patients with chronic obstructive pulmonary disease due to cigarette smoking. *BMC Pulm Med*. 2016;16:27.
76. Ryter SW, Rosas IO, Owen CA, Martinez FJ, Choi ME, Lee CG, et al. Mitochondrial Dysfunction as a Pathogenic Mediator of Chronic Obstructive Pulmonary Disease and Idiopathic Pulmonary Fibrosis. *Ann Am Thorac Soc*. 2018;15(Suppl 4):S266-S72.
77. van der Toorn M, Slebos DJ, de Bruin HG, Leuvenink HG, Bakker SJ, Gans RO, et al. Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(5):L1211-8.
78. Nam HS, Izumchenko E, Dasgupta S, Hoque MO. Mitochondria in chronic obstructive pulmonary disease and lung cancer: where are we now? *Biomark Med*. 2017;11(6):475-89.
79. Mizumura K, Cloonan SM, Nakahira K, Bhashyam AR, Cervo M, Kitada T, et al. Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD. *J Clin Invest*. 2014;124(9):3987-4003.
80. Chen H, Li Z, Dong L, Wu Y, Shen H, Chen Z. Lipid metabolism in chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. 2019;14:1009-18.
81. Jiang Z, Knudsen NH, Wang G, Qiu W, Naing ZZC, Bai Y, et al. Genetic Control of Fatty Acid beta-Oxidation in Chronic Obstructive Pulmonary Disease. *Am J Respir Cell Mol Biol*. 2017;56(6):738-48.

82. Chen Y, Chen P, Hanaoka M, Droma Y, Kubo K. Enhanced levels of prostaglandin E2 and matrix metalloproteinase-2 correlate with the severity of airflow limitation in stable COPD. *Respirology*. 2008;13(7):1014-21.
83. Chakinala RC, Khatri A, Gupta K, Koike K, Epelbaum O. Sphingolipids in COPD. *Eur Respir Rev*. 2019;28(154).
84. Telenga ED, Hoffmann RF, Ruben tK, Hoonhorst SJ, Willemse BW, van Oosterhout AJ, et al. Untargeted lipidomic analysis in chronic obstructive pulmonary disease. Uncovering sphingolipids. *Am J Respir Crit Care Med*. 2014;190(2):155-64.
85. Scarpa MC, Baraldo S, Marian E, Turato G, Calabrese F, Saetta M, et al. Ceramide expression and cell homeostasis in chronic obstructive pulmonary disease. *Respiration*. 2013;85(4):342-9.
86. Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, et al. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med*. 2005;11(5):491-8.
87. Shen Y, Huang S, Kang J, Lin J, Lai K, Sun Y, et al. Management of airway mucus hypersecretion in chronic airway inflammatory disease: Chinese expert consensus (English edition). *Int J Chron Obstruct Pulmon Dis*. 2018;13:399-407.
88. Burgel PR, Martin C. Mucus hypersecretion in COPD: should we only rely on symptoms? *Eur Respir Rev*. 2010;19(116):94-6.
89. Burgel PR, Nesme-Meyer P, Chanez P, Caillaud D, Carre P, Perez T, et al. Cough and sputum production are associated with frequent exacerbations and hospitalizations in COPD subjects. *Chest*. 2009;135(4):975-82.
90. Hogg JC, Macklem PT, Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med*. 1968;278(25):1355-60.
91. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med*. 2004;350(26):2645-53.

92. Higham A, Quinn AM, Cancado JED, Singh D. The pathology of small airways disease in COPD: historical aspects and future directions. *Respir Res.* 2019;20(1):49.
93. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, et al. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *N Engl J Med.* 2011;365(17):1567-75.
94. Vestbo J, Hurd SS, Rodriguez-Roisin R. The 2011 revision of the global strategy for the diagnosis, management and prevention of COPD (GOLD)--why and what? *Clin Respir J.* 2012;6(4):208-14.
95. Pascoe S, Locantore N, Dransfield MT, Barnes NC, Pavord ID. Blood eosinophil counts, exacerbations, and response to the addition of inhaled fluticasone furoate to vilanterol in patients with chronic obstructive pulmonary disease: a secondary analysis of data from two parallel randomised controlled trials. *Lancet Respir Med.* 2015;3(6):435-42.
96. Chong J, Leung B, Poole P. Phosphodiesterase 4 inhibitors for chronic obstructive pulmonary disease. *Cochrane Database Syst Rev.* 2017;9:CD002309.
97. Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, et al. Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. *JAMA.* 1994;272(19):1497-505.
98. Gellert C, Schottker B, Brenner H. Smoking and all-cause mortality in older people: systematic review and meta-analysis. *Arch Intern Med.* 2012;172(11):837-44.
99. Scanlon PD, Connett JE, Waller LA, Altose MD, Bailey WC, Buist AS, et al. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am J Respir Crit Care Med.* 2000;161(2 Pt 1):381-90.
100. Stead LF, Lancaster T. Combined pharmacotherapy and behavioural interventions for smoking cessation. *Cochrane Database Syst Rev.* 2012;10:CD008286.
101. Yang JJ, Yu D, Wen W, Shu XO, Saito E, Rahman S, et al. Tobacco Smoking and Mortality in Asia: A Pooled Meta-analysis. *JAMA Netw Open.* 2019;2(3):e191474.
102. WHO report on the global tobacco epidemic 2019. Available at : https://www.who.int/tobacco/global_report/en/.

103. Lin H, Xiao D, Liu Z, Shi Q, Hajek P, Wang C. National survey of smoking cessation provision in China. *Tob Induc Dis*. 2019;17:25.
104. Cheng HM, Liu WC, Chua G, Liew CF, Li W, Choo W, et al. Impact of a pharmacy-led smoking cessation clinic in a dermatology centre. *Singapore Med J*. 2019;60(1):31-3.
105. Poole PJ, Chacko E, Wood-Baker RW, Cates CJ. Influenza vaccine for patients with chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2000(4):CD002733.
106. Walters JA, Tang JN, Poole P, Wood-Baker R. Pneumococcal vaccines for preventing pneumonia in chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2017;1:CD001390.
107. Alfageme I, Vazquez R, Reyes N, Munoz J, Fernandez A, Hernandez M, et al. Clinical efficacy of anti-pneumococcal vaccination in patients with COPD. *Thorax*. 2006;61(3):189-95.
108. Sheldenkar A, Lim F, Yung CF, Lwin MO. Acceptance and uptake of influenza vaccines in Asia: A systematic review. *Vaccine*. 2019;37(35):4896-905.
109. Spruit MA, Singh SJ, Garvey C, ZuWallack R, Nici L, Rochester C, et al. An official American Thoracic Society/European Respiratory Society statement: key concepts and advances in pulmonary rehabilitation. *Am J Respir Crit Care Med*. 2013;188(8):e13-64.
110. McCarthy B, Casey D, Devane D, Murphy K, Murphy E, Lacasse Y. Pulmonary rehabilitation for chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2015(2):CD003793.
111. Puhan MA, Gimeno-Santos E, Cates CJ, Troosters T. Pulmonary rehabilitation following exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2016;12:CD005305.
112. do Nascimento ES, Sampaio LM, Peixoto-Souza FS, Dias FD, Gomes EL, Greiffo FR, et al. Home-based pulmonary rehabilitation improves clinical features and systemic inflammation in chronic obstructive pulmonary disease patients. *Int J Chron Obstruct Pulmon Dis*. 2015;10:645-53.
113. Laveneziana P, Palange P, Faculty ERSRS. Physical activity, nutritional status and systemic inflammation in COPD. *Eur Respir J*. 2012;40(3):522-9.

114. Camp PG, Hernandez P, Bourbeau J, Kirkham A, Debigare R, Stickland MK, et al. Pulmonary rehabilitation in Canada: A report from the Canadian Thoracic Society COPD Clinical Assembly. *Can Respir J*. 2015;22(3):147-52.
115. Wadell K, Janaudis Ferreira T, Arne M, Lisspers K, Stallberg B, Emtner M. Hospital-based pulmonary rehabilitation in patients with COPD in Sweden--a national survey. *Respir Med*. 2013;107(8):1195-200.
116. Zainuldin R, Sasiadek KM, Abdul Raub NA, Tay NW. An Evaluation on the Effects of Inpatient Pulmonary Rehabilitation Following Acute Exacerbation of Chronic Obstructive Pulmonary Disease in a Singapore Hospital. *Ann Acad Med Singapore*. 2016;45(4):169-71.
117. Levack WMM, Watson J, Hay-Smith EJC, Davies C, Ingham T, Jones B, et al. Factors influencing referral to and uptake and attendance of pulmonary rehabilitation for chronic obstructive pulmonary disease: a qualitative evidence synthesis of the experiences of service users, their families, and healthcare providers. *Cochrane Database of Systematic Reviews*. 2018.
118. Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council Working Party. *Lancet*. 1981;1(8222):681-6.
119. Continuous or nocturnal oxygen therapy in hypoxemic chronic obstructive lung disease: a clinical trial. Nocturnal Oxygen Therapy Trial Group. *Ann Intern Med*. 1980;93(3):391-8.
120. Marin JM, Soriano JB, Carrizo SJ, Boldova A, Celli BR. Outcomes in patients with chronic obstructive pulmonary disease and obstructive sleep apnea: the overlap syndrome. *Am J Respir Crit Care Med*. 2010;182(3):325-31.
121. Murphy PB, Rehal S, Arbane G, Bourke S, Calverley PMA, Crook AM, et al. Effect of Home Noninvasive Ventilation With Oxygen Therapy vs Oxygen Therapy Alone on Hospital Readmission or Death After an Acute COPD Exacerbation: A Randomized Clinical Trial. *JAMA*. 2017;317(21):2177-86.
122. Galli JA, Krahne JS, James Mamary A, Shenoy K, Zhao H, Criner GJ. Home non-invasive ventilation use following acute hypercapnic respiratory failure in COPD. *Respir Med*. 2014;108(5):722-8.

123. Kohnlein T, Windisch W, Kohler D, Drabik A, Geiseler J, Hartl S, et al. Non-invasive positive pressure ventilation for the treatment of severe stable chronic obstructive pulmonary disease: a prospective, multicentre, randomised, controlled clinical trial. *Lancet Respir Med*. 2014;2(9):698-705.
124. Sciurba FC, Criner GJ, Strange C, Shah PL, Michaud G, Connolly TA, et al. Effect of Endobronchial Coils vs Usual Care on Exercise Tolerance in Patients With Severe Emphysema: The RENEW Randomized Clinical Trial. *JAMA*. 2016;315(20):2178-89.
125. Snell G, Herth FJ, Hopkins P, Baker KM, Witt C, Gotfried MH, et al. Bronchoscopic thermal vapour ablation therapy in the management of heterogeneous emphysema. *Eur Respir J*. 2012;39(6):1326-33.
126. Criner GJ, Sue R, Wright S, Dransfield M, Rivas-Perez H, Wiese T, et al. A Multicenter Randomized Controlled Trial of Zephyr Endobronchial Valve Treatment in Heterogeneous Emphysema (LIBERATE). *Am J Respir Crit Care Med*. 2018;198(9):1151-64.
127. Buhl R, Maltais F, Abrahams R, Bjerner L, Derom E, Ferguson G, et al. Tiotropium and olodaterol fixed-dose combination versus mono-components in COPD (GOLD 2-4). *Eur Respir J*. 2015;45(4):969-79.
128. Vogelmeier C, Hederer B, Glaab T, Schmidt H, Rutten-van Molken MP, Beeh KM, et al. Tiotropium versus salmeterol for the prevention of exacerbations of COPD. *N Engl J Med*. 2011;364(12):1093-103.
129. Martinez FJ, Calverley PM, Goehring UM, Brose M, Fabbri LM, Rabe KF. Effect of roflumilast on exacerbations in patients with severe chronic obstructive pulmonary disease uncontrolled by combination therapy (REACT): a multicentre randomised controlled trial. *Lancet*. 2015;385(9971):857-66.
130. Martinez FJ, Vestbo J, Anderson JA, Brook RD, Celli BR, Cowans NJ, et al. Effect of Fluticasone Furoate and Vilanterol on Exacerbations of Chronic Obstructive Pulmonary Disease in Patients with Moderate Airflow Obstruction. *Am J Respir Crit Care Med*. 2017;195(7):881-8.

131. Farne HA, Cates CJ. Long-acting beta2-agonist in addition to tiotropium versus either tiotropium or long-acting beta2-agonist alone for chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2015(10):CD008989.
132. Rodrigo GJ, Plaza V. Efficacy and safety of a fixed-dose combination of indacaterol and Glycopyrronium for the treatment of COPD: a systematic review. *Chest*. 2014;146(2):309-17.
133. Hashimoto S, Ikeuchi H, Murata S, Kitawaki T, Ikeda K, Banerji D. Efficacy and safety of indacaterol/glycopyrronium in Japanese patients with COPD: a subgroup analysis from the SHINE study. *Int J Chron Obstruct Pulmon Dis*. 2016;11:2543-51.
134. Lipson DA, Barnhart F, Brealey N, Brooks J, Criner GJ, Day NC, et al. Once-Daily Single-Inhaler Triple versus Dual Therapy in Patients with COPD. *N Engl J Med*. 2018;378(18):1671-80.
135. Ernst P, Gonzalez AV, Brassard P, Suissa S. Inhaled corticosteroid use in chronic obstructive pulmonary disease and the risk of hospitalization for pneumonia. *Am J Respir Crit Care Med*. 2007;176(2):162-6.
136. Suissa S, Patenaude V, Lapi F, Ernst P. Inhaled corticosteroids in COPD and the risk of serious pneumonia. *Thorax*. 2013;68(11):1029-36.
137. Albert RK, Connett J, Bailey WC, Casaburi R, Cooper JA, Jr., Criner GJ, et al. Azithromycin for prevention of exacerbations of COPD. *N Engl J Med*. 2011;365(8):689-98.
138. Uzun S, Djamin RS, Kluytmans JA, Mulder PG, van't Veer NE, Ermens AA, et al. Azithromycin maintenance treatment in patients with frequent exacerbations of chronic obstructive pulmonary disease (COLUMBUS): a randomised, double-blind, placebo-controlled trial. *Lancet Respir Med*. 2014;2(5):361-8.
139. Poole PJ. Role of mucolytics in the management of COPD. *Int J Chron Obstruct Pulmon Dis*. 2006;1(2):123-8.
140. Cazzola M, Calzetta L, Page C, Jardim J, Chuchalin AG, Rogliani P, et al. Influence of N-acetylcysteine on chronic bronchitis or COPD exacerbations: a meta-analysis. *Eur Respir Rev*. 2015;24(137):451-61.

141. Lim S, Lam DC, Muttalif AR, Yunus F, Wongtim S, Lan le TT, et al. Impact of chronic obstructive pulmonary disease (COPD) in the Asia-Pacific region: the EPIC Asia population-based survey. *Asia Pac Fam Med*. 2015;14(1):4.
142. Dong YH, Hsu CL, Li YY, Chang CH, Lai MS. Bronchodilators use in patients with COPD. *Int J Chron Obstruct Pulmon Dis*. 2015;10:1769-79.
143. Davies L, Angus RM, Calverley PM. Oral corticosteroids in patients admitted to hospital with exacerbations of chronic obstructive pulmonary disease: a prospective randomised controlled trial. *Lancet*. 1999;354(9177):456-60.
144. Niewoehner DE, Erbland ML, Deupree RH, Collins D, Gross NJ, Light RW, et al. Effect of systemic glucocorticoids on exacerbations of chronic obstructive pulmonary disease. Department of Veterans Affairs Cooperative Study Group. *N Engl J Med*. 1999;340(25):1941-7.
145. Ram FS, Rodriguez-Roisin R, Granados-Navarrete A, Garcia-Aymerich J, Barnes NC. Antibiotics for exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2006(2):CD004403.
146. Mathioudakis AG, Chatzimavridou-Grigoriadou V, Corlateanu A, Vestbo J. Procalcitonin to guide antibiotic administration in COPD exacerbations: a meta-analysis. *Eur Respir Rev*. 2017;26(143).
147. Butler CC, Gillespie D, White P, Bates J, Lowe R, Thomas-Jones E, et al. C-Reactive Protein Testing to Guide Antibiotic Prescribing for COPD Exacerbations. *N Engl J Med*. 2019;381(2):111-20.
148. Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann Intern Med*. 1987;106(2):196-204.
149. Lee KH, Song Y, Wu W, Yu K, Zhang G. The gut microbiota, environmental factors, and links to the development of food allergy. *Clin Mol Allergy*. 2020;18:5.
150. Pascal M, Perez-Gordo M, Caballero T, Escribese MM, Lopez Longo MN, Luengo O, et al. Microbiome and Allergic Diseases. *Front Immunol*. 2018;9:1584.

151. Chen YC, Ho WC, Yu YH. Adolescent lung function associated with incense burning and other environmental exposures at home. *Indoor air*. 2017;27(4):746-52.
152. Hsu NY, Wang JY, Wu PC, Su HJ. Paternal heredity and housing characteristics affect childhood asthma and allergy morbidity. *Arch Environ Occup Health*. 2012;67(3):155-62.
153. Yang CY, Chiu JF, Cheng MF, Lin MC. Effects of indoor environmental factors on respiratory health of children in a subtropical climate. *Environ Res*. 1997;75(1):49-55.
154. Chen YC. Chinese values, health and nursing. *J Adv Nurs*. 2001;36(2):270-3.
155. Waldron I. Patterns and causes of gender differences in smoking. *Soc Sci Med*. 1991;32(9):989-1005.
156. Waldron I, Bratelli G, Carriker L, Sung WC, Vogeli C, Waldman E. Gender differences in tobacco use in Africa, Asia, the Pacific, and Latin America. *Soc Sci Med*. 1988;27(11):1269-75.
157. Teramoto S, Yamamoto H, Yamaguchi Y, Matsuse T, Ouchi Y. Global burden of COPD in Japan and Asia. *Lancet*. 2003;362(9397):1764-5.
158. Bhome AB, Brashier B. Profiles of chronic obstructive lung disease: characteristics of stable chronic obstructive lung disease in different parts of Asia. *Curr Opin Pulm Med*. 2014;20(2):165-72.
159. Adeloye D, Chua S, Lee C, Basquill C, Papana A, Theodoratou E, et al. Global and regional estimates of COPD prevalence: Systematic review and meta-analysis. *J Glob Health*. 2015;5(2):020415.
160. Regional CWG. COPD prevalence in 12 Asia-Pacific countries and regions: projections based on the COPD prevalence estimation model. *Respirology*. 2003;8(2):192-8.
161. Zhong N, Wang C, Yao W, Chen P, Kang J, Huang S, et al. Prevalence of chronic obstructive pulmonary disease in China: a large, population-based survey. *Am J Respir Crit Care Med*. 2007;176(8):753-60.
162. Fang L, Gao P, Bao H, Tang X, Wang B, Feng Y, et al. Chronic obstructive pulmonary disease in China: a nationwide prevalence study. *Lancet Respir Med*. 2018;6(6):421-30.

163. Wang C, Xu J, Yang L, Xu Y, Zhang X, Bai C, et al. Prevalence and risk factors of chronic obstructive pulmonary disease in China (the China Pulmonary Health [CPH] study): a national cross-sectional study. *Lancet*. 2018;391(10131):1706-17.
164. Yoo KH, Kim YS, Sheen SS, Park JH, Hwang YI, Kim SH, et al. Prevalence of chronic obstructive pulmonary disease in Korea: the fourth Korean National Health and Nutrition Examination Survey, 2008. *Respirology*. 2011;16(4):659-65.
165. Matsumoto K, Seki N, Fukuyama S, Moriwaki A, Kan-o K, Matsunaga Y, et al. Prevalence of asthma with airflow limitation, COPD, and COPD with variable airflow limitation in older subjects in a general Japanese population: the Hisayama Study. *Respir Investig*. 2015;53(1):22-9.
166. Giovino GA, Mirza SA, Samet JM, Gupta PC, Jarvis MJ, Bhala N, et al. Tobacco use in 3 billion individuals from 16 countries: an analysis of nationally representative cross-sectional household surveys. *Lancet*. 2012;380(9842):668-79.
167. Nguyen Viet N, Yunus F, Nguyen Thi Phuong A, Dao Bich V, Damayanti T, Wiyono WH, et al. The prevalence and patient characteristics of chronic obstructive pulmonary disease in non-smokers in Vietnam and Indonesia: An observational survey. *Respirology*. 2015;20(4):602-11.
168. Oxman AD, Muir DC, Shannon HS, Stock SR, Hnizdo E, Lange HJ. Occupational dust exposure and chronic obstructive pulmonary disease. A systematic overview of the evidence. *Am Rev Respir Dis*. 1993;148(1):38-48.
169. Kim WJ, Yim JJ, Kim DK, Lee MG, Fuhlbrigge AL, Sliwinski P, et al. Severe COPD cases from Korea, Poland, and USA have substantial differences in respiratory symptoms and other respiratory illnesses. *Int J Chron Obstruct Pulmon Dis*. 2017;12:3415-23.
170. WHO Global Tuberculosis report 2019. Available From: https://www.who.int/tb/publications/global_report/en/.
171. Hnizdo E, Singh T, Churchyard G. Chronic pulmonary function impairment caused by initial and recurrent pulmonary tuberculosis following treatment. *Thorax*. 2000;55(1):32-8.

172. Hwang YI, Kim JH, Lee CY, Park S, Park YB, Jang SH, et al. The association between airflow obstruction and radiologic change by tuberculosis. *J Thorac Dis.* 2014;6(5):471-6.
173. Yakar HI, Gunen H, Pehlivan E, Aydogan S. The role of tuberculosis in COPD. *Int J Chron Obstruct Pulmon Dis.* 2017;12:323-9.
174. Everaerts S, Lagrou K, Dubbeldam A, Lorent N, Vermeersch K, Van Hoeyveld E, et al. Sensitization to *Aspergillus fumigatus* as a risk factor for bronchiectasis in COPD. *Int J Chron Obstruct Pulmon Dis.* 2017;12:2629-38.
175. Martinez-Garcia MA, de la Rosa Carrillo D, Soler-Cataluna JJ, Donat-Sanz Y, Serra PC, Lerma MA, et al. Prognostic value of bronchiectasis in patients with moderate-to-severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2013;187(8):823-31.
176. Huang CT, Tsai YJ, Wu HD, Wang JY, Yu CJ, Lee LN, et al. Impact of non-tuberculous mycobacteria on pulmonary function decline in chronic obstructive pulmonary disease. *Int J Tuberc Lung Dis.* 2012;16(4):539-45.
177. Fukuchi Y, Nishimura M, Ichinose M, Adachi M, Nagai A, Kuriyama T, et al. COPD in Japan: the Nippon COPD Epidemiology study. *Respirology.* 2004;9(4):458-65.
178. Loh LC. Chronic bronchitis is not necessary to define COPD. *Respirology.* 2011;16(3):574.
179. Lu M, Yao W, Zhong N, Zhou Y, Wang C, Chen P, et al. Chronic obstructive pulmonary disease in the absence of chronic bronchitis in China. *Respirology.* 2010;15(7):1072-8.
180. Hurst JR, Vestbo J, Anzueto A, Locantore N, Mullerova H, Tal-Singer R, et al. Susceptibility to exacerbation in chronic obstructive pulmonary disease. *N Engl J Med.* 2010;363(12):1128-38.
181. Wise RA, Anzueto A, Cotton D, Dahl R, Devins T, Disse B, et al. Tiotropium Respimat inhaler and the risk of death in COPD. *N Engl J Med.* 2013;369(16):1491-501.
182. Zhong N, Moon HS, Lee KH, Mahayiddin AA, Boonsawat W, Isidro MG, et al. TIOtropium Safety and Performance In Respimat((R)) (TIOspir(TM)): Analysis of Asian cohort of COPD patients. *Respirology.* 2016;21(8):1397-403.

183. Suzuki M, Makita H, Konno S, Shimizu K, Kimura H, Kimura H, et al. Asthma-like Features and Clinical Course of Chronic Obstructive Pulmonary Disease. An Analysis from the Hokkaido COPD Cohort Study. *Am J Respir Crit Care Med*. 2016;194(11):1358-65.
184. Greaves L. The meanings of smoking to women and their implications for cessation. *Int J Environ Res Public Health*. 2015;12(2):1449-65.
185. Guo Y, Zhang T, Wang Z, Yu F, Xu Q, Guo W, et al. Body mass index and mortality in chronic obstructive pulmonary disease: A dose-response meta-analysis. *Medicine*. 2016;95(28):e4225.
186. Celli BR, Cote CG, Marin JM, Casanova C, Montes de Oca M, Mendez RA, et al. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med*. 2004;350(10):1005-12.
187. Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax*. 2002;57(10):847-52.
188. Hoogendoorn M, Hoogenveen RT, Rutten-van Molken MP, Vestbo J, Feenstra TL. Case fatality of COPD exacerbations: a meta-analysis and statistical modelling approach. *Eur Respir J*. 2011;37(3):508-15.
189. Keir HR, Dicker A, Lonergan M, Crichton M, Miller BE, Tal-Singer R, et al. Clinical endotypes of exacerbation are associated with differences in microbial composition and diversity in COPD. *Eur Respir J*. 2020.
190. Kang CI, Song JH. Antimicrobial resistance in Asia: current epidemiology and clinical implications. *Infect Chemother*. 2013;45(1):22-31.
191. Putcha N, Drummond MB, Wise RA, Hansel NN. Comorbidities and Chronic Obstructive Pulmonary Disease: Prevalence, Influence on Outcomes, and Management. *Semin Respir Crit Care Med*. 2015;36(4):575-91.
192. Berger JS, Sanborn TA, Sherman W, Brown DL. Effect of chronic obstructive pulmonary disease on survival of patients with coronary heart disease having percutaneous coronary intervention. *Am J Cardiol*. 2004;94(5):649-51.

193. Vanfleteren LE, Spruit MA, Groenen M, Gaffron S, van Empel VP, Bruijnzeel PL, et al. Clusters of comorbidities based on validated objective measurements and systemic inflammation in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2013;187(7):728-35.
194. Burgel PR, Paillasseur JL, Caillaud D, Tillie-Leblond I, Chanez P, Escamilla R, et al. Clinical COPD phenotypes: a novel approach using principal component and cluster analyses. *Eur Respir J*. 2010;36(3):531-9.
195. Burgel PR, Paillasseur JL, Peene B, Dusser D, Roche N, Coolen J, et al. Two distinct chronic obstructive pulmonary disease (COPD) phenotypes are associated with high risk of mortality. *PloS one*. 2012;7(12):e51048.
196. Lee H, Shin SH, Gu S, Zhao D, Kang D, Joi YR, et al. Racial differences in comorbidity profile among patients with chronic obstructive pulmonary disease. *BMC Med*. 2018;16(1):178.
197. Le Rouzic O, Roche N, Cortot AB, Tillie-Leblond I, Masure F, Perez T, et al. Defining the "Frequent Exacerbator" Phenotype in COPD: A Hypothesis-Free Approach. *Chest*. 2018;153(5):1106-15.
198. Miller J, Edwards LD, Agusti A, Bakke P, Calverley PM, Celli B, et al. Comorbidity, systemic inflammation and outcomes in the ECLIPSE cohort. *Respir Med*. 2013;107(9):1376-84.
199. Perera WR, Hurst JR, Wilkinson TM, Sapsford RJ, Mullerova H, Donaldson GC, et al. Inflammatory changes, recovery and recurrence at COPD exacerbation. *Eur Respir J*. 2007;29(3):527-34.
200. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 2013;187(4):347-65.
201. Sasaki T, Nakayama K, Yasuda H, Yoshida M, Asamura T, Ohnishi T, et al. A randomized, single-blind study of lansoprazole for the prevention of exacerbations of chronic obstructive pulmonary disease in older patients. *J Am Geriatr Soc*. 2009;57(8):1453-7.

202. Suissa S, Dell'Aniello S, Ernst P. Long-term natural history of chronic obstructive pulmonary disease: severe exacerbations and mortality. *Thorax*. 2012;67(11):957-63.
203. Fukuchi Y, Fernandez L, Kuo HP, Mahayiddin A, Celli B, Decramer M, et al. Efficacy of tiotropium in COPD patients from Asia: a subgroup analysis from the UPLIFT trial. *Respirology*. 2011;16(5):825-35.
204. Ishii T, Nishimura M, Akimoto A, James MH, Jones P. Understanding low COPD exacerbation rates in Japan: a review and comparison with other countries. *Int J Chron Obstruct Pulmon Dis*. 2018;13:3459-71.
205. Bafadhel M, McKenna S, Terry S, Mistry V, Reid C, Haldar P, et al. Acute exacerbations of chronic obstructive pulmonary disease: identification of biologic clusters and their biomarkers. *Am J Respir Crit Care Med*. 2011;184(6):662-71.
206. Siddiqui SH, Guasconi A, Vestbo J, Jones P, Agusti A, Paggiaro P, et al. Blood Eosinophils: A Biomarker of Response to Extrafine Beclomethasone/Formoterol in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med*. 2015;192(4):523-5.
207. Bafadhel M, McKenna S, Terry S, Mistry V, Pancholi M, Venge P, et al. Blood eosinophils to direct corticosteroid treatment of exacerbations of chronic obstructive pulmonary disease: a randomized placebo-controlled trial. *Am J Respir Crit Care Med*. 2012;186(1):48-55.
208. Pavord ID, Lettis S, Locantore N, Pascoe S, Jones PW, Wedzicha JA, et al. Blood eosinophils and inhaled corticosteroid/long-acting beta-2 agonist efficacy in COPD. *Thorax*. 2016;71(2):118-25.
209. Casanova C, Celli BR, de-Torres JP, Martinez-Gonzalez C, Cosio BG, Pinto-Plata V, et al. Prevalence of persistent blood eosinophilia: relation to outcomes in patients with COPD. *Eur Respir J*. 2017;50(5).
210. Kerkhof M, Sonnappa S, Postma DS, Brusselle G, Agusti A, Anzueto A, et al. Blood eosinophil count and exacerbation risk in patients with COPD. *Eur Respir J*. 2017;50(1).
211. Vedel-Krogh S, Nielsen SF, Lange P, Vestbo J, Nordestgaard BG. Blood Eosinophils and Exacerbations in Chronic Obstructive Pulmonary Disease. The Copenhagen General Population Study. *Am J Respir Crit Care Med*. 2016;193(9):965-74.

212. Morgenstern V, Zutavern A, Cyrys J, Brockow I, Koletzko S, Kramer U, et al. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am J Respir Crit Care Med*. 2008;177(12):1331-7.
213. D'Amato G, Liccardi G, D'Amato M, Cazzola M. Outdoor air pollution, climatic changes and allergic bronchial asthma. *Eur Respir J*. 2002;20(3):763-76.
214. Strachan DP. Hay fever, hygiene, and household size. *BMJ*. 1989;299(6710):1259-60.
215. Liu AH, Murphy JR. Hygiene hypothesis: fact or fiction? *J Allergy Clin Immunol*. 2003;111(3):471-8.
216. Lambrecht BN, Hammad H. The immunology of the allergy epidemic and the hygiene hypothesis. *Nat Immunol*. 2017;18(10):1076-83.
217. Tham EH, Lee AJ, Bever HV. Aeroallergen sensitization and allergic disease phenotypes in Asia. *Asian Pac J Allergy Immunol*. 2016;34(3):181-9.
218. Chew FT, Lim SH, Goh DY, Lee BW. Sensitization to local dust-mite fauna in Singapore. *Allergy*. 1999;54(11):1150-9.
219. Minami T, Fukutomi Y, Inada R, Tsuda M, Sekiya K, Miyazaki M, et al. Regional differences in the prevalence of sensitization to environmental allergens: Analysis on IgE antibody testing conducted at major clinical testing laboratories throughout Japan from 2002 to 2011. *Allergol Int*. 2019;68(4):440-9.
220. Andiappan AK, Puan KJ, Lee B, Nardin A, Poidinger M, Connolly J, et al. Allergic airway diseases in a tropical urban environment are driven by dominant mono-specific sensitization against house dust mites. *Allergy*. 2014;69(4):501-9.
221. Maes T, Provoost S, Lanckacker EA, Cataldo DD, Vanoirbeek JA, Nemery B, et al. Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. *Respir Res*. 2010;11:7.
222. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, et al. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med*. 2012;209(8):1505-17.

223. van Ree R, Hummelshoj L, Plantinga M, Poulsen LK, Swindle E. Allergic sensitization: host-immune factors. *Clin Transl Allergy*. 2014;4(1):12.
224. Platts-Mills TA. The role of immunoglobulin E in allergy and asthma. *Am J Respir Crit Care Med*. 2001;164(8 Pt 2):S1-5.
225. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med*. 2012;18(5):693-704.
226. Zhao J, Lloyd CM, Noble A. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol*. 2013;6(2):335-46.
227. Bafadhel M, McKenna S, Agbetile J, Fairs A, Desai D, Mistry V, et al. *Aspergillus fumigatus* during stable state and exacerbations of COPD. *The European respiratory journal*. 2014;43(1):64-71.
228. Queiroz CF, Lemos AC, Bastos ML, Neves MC, Camelier AA, Carvalho NB, et al. Inflammatory and immunological profiles in patients with COPD: relationship with FEV 1 reversibility. *J Bras Pneumol*. 2016;42(4):241-7.
229. Neves MC, Neves YC, Mendes CM, Bastos MN, Camelier AA, Queiroz CF, et al. Evaluation of atopy in patients with COPD. *J Bras Pneumol*. 2013;39(3):296-305.
230. Goh KJ, Yip ACA, Lapperre TS, Chan AK, Chew FT, Chotirmall SH, et al. Sensitization to *Aspergillus* species is associated with frequent exacerbations in severe asthma. *Journal of asthma and allergy*. 2017;10:131-40.
231. Jin J, Liu X, Sun Y. The prevalence of increased serum IgE and *Aspergillus* sensitization in patients with COPD and their association with symptoms and lung function. *Respir Res*. 2014;15:130.
232. Global Initiative for Asthma. Global Management and Prevention 2014. www.ginasthma.org.
233. Alshabanat A, Zafari Z, Albanyan O, Dairi M, FitzGerald JM. Asthma and COPD Overlap Syndrome (ACOS): A Systematic Review and Meta Analysis. *PLoS One*. 2015;10(9):e0136065.

234. Kauppi P, Kupiainen H, Lindqvist A, Tammilehto L, Kilpelainen M, Kinnula VL, et al. Overlap syndrome of asthma and COPD predicts low quality of life. *J Asthma*. 2011;48(3):279-85.
235. Gibson PG, Simpson JL. The overlap syndrome of asthma and COPD: what are its features and how important is it? *Thorax*. 2009;64(8):728-35.
236. Lange P, Colak Y, Ingebrigtsen TS, Vestbo J, Marott JL. Long-term prognosis of asthma, chronic obstructive pulmonary disease, and asthma-chronic obstructive pulmonary disease overlap in the Copenhagen City Heart study: a prospective population-based analysis. *Lancet Respir Med*. 2016;4(6):454-62.
237. Sin DD, Miravittles M, Mannino DM, Soriano JB, Price D, Celli BR, et al. What is asthma-COPD overlap syndrome? Towards a consensus definition from a round table discussion. *Eur Respir J*. 2016;48(3):664-73.
238. Chandrasekaran R, Mac Aogain M, Chalmers JD, Elborn SJ, Chotirmall SH. Geographic variation in the aetiology, epidemiology and microbiology of bronchiectasis. *BMC pulmonary medicine*. 2018;18(1):83.
239. Polverino E, Goeminne PC, McDonnell MJ, Aliberti S, Marshall SE, Loebinger MR, et al. European Respiratory Society guidelines for the management of adult bronchiectasis. *Eur Respir J*. 2017;50(3).
240. Chalmers JD, Goeminne P, Aliberti S, McDonnell MJ, Lonni S, Davidson J, et al. The bronchiectasis severity index. An international derivation and validation study. *Am J Respir Crit Care Med*. 2014;189(5):576-85.
241. Chalmers JD. Bronchiectasis and COPD Overlap: A Case of Mistaken Identity? *Chest*. 2017;151(6):1204-6.
242. Naidich DP, McCauley DI, Khouri NF, Stitik FP, Siegelman SS. Computed tomography of bronchiectasis. *J Comput Assist Tomogr*. 1982;6(3):437-44.
243. Polverino E, Dimakou K, Hurst J, Martinez-Garcia MA, Miravittles M, Paggiaro P, et al. The overlap between bronchiectasis and chronic airway diseases: state of the art and future directions. *Eur Respir J*. 2018;52(3).

244. Tan WC, Hague CJ, Leipsic J, Bourbeau J, Zheng L, Li PZ, et al. Findings on Thoracic Computed Tomography Scans and Respiratory Outcomes in Persons with and without Chronic Obstructive Pulmonary Disease: A Population-Based Cohort Study. *PLoS One*. 2016;11(11):e0166745.
245. Agusti A, Calverley PM, Celli B, Coxson HO, Edwards LD, Lomas DA, et al. Characterisation of COPD heterogeneity in the ECLIPSE cohort. *Respir Res*. 2010;11:122.
246. Ni Y, Shi G, Yu Y, Hao J, Chen T, Song H. Clinical characteristics of patients with chronic obstructive pulmonary disease with comorbid bronchiectasis: a systemic review and meta-analysis. *Int J Chron Obstruct Pulmon Dis*. 2015;10:1465-75.
247. Patel IS, Vlahos I, Wilkinson TM, Lloyd-Owen SJ, Donaldson GC, Wilks M, et al. Bronchiectasis, exacerbation indices, and inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2004;170(4):400-7.
248. Fletcher C, Peto R. The natural history of chronic airflow obstruction. *British medical journal*. 1977;1(6077):1645-8.
249. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *The New England journal of medicine*. 2008;359(22):2355-65.
250. Donaldson GC, Seemungal T, Jeffries DJ, Wedzicha JA. Effect of temperature on lung function and symptoms in chronic obstructive pulmonary disease. *Eur Respir J*. 1999;13(4):844-9.
251. Matkovic Z, Miravittles M. Chronic bronchial infection in COPD. Is there an infective phenotype? *Respir Med*. 2013;107(1):10-22.
252. Berenson CS, Kruzel RL, Eberhardt E, Sethi S. Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. *J Infect Dis*. 2013;208(12):2036-45.
253. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha JA, et al. Defective macrophage phagocytosis of bacteria in COPD. *The European respiratory journal*. 2010;35(5):1039-47.

254. King PT, Sharma R, O'Sullivan K, Selemidis S, Lim S, Radhakrishna N, et al. Nontypeable *Haemophilus influenzae* induces sustained lung oxidative stress and protease expression. *PLoS One*. 2015;10(3):e0120371.
255. Sethi S, Evans N, Grant BJ, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *N Engl J Med*. 2002;347(7):465-71.
256. White AJ, Gompertz S, Bayley DL, Hill SL, O'Brien C, Unsal I, et al. Resolution of bronchial inflammation is related to bacterial eradication following treatment of exacerbations of chronic bronchitis. *Thorax*. 2003;58(8):680-5.
257. Seemungal T, Harper-Owen R, Bhowmik A, Moric I, Sanderson G, Message S, et al. Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2001;164(9):1618-23.
258. Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2000;161(5):1608-13.
259. Wedzicha JA. Role of viruses in exacerbations of chronic obstructive pulmonary disease. *Proc Am Thorac Soc*. 2004;1(2):115-20.
260. Seemungal TA, Harper-Owen R, Bhowmik A, Jeffries DJ, Wedzicha JA. Detection of rhinovirus in induced sputum at exacerbation of chronic obstructive pulmonary disease. *Eur Respir J*. 2000;16(4):677-83.
261. Nichol KL, Baken L, Nelson A. Relation between influenza vaccination and outpatient visits, hospitalization, and mortality in elderly persons with chronic lung disease. *Ann Intern Med*. 1999;130(5):397-403.
262. Mohan A, Chandra S, Agarwal D, Guleria R, Broor S, Gaur B, et al. Prevalence of viral infection detected by PCR and RT-PCR in patients with acute exacerbation of COPD: a systematic review. *Respirology*. 2010;15(3):536-42.
263. McCullers JA. The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat Rev Microbiol*. 2014;12(4):252-62.

264. Bellinghausen C, Gulraiz F, Heinzmann AC, Dentener MA, Savelkoul PH, Wouters EF, et al. Exposure to common respiratory bacteria alters the airway epithelial response to subsequent viral infection. *Respir Res*. 2016;17(1):68.
265. Wark PA, Tooze M, Powell H, Parsons K. Viral and bacterial infection in acute asthma and chronic obstructive pulmonary disease increases the risk of readmission. *Respirology*. 2013;18(6):996-1002.
266. Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, et al. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am J Respir Crit Care Med*. 2006;173(10):1114-21.
267. Ko FW, Chan KP, Hui DS, Goddard JR, Shaw JG, Reid DW, et al. Acute exacerbation of COPD. *Respirology*. 2016;21(7):1152-65.
268. Berenson CS, Wrona CT, Grove LJ, Maloney J, Garlipp MA, Wallace PK, et al. Impaired alveolar macrophage response to *Haemophilus* antigens in chronic obstructive lung disease. *Am J Respir Crit Care Med*. 2006;174(1):31-40.
269. Bresser P, Out TA, van Alphen L, Jansen HM, Lutter R. Airway inflammation in nonobstructive and obstructive chronic bronchitis with chronic *haemophilus influenzae* airway infection. Comparison with noninfected patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2000;162(3 Pt 1):947-52.
270. Sethi S, Maloney J, Grove L, Wrona C, Berenson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2006;173(9):991-8.
271. Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am J Med*. 2000;109(4):288-95.
272. Desai H, Eschberger K, Wrona C, Grove L, Agrawal A, Grant B, et al. Bacterial colonization increases daily symptoms in patients with chronic obstructive pulmonary disease. *Ann Am Thorac Soc*. 2014;11(3):303-9.

273. Banerjee D, Khair OA, Honeybourne D. Impact of sputum bacteria on airway inflammation and health status in clinical stable COPD. *Eur Respir J*. 2004;23(5):685-91.
274. St Geme JW, 3rd, Falkow S, Barenkamp SJ. High-molecular-weight proteins of nontypable *Haemophilus influenzae* mediate attachment to human epithelial cells. *Proc Natl Acad Sci U S A*. 1993;90(7):2875-9.
275. Reddy MS, Bernstein JM, Murphy TF, Faden HS. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infect Immun*. 1996;64(4):1477-9.
276. Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. *Clin Microbiol Rev*. 2001;14(2):336-63.
277. Berenson CS, Murphy TF, Wrona CT, Sethi S. Outer membrane protein P6 of nontypeable *Haemophilus influenzae* is a potent and selective inducer of human macrophage proinflammatory cytokines. *Infect Immun*. 2005;73(5):2728-35.
278. Sethi S, Mallia P, Johnston SL. New paradigms in the pathogenesis of chronic obstructive pulmonary disease II. *Proc Am Thorac Soc*. 2009;6(6):532-4.
279. Moghaddam SJ, Clement CG, De la Garza MM, Zou X, Travis EL, Young HW, et al. *Haemophilus influenzae* lysate induces aspects of the chronic obstructive pulmonary disease phenotype. *Am J Respir Cell Mol Biol*. 2008;38(6):629-38.
280. Miravittles M, Espinosa C, Fernandez-Laso E, Martos JA, Maldonado JA, Gallego M. Relationship between bacterial flora in sputum and functional impairment in patients with acute exacerbations of COPD. Study Group of Bacterial Infection in COPD. *Chest*. 1999;116(1):40-6.
281. Tufvesson E, Bjermer L, Ekberg M. Patients with chronic obstructive pulmonary disease and chronically colonized with *Haemophilus influenzae* during stable disease phase have increased airway inflammation. *Int J Chron Obstruct Pulmon Dis*. 2015;10:881-9.
282. Monso E, Ruiz J, Rosell A, Manterola J, Fiz J, Morera J, et al. Bacterial infection in chronic obstructive pulmonary disease. A study of stable and exacerbated outpatients using the protected specimen brush. *Am J Respir Crit Care Med*. 1995;152(4 Pt 1):1316-20.

283. Parameswaran GI, Wrona CT, Murphy TF, Sethi S. *Moraxella catarrhalis* acquisition, airway inflammation and protease-antiprotease balance in chronic obstructive pulmonary disease. *BMC Infect Dis.* 2009;9:178.
284. Bogaert D, van der Valk P, Ramdin R, Sluijter M, Monninkhof E, Hendrix R, et al. Host-pathogen interaction during pneumococcal infection in patients with chronic obstructive pulmonary disease. *Infect Immun.* 2004;72(2):818-23.
285. Murphy TF, Brauer AL, Grant BJ, Sethi S. *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. *Am J Respir Crit Care Med.* 2005;172(2):195-9.
286. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr Pulmonol.* 2002;34(2):91-100.
287. Evans SA, Turner SM, Bosch BJ, Hardy CC, Woodhead MA. Lung function in bronchiectasis: the influence of *Pseudomonas aeruginosa*. *The European respiratory journal.* 1996;9(8):1601-4.
288. Garcia-Vidal C, Almagro P, Romani V, Rodriguez-Carballeira M, Cuchi E, Canales L, et al. *Pseudomonas aeruginosa* in patients hospitalised for COPD exacerbation: a prospective study. *Eur Respir J.* 2009;34(5):1072-8.
289. Boutou AK, Raste Y, Reid J, Alshafi K, Polkey MI, Hopkinson NS. Does a single *Pseudomonas aeruginosa* isolation predict COPD mortality? *Eur Respir J.* 2014;44(3):794-7.
290. Keicho N, Elliott WM, Hogg JC, Hayashi S. Adenovirus E1A gene dysregulates ICAM-1 expression in transformed pulmonary epithelial cells. *Am J Respir Cell Mol Biol.* 1997;16(1):23-30.
291. Matsuse T, Hayashi S, Kuwano K, Keunecke H, Jefferies WA, Hogg JC. Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *Am Rev Respir Dis.* 1992;146(1):177-84.

292. Wilkinson TM, Donaldson GC, Johnston SL, Openshaw PJ, Wedzicha JA. Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2006;173(8):871-6.
293. Keicho N, Elliott WM, Hogg JC, Hayashi S. Adenovirus E1A upregulates interleukin-8 expression induced by endotoxin in pulmonary epithelial cells. *Am J Physiol*. 1997;272(6 Pt 1):L1046-52.
294. McManus TE, Marley AM, Baxter N, Christie SN, Elborn JS, Heaney LG, et al. Acute and latent adenovirus in COPD. *Respir Med*. 2007;101(10):2084-90.
295. Retamales I, Elliott WM, Meshi B, Coxson HO, Pare PD, Sciurba FC, et al. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med*. 2001;164(3):469-73.
296. Meshi B, Vitalis TZ, Ionescu D, Elliott WM, Liu C, Wang XD, et al. Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. *Am J Respir Cell Mol Biol*. 2002;26(1):52-7.
297. Nam HH, Ison MG. Respiratory syncytial virus infection in adults. *BMJ*. 2019;366:l5021.
298. Zhang L, Peebles ME, Boucher RC, Collins PL, Pickles RJ. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J Virol*. 2002;76(11):5654-66.
299. Hall CB. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med*. 2001;344(25):1917-28.
300. Utokeparch S, Sze MA, Gosselink JV, McDonough JE, Elliott WM, Hogg JC, et al. Respiratory viral detection and small airway inflammation in lung tissue of patients with stable, mild COPD. *COPD*. 2014;11(2):197-203.
301. Guinea J, Torres-Narbona M, Gijon P, Munoz P, Pozo F, Pelaez T, et al. Pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: incidence, risk factors, and outcome. *Clin Microbiol Infect*. 2010;16(7):870-7.

302. Maturu VN, Agarwal R. Prevalence of *Aspergillus* sensitization and allergic bronchopulmonary aspergillosis in cystic fibrosis: systematic review and meta-analysis. *Clin Exp Allergy*. 2015;45(12):1765-78.
303. Agbetile J, Fairs A, Desai D, Hargadon B, Bourne M, Mutalithas K, et al. Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV1. *Clin Exp Allergy*. 2012;42(5):782-91.
304. Fitzpatrick ME, Tedrow JR, Hillenbrand ME, Lucht L, Richards T, Norris KA, et al. *Pneumocystis jirovecii* colonization is associated with enhanced Th1 inflammatory gene expression in lungs of humans with chronic obstructive pulmonary disease. *Microbiol Immunol*. 2014;58(3):202-11.
305. Han MK, Huang YJ, Lipuma JJ, Boushey HA, Boucher RC, Cookson WO, et al. Significance of the microbiome in obstructive lung disease. *Thorax*. 2012;67(5):456-63.
306. Budden KF, Shukla SD, Rehman SF, Bowerman KL, Keely S, Hugenholtz P, et al. Functional effects of the microbiota in chronic respiratory disease. *Lancet Respir Med*. 2019.
307. Wang Z, Bafadhel M, Haldar K, Spivak A, Mayhew D, Miller BE, et al. Lung microbiome dynamics in COPD exacerbations. *Eur Respir J*. 2016;47(4):1082-92.
308. Lynch SV. The Lung Microbiome and Airway Disease. *Ann Am Thorac Soc*. 2016;13 Suppl 2:S462-S5.
309. Lim MY, Yoon HS, Rho M, Sung J, Song YM, Lee K, et al. Analysis of the association between host genetics, smoking, and sputum microbiota in healthy humans. *Sci Rep*. 2016;6:23745.
310. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. *PLoS One*. 2010;5(1):e8578.
311. Cabrera-Rubio R, Garcia-Nunez M, Seto L, Anto JM, Moya A, Monso E, et al. Microbiome diversity in the bronchial tracts of patients with chronic obstructive pulmonary disease. *J Clin Microbiol*. 2012;50(11):3562-8.
312. Tiew PY, Jaggi TK, Chan LLY, Chotirmall SH. The airway microbiome in COPD, bronchiectasis and bronchiectasis-COPD overlap. *Clin Respir J*. 2020.

313. Wang Z, Maschera B, Lea S, Kolsum U, Michalovich D, Van Horn S, et al. Airway host-microbiome interactions in chronic obstructive pulmonary disease. *Respir Res*. 2019;20(1):113.
314. Mayhew D, Devos N, Lambert C, Brown JR, Clarke SC, Kim VL, et al. Longitudinal profiling of the lung microbiome in the AERIS study demonstrates repeatability of bacterial and eosinophilic COPD exacerbations. *Thorax*. 2018;73(5):422-30.
315. Galiana A, Aguirre E, Rodriguez JC, Mira A, Santibanez M, Candela I, et al. Sputum microbiota in moderate versus severe patients with COPD. *Eur Respir J*. 2014;43(6):1787-90.
316. Garcia-Nunez M, Millares L, Pomares X, Ferrari R, Perez-Brocal V, Gallego M, et al. Severity-related changes of bronchial microbiome in chronic obstructive pulmonary disease. *J Clin Microbiol*. 2014;52(12):4217-23.
317. Dicker AJ, Huang JT, Lonergan M, Keir HR, Fong CJ, Tan B, et al. The Sputum Microbiome, Airway Inflammation and Mortality in Chronic Obstructive Pulmonary Disease. *J Allergy Clin Immunol*. 2020.
318. Wang Z, Singh R, Miller BE, Tal-Singer R, Van Horn S, Tomsho L, et al. Sputum microbiome temporal variability and dysbiosis in chronic obstructive pulmonary disease exacerbations: an analysis of the COPD MAP study. *Thorax*. 2018;73(4):331-8.
319. Bouquet J, Tabor DE, Silver JS, Nair V, Tovchigrechko A, Griffin MP, et al. Microbial burden and viral exacerbations in a longitudinal multicenter COPD cohort. *Respir Res*. 2020;21(1):77.
320. Jubinville E, Veillette M, Milot J, Maltais F, Comeau AM, Levesque RC, et al. Exacerbation induces a microbiota shift in sputa of COPD patients. *PLoS One*. 2018;13(3):e0194355.
321. Leitao Filho FS, Alotaibi NM, Ngan D, Tam S, Yang J, Hollander Z, et al. Sputum Microbiome Is Associated with 1-Year Mortality after Chronic Obstructive Pulmonary Disease Hospitalizations. *Am J Respir Crit Care Med*. 2019;199(10):1205-13.
322. Su J, Liu HY, Tan XL, Ji Y, Jiang YX, Prabhakar M, et al. Sputum Bacterial and Fungal Dynamics during Exacerbations of Severe COPD. *PLoS One*. 2015;10(7):e0130736.

323. Park H, Shin JW, Park SG, Kim W. Microbial communities in the upper respiratory tract of patients with asthma and chronic obstructive pulmonary disease. *PLoS One*. 2014;9(10):e109710.
324. Liu HY, Zhang SY, Yang WY, Su XF, He Y, Zhou HW, et al. Oropharyngeal and Sputum Microbiomes Are Similar Following Exacerbation of Chronic Obstructive Pulmonary Disease. *Front Microbiol*. 2017;8:1163.
325. Diao W, Shen N, Du Y, Erb-Downward JR, Sun X, Guo C, et al. Symptom-related sputum microbiota in stable chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. 2018;13:2289-99.
326. Tiew PY, Mac Aogain M, Ali N, Thng KX, Goh K, Lau KJX, et al. The Mycobioome in Health and Disease: Emerging Concepts, Methodologies and Challenges. *Mycopathologia*. 2020.
327. Mac Aogain M, Chandrasekaran R, Lim AYH, Low TB, Tan GL, Hassan T, et al. Immunological corollary of the pulmonary mycobioome in bronchiectasis: the CAMEB study. *Eur Respir J*. 2018;52(1).
328. Cui L, Lucht L, Tipton L, Rogers MB, Fitch A, Kessinger C, et al. Topographic diversity of the respiratory tract mycobioome and alteration in HIV and lung disease. *Am J Respir Crit Care Med*. 2015;191(8):932-42.
329. Mitchell AB, Oliver BG, Glanville AR. Translational Aspects of the Human Respiratory Virome. *Am J Respir Crit Care Med*. 2016;194(12):1458-64.
330. Willner D, Furlan M, Haynes M, Schmieder R, Angly FE, Silva J, et al. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS One*. 2009;4(10):e7370.
331. van Rijn AL, van Boheemen S, Sidorov I, Carbo EC, Pappas N, Mei H, et al. The respiratory virome and exacerbations in patients with chronic obstructive pulmonary disease. *PLoS One*. 2019;14(10):e0223952.

332. Elbehery AHA, Feichtmayer J, Singh D, Griebler C, Deng L. The Human Virome Protein Cluster Database (HVPC): A Human Viral Metagenomic Database for Diversity and Function Annotation. *Front Microbiol.* 2018;9:1110.
333. Reece E, Doyle S, Greally P, Renwick J, McClean S. *Aspergillus fumigatus* Inhibits *Pseudomonas aeruginosa* in Co-culture: Implications of a Mutually Antagonistic Relationship on Virulence and Inflammation in the CF Airway. *Front Microbiol.* 2018;9:1205.
334. Kruger W, Vielreicher S, Kapitan M, Jacobsen ID, Niemiec MJ. Fungal-Bacterial Interactions in Health and Disease. *Pathogens.* 2019;8(2).
335. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, et al. *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol Lett.* 2010;313(2):96-102.
336. Yonezawa M, Sugiyama H, Kizawa K, Hori R, Mitsuyama J, Araki H, et al. A new model of pulmonary superinfection with *Aspergillus fumigatus* and *Pseudomonas aeruginosa* in mice. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy.* 2000;6(3):155-61.
337. Reece E, Segurado R, Jackson A, McClean S, Renwick J, Greally P. Co-colonisation with *Aspergillus fumigatus* and *Pseudomonas aeruginosa* is associated with poorer health in cystic fibrosis patients: an Irish registry analysis. *BMC Pulm Med.* 2017;17(1):70.
338. Ferreira JA, Penner JC, Moss RB, Haagensen JA, Clemons KV, Spormann AM, et al. Inhibition of *Aspergillus fumigatus* and Its Biofilm by *Pseudomonas aeruginosa* Is Dependent on the Source, Phenotype and Growth Conditions of the Bacterium. *PLoS One.* 2015;10(8):e0134692.
339. Hanada S, Pirzadeh M, Carver KY, Deng JC. Respiratory Viral Infection-Induced Microbiome Alterations and Secondary Bacterial Pneumonia. *Front Immunol.* 2018;9:2640.
340. Molyneaux PL, Mallia P, Cox MJ, Footitt J, Willis-Owen SA, Homola D, et al. Outgrowth of the bacterial airway microbiome after rhinovirus exacerbation of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine.* 2013;188(10):1224-31.

341. Chotirmall SH, Gellatly SL, Budden KF, Mac Aogain M, Shukla SD, Wood DL, et al. Microbiomes in respiratory health and disease: An Asia-Pacific perspective. *Respirology*. 2017;22(2):240-50.
342. Takeshita T, Matsuo K, Furuta M, Shibata Y, Fukami K, Shimazaki Y, et al. Distinct composition of the oral indigenous microbiota in South Korean and Japanese adults. *Sci Rep*. 2014;4:6990.
343. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet*. 1997;349(9064):1498-504.
344. Sullivan SD, Ramsey SD, Lee TA. The economic burden of COPD. *Chest*. 2000;117(2 Suppl):5S-9S.
345. Ko FW, Hui DS, Lai CK. Worldwide burden of COPD in high- and low-income countries. Part III. Asia-Pacific studies. *Int J Tuberc Lung Dis*. 2008;12(7):713-7.
346. Davis J, Tam E, Taira D. Disparate Rates of Utilization and Progression to Combined Heart Failure and Chronic Obstructive Pulmonary Disease among Asians and Pacific Islanders in Hawai'i. *Hawaii J Med Public Health*. 2016;75(8):228-34.
347. Gilkes A, Hull S, Durbaba S, Schofield P, Ashworth M, Mathur R, et al. Ethnic differences in smoking intensity and COPD risk: an observational study in primary care. *NPJ Prim Care Respir Med*. 2017;27(1):50.
348. Gilkes A, Ashworth M, Schofield P, Harries TH, Durbaba S, Weston C, et al. Does COPD risk vary by ethnicity? A retrospective cross-sectional study. *Int J Chron Obstruct Pulmon Dis*. 2016;11:739-46.
349. Tran HN, Siu S, Iribarren C, Udaltsova N, Klatsky AL. Ethnicity and risk of hospitalization for asthma and chronic obstructive pulmonary disease. *Ann Epidemiol*. 2011;21(8):615-22.
350. Burgel PR, Paillasseur JL, Janssens W, Piquet J, Ter Riet G, Garcia-Aymerich J, et al. A simple algorithm for the identification of clinical COPD phenotypes. *Eur Respir J*. 2017;50(5).

351. Garcia-Aymerich J, Gomez FP, Benet M, Farrero E, Basagana X, Gayete A, et al. Identification and prospective validation of clinically relevant chronic obstructive pulmonary disease (COPD) subtypes. *Thorax*. 2011;66(5):430-7.
352. Kim WJ, Gupta V, Nishimura M, Makita H, Idolor L, Roa C, et al. Identification of chronic obstructive pulmonary disease subgroups in 13 Asian cities. *Int J Tuberc Lung Dis*. 2018;22(7):820-6.
353. Burgel PR, Paillasseur JL, Roche N. Identification of clinical phenotypes using cluster analyses in COPD patients with multiple comorbidities. *Biomed Res Int*. 2014;2014:420134.
354. Chubachi S, Sato M, Kameyama N, Tsutsumi A, Sasaki M, Tateno H, et al. Identification of five clusters of comorbidities in a longitudinal Japanese chronic obstructive pulmonary disease cohort. *Respir Med*. 2016;117:272-9.
355. Raheison C, Ouailaya EH, Bernady A, Casteigt J, Nocent-Eijnani C, Falque L, et al. Comorbidities and COPD severity in a clinic-based cohort. *BMC Pulm Med*. 2018;18(1):117.
356. Ng TP, Niti M, Tan WC. Trends and ethnic differences in COPD hospitalization and mortality in Singapore. *COPD*. 2004;1(1):5-11.
357. Reilly KH, Gu D, Duan X, Wu X, Chen CS, Huang J, et al. Risk factors for chronic obstructive pulmonary disease mortality in Chinese adults. *Am J Epidemiol*. 2008;167(8):998-1004.
358. Lopez AD, Shibuya K, Rao C, Mathers CD, Hansell AL, Held LS, et al. Chronic obstructive pulmonary disease: current burden and future projections. *Eur Respir J*. 2006;27(2):397-412.
359. Bhatt SP, Balte PP, Schwartz JE, Cassano PA, Couper D, Jacobs DR, Jr., et al. Discriminative Accuracy of FEV1:FVC Thresholds for COPD-Related Hospitalization and Mortality. *JAMA*. 2019;321(24):2438-47.
360. Divo M, Cote C, de Torres JP, Casanova C, Marin JM, Pinto-Plata V, et al. Comorbidities and risk of mortality in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2012;186(2):155-61.

361. Morris JF, Koski A, Johnson LC. Spirometric standards for healthy nonsmoking adults. *Am Rev Respir Dis.* 1971;103(1):57-67.
362. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. *Eur Respir J.* 2005;26(5):948-68.
363. Murray PR, Washington JA. Microscopic and bacteriologic analysis of expectorated sputum. *Mayo Clin Proc.* 1975;50(6):339-44.
364. Van Scoy RE. Bacterial sputum cultures. A clinician's viewpoint. *Mayo Clin Proc.* 1977;52(1):39-41.
365. Peay KG, Kennedy PG, Talbot JM. Dimensions of biodiversity in the Earth mycobiome. *Nat Rev Microbiol.* 2016;14(7):434-47.
366. Kirchhof P, Benussi S, Kotecha D, Ahlsson A, Atar D, Casadei B, et al. 2016 ESC Guidelines for the management of atrial fibrillation developed in collaboration with EACTS. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery.* 2016;50(5):e1-e88.
367. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail.* 2016;18(8):891-975.
368. Task Force M, Montalescot G, Sechtem U, Achenbach S, Andreotti F, Arden C, et al. 2013 ESC guidelines on the management of stable coronary artery disease: the Task Force on the management of stable coronary artery disease of the European Society of Cardiology. *Eur Heart J.* 2013;34(38):2949-3003.
369. American Diabetes A. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. *Diabetes Care.* 2018;41(Suppl 1):S13-S27.
370. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet.* 2008;371(9615):838-51.

371. Committee ASoP, Banerjee S, Cash BD, Dominitz JA, Baron TH, Anderson MA, et al. The role of endoscopy in the management of patients with peptic ulcer disease. *Gastrointest Endosc*. 2010;71(4):663-8.
372. Salvi S, Holgate ST. Mechanisms of particulate matter toxicity. *Clin Exp Allergy*. 1999;29(9):1187-94.
373. Sacco RL, Kasner SE, Broderick JP, Caplan LR, Connors JJ, Culebras A, et al. An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2013;44(7):2064-89.
374. Gerhard-Herman MD, Gornik HL, Barrett C, Barshes NR, Corriere MA, Drachman DE, et al. 2016 AHA/ACC Guideline on the Management of Patients With Lower Extremity Peripheral Artery Disease: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation*. 2017;135(12):e686-e725.
375. Hong JY, Kim M, Sol IS, Kim KW, Lee CM, Elias JA, et al. Chitotriosidase inhibits allergic asthmatic airways via regulation of TGF-beta expression and Foxp3(+) Treg cells. *Allergy*. 2018;73(8):1686-99.
376. Lewinsohn DM, Leonard MK, LoBue PA, Cohn DL, Daley CL, Desmond E, et al. Official American Thoracic Society/Infectious Diseases Society of America/Centers for Disease Control and Prevention Clinical Practice Guidelines: Diagnosis of Tuberculosis in Adults and Children. *Clin Infect Dis*. 2017;64(2):111-5.
377. Jung KH, Kim SJ, Shin C, Kim JH. The considerable, often neglected, impact of pulmonary tuberculosis on the prevalence of COPD. *Am J Respir Crit Care Med*. 2008;178(4):431; author reply 2-3.
378. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. *Am J Respir Crit Care Med*. 2000;161(4 Pt 1):1376-95.

379. Compston J, Cooper A, Cooper C, Gittos N, Gregson C, Harvey N, et al. UK clinical guideline for the prevention and treatment of osteoporosis. *Arch Osteoporos*. 2017;12(1):43.
380. Agusti A, Edwards LD, Rennard SI, MacNee W, Tal-Singer R, Miller BE, et al. Persistent systemic inflammation is associated with poor clinical outcomes in COPD: a novel phenotype. *PLoS One*. 2012;7(5):e37483.
381. Ravimohan S, Kornfeld H, Weissman D, Bisson GP. Tuberculosis and lung damage: from epidemiology to pathophysiology. *Eur Respir Rev*. 2018;27(147).
382. Byrne AL, Marais BJ, Mitnick CD, Lecca L, Marks GB. Tuberculosis and chronic respiratory disease: a systematic review. *Int J Infect Dis*. 2015;32:138-46.
383. O'Leary SM, Coleman MM, Chew WM, Morrow C, McLaughlin AM, Gleeson LE, et al. Cigarette smoking impairs human pulmonary immunity to *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med*. 2014;190(12):1430-6.
384. Park HJ, Byun MK, Kim HJ, Ahn CM, Kim DK, Kim YI, et al. History of pulmonary tuberculosis affects the severity and clinical outcomes of COPD. *Respirology*. 2018;23(1):100-6.
385. Oh JY, Lee YS, Min KH, Hur GY, Lee SY, Kang KH, et al. Difference in systemic inflammation and predictors of acute exacerbation between smoking-associated COPD and tuberculosis-associated COPD. *Int J Chron Obstruct Pulmon Dis*. 2018;13:3381-7.
386. Coussens AK, Wilkinson RJ, Nikolayevskyy V, Elkington PT, Hanifa Y, Islam K, et al. Ethnic variation in inflammatory profile in tuberculosis. *PLoS Pathog*. 2013;9(7):e1003468.
387. Pareek M, Evans J, Innes J, Smith G, Hingley-Wilson S, Loughheed KE, et al. Ethnicity and mycobacterial lineage as determinants of tuberculosis disease phenotype. *Thorax*. 2013;68(3):221-9.
388. Kubota Y, Tay WT, Asai K, Murai K, Nakajima I, Hagiwara N, et al. Chronic obstructive pulmonary disease and beta-blocker treatment in Asian patients with heart failure. *ESC Heart Fail*. 2018;5(2):297-305.

389. Ho TW, Huang CT, Ruan SY, Tsai YJ, Lai F, Yu CJ. Diabetes mellitus in patients with chronic obstructive pulmonary disease-The impact on mortality. *PLoS One*. 2017;12(4):e0175794.
390. Mannino DM, Thorn D, Swensen A, Holguin F. Prevalence and outcomes of diabetes, hypertension and cardiovascular disease in COPD. *Eur Respir J*. 2008;32(4):962-9.
391. Khan NA, Wang H, Anand S, Jin Y, Campbell NR, Pilote L, et al. Ethnicity and sex affect diabetes incidence and outcomes. *Diabetes Care*. 2011;34(1):96-101.
392. Josephs L, Culliford D, Johnson M, Thomas M. Improved outcomes in ex-smokers with COPD: a UK primary care observational cohort study. *Eur Respir J*. 2017;49(5).
393. Godtfredsen NS, Lam TH, Hansel TT, Leon ME, Gray N, Dresler C, et al. COPD-related morbidity and mortality after smoking cessation: status of the evidence. *Eur Respir J*. 2008;32(4):844-53.
394. Wu J, Sin DD. Improved patient outcome with smoking cessation: when is it too late? *Int J Chron Obstruct Pulmon Dis*. 2011;6:259-67.
395. Agusti A, Edwards LD, Rennard SI, MacNee W, Tal-Singer R, Miller BE, et al. Persistent systemic inflammation is associated with poor clinical outcomes in COPD: a novel phenotype. *PLoS One*. 2012;7(5):e37483.
396. Kofler S, Nickel T, Weis M. Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clin Sci (Lond)*. 2005;108(3):205-13.
397. Mehra VC, Ramgolam VS, Bender JR. Cytokines and cardiovascular disease. *J Leukoc Biol*. 2005;78(4):805-18.
398. Feldman AM, Combes A, Wagner D, Kadakomi T, Kubota T, Li YY, et al. The role of tumor necrosis factor in the pathophysiology of heart failure. *J Am Coll Cardiol*. 2000;35(3):537-44.
399. Harding JS, Herbath M, Chen Y, Rayasam A, Ritter A, Csoka B, et al. VEGF-A from Granuloma Macrophages Regulates Granulomatous Inflammation by a Non-angiogenic Pathway during Mycobacterial Infection. *Cell Rep*. 2019;27(7):2119-31 e6.

400. Datta M, Via LE, Kamoun WS, Liu C, Chen W, Seano G, et al. Anti-vascular endothelial growth factor treatment normalizes tuberculosis granuloma vasculature and improves small molecule delivery. *Proc Natl Acad Sci U S A*. 2015;112(6):1827-32.
401. Matsuyama W, Hashiguchi T, Matsumuro K, Iwami F, Hirotsu Y, Kawabata M, et al. Increased serum level of vascular endothelial growth factor in pulmonary tuberculosis. *Am J Respir Crit Care Med*. 2000;162(3 Pt 1):1120-2.
402. Alatas F, Alatas O, Metintas M, Ozarslan A, Erginel S, Yildirim H. Vascular endothelial growth factor levels in active pulmonary tuberculosis. *Chest*. 2004;125(6):2156-9.
403. Kumar NP, Banurekha VV, Nair D, Babu S. Circulating Angiogenic Factors as Biomarkers of Disease Severity and Bacterial Burden in Pulmonary Tuberculosis. *PLoS One*. 2016;11(1):e0146318.
404. Harrison MT, Short P, Williamson PA, Singanayagam A, Chalmers JD, Schembri S. Thrombocytosis is associated with increased short and long term mortality after exacerbation of chronic obstructive pulmonary disease: a role for antiplatelet therapy? *Thorax*. 2014;69(7):609-15.
405. Fawzy A, Putcha N, Paulin LM, Aaron CP, Labaki WW, Han MK, et al. Association of thrombocytosis with COPD morbidity: the SPIROMICS and COPDGene cohorts. *Respir Res*. 2018;19(1):20.
406. Noskovicova N, Petrek M, Eickelberg O, Heinzelmann K. Platelet-derived growth factor signaling in the lung. From lung development and disease to clinical studies. *Am J Respir Cell Mol Biol*. 2015;52(3):263-84.
407. Churg A, Tai H, Coulthard T, Wang R, Wright JL. Cigarette smoke drives small airway remodeling by induction of growth factors in the airway wall. *Am J Respir Crit Care Med*. 2006;174(12):1327-34.
408. Raines EW. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev*. 2004;15(4):237-54.
409. Bhatt SP, Balte PP, Schwartz JE, Cassano PA, Couper D, Jacobs DR, Jr, et al. Discriminative Accuracy of FEV1:FVC Thresholds for COPD-Related Hospitalization and

Mortality Discriminative Accuracy of FEV1:FVC Thresholds for COPD-Related Hospitalization and Mortality Discriminative Accuracy of FEV1:FVC Thresholds for COPD-Related Hospitalization and Mortality. *JAMA*. 2019;321(24):2438-47.

410. Puhan MA, Hansel NN, Sobradillo P, Enright P, Lange P, Hickson D, et al. Large-scale international validation of the ADO index in subjects with COPD: an individual subject data analysis of 10 cohorts. *BMJ Open*. 2012;2(6).

411. Cosio MG, Saetta M, Agusti A. Immunologic aspects of chronic obstructive pulmonary disease. *N Engl J Med*. 2009;360(23):2445-54.

412. Hoenderdos K, Condliffe A. The neutrophil in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol*. 2013;48(5):531-9.

413. Itabashi S, Fukushima T, Aikawa T, Yanai M, Sekizawa K, Sasaki H, et al. Allergic sensitization in elderly patients with chronic obstructive pulmonary disease. *Respiration*. 1990;57(6):384-8.

414. Kidon MI, See Y, Goh A, Chay OM, Balakrishnan A. Aeroallergen sensitization in pediatric allergic rhinitis in Singapore: is air-conditioning a factor in the tropics? *Pediatr Allergy Immunol*. 2004;15(4):340-3.

415. Oluwole O, Kirychuk SP, Lawson JA, Karunanayake C, Cockcroft DW, Willson PJ, et al. Indoor mold levels and current asthma among school-aged children in Saskatchewan, Canada. *Indoor air*. 2017;27(2):311-9.

416. Karvonen AM, Hyvarinen A, Korppi M, Haverinen-Shaughnessy U, Renz H, Pfefferle PI, et al. Moisture damage and asthma: a birth cohort study. *Pediatrics*. 2015;135(3):e598-606.

417. Atkinson RW, Strachan DP. Role of outdoor aeroallergens in asthma exacerbations: epidemiological evidence. *Thorax*. 2004;59(4):277-8.

418. Pulimood TB, Corden JM, Bryden C, Sharples L, Nasser SM. Epidemic asthma and the role of the fungal mold *Alternaria alternata*. *J Allergy Clin Immunol*. 2007;120(3):610-7.

419. Chew FT, Lim SH, Shang HS, Dahlia MD, Goh DY, Lee BW, et al. Evaluation of the allergenicity of tropical pollen and airborne spores in Singapore. *Allergy*. 2000;55(4):340-7.

420. Gusareva ES, Acerbi E, Lau KJX, Luhung I, Premkrishnan BNV, Kolundzija S, et al. Microbial communities in the tropical air ecosystem follow a precise diel cycle. *Proc Natl Acad Sci U S A*. 2019;116(46):23299-308.
421. Sánchez-Muñoz M, Muñoz-Vicente M, Cobas G, Portela R, Amils R, Sánchez B. Comparison of three high-flow single-stage impaction-based air samplers for bacteria quantification: DUO SAS SUPER 360, SAMPL’AIR and SPIN AIR. *Analytical Methods*. 2012;4.
422. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. 1999;41:95-8.
423. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997;25(17):3389-402.
424. Kidon MI, Chiang WC, Liew WK, Ong TC, Tiong YS, Wong KN, et al. Mite component-specific IgE repertoire and phenotypes of allergic disease in childhood: the tropical perspective. *Pediatr Allergy Immunol*. 2011;22(2):202-10.
425. Chan SL, Ong TC, Gao YF, Tiong YS, Wang de Y, Chew FT, et al. Nuclear magnetic resonance structure and IgE epitopes of Blo t 5, a major dust mite allergen. *J Immunol*. 2008;181(4):2586-96.
426. Gao YF, Wang de Y, Ong TC, Tay SL, Yap KH, Chew FT. Identification and characterization of a novel allergen from *Blomia tropicalis*: Blo t 21. *J Allergy Clin Immunol*. 2007;120(1):105-12.
427. Batard T, Hrabina A, Bi XZ, Chabre H, Lemoine P, Couret MN, et al. Production and proteomic characterization of pharmaceutical-grade *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* extracts for allergy vaccines. *Int Arch Allergy Immunol*. 2006;140(4):295-305.
428. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. . *EMBnet Journal*. 2011;17:10-2.

429. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. *Genome research*. 2007;17(3):377-86.
430. Pomes A, Davies JM, Gadermaier G, Hilger C, Holzhauser T, Lidholm J, et al. WHO/IUIS Allergen Nomenclature: Providing a common language. *Molecular immunology*. 2018;100:3-13.
431. Paradis E, Schliep K. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*. 2019;35(3):526-8.
432. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;9(4):357-9.
433. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-9.
434. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*. 2010;26(6):841-2.
435. Kaminski J, Gibson MK, Franzosa EA, Segata N, Dantas G, Huttenhower C. High-Specificity Targeted Functional Profiling in Microbial Communities with ShortBRED. *PLoS computational biology*. 2015;11(12):e1004557.
436. Kurup VP, Banerjee B, Hemmann S, Greenberger PA, Blaser K, Cramer R. Selected recombinant *Aspergillus fumigatus* allergens bind specifically to IgE in ABPA. *Clin Exp Allergy*. 2000;30(7):988-93.
437. Agarwal R, Hazarika B, Gupta D, Aggarwal AN, Chakrabarti A, Jindal SK. *Aspergillus* hypersensitivity in patients with chronic obstructive pulmonary disease: COPD as a risk factor for ABPA? *Med Mycol*. 2010;48(7):988-94.
438. Atkinson RW, Strachan DP, Anderson HR, Hajat S, Emberlin J. Temporal associations between daily counts of fungal spores and asthma exacerbations. *Occup Environ Med*. 2006;63(9):580-90.
439. Delfino RJ, Zeiger RS, Seltzer JM, Street DH, Matteucci RM, Anderson PR, et al. The effect of outdoor fungal spore concentrations on daily asthma severity. *Environmental health perspectives*. 1997;105(6):622-35.

440. O'Hollaren MT, Yunginger JW, Offord KP, Somers MJ, O'Connell EJ, Ballard DJ, et al. Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. *N Engl J Med*. 1991;324(6):359-63.
441. Sharpe RA, Bearman N, Thornton CR, Husk K, Osborne NJ. Indoor fungal diversity and asthma: a meta-analysis and systematic review of risk factors. *J Allergy Clin Immunol*. 2015;135(1):110-22.
442. Viswanathan RK, Mathur SK. Role of allergen sensitization in older adults. *Curr Allergy Asthma Rep*. 2011;11(5):427-33.
443. Havstad SL, Johnson CC, Zoratti EM, Ezell JM, Woodcroft K, Ownby DR, et al. Tobacco smoke exposure and allergic sensitization in children: a propensity score analysis. *Respirology*. 2012;17(7):1068-72.
444. Lannero E, Wickman M, van Hage M, Bergstrom A, Pershagen G, Nordvall L. Exposure to environmental tobacco smoke and sensitisation in children. *Thorax*. 2008;63(2):172-6.
445. Garnacho-Montero J, Amaya-Villar R, Ortiz-Leyba C, Leon C, Alvarez-Lerma F, Nolla-Salas J, et al. Isolation of *Aspergillus* spp. from the respiratory tract in critically ill patients: risk factors, clinical presentation and outcome. *Crit Care*. 2005;9(3):R191-9.
446. Palmer LB, Greenberg HE, Schiff MJ. Corticosteroid treatment as a risk factor for invasive aspergillosis in patients with lung disease. *Thorax*. 1991;46(1):15-20.
447. Bulpa P, Dive A, Sibille Y. Invasive pulmonary aspergillosis in patients with chronic obstructive pulmonary disease. *Eur Respir J*. 2007;30(4):782-800.
448. Nash AK, Auchtung TA, Wong MC, Smith DP, Gesell JR, Ross MC, et al. The gut mycobiome of the Human Microbiome Project healthy cohort. *Microbiome*. 2017;5(1):153.
449. Delhaes L, Monchy S, Frealle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community--implications for therapeutic management. *PLoS One*. 2012;7(4):e36313.

450. Fraczek MG, Chishimba L, Niven RM, Bromley M, Simpson A, Smyth L, et al. Corticosteroid treatment is associated with increased filamentous fungal burden in allergic fungal disease. *J Allergy Clin Immunol*. 2018;142(2):407-14.
451. Pragman AA, Kim HB, Reilly CS, Wendt C, Isaacson RE. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *PLoS One*. 2012;7(10):e47305.
452. Einarsson GG, Comer DM, McIlreavey L, Parkhill J, Ennis M, Tunney MM, et al. Community dynamics and the lower airway microbiota in stable chronic obstructive pulmonary disease, smokers and healthy non-smokers. *Thorax*. 2016;71(9):795-803.
453. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG. Sputum *Candida albicans* presages FEV(1) decline and hospital-treated exacerbations in cystic fibrosis. *Chest*. 2010;138(5):1186-95.
454. Coughlan CA, Chotirmall SH, Renwick J, Hassan T, Low TB, Bergsson G, et al. The effect of *Aspergillus fumigatus* infection on vitamin D receptor expression in cystic fibrosis. *American journal of respiratory and critical care medicine*. 2012;186(10):999-1007.
455. White TJB, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA Genes for Phylogenetics. Elsevier Inc: Amsterdam. 1990:315-22.
456. Usyk M, Zolnik CP, Patel H, Levi MH, Burk RD. Novel ITS1 Fungal Primers for Characterization of the Mycobiome. *mSphere*. 2017;2(6).
457. Nilsson RH, Anslan S, Bahram M, Wurzbacher C, Baldrian P, Tedersoo L. Mycobiome diversity: high-throughput sequencing and identification of fungi. *Nat Rev Microbiol*. 2019;17(2):95-109.
458. Ihrmark K, Bodeker IT, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, et al. New primers to amplify the fungal ITS2 region--evaluation by 454-sequencing of artificial and natural communities. *FEMS microbiology ecology*. 2012;82(3):666-77.
459. Ward TL, Dominguez-Bello MG, Heisel T, Al-Ghalith G, Knights D, Gale CA. Development of the Human Mycobiome over the First Month of Life and across Body Sites. *mSystems*. 2018;3(3).

460. Heisel T, Podgorski H, Staley CM, Knights D, Sadowsky MJ, Gale CA. Complementary amplicon-based genomic approaches for the study of fungal communities in humans. *PLoS One*. 2015;10(2):e0116705.
461. Ali N, Mac Aogain M, Morales RF, Tiew PY, Chotirmall SH. Optimisation and Benchmarking of Targeted Amplicon Sequencing for Mycobiome Analysis of Respiratory Specimens. *International journal of molecular sciences*. 2019;20(20).
462. Koljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, et al. Towards a unified paradigm for sequence-based identification of fungi. *Mol Ecol*. 2013;22(21):5271-7.
463. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007;73(16):5261-7.
464. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*. 2018;6(1):226.
465. Hennig C. Dissolution point and isolation robustness: Robustness criteria for general cluster analysis methods. *Journal of Multivariate Analysis*. 2008;99(6):1154-76.
466. Faust K, Sathirapongsasuti JF, Izard J, Segata N, Gevers D, Raes J, et al. Microbial co-occurrence relationships in the human microbiome. *PLoS computational biology*. 2012;8(7):e1002606.
467. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*. 2003;13(11):2498-504.
468. Huseyin CE, O'Toole PW, Cotter PD, Scanlan PD. Forgotten fungi-the gut mycobiome in human health and disease. *FEMS microbiology reviews*. 2017;41(4):479-511.
469. Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC microbiology*. 2010;10:189.

470. Op De Beeck M, Lievens B, Busschaert P, Declerck S, Vangronsveld J, Colpaert JV. Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PLoS One*. 2014;9(6):e97629.
471. Tedersoo L, Lindahl B. Fungal identification biases in microbiome projects. *Environ Microbiol Rep*. 2016;8(5):774-9.
472. Yang RH, Su JH, Shang JJ, Wu YY, Li Y, Bao DP, et al. Evaluation of the ribosomal DNA internal transcribed spacer (ITS), specifically ITS1 and ITS2, for the analysis of fungal diversity by deep sequencing. *PLoS One*. 2018;13(10):e0206428.
473. Pragman AA, Knutson KA, Gould TJ, Isaacson RE, Reilly CS, Wendt CH. Chronic obstructive pulmonary disease upper airway microbiota alpha diversity is associated with exacerbation phenotype: a case-control observational study. *Respir Res*. 2019;20(1):114.
474. Kim SH, Clark ST, Surendra A, Copeland JK, Wang PW, Ammar R, et al. Global Analysis of the Fungal Microbiome in Cystic Fibrosis Patients Reveals Loss of Function of the Transcriptional Repressor Nrg1 as a Mechanism of Pathogen Adaptation. *PLoS Pathog*. 2015;11(11):e1005308.
475. Willger SD, Grim SL, Dolben EL, Shipunova A, Hampton TH, Morrison HG, et al. Characterization and quantification of the fungal microbiome in serial samples from individuals with cystic fibrosis. *Microbiome*. 2014;2:40.
476. Kramer R, Sauer-Heilborn A, Welte T, Guzman CA, Abraham WR, Hofle MG. Cohort Study of Airway Mycobiome in Adult Cystic Fibrosis Patients: Differences in Community Structure between Fungi and Bacteria Reveal Predominance of Transient Fungal Elements. *J Clin Microbiol*. 2015;53(9):2900-7.
477. Charlson ES, Diamond JM, Bittinger K, Fitzgerald AS, Yadav A, Haas AR, et al. Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant. *Am J Respir Crit Care Med*. 2012;186(6):536-45.
478. van Woerden HC, Gregory C, Brown R, Marchesi JR, Hoogendoorn B, Matthews IP. Differences in fungi present in induced sputum samples from asthma patients and non-atopic controls: a community based case control study. *BMC Infect Dis*. 2013;13:69.

479. Binte Mohamed Ali NA, Ivan FX, Aogain MM, Narayana JK, Lee SY, Lim CL, et al.
The Healthy Airway Mycobiome in Individuals of Asian Descent. *Chest*. 2020.

APPENDIX: PUBLICATIONS AND AWARD