

RSV Associated COPD Exacerbation Prevalence and Severity

A thesis submitted to Imperial College London for the degree of Doctor of Philosophy by

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Declaration of Originality

All the work presented in this thesis was carried out by myself, as part of my PhD at Imperial College London, unless stated and referenced in the text.

Specifically, I booked and undertook almost all the participant visits, collecting the samples and measurements during these visits. I recruited nearly all the patients into the study, using various methods, including advertisements, with the EXCEL London COPD cohort being used as a foundation for these recruitments. I was assisted by Dr Andrew Richie, Dr Mairi Macleod, Dr Lydia Finney, Dr Faisal Kamal and Julie Gent with visits and sample collection. Professor Wisia Wedzicha and Professor Gavin Donaldson oversaw the running of the study.

Dr Ryan Thwaites, Ashley Sanchez Sevilla Uruchurtu and I undertook all the Pentaplex experiments. I was involved with conducting all these experiments and learnt the technique from Ashley Sanchez Sevilla Uruchurtu.

Professor Peter Openshaw helped formulate a plan of analysis with the serological RSV data.

GSK kindly undertook the PCR analysis unless otherwise stated. The results were sent to me for analysis.

Jansen kindly undertook the Pre + Post F experiments. The results were sent to me for analysis.

The Royal Brompton hospital laboratory kindly undertook the microbiology, blood examination (for FBC, U/E's, CRP etc) and performed the hospital respiratory virus PCR panel.

Professor Gavin Donaldson assisted with the statistical analysis of this thesis.

Dr Hassan Shahbakhti and Dr Raymond Sapsford assisted with sample processing and storage as managers of Professor Wedzicha's laboratory. Dr Hassan Shahbakhti aided with development of the in-house ELISA as described in the methods chapter.

Prof Van Den Berg and his team at Groningen undertook the patient visits and data collection at the Groningen site. They also sent the samples for Pre + Post F ELISA analysis to

Janssen and samples to GSK for PCR analysis. The samples from Groningen and Imperial were tested together in the same batch. Dr Ben Ditz was involved with collecting the data from the cohort at the Groningen site.

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Morris T, Carswell O, Tate J et al. 2023. Policy priorities for protecting adults at risk of severe illness from RSV. London: The Health Policy Partnership⁶

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Abstracts Arising from this Thesis

Immunological and Inflammatory Biomarkers of Susceptibility and Severity in Adult Respiratory Syncytial Virus (RSV) Infections⁷ – ATS 2020 Poster

Respiratory Syncytial Virus (RSV) Detection Is Associated with an Increased Inflammatory Response in Stable (non-Exacerbating) Chronic Obstructive Pulmonary Disease (COPD) Patients⁸ – ATS 2019 Poster (won Assembly on Clinical Problems COPD Abstract Scholarship Award)

Comparison of Paper and Tablet Based App Diary Cards in COPD Patients⁹ – ATS 2019 Poster (not first author)

Activity Monitoring in COPD: A Feasibility Study¹⁰ – ATS 2019 Poster (not first author)

Use of a Sample Tracking Log as a Measure of Chronic Bronchitis¹¹ – ATS 2020 Poster (not first author)

Duration of Persistent Sputum Production Is Related to Lung Function Decline¹² – ATS 2020 (not first Author)

Relationship of cardiac biomarkers in COPD to exacerbation and hyperinflation¹³ – ERS 2020 (not first author)

Abstract

Chronic Obstructive Pulmonary Disease (COPD) is an increasing global problem, often with devastating consequences for those affected, their families and communities. Respiratory Syncytial Virus (RSV) is a common respiratory pathogen, increasingly recognised as a scourge not only of children but also of frail older adults. The aim of my work was to investigate the role of RSV in exacerbations of COPD in a well-characterised cohort of patients. I also investigated the severity of RSV associated exacerbations as compared to other exacerbation causes.

One hundred and seventy-seven patients were recruited into the study at the Imperial site and followed for an average of 748 days, giving a total of 296.8 patient-years of data. Virus associated exacerbations were detected by PCR analysis of nasopharyngeal swabs and sputum, and by serological investigations. A novel pentaplex RSV antibody test was used for the first time in a clinical study of this kind.

RSV infection was identified in 32 exacerbations, 7.4% of the total. Thirty of these RSV-associated exacerbations were detected using the new serological method; 3 were discovered by PCR of 175 nasopharyngeal specimens and 5 by PCR of 93 sputum samples taken at exacerbation. In total, only 2 additional RSV-associated exacerbations were found by PCR, calling into question the validity and justification for the direct viral detection in studies of this kind.

26 attended exacerbations were identified as being Rhinovirus related, 9 flu related, 18 RSV related, 24 bacterial related, 16 related to other viruses and 15 having both viruses and bacteria present. 64 attended exacerbations were without any viruses or bacteria detected.

When investigating the exacerbation severity, RSV associated exacerbations had a higher trend to CRP and CAT score change, but a shorter duration of illness, of 10.3 days. The duration of illness difference was statistically significant to all other exacerbation causes (10.3 vs 20.48 $p < 0.05$ using Dunn's test).

In conclusion, RSV is a significant cause of COPD exacerbations. The introduction of a safe and effective RSV vaccines will, it is hoped, soon show the benefits of preventing RSV disease in vulnerable older adults with chronic respiratory disease.

Abbreviations

Ab	Antibody
AECOPD	Acute Exacerbation of Chronic Obstructive Pulmonary Disease
ATS	American Thoracic Society
BAL	Bronchoalveolar Lavage
BP	Blood Pressure
CAT	COPD Assessment Test
cDNA	Complimentary Deoxyribose Nucleic Acid
COPD	Chronic Obstructive Pulmonary Disease
COPD GOLD	Global Initiative for Chronic Obstructive Lung Disease
COVID-19	Coronavirus Disease 2019
CT	Computer Tomography
CXCL-10	C-X-C Motif Chemokine Ligand 10
DNA	Deoxyribose Nucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
ECDC	European Centre for Disease Control
ECG	Electrocardiogram
ECRF	Electronic Case Report Form
ELISA	Enzyme-linked Immunosorbent Assay
ERS	European Respiratory Society
FBC	Full Blood Count
FEV1	Forced Expiratory Volume in 1 Second
FVC	Forced Vital Capacity
Ga Ab	RSV Ga protein specific Antibody
Gb Ab	RSV Gb protein specific Antibody
GDPR	General Data Protection Regulation
GSK	Glaxo-Smith-Kline
Hba1c	Glycated Hemoglobin
HMV	Human Metapneumovirus

HPA	Health Protection Authority
HR	Heart Rate
HRV	Human Rhinovirus
ICS	Inhaled Corticosteroids
Ig	Immunoglobulin
Ig A	Immunoglobulin A
Ig G	Immunoglobulin G
IL	Interleukin
IQR	Inter-quartile range
IRAS	Integrated Research Application System
LFT	Liver Function Tests
LIMIC	Lower + Middle Income Country
MPO	Human Myeloperoxidase
N Ab	RSV N protein specific Antibody
NHLI	National Heart and Lung Institute
NHS	National Health Service
NP	Naso-Pharyngeal
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIS	Patient Information Sheet
PIV	Parainfluenza Viruses
Post-F Ab	RSV Post-F protein specific Antibody
PPE	Personal Protective Equipement
PPM	Potentially Pathogenic Microbes
Pre-F Ab	RSV Pre-F protein specific Antibody
QLAY	Quality-Adjusted Life Years
qPCR	Quantitative Polymerase Chain Reaction
RBH	Royal Brompton Hospital
REC	Research Ethics Committee
RESCEU	REspiratory Syncytial virus Consortium in EUrope
RIVM	The Dutch National Institute for Public Health and the Environment

RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
SATS	Oxygen Saturations (Peripheral)
SGRQ	St. George's Respiratory Questionnaire
SP-D	Surfactant protein D
TFT	Thyroid Function Tests
TNF- α	Tumour Necrosis Factor Alpha
U/E	Urea and Electrolytes
WP	Work Package

Table 1 - Abbreviations

Contents

1	Introduction	25
1.1	Overview	25
1.2	COPD.....	27
1.2.1	Definition of COPD	27
1.2.2	Prevalence, and disease burden	27
1.3	COPD exacerbations.....	28
1.3.1	COPD Exacerbation Definition	28
1.3.2	Clinical importance	28
1.3.3	Risk factors.....	30
1.3.4	Causes	30
1.3.5	Microbiome in COPD Exacerbations	32
1.4	Biomarkers in AECOPD:.....	32
1.4.1	Common Biomarkers	33
1.4.2	Biomarkers For Bacterial Associated AECOPD:.....	35
1.4.3	Biomarkers For Sputum Eosinophilia Associated AECOPD:.....	35
1.4.4	Biomarkers For Frequent Exacerbators (defined here as 2 or more per year):	36
1.5	RSV.....	36
1.5.1	RSV Structure	37
1.5.2	RSV in COPD	37
1.5.3	RSV in Children.....	38
1.5.4	Symptoms of RSV in Adults.....	39
1.5.5	Seasonality of RSV in the UK.....	42
1.5.6	RSV Immunology in older Adults	44
1.6	Hypothesis, aims and Objectives	45
1.6.1	Hypothesis.....	45

1.6.2	Aims + Objectives.....	45
1.7	Summary	46
2	Methods.....	47
2.1	Recruitment.....	47
2.1.1	Recruitment Goals and Power Calculation	47
2.1.2	Initial Contact with Potential Participant.....	47
2.1.3	Telephone Screening	47
2.1.4	Recruitment Visit	48
2.1.5	Future Visits	51
2.1.6	Home Diary Cards and Exacerbation Reporting	51
2.1.7	Exacerbations.....	51
2.2	Regulatory permissions and consent	53
2.3	Sample Processing and Hard-Copy Storage:	53
2.3.1	Sample Processing	53
2.3.2	Study Data Storage	54
2.3.3	Sample Storage	55
2.4	Pentaplex.....	56
2.4.1	Equipment + Reagents Needed	56
2.4.2	Preparation of Reagents and Samples.....	57
2.4.3	Preparing the plate	59
2.4.4	Plate Incubation	60
2.4.5	Reading the plates:	60
2.4.6	Example of processed pentaplex plate with raw results:.....	61
2.5	ELISA (In-House Designed):	62
2.5.1	Equipment needed:	62
2.5.2	Preparation of reagents	62

2.5.3	Incubation and Development of Plates	64
2.5.4	Plate Data Analysis.....	65
2.5.5	Example of processed ELISA plate with raw results	65
2.6	Janssen ELISA.....	66
2.7	Multiplex PCR and RSV qPCR (sent to GSK).....	66
2.8	In-House qPCR (used in Pilot data)	66
2.8.1	Sputum Preparation and RNA Extraction	66
2.8.2	cDNA Conversion	66
2.8.3	qPCR Analysis	66
2.9	Statistical analysis.....	67
2.10	Groningen Protocols.....	67
2.11	Summary:	67
3	Literature Search.....	68
3.1	Introduction.....	68
3.2	Methods	69
3.3	Results	70
3.3.1	Humoral Immunity:.....	72
3.3.2	Cellular Immunity:.....	74
3.3.3	Viral Load:	76
3.3.4	Innate Inflammatory Response:	76
3.4	Biomarkers of RSV Severity and Susceptibility in Adults - Discussion	78
4	Preliminary Data – RSV in the Non-Exacerbating State.....	84
4.1	Methods:	84
4.2	Results:	85
4.3	Summary:	89
5	Results.....	90

5.1	Recruitment:.....	90
5.1.1	Recruitment Introduction:	90
5.1.2	Recruitment Methods and Success Rates:	90
5.1.3	Recruitment Method Success Overview:.....	94
5.2	Recruitment results:.....	94
5.3	Visit Statistics:	96
5.4	NP swab and Sputum Results.....	96
5.4.1	Samples Tested for viral PCR:	96
5.4.2	PCR Results (GSK):.....	97
5.4.3	PCR RSV Results (Royal Brompton PCR):	100
5.4.4	RBH Sputum Bacteriology Results:	102
5.5	RSV Antibody measurements:.....	104
5.5.1	Pentaplex Specific Antibody measurements:	104
5.5.2	PCR Positive Cases Pentaplex results:.....	105
5.5.3	N Antibody rises:.....	107
5.6	Pentaplex Results Validation:.....	109
5.6.1	RSV vs Rhinovirus associated Exacerbations	109
5.6.2	Pentaplex Results:.....	115
5.7	PCR vs Serological diagnosis of RSV associated events:	116
5.8	RIVM Pentaplex and Janssen ELISA:.....	117
5.8.1	Time to Recovery – RSV vs Rhinovirus.....	120
5.8.2	FEV1% change Analysis – RSV vs Rhinovirus.....	121
5.8.3	Other Markers of Severity – RSV vs Rhinovirus	122
5.9	RSV vs All Other Exacerbation Causes:.....	126
5.9.1	Plan of analysis.....	126
5.9.2	Time to Recovery Analysis – RSV vs All Causes:.....	127

5.9.3	FEV1 Change Analysis – RSV vs All Causes:.....	131
5.9.4	Other Markers of Severity Analysis – RSV vs All Causes:.....	133
5.10	RSV Cases and Exacerbation Frequency:	136
5.10.1	Why is COPD Exacerbation Frequency important in RSV related exacerbations? 136	
5.10.2	Plan of analysis.....	137
5.10.3	Results – RSV related Exacerbations vs Other exacerbation Causes.....	137
5.10.4	Rhinovirus Cases Vs Exacerbation Frequency:	138
5.10.5	Difference in Exacerbation Frequency – RSV and Rhinovirus	139
5.11	Infrequent / Frequent Exacerbators - RSV and Rhinovirus:.....	140
5.12	Other Associations Between RSV and Rhinovirus Related Exacerbations.....	141
5.12.1	Smoking History Vs RSV and Rhinovirus:	141
5.12.2	Lung Function Vs RSV and Rhinovirus:.....	141
5.13	Overall RSV numbers at Imperial:	141
5.14	Summary	143
6	Results from Groningen and Imperial Cohorts	144
6.1	Introduction.....	144
6.2	Demographics.....	144
6.3	RSV Positivity Data	146
6.4	Totals Across Both Cohorts	146
6.5	Other Virus Positivity Data (From Multiplex PCR)	148
6.6	Conclusions.....	150
6.7	Summary	151
7	Discussion.....	152
7.1	Why RSV matters in older adults and why Diagnosis is Important.....	152
7.2	Diagnosis of RSV in adults – an impossible task? Lessons learned from this study	153

7.3	RSV and development of COPD.....	156
7.4	Comparing RSV Related COPD Exacerbations to Rhinovirus Related	157
7.5	RSV severity compared to other exacerbation causes: Is RSV or Rhinovirus the Outlier?	158
7.6	Exacerbation Frequency and viral susceptibility.....	159
7.7	Biomarkers	159
7.8	Other findings from the Results	160
7.8.1	Recruitment	160
7.8.2	Baseline (non-exacerbating) Samples.....	160
7.9	Limitations of the Study	161
7.10	RSV Vaccines – is this the future?	163
7.11	Extrapolation of Imperial Results.....	166
7.12	RSV in a Post Covid-19 world	166
7.13	Ideas For Future Research Arising from this Thesis	167
8	References	168
9	Appendix	191
9.1	Demographics from Chapter 3	191
9.2	Example Diary Card	192
9.3	Annual Review Form	194
9.5	Exacerbation Form	198
9.7	Sputum Processing: STANDARD OPERATING PROCEDURE.....	200
9.8	CAT Questionnaire	202
9.9	Recruitment Advert.....	203

List of Figures

Figure 1 - COPD Exacerbation cartoon designed by Dr Dexter Wiseman, an original Illustration by Mr Benjamin Windibank (The snowball represents recurrent AECOPD episodes, and the increased morbidity associated with these events. The falling snow represents triggers for exacerbation.)	29
Figure 2 - RSV Viral Structure (created in BioRender)	37
Figure 3 – Symptom prevalence for 5 common respiratory viruses in adults from Influenza-65 trial ¹⁰⁰ . *Coronaviruses pre Covid-19.....	41
Figure 4 – RSV cases in <1 years old vs 15+ years old by percentage of positive cases. Data from HPA and NHS Laboratories.....	42
Figure 5 – RSV cases in <1 year olds vs 15+ years old – total cases reported	43
Figure 6 – Plate Layout for Pentaplex.....	57
Figure 7 - Example of Pentaplex Readings.....	61
Figure 8 – Plate Layout for ELISA	64
Figure 9 - Example of processed ELISA plate with raw results	65
Figure 10 – Search Results - adapted from Wiseman, D.J. et al ¹	71
Figure 11 – Serum fibrinogen level at RSV -ve (n=48) vs RSV +ve (n=17) baseline visits. There are three outlying levels in the sputum positive group (P=0.0332 using an unpaired T-Test)87	87
Figure 12 – Log10 Serum CRP at RSV -ve (n=48) vs RSV +ve (n=17) baseline visits. There are four outlying levels in the sputum positive group. (P=0.0383 using an unpaired T-Test)	88
Figure 13 – Discover Register Recruitment	92
Figure 14 – Overall Recruitment Success Rates by Method	94
Figure 15 – Study Completion Numbers.....	96
Figure 16 – NP Exacerbation Swabs Viral PCR Results (GSK).....	98
Figure 17 – Exacerbation Sputum Viral PCR Result (GSK).....	99
Figure 18 – NP Exacerbation Swabs Viral PCR Results (RBH)	101
Figure 19 – Sputum Microbiology Results	103
Figure 20 – 4-fold cumulative antibody rises for ‘positive’ results	105
Figure 21 – Fold change (x-axis) vs time in days (y-axis) for participant 1. Day 0 is when PCR was positive for RSV.....	106

Figure 22 – Fold change (x-axis) vs time in days (y-axis) for participant 2. Day 0 is when PCR was positive for RSV.....	107
Figure 23 – Fold change (x-axis) vs time in days (y-axis) for participant 3. Day 0 is when PCR was positive for RSV.....	107
Figure 24 – Fold change for RSV specific protein N Ab for 5 participants with positive PCR for RSV. Day 0 is day of positive PCR test.....	108
Figure 25 – Fold change in RSV specific Pre-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.011 using Mann Whitney U-Test (RSV n=5 Rhino n=14)	111
Figure 26 – Fold change in RSV specific Post-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.011 using Mann Whitney U-Test. 0=Rhinovirus associated Exacerbations, 1=RSV associated Exacerbations. (RSV n=5 Rhino n=14).....	112
Figure 27 – Fold change in RSV specific N protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.006 using Mann Whitney U-Test (RSV n=5 Rhino n=14)	113
Figure 28 – Fold change in RSV specific Pre-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.339 using Mann Whitney U-Test (RSV n=5 Rhino n=14)	114
Figure 29 – Fold change in RSV specific Pre-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.167 using Mann Whitney U-Test (RSV n=5 Rhino n=14)	115
Figure 30 – Time to recovery (days) in RSV associated exacerbations by diagnostic method. P=0.947 using Mann Whitney U-Test (serology diagnosis n=13 PCR diagnosis n=5).....	116
Figure 31 – FEV1 (%) Change in RSV associated exacerbations by diagnostic method. P=0.947 using Mann Whitney U-Test (serology diagnosis n=13 PCR diagnosis n=5).....	117
Figure 32 – Bland Altman Plot showing the agreement of Pre-F measurements between the Janssen and Pentaplex methods (data log transformed before analysis). $\log_{10} \text{janssenelisa} = \text{Janssen data}$ $\log_{10} \text{pentaplex} = \text{pentaplex data}$	119
Figure 33 – Bland Altman Plot showing the agreement of Post-F measurements between the Janssen and Pentaplex methods (data log transformed before analysis). $\log_{10} \text{janssenelisa} = \text{Janssen data}$ $\log_{10} \text{pentaplex} = \text{pentaplex data}$ Severity Analysis - Rhinovirus Vs RSV	120
Figure 34 – Time to Recovery (days) RSV associated exacerbation Vs Rhinovirus associated Exacerbation. P=0.021 using Mann Whitney U-Test (RSV n=18 Rhinovirus n=14)	121
Figure 35 – FEV1 (%) Change in RSV associated exacerbation Vs Rhinovirus associated Exacerbation. P=0.138 using Mann Whitney U-Test (RSV n=15 Rhinovirus n=14)	122

Figure 36 – CRP (mg/L) in RSV associated exacerbation Vs Rhinovirus associated Exacerbation. P=0.41 using Mann Whitney U-Test (RSV n=18 Rhinovirus n=14)	123
Figure 37 – Fibrinogen (g/L) RSV associated exacerbation Vs Rhinovirus associated Exacerbation. P=0.64 using Mann Whitney U-Test (RSV n=18 Rhinovirus n=14)	124
Figure 38 – CAT Score Change in RSV associated exacerbation Vs Rhinovirus associated Exacerbation. P=0.391 using Mann Whitney U-Test (RSV n=18 Rhinovirus n=14)	125
Figure 39 – Time to Recovery (days) for different exacerbation causes	129
Figure 40 – Days to recovery for exacerbation groupings (none n=64 virus n=69 bacteria n=24 virus + bacteria n=15)	131
Figure 41 - FEV1 (%) Change during RSV associated exacerbations vs other exacerbation causes.....	133
Figure 42 – CRP (mg/L) during RSV associated exacerbations vs other exacerbation causes	134
Figure 43 - CAT score change during RSV associated exacerbations vs other exacerbation causes.....	135
Figure 44 - Exacerbation Frequency over the course of the study in participants who had an RSV associated exacerbation vs those that had an exacerbation by another pathogen or unknown pathogen. P=0.656 using Mann Whitney Test (all other n=146 RSV n=32)	137
Figure 45 - Exacerbation Frequency over the course of the study in participants who had RSV diagnosed vs those that never did. P=0.003 using Mann Whitney Test (all other n=111 RSV n=31)	138
Figure 46 - Exacerbation Frequency over the course of the study in participants who had Rhinovirus diagnosed vs those that never did. P=0.003 using Mann Whitney Test (ever rhinovirus n=24 never rhinovirus n=118)	139
Figure 47 - Exacerbation Frequency over the course of the study in participants who had RSV diagnosed vs those that never did (those participants that never exacerbated excluded). P=0.174 using Mann Whitney U-Test	140
Figure 48 – Total RSV associated exacerbation events diagnosed during the study	142
Figure 49 - Graph showing PCR positivity from NP swabs at exacerbation, Groningen in orange, Imperial in blue. Imperial (N=175) Groningen (N=122)	148
Figure 50 - Graph showing PCR positivity from Sputum Samples at exacerbation, Groningen in orange, Imperial in blue. Imperial (N=93) Groningen (N=22).....	149

Figure 51- RSV Totals Across Both Cohorts by Diagnostic Method	151
Figure 52 Vaccine and mAB snapshot indicating progress of vaccine and mAB candidates. Published online by PATH (as of January 2023) – permission to include sought.....	165
Figure 53 - Front of Diary Card giving an example of an exacerbation	192
Figure 54 – Rear of Diary Card	193
Figure 55 – CAT Score Questionnaire	202
Figure 56 - Recruitment Advert	203

List of Tables

Table 1 - Abbreviations	13
Table 2 – List of equipment and reagents needed for the Pentaplex assay	57
Table 3 – List of equipment and reagents needed for the in-house ELISA	62
Table 4– Eligibility and Exclusion Criteria – adapted from Wiseman, D.J. et al ¹	70
Table 5 – adapted from Wiseman DJ et al ¹ : Study Characteristics	79
Table 6 – adapted from Wiseman DJ et al ¹ : Summary of RSV Biomarkers in Adults	81
Table 7 – Limitations With Current Research and Future Research Options	83
Table 8– Cohort Demographics	95
Table 9 – Cohen’s Kappa agreement of Pentaplex vs Janssen methods for assessing fold change in Pre-f and Post-F RSV specific protein antibodies	118
Table 10 - Kruskal-Wallis and Dunn’s test to give significance of time to recovery (days) in RSV associated exacerbations vs other exacerbation causes	128
Table 11 - – Mean days to recovery for different exacerbation causes	130
Table 12 - - Kruskal-Wallis and Dunn’s test to give significance of FEV1 (%) Change in RSV associated exacerbations vs other exacerbation causes	132
Table 13 - FEV1 (%) Change in RSV associated exacerbations vs other exacerbation causes	132
Table 14 - Markers of Exacerbation severity by Exacerbation Cause Kruskal-Wallis and Dunn’s test to give significance. If significance when compared to RSV related exacerbations, these are highlighted in bold. Data shown as Median + IQR	136
Table 15 – Demographics of Imperial participants against Groningen participants with statistical difference measured	145
Table 16 - Demographics for the patients in Chapter 3 – taken from F. Kamal et al 2021 ⁴ ..	191
Table 17 – Annual Review Form	197
Table 18 – Exacerbation Form	199

1 Introduction

1.1 Overview

The overall objective of my PhD is to investigate the prevalence and role at exacerbation of respiratory syncytial virus (RSV) in patients with chronic obstructive pulmonary disease (COPD). This thesis will mainly focus on RSV prevalence, diagnostics and investigating disease severity in this particular cohort of patients.

Previous studies have investigated the prevalence of RSV at COPD exacerbation, and the reported rates vary between 1% and 40%¹⁴. In the UK the primary cause of COPD is smoking related damage¹⁵. Exacerbations are important as they cause worsening morbidity, mortality and accelerate disease progression¹⁶. This study will be using the latest molecular techniques for RSV identification and will utilize the significant expertise available in Professor Openshaw and Dr Ryan Thwaites team with these methodologies. This study will also follow a cohort of patients over 3 years, with the aim of developing a more accurate picture of RSV prevalence amongst COPD patients over time. This will involve working with Professors Wedzicha, Professor Donaldson and the well-established London EXCEL COPD cohort.

A parallel cohort has been set up at Groningen University, with Professor Maarten van den Bergs team recruiting ~ 200 COPD participants. The inclusion / exclusion criteria and visit protocols are identical. We will have access to some of this data to be able to compare across the two cohorts. This has been agreed with Professor van den Berg.

We are recruiting a cohort of ~ 200 participants at Imperial college London to investigate RSV both at baseline visits and at exacerbation. We aim to establish the burden of RSV disease amongst this cohort and then determine the severity of these infections as compared to other causes of COPD exacerbations.

We additionally aim to evaluate biomarkers of disease severity. This will involve a literature search to identify those biomarkers found to date in RSV infection amongst adults. Working with the RESCEU (Respiratory Syncytial Virus in Europe) consortium, we will also identify

biomarkers from the extensive published paediatric literature that could also be evaluated as potential biomarkers of disease severity and susceptibility in adults. Molecular investigations by PCR (Polymerase Chain Reaction) for RSV are time consuming and incur significant expense. A simple biomarker, or combination of biomarkers could provide an earlier diagnosis and indicate likely disease severity, thus benefitting clinical patient care.

There is little published data as to the prevalence of RSV in COPD patients during the stable state (non-exacerbating), but the data so far indicates that RSV may chronically infect a subset of patients during the stable state¹⁷. It has been shown that those patients with chronic RSV infection do have a greater decline in their FEV1 (Forced Expiratory Volume in 1 second, a measure of respiratory obstruction) over time when compared to those not chronically infected with RSV, by as much as 50ml/year¹⁸. The pilot data will utilise previously collected samples from a cohort of COPD patients that have been tested for multiple respiratory viruses.

Information is still lacking as to the significance RSV plays during exacerbations of COPD in Europe specifically, or how much of a role it plays in increasing susceptibility to bacterial infections. There are no studies focusing on severity of exacerbations of COPD caused by RSV disease compared to other exacerbation causes.

Investigating the RSV burden in COPD patients has gained importance in recent years due to a number of vaccines and anti-virals currently being developed. Some of these interventions are likely to be available and on the market within the next year. It will therefore be increasingly important to know which patient groups would most benefit from being vaccinated against RSV.

This project aims to generate substantial data on RSV prevalence and disease severity amongst COPD patients. We also aim to collect data on other causes of COPD exacerbation including other viruses and bacteria. We will utilise patient diary cards, questionnaires, collect detailed demographical data and biological samples. We can therefore potentially perform several exploratory analyses including investigating RSV related exacerbations in comparison to other exacerbation causes. We will be able to utilize the extensive expertise at the NHLI

(National Heart and Lung Institute) when performing this study, at both the St Mary's and Brompton campuses.

This research will form part of the output of the RSECEU consortium¹⁹, which has the aim to describe the clinical and economic burden of RSV throughout Europe. RESCEU was split into 6 work packages (WP's). I was mainly collaborating with WP4, the prospective data collection group and WP5, the biomarker discovery group. The literature search has previously been published and the clinical study findings from this thesis will be published soon.

1.2 COPD

1.2.1 Definition of COPD

COPD is a common, preventable condition that results in decline in function of the lungs and is usually progressive²⁰. COPD is defined as airflow obstruction, as proven by spirometry (with an FEV1/FVC (forced vital capacity) ratio <0.7), that is not reversible, or poorly reversible. Obstruction on spirometry along with symptoms suggestive of COPD and a history of chronic exposure to causative agents is usually enough to make a diagnosis.

The disease is a spectrum which includes emphysema and small airway obstruction, with both often present²¹. Smoking is the most common cause of COPD in Western countries, but it is estimated that $\frac{3}{4}$ of COPD cases are to be found in lower and middle income countries (LMIC's)²² where exposure to biomass fuels is a common cause²³.

1.2.2 Prevalence, and disease burden

COPD is found world-wide and is a disease that confers significant burden to healthcare systems and has a huge impact, causing a great degree of mortality and morbidity. Around 2% of the UK population are thought to have a diagnosis of COPD²⁴, but with undiagnosed cases the prevalence could be considerably higher.

1.3 COPD exacerbations

1.3.1 COPD Exacerbation Definition

COPD exacerbations (often referred to as Acute Exacerbation of COPD (AECOPD)) are defined as acute episodes of worsening symptoms, in particular: increased dyspnoea, a change in sputum colour and increased sputum production²⁵.

1.3.2 Clinical importance

Exacerbations of COPD can be severe, debilitating and sometimes fatal. It is estimated that 2.4% of all acute hospitalisations in England are attributed to COPD exacerbations and the median length of stay is 7 days^{26 27 28}. Mortality for AECOPD requiring a hospital admission is approximately 11.6%. This mortality rate increases to 37% for readmissions²⁹. Only 50% of COPD exacerbations are ever reported to healthcare professionals³⁰, but even unreported exacerbations are known to have an effect on disease trajectory.

COPD Exacerbations have been shown to progress the disease by increasing the speed of decline in FEV1 over time³¹. Frequent exacerbators (3+/year) generally have worse quality of life scores as measured in the St George's Respiratory Questionnaire (SGRQ) score¹⁶. Any factors that can reduce the frequency of COPD exacerbations could therefore reduce disease progression and improve symptoms. RSV is a known cause of exacerbations in COPD³².

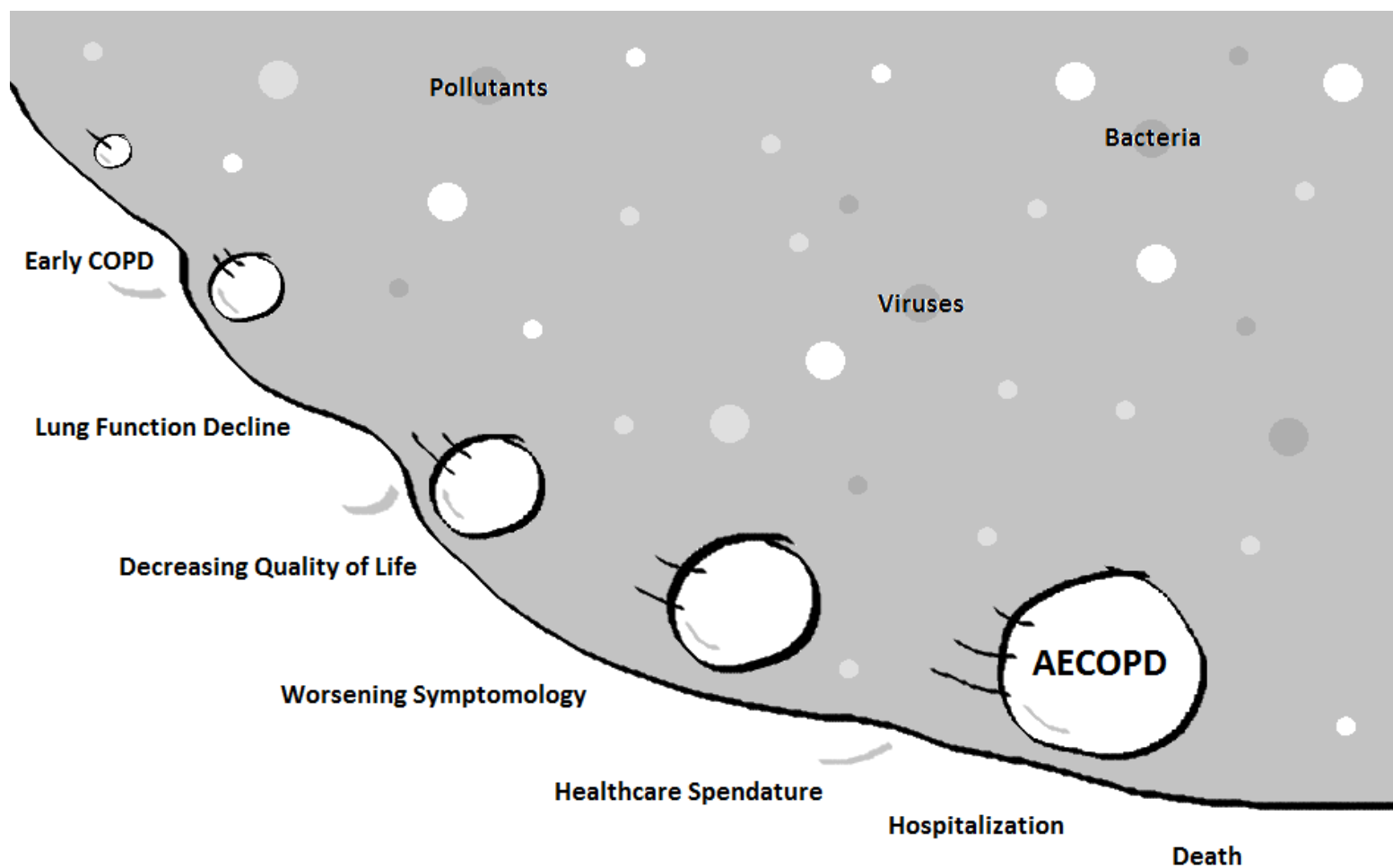


Figure 1 - COPD Exacerbation cartoon designed by Dr Dexter Wiseman, an original Illustration by Mr Benjamin Windibank (The snowball represents recurrent AECOPD episodes, and the increased morbidity associated with these events. The falling snow represents triggers for exacerbation.)

1.3.3 Risk factors

The main risk factor for having an acute exacerbation of COPD is a history of recurrent exacerbations of COPD. A higher global initiative for chronic obstructive lung disease (GOLD) stage³³ / poorer lung function, the absence of inhaled corticosteroid (ICS) treatment and female sex are the other known risk factors³⁴.

1.3.4 Causes

There is often no pathogen found as a cause for an acute exacerbation of COPD despite detailed analysis of sputum, sampling from respiratory passages and performing microbiological culture. Multiple other causes of COPD exacerbation are documented and described below.

1.3.4.1 Pollutants

There has previously been debate as to the importance of pollutants as a cause of exacerbations of COPD. More recently however, the evidence has been more convincing for a link. An Austrian study from Neuberger et al³⁵ found that COPD patients were amongst the most likely to have a death related to respiratory complications 0-14 days after high levels of pollution were detected in the air. A meta-analysis from 2016 reviewed 59 studies and found that there was a significant risk from short-term pollution exposure and developing a COPD exacerbation^{36 37}.

1.3.4.2 Bronchiectasis

Bronchiectasis is condition where the airways of the lungs become abnormally and irreversibly widened, which often results in a subsequent build-up of mucus. COPD is one of the known causes³⁸. A meta-analysis was performed by Ni et al³⁹ in 2015 reviewing 6 manuscripts investigating the link between bronchiectasis diagnosed on CT (computer tomography) scans and exacerbation frequency. Not only did the participants with bronchiectasis seen on CT scan have more frequent exacerbations, they also had higher levels of inflammatory biomarkers and poorer lung function.

1.3.4.3 Viral Pathogens

Multiple studies have found that viral infections are the most important pathogenic risk factor for exacerbations of COPD. One case control study by Rodhe et al⁴⁰ found viruses in the respiratory specimens of 56% of hospitalised COPD patients when compared to 19 % of controls.

A detailed review by Hewitt et al identified the most common viral pathogens to cause acute exacerbations of COPD to include influenza (Flu) viruses, coronaviruses, human rhinoviruses (HRV), and respiratory syncytial virus (RSV)⁴¹.

Diagnosing and identifying viral pathogens is not always possible, even when using sensitive PCR based techniques, therefore many viral-related exacerbations will go unlabelled as such. The TORCH study (Towards a Revolution in COPD Health) found a two-fold increase in COPD exacerbations during the winter months. The authors postulate that one of the main drivers behind this will be an increase in viral pathogens amongst the general population during these months⁴².

1.3.4.4 Bacterial Pathogens

Bacteria are also known to cause exacerbations of COPD. A paper by Shimizu et al in 2015 analysed respiratory secretions by PCR and found that infectious aetiologies (either viral or bacterial) were present in 88% of exacerbations, with bacteria present in 62% of exacerbations using both PCR and culture techniques⁴³.

A paper by Papi et al from 2006 examined 64 patients hospitalised with COPD for bacteria in respiratory secretions and discovered 55% of patients had bacteria present in sputum using culture techniques⁴³. From Sethi et al's paper from 2002, the three bacterial species predominantly isolated at COPD exacerbation were *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*⁴⁴. Rosell et al sampled 86 COPD exacerbations using bronchoscopy and *Haemophilus influenzae* and *P aeruginosa* were the most common bacteria isolated from those samples using culture techniques⁴⁵.

1.3.4.5 Eosinophilic Exacerbations

Eosinophils are raised in some, but not all COPD patients. It has been postulated, as in asthma, that this could represent a potential future treatable trait in COPD⁴⁶ (using medications such as IL-5 (interleukin 5) inhibitors). Some studies have demonstrated that those COPD patients with higher eosinophil levels have worse outcomes and higher rates of exacerbations⁴⁷. It has also been postulated that treatment of COPD exacerbations could become more personalised, with corticosteroids for example offered to those patients with a high eosinophil count⁴⁸. This is still a relatively novel area of research and has not as yet changed clinical practice.

1.3.5 Microbiome in COPD Exacerbations

It used to be thought that the lower airways and the lungs were a sterile environment with no microbes present, unless during a disease state such as pneumonia. This was because it is not always possible to culture microbes from lower airway samples. Since the implementation of newer diagnostics, such as ribosomal DNA (deoxyribose nucleic acid) analysis, it has now been demonstrated that there are multiple microbes present in all lungs⁴⁹ during health.

Several studies also demonstrate a difference in the microbiome between health and COPD⁵⁰. There are also theories that altering the microbiome may potentially affect the course of the disease⁵¹. The microbiome in COPD has been shown to alter during exacerbations, with potentially pathogenic organisms (PPM's) detected and showing overgrowth⁵².

1.4 Biomarkers in AECOPD:

A reliable and objective biomarker would be invaluable to aid in diagnosis and treatment of an AECOPD, and to use as an outcome measure for clinical trials. The samples most investigated to date are serum or plasma, but urine, sputum or exhaled breath may also contain useful biomarkers.

One of the main reasons there has been little success in finding a universal biomarker for an AECOPD is because of the different etiological causes of exacerbations and the consequent

heterogeneity in the associated biological and inflammatory changes. Distinguishing between these causes will greatly aid in targeting treatment and help prevent un-necessary antibiotic prescribing.

1.4.1 Common Biomarkers

1.4.1.1 CRP (C-reactive protein):

C-reactive protein (CRP) is possibly the most widely used biomarker when investigating and monitoring a chest infection, or indeed infection in general. CRP is raised consistently in AECOPD in multiple studies when compared to the convalescent state⁵³. There is a school of thought that a raised CRP may distinguish a bacterial from a viral triggered AECOPD; however, studies have thus far shown conflicting results. A 2010 study examined the CRP level in 86 patients during AECOPD found that the CRP levels did not distinguish viral from bacterial causes⁵⁴. A larger study investigating 118 patients over a one-year period did find that there was a slightly higher level of CRP in bacterial compared to viral AECOPD or cases when no pathogen was identified (58.3 mg/L IQR 21-28.2 vs 37.3 IQR 18.6-79.1). AECOPD caused by *H. Influenzae* or *Streptococcus Pneumonia* incurred the highest CRP levels⁵⁵.

1.4.1.2 PCT (Procalcitonin):

Evidence shows that Procalcitonin (PCT) is released from different tissues in response to bacterial infections or inflammation. Levels of PCT ≥ 0.25 ng/ml have been shown to indicate an AECOPD requiring hospital admission for ≥ 7 days⁵⁶. A meta-analysis investigating procalcitonin-based protocols in guiding antibiotic usage during an AECOPD found that they were clinically effective and safe⁵⁷, however the available studies were of low quality. More rigorous studies that evaluate PCT specifically in AECOPD as a prognostic and diagnostic biomarker are needed⁵⁸. It would fit that PCT would be a good distinguishing biomarker for bacterial triggered AECOPD. However as COPD patients often have background inflammation in the airways this hypothesis might not ring true. Further studies are needed in this area.

1.4.1.3 BNP (Brain natriuretic peptide):

Brain natriuretic peptide (BNP) has been shown to increase in AECOPD, where higher levels indicate a more severe exacerbation and are associated with a longer hospital stay^{59 60}. One study looking at 60 patients with COPD (17 exacerbation episodes) found BNP was significantly elevated in an AECOPD (79.9+/-16.2pg/ml at exacerbation vs 41.2+/- 8.7pg/ml at stable state)⁶¹.

1.4.1.4 Plasma Fibrinogen + IL-6 (interleukin-6):

Fibrinogen increases during COPD exacerbation (0.36 g/L SD=0.74), and then returns to the patient's baseline over a time period of two to six weeks^{62 63}. This is associated with a concurrent rise in interleukin-6 (IL-6). A large meta-analysis of over 154,000 participants indicates that a 1-g/L increase in plasma fibrinogen resulted in a 3.7-fold increase in COPD-specific mortality⁶⁴. IL-6 has been shown to be a better predictor of mortality than both CRP and plasma fibrinogen⁶⁵.

1.4.1.5 Urine Metabolomics:

There have been very few biomarkers that have been isolated in the urine which would be clinically useful in AECOPD. One study which shows promise for the future has however shown that certain metabolites can be used to differentiate COPD from asthma with a greater than 90% accuracy⁶⁶.

1.4.1.6 Exhaled Nitric Oxide:

Exhaled Nitric Oxide increases during an AECOPD, as shown in a number of studies, with one demonstrating a rise of 1.9 (-0.4-4.0) ppb at exacerbation^{67 68}. This may have uses in the future with regards to tailoring COPD treatment⁶⁹.

1.4.2 Biomarkers For Bacterial Associated AECOPD:

A large study published in 2011 (Bafadhel et al) investigated biomarkers in AECOPD, following 145 patients over a one-year period. The design of the study was to investigate biomarkers that could help determine the cause of an AECOPD. The best biomarker for determining bacterial associated AECOPD was found to be sputum interleukin 1 beta (IL-1 β), with a cut-off of 125pg/ml having a specificity of 80% and sensitivity of 90%. The serum biomarker best suited for distinguishing a bacterial cause in this study was CRP at a cut-off of 10mg/l, having a specificity of 70% and sensitivity of 60%⁷⁰. An E-Nose looking for volatile organic compounds has recently been shown in a small study to be able to distinguish bacterial from viral COPD exacerbations⁷¹. As this technology develops, and new research is published, this may well form part of the future of outpatient diagnostics. A study by Dal Negro et al looking at causes of AECOPD found that in the sputum, high Tumour necrosis factor α (TNF- α) was associated with *Pseudomonas* related exacerbations, and in those subjects without high TNF- α , high levels of interleukin 8 (IL-8) and IL-1 β in the sputum distinguished bacterial from viral and non-infective exacerbations⁷².

1.4.2.1 Biomarkers For Viral Associated AECOPD:

The best biomarker from Bafadhel et al's study for distinguishing a viral cause is serum CXCL10 (C-X-C motif chemokine ligand 10 or IP-10) at a cut-off of 56pg/ml, giving a specificity of 65% and sensitivity of 75%⁷⁰. In a separate study looking at viral causes of AECOPD, it was found that IL-6, monocyte chemoattractant protein 1 (MCP-1) and TNF- α were all raised in viral associated AECOPD when compared to viral negative subjects and controls⁷³. A similar study also found CXCL10 and Interferon Gamma (IFN- γ) were significantly raised in viral associated AECOPD when compared to viral negative exacerbations or stable COPD⁷⁴. PCT has been suggested as a possible determinant of viral associated AECOPD, but the evidence so far is equivocal⁷⁵.

1.4.3 Biomarkers For Sputum Eosinophilia Associated AECOPD:

Serum peripheral blood eosinophil count at a cut-off of 2% was the best measure at detecting an eosinophilic inflammatory cause from Bafadhel et al with a specificity of 60% and

sensitivity of 90%⁷⁰. An Egyptian study has linked a blood Eosinophil count of >2% as benefiting from oral corticosteroids at exacerbation⁷⁶.

1.4.4 Biomarkers For Frequent Exacerbators (defined here as 2 or more per year):

A Danish study investigating biomarkers indicating frequent exacerbators (defined as patients experiencing 2 or more exacerbations per year) discovered that: simultaneously elevated fibrinogen, CRP and leukocytes indicated an increased risk of frequent exacerbation amongst those with stable COPD⁷⁷. Raised plasma fibrinogen has also been shown to indicate patients who are at risk of frequent exacerbation – this has been repeated in multiple studies^{78 63}. High levels of serum surfactant protein D (SP-D) have been demonstrated to predict exacerbations when at highest levels⁷⁹. Unless otherwise stated, frequent exacerbators are defined as 3 or more exacerbations per year in this thesis.

1.5 RSV

Respiratory syncytial virus (RSV) is a negative-sense single stranded ribonucleic acid (RNA) virus of the *orthopneumovirus* family. It is known as the most common cause of bronchiolitis in young infants. 50% of children are infected by the 1st year of age with hospitalization rates between 2-10%⁸⁰. However, there is growing evidence that RSV is also a significant pathogen in older adults, particularly in those with comorbidities such as COPD⁸¹.

Until recently there has been controversy about the best way of diagnosing RSV infection in adults. Although the best method is still not completely clear, testing has improved substantially in recent years and it has also been demonstrated that a 4-fold increase in antibody to any RSV antigen indicates an RSV infection during that season⁸². RSV diagnostics in adults is discussed in more detail later in this thesis.

1.5.1 RSV Structure

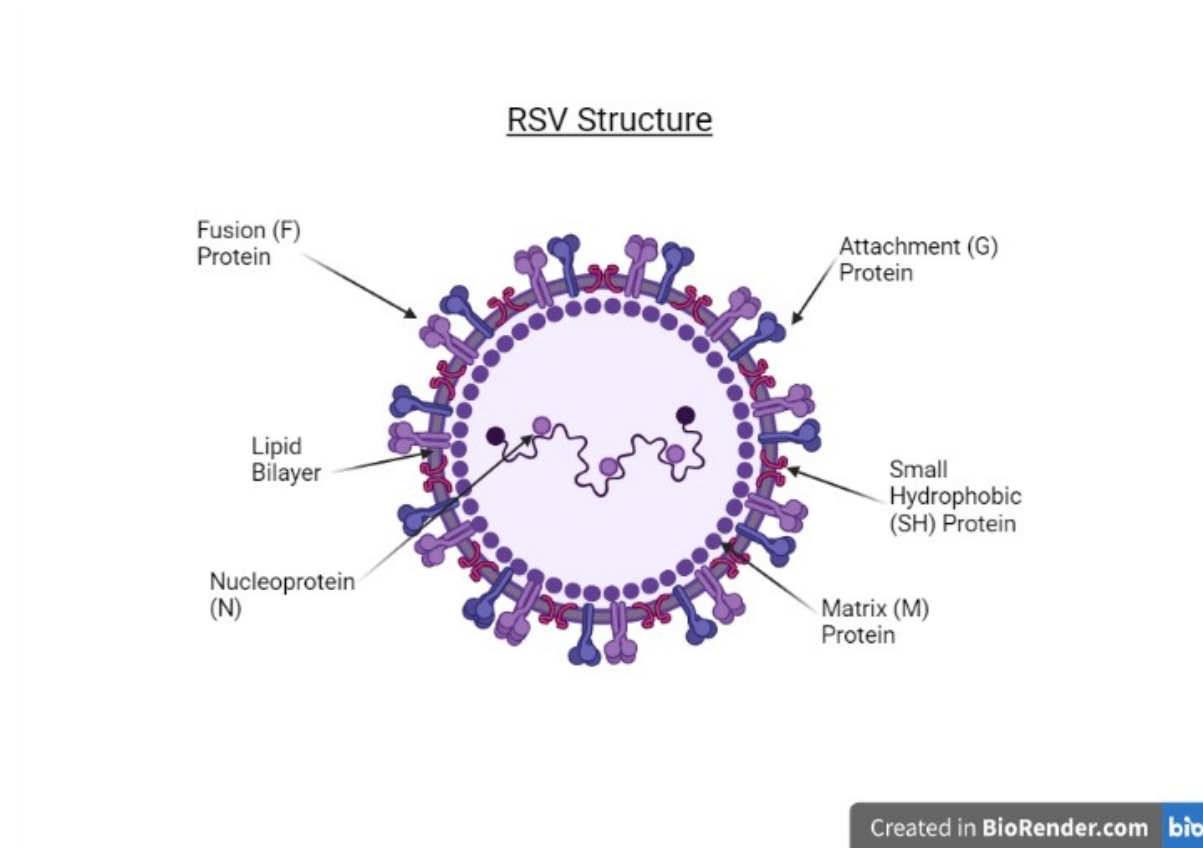


Figure 2 - RSV Viral Structure (created in BioRender)

Figure 2 shows the RSV viral structure, with surface and internal proteins labelled. The Fusion (F) protein has two forms, pre-F and post-F. This protein changes shape and is involved with assisting the virus to fuse with and invaginate into cells. There are epitopes on the F protein that are preserved across both structural forms, and therefore promising potential vaccine candidate targets. Attachment (G) protein is involved with viral attachment to ciliated cells and is either in a Ga or Gb form depending on the viral subtype. The nucleoprotein (N) serves to protect the viral RNA, with the matrix (M) and small hydrophobic (SH) proteins forming parts of the viral membrane.

1.5.2 RSV in COPD

RSV is known to cause exacerbations of COPD. A 2004 study by Walsh et al investigated RSV over four consecutive winters. RSV was common in the cohort of high-risk adults (those with chronic heart or lung disease) and patients ≥ 65 hospitalized with acute cardiopulmonary

conditions. RSV was also responsible for 11.4% of COPD admissions and 7.2% of Asthma admissions⁸³.

Previous studies have demonstrated the presence of RSV in the stable COPD patient population¹⁷. This is discussed in more detail in the preliminary data chapter and the discussion. Wilkinson et al found persistent RSV detection from sputum of COPD patients when not exacerbating (32% of sputum samples)⁸. Presence of RSV in this study was associated with airway inflammation and decline in lung function.

There have been a few studies to look into possible biomarkers for RSV infection, but mainly in children with Bronchiolitis, and no large studies exclusively in COPD patients⁸⁴.

There is a hypothesis that there is genetic susceptibility to RSV. This has been investigated previously in pre-term children suffering from bronchiolitis with RSV⁸⁵. Infants with single nucleotide polymorphisms (SNP's) in genes coding for the innate immune response have been shown to have a higher propensity for life-threatening RSV illness⁸⁶. The genetic susceptibility to RSV has never been investigated in COPD patients.

1.5.3 RSV in Children

Paediatricians are very much enlightened to the danger RSV poses seasonally. The number one cause of inpatient stay in infancy is Bronchiolitis⁸⁰, and in studies RSV is shown to be the cause of between 50-80% of cases of infant Bronchiolitis during the RSV season in North America⁸⁷. Shi T et al stated that "There is an estimated 0.86-0.94 RSV attributable [deaths] per 1000 livebirths world-wide. RSV caused 54,384 occupied bed days in the 2010/11 RSV season amongst infants $\leq 18/12$ "⁸⁸. By the age of 2 nearly all children will have been infected with RSV. The peak time for this is between the second and third month of life⁸⁹.

As RSV is so prevalent and causes such significant morbidity and mortality in children, there has been a lot of emphasis on trials and studies looking into prophylaxis and treatment of RSV infection in infants. A 2011 systematic review of Prophylaxis with Palivizumab found that there was not enough evidence to state its effectiveness based on the UK quality-adjusted life year (QALY) threshold, unless used in the CHD (Congenital Heart Disease), CLD (Chronic Lung

Disease) groups of patients, and only then when other factors were taken into account (or Infants without CHD / CLD in specific circumstances⁹⁰). This is also discussed in the most recent American Academy of Pediatrics (AAP) guidelines for Palivizumab prophylaxis for “children at increased risk of hospitalization for RSV”⁹¹.

The mainstay of treatment for RSV infection in children remains supportive – standard pediatric hemodynamic and respiratory support. Ribavirin (a nucleoside analogue which shows good in vitro activity against RSV) has mixed evidence for its use^{92 5}.

One interesting study has shown that children who suffer from RSV associated wheeze when aged less than 24 months are more likely to be labelled as asthmatic, or to develop abnormal lung function later on in life⁹³.

1.5.4 Symptoms of RSV in Adults

Diagnosing RSV infection in adults is a challenge. The viral load is lower in adult than in infant infections and symptoms may be most evident late in disease. Viral swabs do not necessarily pick up every infection⁸². It is therefore important to know what symptoms to look out for, and to know which patient samples to focus on.

RSV presents in children initially with a blocked / runny nose and fever, but this can then progress in some cases to a lower respiratory tract infection. In severe cases it can eventually lead to bronchiolitis with signs of respiratory distress, and in the most severe cases apnea and death⁸⁷.

Symptoms of RSV in adults are very similar to, and often mistaken for, flu and other respiratory viruses. In order of occurrence, the most common symptoms in RSV infection are cough (82% of symptomatic infections), followed by dyspnea (81%), wheeze (69%), nasal congestion (58%) and feeling feverish (46%)⁹⁴. These symptoms typically last around 6-7 days, with RSV having a longer prodrome when compared to Influenza A, and a longer recovery period⁹⁵. Around 90% of adults showing serologic evidence of RSV in one US prospective study had symptoms of the infection⁹⁶.

Sundaram et al's paper⁹⁷ from 2014 recruited patients ≥ 50 years old with acute respiratory illness, who had concurrently been recruited for studies evaluating influenza vaccine effectiveness from 2004 through to 2010 in Wisconsin. Out of the 184 RSV infections studied, the main symptoms were: a cough in 99%, fever in 61% and wheeze in 67%. Gu Yoon et al⁹⁸ collected medical records of hospitalized adult patients who were diagnosed with RSV infection from January 2012 to December 2015 from 3 tertiary hospitals in South Korea. 132 patients ≥ 65 admitted with RSV during this period. Of those 132, 65% had fever, 65% cough, 68% productive of sputum. Only 15% had rhinorrhoea and 41% dyspnoea. Interestingly the average length of stay for ≥ 65 was 20 days, 10% of the RSV admissions passed away and the cost of an RSV admission in South Korea had a median sum of 2,800 USD. Walsh summarized the outpatient symptoms in older adults as being: Nasal congestion (83%), cough (79%) and sputum production (64%), with dyspnoea (9%), fever (18%) and wheeze (23%) being less common⁹⁹

Diagnosis from a purely symptomatic perspective is not possible, as the symptoms for RSV and other respiratory viruses overlap and are incredibly similar. Viral symptoms from community dwelling adults from the Influenza-65 trial¹⁰⁰ have been described in detail. This included over 36,000 adults from America (Canada, Mexico, and the US), Europe (Belgium, Czech Republic, Estonia, France, Germany, Norway, Poland, Romania Russia, the Netherlands, UK), and East Asia (Taiwan). Looking across the graph at symptoms (figure 3) of RSV, Influenza, metapneumovirus, Rhinovirus and coronavirus infection, they all appear remarkably similar. This detailed symptom list found cough, fatigue, nasal congestion and headache to be the most common symptoms in RSV. Those symptoms are in the top 10 symptoms of Covid-19 according to the ZOE Covid study in the UK, which is an app based symptom tracking study¹⁰¹

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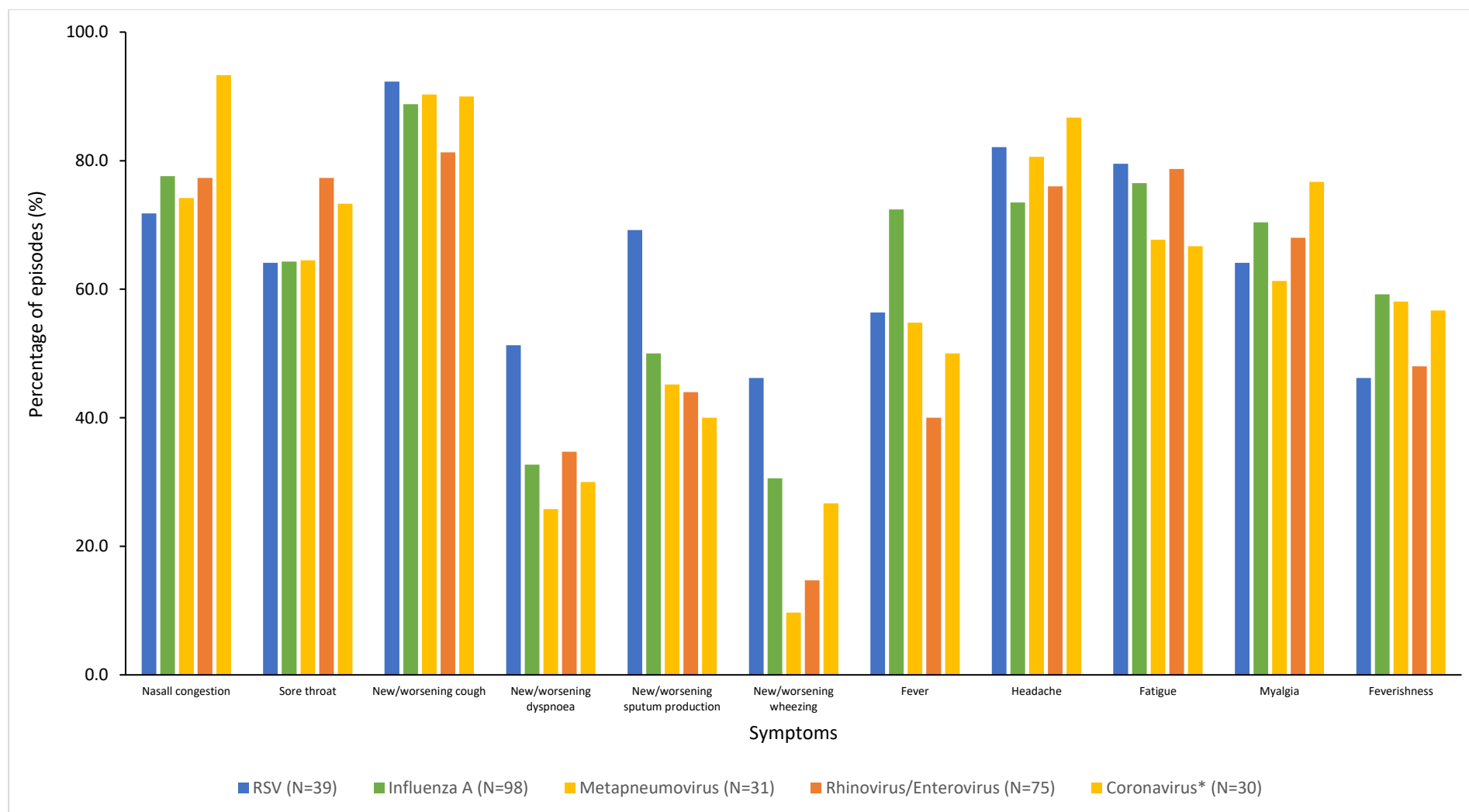


Figure 3 – Symptom prevalence for 5 common respiratory viruses in adults from Influenza-65 trial¹⁰⁰. *Coronaviruses pre Covid-19

By measuring patient symptoms using diary cards on a daily basis and educating our patients on what to look out for, we should be able to sample a good number of symptomatic RSV infections during this trial. By measuring antibody levels pre + post season in a paired analysis we should be able to pick up the 'missed' infections.

1.5.5 Seasonality of RSV in the UK

The UK national archives + gov.uk website hold data on all viral infections reported to the Health Protection Agency (HPA) and national health service (NHS) Laboratories. This dataset goes back in detail over more than 50 years. We have analysed the RSV data from the beginning of 2015 up until May 2018 (latest available at the time of analysis). We then extracted the number of cases and percentage of cases of RSV reported in different age groups over this time period.

Interestingly there is less of a swing in seasonality for RSV positivity in those over 15 years of age than observed in the <1 and 1-4 year age groups. The reason 15 years was used as a cut-off is because the data is reported in sub-groups of age: <1, 1-4, 4-15, 15-45, 45-65 and >65. Depicted is a chart looking at the percentage of cases (if <1 age group + 15+ age group added together =100%) in the <1 vs +15 age groups from 2015-2018 (figure 4).

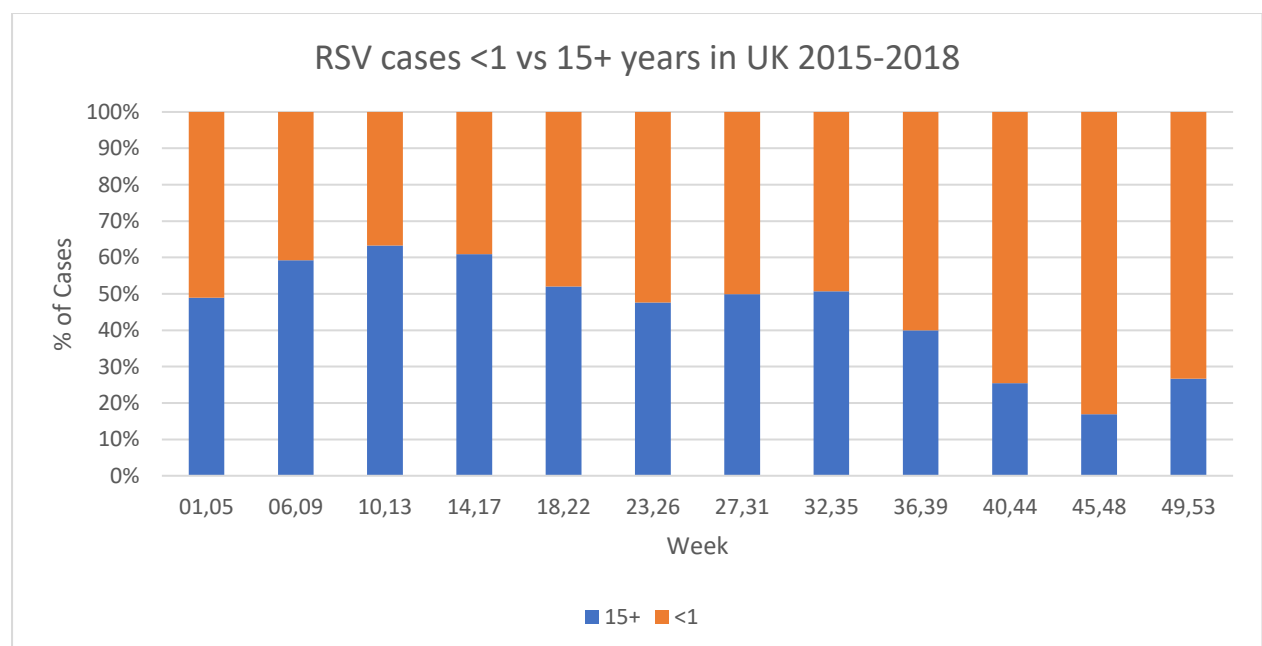


Figure 4 – RSV cases in <1 years old vs 15+ years old by percentage of positive cases. Data from HPA and NHS Laboratories.

Figure 5 shows the number of RSV cases per month over the course of the year for both <1 and 15+ year old patient groups. The time periods with the fewest reported cases occur from weeks 22-39 (~ May to October). From high to low season the difference in positive cases for the 15+ age bracket is 17 fold, whereas in the <1 year bracket the difference increases to 50 fold more positive cases.

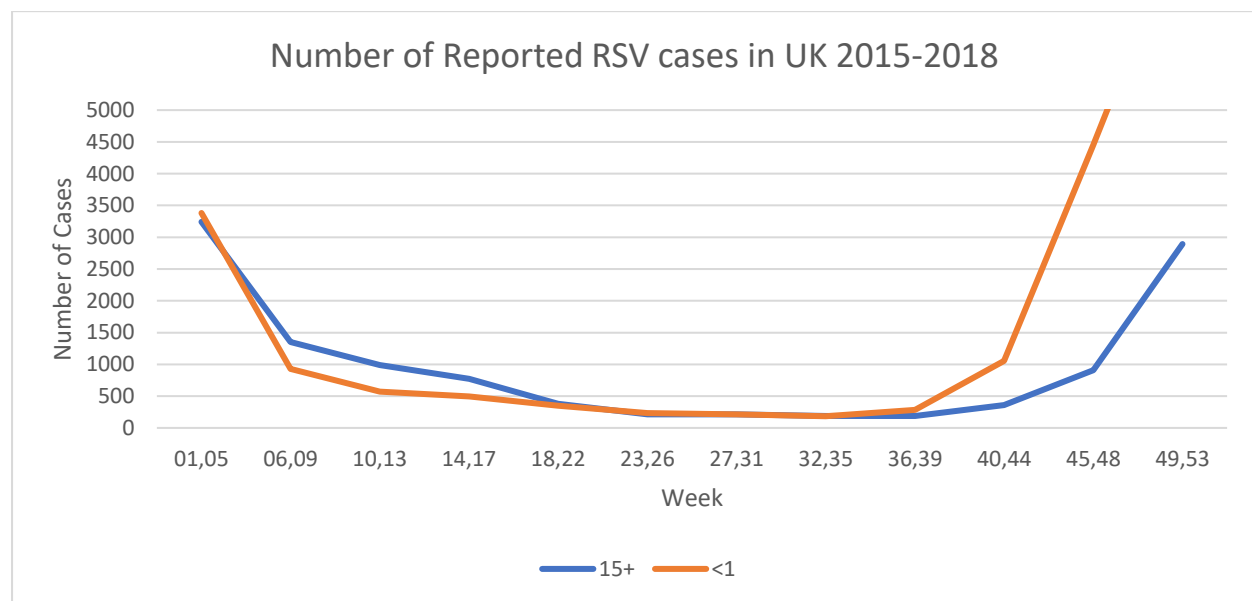


Figure 5 – RSV cases in <1 year olds vs 15+ years old – total cases reported

There are likely to be a number of reasons for this difference, including more swabbing and sampling for viruses taking place during the winter RSV / Flu viral season. This seasonality data correlates with the information previously published looking at data hosted at the ECDC (European Centre for Disease Prevention and Control) for RSV seasonality in the UK¹⁰². It does therefore appear that (although perhaps not as extreme as in the <1 year age group) the adult RSV season follows a very similar trajectory to that of infants. The seasonality in the European countries evaluated in this report all follow a very similar pattern, as indeed does most of the RSV seasonality data from the Northern hemisphere¹⁰³. In equatorial countries the season is not so clear cut, and the seasonality in the Southern hemisphere tends to follow a summer / winter trough and peak¹⁰⁴.

From previously completed studies, it is suggested that patients with COPD can become chronically infected¹⁸ with RSV, so it is not as clear if the seasonality of RSV infection amongst this specific group of patients would follow the same pattern. The national archive data is not

broken down into comorbidities, so it will be interesting to see if the patients recruited here, and in the aligned RESCEU COPD cohort in Groningen, show the same seasonality trend for RSV infection.

Another observation noted from the UK government data is that RSV testing, or at least reporting to the HPA has increased significantly in adult patients (15+ as per age breakdowns) over the last 10 years. At the height of the season in 2009 there were 137 cases in the 15+ age group reported compared to 1996 in the <1 age group, or 6% of total cases. By 2017, this had increased to 1066 in the 15+ age group, which was 24% of the total number of positive cases for that month.

1.5.6 RSV Immunology in older Adults

There is evidence that immunosenescence (the decreased effectiveness of the immune system associated with increasing age) plays a role in RSV becoming a serious pathogen again during later life. In a Canadian study, it was found that in peripheral blood mononuclear cell (PBMC) analysis of elderly nursing home residents, “high [cytomegalovirus] CMV-reactive CD4+ T-cells were associated with an increased risk and high [regulatory T cells] T-regs were associated with a reduced risk of respiratory viral infection”¹⁰⁵. It has also been shown in mouse studies, that low levels of T-regs slow down the clearance of the RSV virus from the lung tissue¹⁰⁶.

1.5.6.1 Antibody response in adults

Antibody rise occurs post RSV infection even in elderly frail patients and it is therefore a useful way to diagnose an RSV related exacerbation. The four-fold rise in antibody (from baseline) in Pre-F and Post-F RSV specific RSV protein Immunoglobulin G (IgG) has been used in literature as a diagnostic method in RSV for decades¹⁰⁷.

A 1998 study looked at serum antibodies in symptomatic RSV infection, and found that older adults with lower serum neutralizing-antibody titers were more likely to develop symptomatic RSV infection¹⁰⁸. Serum IgG and nasal Immunoglobulin A (IgA) levels to F and G protein were also found to be lower in symptomatic RSV infected subjects compared to controls in a 2004

paper¹⁰⁹. Levels of nasal IgA do seem to be a very important factor in the prevention of developing RSV infection, as shown in a number of papers^{110 111}.

There is evidence that the antibody response to RSV is similar in COPD patients to that of age-matched healthy controls¹¹². The quality of the antibodies, using avidity measurements, also appear similar in the COPD patients as compared to the healthy control groups¹¹².

Biomarkers for RSV susceptibility and severity in adults is described in more detail in the literature review chapter. There will be details of further issues regarding immunosenescence in the discussion chapter.

1.6 Hypothesis, aims and Objectives

1.6.1 Hypothesis

- 1) Antibodies to RSV other than Pre-F and Post-F can be used in diagnostics for RSV in adults with COPD.
- 2) RSV diagnosis by PCR from sputum samples will be more sensitive than PCR from nasopharyngeal (NP) swabs.
- 3) RSV will be as important as other causes of COPD exacerbation when looking at markers of exacerbation severity.
- 4) RSV prevalence will be similar in the UK and the Netherlands when comparing the two cohorts.
- 5) RSV will be found as a cause of COPD exacerbations in ~ 5-10% of cases. This would fit with previous literature and help to demonstrate accuracy of methodology in this study.

1.6.2 Aims + Objectives

We aim to follow a cohort of COPD patients for at least 2 RSV seasons and describe exacerbations using both standard and novel diagnostic techniques. We also aim to describe these exacerbations and compare the severity of RSV associated exacerbations to exacerbations associated with other pathogens. This cohort will have a mirrored cohort in

Groningen (The Netherlands) and RSV prevalence can be compared across the two locations and the methods validated.

1.7 Summary

The introduction chapter of this thesis discusses the burden of RSV, COPD, the clinical importance of COPD exacerbations and causes. The introduction includes discussion on the evidence behind biomarkers related to COPD exacerbation severity. There is also discussion of RSV prevalence and healthcare utilisation caused by RSV amongst adults. The introduction describes the importance of RSV as a cause of COPD exacerbations and the poor outcomes COPD patients have when they contract RSV. The chapter goes on to discuss the immune response to RSV in adults generally, and the factors most important with regards to COPD.

It is important to understand this foundation knowledge for the conclusions in this thesis and the importance of the objectives of the study to become clear. It also aids in understanding as to why adult RSV vaccine development is so important, especially amongst those with COPD.

2 Methods

2.1 Recruitment

The majority of the participants were recruited into the study directly from the London EXCEL COPD cohort. Three other methods were used to recruit additional participants. The success of each method has been described in the beginning of the results chapter.

2.1.1 Recruitment Goals and Power Calculation

RSV is estimated to cause between 5 and 10 % of COPD exacerbations^{113 114}. We estimate that 15 to 20 RSV associated exacerbations would need to be studied to get a clear idea of antibody response based on previous human challenge studies¹¹⁵. We would therefore need between 200 and 400 exacerbations to occur during the study period. Based on previous literature using similar cohorts, the exacerbation rate of COPD participants in such studies is around 2 exacerbations per year¹¹⁶. We would therefore estimate we would need to recruit between 100 and 200 patients into the study.

To estimate prevalence to a 5% accuracy at 80% power to observe a significant difference with $P=0.05$ (type I error rate alpha 5%) we would need to recruit 185 participants (using ClinCalc¹¹⁷ power calculator).

2.1.2 Initial Contact with Potential Participant

After identifying a potential participant for recruitment, via any of the methods discussed, a phone call was arranged. Initially the research fellow or recruitment nurse called the patient and briefly described the study and what joining the study would entail. If the potential participant indicated an interest in joining the study, then a short telephone screening would occur at an agreed time.

2.1.3 Telephone Screening

The potential participant is telephone screened after verbally consenting to the principal outline of the study. We would then assess their pack-year history, age and enquire about

any other relevant medical diagnosis (as per inclusion / exclusion criteria detailed later). If they appear to meet all the inclusion criteria, and do not meet any of the exclusion criteria, we then send them the study Participant Information Sheet (PIS) and invite them for an initial study visit.

2.1.4 Recruitment Visit

At the visit, which would be at least 2 weeks after sending the PIS, we complete the recruitment process and officially the participant is then recruited into the study. This visit counts as their first day in the study.

During the initial recruitment visit, the first thing the recruiter (usually one of the clinical fellows or research nurse) would do is confirm the participants details including name, date of birth, address and GP details. The participant must have read the PIS and we would answer any questions they may have and then ask them to sign the PIS consent form in front of us. After this we would go through and verbally re-check the inclusion criteria are met, and that none of the exclusion criteria are met.

2.1.4.1 Inclusion Criteria

The inclusion criteria for this study are: having capacity and ability to consent, being equal to or over the age of 40, having a 10 pack-year smoking history and having evidence of COPD on spirometry ($FEV_1/FVC < 0.7$).

2.1.4.2 Exclusion Criteria

Exclusion criteria are: having a diagnosis of asthma, having significant bronchiectasis or other significant respiratory disease including lung cancer, or any immunosuppressed patients such as those on active cancer therapy, on immunosuppressive medications or long term steroid therapy ($\geq 10\text{mg}$ prednisolone /day).

2.1.4.3 Initial Observations and Electrocardiogram (ECG)

After signing the PIS consent form the visit is started by performing an Electrocardiogram (ECG), measuring a Blood Pressure (BP), heart rate (HR) and peripheral saturations (SATS). Then the participants height and weight are measured. The ECG is analysed prior to spirometry for any sign of arrhythmia or infarction. The HR, BP and SATS are referred to as the observations (OBS).

2.1.4.4 Spirometry

Initially any contraindications to spirometry are checked¹¹⁸. If the participant does not meet any of the contraindications, then following the European respiratory society (ERS) / American thoracic society (ATS) criteria spirometry is performed¹¹⁹. The participant can then be confirmed that they meet the inclusion criteria for having spirometry evidence of COPD ($FEV_1/FVC < 0.7$). At this point their entry into the study is finalised (as this inclusion criteria cannot be assessed over the phone, and the PIS has to be signed before the participation commences). If the participants spirometry readings fall outside of the inclusion criteria, then the signed PIS is filed, but the participant is not included into the study and will not be counted in recruitment numbers.

2.1.4.5 Questionnaires

Following the spirometry the participant is asked to fill out a St George's respiratory questionnaire (SGRQ) and a COPD assessment test (CAT) form. If they have any questions while filling out the questionnaires, there will be a research nurse or fellow there to assist them.

2.1.4.6 Clinical Blood Samples

Clinical blood samples are taken after the questionnaires are completed. These include bloods to send to the hospital lab for full blood count (FBC), urea and electrolytes (U/E's), CRP, Fibrinogen, liver function tests (LFT's), thyroid function tests (TFT's) and Glycated haemoglobin (HbA1c). The blood is collected as per manufacturers guidelines and local

hospital protocol. Samples are sent to the hospital laboratory and processed per local protocols.

2.1.4.7 Research Blood Samples

Research blood samples are collected at the same time as the clinical blood samples. These are collected in a 10ml 'red topped' clotting tubes as per manufacturers guidelines and processed as detailed later in the sample processing section.

2.1.4.8 Sputum

We do not induce sputum as part of this study. If the participant is able to bring up (expectorate) any sputum at the visit they are asked to do so. At later visits (after the recruitment visit) we ask them to bring that morning's sputum with them if they have expectorated any. The sputum is separated from saliva and split into 2 samples. One is sent for microbiological analysis in the hospital lab as per local protocols. The second is sent for processing as a research sample as detailed later in the sample processing section of this methods chapter.

2.1.4.9 Nasopharyngeal (NP) Swabs

The swabs used are flocked nasal swabs and the practitioner taking the samples must wear appropriate personal protective equipment (PPE) as per local hospital guidelines. Firstly, the participant is asked if they had suffered from any nose bleeds or nasal discomfort recently and if so, if it was one particular nostril (if it was one in particular then that nostril is avoided). Next the participant is asked to blow their nose into a tissue and sit back, with neck extended and head resting on a pillow. A visual inspection of the nasal passage is carried out by the clinical fellow or nurse, looking for any obvious obstruction. The swab is then gently inserted into the nostril along the inferior aspect of the nasal passage. It is inserted until gentle resistance is felt (usually half the distance between the participants nostril and ear). The swab is then rotated 3 – 4 times and placed directly into the collection tube with MicroTest 4¹²⁰ (M4RT) media.

After swabbing, the tip is cut with recently sterilized scissors and placed directly into M4RT media for later processing, as detailed later in the sample processing section of this methods chapter.

2.1.5 Future Visits

Participants are seen at 3-6 monthly intervals, where spirometry, bloods, sputum (if expectorating), the OBS, CAT, SGRQ and Sputum Score are taken. Yearly the participants are seen for an annual review, where social questions are also asked (see annual review in appendix).

2.1.6 Home Diary Cards and Exacerbation Reporting

Diary cards are completed daily. Changes in symptoms, treatments and daily peak flow measurements are recorded (see example diary card in appendix). The participants are educated in how to fill out the diary cards during the recruitment visit. The cards are inspected when participants return to the clinic and any irregularities are questioned. If there is an exacerbation recorded on the diary card (see exacerbation definition) then the participant is questioned about this including if any rescue medication (antibiotics and / or steroids) was taken. If there are any errors in filling out the diary cards then the participants are re-educated in how to fill them out.

2.1.7 Exacerbations

2.1.7.1 Exacerbation Visits

Participants have a phone number to call if experiencing an exacerbation, which is staffed 24/7. They are brought into the study centre on the next working day if possible. They are reviewed clinically by a clinical fellow and decision to admit, offer treatment or supportive therapy is made. OBS, spirometry, blood and sputum samples are taken as described above. They are asked to complete a CAT score, sputum score and exposure questions (see exacerbation visit form in appendix).

They are asked to return 2 weeks and 6 weeks later to monitor recovery and re-sample if willing and available.

2.1.7.2 Exacerbation Definition

Exacerbations in this study are defined as two new symptoms (at least one major) for two or more consecutive days. Major symptoms are: increased breathlessness, increased sputum volume or intensified sputum colour. Minor symptoms are: sore throat, fever, worsening cough, increased wheeze or chest tightness and a cold. A mild exacerbation is defined as that meeting the above criteria but which did not require any treatment. A moderate exacerbation required treatment with antibiotics and / or oral corticosteroids and a severe exacerbation required hospital admission. Exacerbations are defined as ending after two consecutive symptom free days. The last day of symptoms is the end date. If another exacerbation starts within 5 days of the end of the previous exacerbation, this is counted as a prolonged exacerbation rather than two separate events.

2.1.7.3 Exacerbation Treatment

Exacerbations are treated as per local hospital guidelines and the attending physician's medical opinion.

2.1.7.4 Exacerbation Severity Measurements

To calculate the percentage change in FEV₁, FEV₁ at exacerbation was divided by the baseline FEV₁ and multiplied that by 100.

Time to recovery was evaluated by examining the participants diary-cards and counting symptomatic days. Once the participant has had two symptom free days, and not suffered from another exacerbation by diary card criteria within five days, the exacerbation was considered to be over.

The CAT score change was calculated by taking the total CAT score at the previous baseline visit (as long as it was more than 5 weeks since the end of the last exacerbation and more

than 2 weeks before the exacerbation visit) and comparing it to the CAT score at the exacerbation visit.

The exacerbation frequency was calculated by dividing the number of exacerbations (both diary-card diagnosed exacerbations and attended exacerbations) by the time in the study in days, then multiplying the answer by 365.25.

2.2 Regulatory permissions and consent

All relevant Research Ethics Committee (REC) and ethics permissions were obtained prior to the start of this project. Any ethics amendments (such as recruitment amendments) were authored and submitted. Integrated Research Application System (IRAS) ID: 232292, REC Reference: 17/LO/1424 (date of REC approval 18/10/2017).

2.3 Sample Processing and Hard-Copy Storage:

2.3.1 Sample Processing

Research serum and sputum samples are sent to our in-house laboratory. They are processed as per our protocols below:

2.3.1.1 Serum

Whole blood is collected into 'red topped' clotting tubes. After waiting between 30 mins and 1 hour the tube is placed in the laboratory centrifuge. The samples are spun at 2000 rpm for 10 mins. The centrifuge is refrigerated to 4 degrees.

The centrifuging results in the serum becoming separated from the blood cells. The serum is then pipetted out into 0.5ml aliquots into Eppendorf tubes which are then snap frozen at -80°C. The remaining blood cells are discarded. The serum is aliquoted and then frozen so that

multiple analyses can be run from the same serum sample without freeze-thawing degrading the sample.

2.3.1.2 Sputum

The sputum plugs are separated from saliva and mixed in an 8-1 ratio with Dulbecco's phosphate-buffered saline (DPBS) (1g of sputum = 8ml DPBS). This is agitated with glass beads on a shaker for 15 mins and then aliquoted into 0.5ml samples. The aliquots are snap frozen and stored at -80°C. Full sputum processing protocol in appendix.

2.3.1.3 Nasopharyngeal Swabs

The NP swab is inserted into the storage media (M4RT) immediately after collection and the tube is labelled with the participants code and the date. This tube with swab in situ is flash-frozen at -80°C for future analysis.

2.3.2 Study Data Storage

Study data is filed as described below. Periodically the data in the patient files is entered into the electronic case report form (ECRF) held on local servers and complying with UK and trust general data protection regulation (GDPR) regulations.

2.3.2.1 Electrocardiogram (ECG)

ECG's are performed in the study centre, on a bed in the study clinical room. A 12 lead ECG machine is used. This is primarily for patient safety and pre-spirometry assessment, but the ECG's are then filed in the participant specific notes for analysis and potential future studies.

2.3.2.2 Study Documents

Clinical data, along with the original questionnaires and ECG's are stored in a participant-specific file in a secure area of the study team office. This will be stored as per UK and local trust GDPR guidelines.

2.3.2.3 Questionnaires

The CAT score, SGRQ, Depression Scores and fatigue score are completed at the study centre by the patient. These are then filed in the participant-specific file (see CAT questionnaire in Appendix).

2.3.3 Sample Storage

All processed serum, sputum, NP swabs and whole blood samples are kept in temperature monitored -80°C storage freezers. The samples are labelled with a letter and number code particular to each participant (but non-patient identifiable without the key) and the date of collection.

2.4 Pentaplex

2.4.1 Equipment + Reagents Needed

Pipettes and Pipette Tips	
2x 96 well polypropylene Plates for Sample Dilution	Thermo Scientific cat 611u96
1.5ml Eppendorf tubes for Standard Preparation	0030.121.589
2 96 well plates with vacuumable bottom filters suitable for reading on Luminex Bio Plex 2000	Thermo Fisher cat EPX-88888-000
Bovine serum albumin (BSA)	Sigma Aldrich cat A2153
Phosphate-buffered saline (PBS)	Tablet Gibco cat 18912-014
Tween-20	Sigma cat P1379-100m
Serum for Standard Curve	Supplied by RIVM (Proprietary)
Control Serum	Supplied by RIVM (Proprietary)
Ga, Gb, N, Pre-F and Post-F Beads	Supplied by RIVM (Proprietary)
RPE (goat anti-human IgG)	Abcam cat ab6858
Vacuum Washer	
Plate Shaker	

Luminex Bio Plex 2000	
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Table 2 – List of equipment and reagents needed for the Pentaplex assay

2.4.2 Preparation of Reagents and Samples

2.4.2.1 Serum

First the serum samples chosen to be analysed are taken out of the storage boxes (kept at -80°C) and defrosted. A template is designed for the plate and samples matched to their position in the plate on a rack.

Plate layout below:

S	S	S	S	S	S	S	S	S	C1	Std1	Std9
S	S	S	S	S	S	S	S	S	C2	Std2	Std10
S	S	S	S	S	S	S	S	S	C3	Std3	Std11
S	S	S	S	S	S	S	S	S	C4	Std4	Std12
S	S	S	S	S	S	S	S	S	C5	Std5	Std13
S	S	S	S	S	S	S	S	S	mAb1	Std6	Std14
S	S	S	S	S	S	S	S	S	mAb2	Std7	B
S	S	S	S	S	S	S	S	S	mAb3	Std8	B

Figure 6 – Plate Layout for Pentaplex

Two plates are prepared for the assay, one with a 1:200 dilution, which allows for detection of the Ga / Gb antibody and a subsequent 1:8000 dilution for the Pre / Post F and N antibodies.

2.4.2.2 Assay Buffer

While the samples are defrosting, the assay buffer is prepared. This is made fresh each day and is essential that it is fresh for the pentaplex. 100ml of PBS is added to 1g of BSA and the solution is agitated till the BSA has dissolved. 100µl of Tween-20 is then added and mixed in thoroughly.

2.4.2.3 Sample Dilutions

Once the assay buffer is prepared and the samples have defrosted, two 96 well dilution / mixing plates are prepared. Three 96 well dilution plates are needed to create this. The first dilution plate is to make up a 1:20 dilution from which the two final dilutions are made. One of the plates is made up to a 1:200 dilution and the second to a 1:8000 dilution.

The method used for the dilutions is to prepare each sample in a 1:20 dilution with assay buffer initially. To make the 1:200 dilution, 10µl of the 1:20 diluted serum sample is mixed with 90µl of buffer (thus creating 1:200 dilution of serum to assay buffer). Next 5µl of the 1:20 dilution is mixed with 195µl of assay buffer (thus creating 1:8000 dilution of serum to assay buffer). These two plates are labelled 200 and 8000. All 3 of the dilution plates maintain the same layout as for the final plate for the reader to minimise errors. The 1:20 dilution plate is placed in a 5°C refrigerator in case any of the plates fail and therefore a repeat analysis can be run later in the same day.

2.4.2.4 Controls

The controls are diluted into a 1:200 and 1:8000 dilution using the same method as to dilute the samples.

2.4.2.5 Standards Preparation

The standard was provided by generous donation from Guy Berbers from the Dutch national institute for public health and the environment¹²¹ (RIVM) for this assay. Initially 4µl of standard was diluted with 96µl of assay buffer, giving an initial dilution of 1:25. This was then 3-fold diluted a further 13 times giving 14 standards in descending concentrations (1:25, 1:75, 1:225, 1:675, 1:2025, 1:6075, 1:18225, 1:54675, 1:164025, 1:492075, 1:1476225, 1:4428675, 1:13286025, 1:39858075). The same 14 standard dilutions are used on both the 1:200 and 1:8000 plates without any further alterations. These are not mixed and diluted in the dilution plates, but in carefully labelled Eppendorf tubes, as the same dilutions are used for both the 1:200 and 1:8000 plate.

2.4.2.6 mAB Preparation

The three human monoclonal antibodies (mAB) used are: Recombinant Human Antibody (D25), Palivizumab and Human Fc (AM 22 - NIH). These came pre-diluted as 1:500. These were further diluted as 5µl in 195 of assay buffer to give a final concentration of 1:20000. The same dilutions are used for both the 1:200 and 1:8000 plate.

2.4.2.7 Bead Solution

The bead mixture is created by adding 20µl of each bead type (Pre-F, Post-F, N, Ga and Gb) to 5ml of assay buffer. It is important to vortex the bead mix before pipetting out into the assay buffer as the beads can clump at the bottom of the solution. Once the beads have been added to the buffer, vortex the whole solution again to re-suspend.

2.4.2.8 Conjugate Solution

10ml of assay buffer is added to 50µl of RPE (goat anti-human IgG). This gives a dilution of 1:200.

2.4.3 Preparing the plate

2.4.3.1 Vacuum Plate Preparation

Two plates should be pre-washed with PBS, to moisten the lower filter. The PBS can then be vacuumed out after 5 minutes. The vacuuming should only occur just before the samples are added, to ensure that the plate filter does not dry out.

2.4.3.2 Adding Beads

Once the pre-wetted plates have been vacuumed and visual check made to ensure no PBS is left in the plate, 25µl of bead mix is added into each well. The bead mix is vortexed again prior to adding and placed in a trough for easy pipetting using a multi-channel pipette.

2.4.3.3 Adding sample, control, mAb or standard

25µl of either sample, mAb, control or standard is then added into each well. At this point each sample is added to its designated well as per the plate layout.

2.4.3.4 Blank Wells

For 2 wells, there will be no sample, mAb, control or standard added. These are blanks and 25µl of assay buffer is added to the plate, with all of the following steps then followed including adding the beads.

2.4.4 Plate Incubation

These 2 plates are then incubated at room temperature, in the dark (or wrapped in tinfoil), for 45 minutes on a shaking platform.

After 45 minutes of incubation on the shaking platform, the plates are then washed and vacuumed 3 times using 200µl of PBS per well. Once the plate has been vacuumed for the third time, 50µl of conjugate is added into each well from a trough using a multi-channel pipette. These 2 plates are then incubated at room temperature, in the dark (or covered in tinfoil), for 20 minutes on a shaking platform.

After 20 minutes of incubation on the shaking platform, the plates are washed and vacuumed once with 200µl of PBS. 100ul of PBS is then added to each well, and the plates are ready to be analysed.

2.4.5 Reading the plates:

The plates are analysed on a 'Luminex Bio Plex 2000', with the settings: High reporter-1 (RP1) Target, 50 beads per region and a 60sec timeout. All the results are then transferred to Microsoft EXCEL for analysis and formation of standard curves for future concentration calculation.

The Luminex reader must be Calibrated each morning before the samples are analysed.

2.4.6 Example of processed pentaplex plate with raw results:

	A	B	C	D	E	F
1	Well	Ga	Gb	Post-F	N	Pre-F
2	A1	2.9122	6.55466	424.2748	369.532	741.5788
3	B1	38.55608	8.0557	247.7292	214.386	321.6268
4	C1	12.52338	60.3423	OOR>	274.2636	167.724
5	D1	6.96036	17.76688	1114.6572	1953.2676	1092.1952
6	E1	3.89106	8.7347	856.7712	1259.4256	716.0396
7	F1	4.38042	4.65106	126.854	185.9596	138.0748
8	G1	21.38002	18.02458	1190.6656	573.8828	1397.8312
9	H1	6.2291	7.17018	OOR<	0.516	OOR<
10	A2	42.5555	60.11224	6870.0372	12150.7508	5969.1916
11	B2	6.2291	8.19682	176.1176	385.3128	227.168
12	C2	3.0827	6.20572	707.5264	555.578	1129.872
13	D2	33.20976	31.94018	6395.0224	11474.6544	4881.3724
14	E2	74.75018	86.9325	OOR>	21408.154	17667.4784
15	F2	3.786	6.70836	545.7368	449.5964	895.3564
16	G2	21.61236	117.72086	279.1008	453.1256	286.7444
17	H2	13.68906	12.5486	307.898	515.0864	856.8484

Figure 7 - Example of Pentaplex Readings

2.5 ELISA (In-House Designed):

2.5.1 Equipment needed:

96 Well ELISA Plate (clear bottom)	Thermo Scientific cat 10468075
96 Well V-Bottomed Plate (for mixing)	Thermo Scientific cat 611u96
PBS	Tablet Gibco cat 18912-014
RSV Antigen	My BioSource cat MBS318663
Dried Milk Powder	Milk brand Marvel
Anti-Human IgG	Life Technologies cat H10007
H2SO4 2N	Made in batches by Imperial labs
Tween-20	Sigma cat P1379-100m
3,3',5,5'-tetramethylbenzidine (TMB)	Life Technologies cat 002023

Table 3 – List of equipment and reagents needed for the in-house ELISA

2.5.2 Preparation of reagents

2.5.2.1 Preparation of Blocking Agent and Dilution Agent

The blocking agent and dilution agent used in this enzyme-linked immunosorbent assay (ELISA) is a PBS / 1% milk mix. They are identical. This solution is made at the beginning of the week that the ELISA being assayed and stored at 4 degrees Celsius when not being used. To make up 200ml of blocking / dilution agent, add 2g of dried milk powder (Marvel) to 200ml of PBS. Label this as 1% milk / PBS, with the date on and keep sealed at 4 degrees Celsius in the dark.

2.5.2.2 Preparation of Washing Agent

Some of the steps use off the shelf PBS for washing, whereas other steps use a PBS / 0.05% Tween washing agent. To prepare 200ml of the PBS / 0.05% tween, add 100µl of Tween-20 to 200ml of PBS.

2.5.2.3 Preparation of Detection Antibody Solution

The solution is made up to 5ml for each plate being read, using the dilution agent created as above. 2.5µl of the IgG detection antibody solution is added to 3997.5µl of the dilution agent. This gives a dilution of 1:1600.

2.5.2.4 Preparation of Samples and Dilutions

The samples are taken out of the -80°C freezer and fully thawed in preparation for the ELISA. Using a separate 96-well v-bottom plate, the samples are diluted in 1:2, then 4-fold serial dilutions across the plate, from left to right. This is done by initially filling the first column (A) with 120µl of dilution agent, and the remaining columns with 180µl of dilution agent. 120µl of serum is added to the first column and then thoroughly mixed. Tips changed between each dilution mix, 60µl of the mixed serum / dilution agent from the first column well is added to the next well with 180µl of dilution agent. This process continues across each column, mixing thoroughly in-between and changing tips.

A multichannel pipette can be used for the serial dilutions once the serum samples are in the first column. The last two columns are left without samples in as negative control wells. The serial dilution serum concentrations are 1:2, 1:8, 1:32, 1:128, 1:512, 1:2048, 1:8192, 1:32768, 1:131072, 1:524288.

Preparation of plate

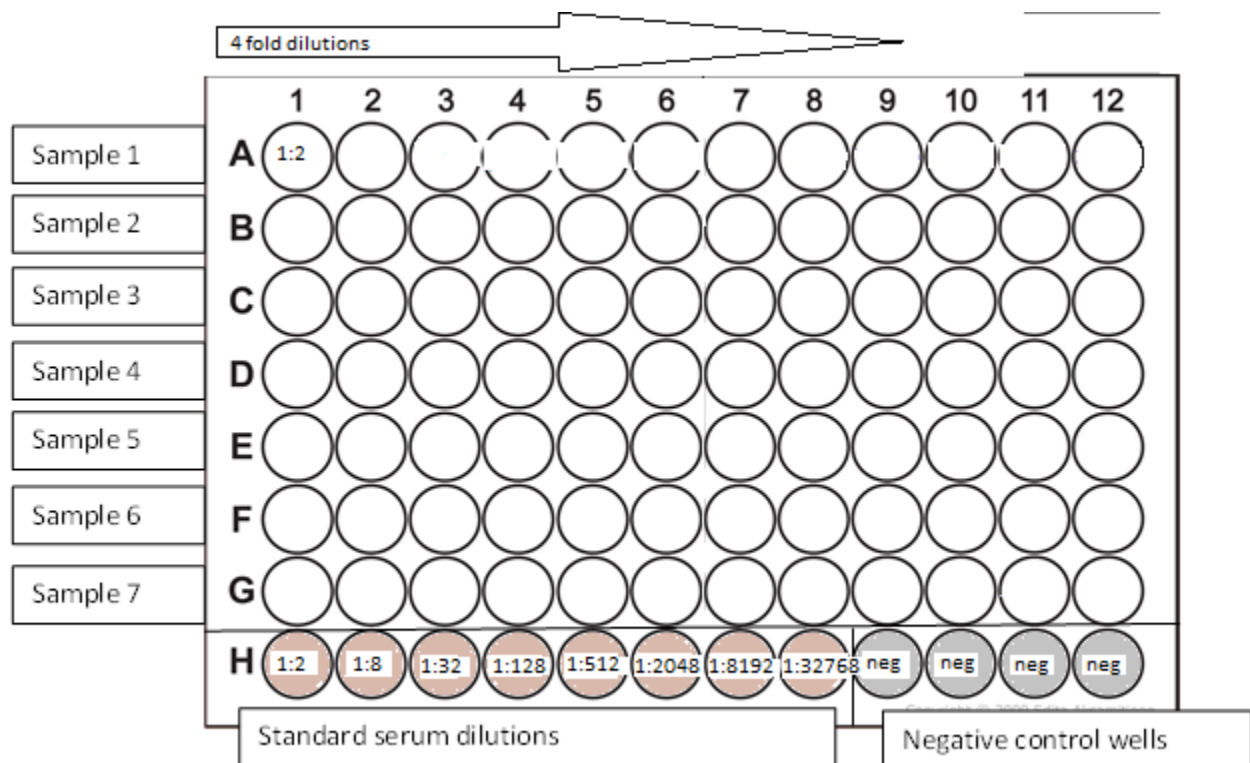


Figure 8 – Plate Layout for ELISA

The day before the plate is analysed, it must be coated with the RSV antigen. An aliquot of the RSV antigen is diluted to a 1:5000 stock with PBS and 50µl is added to each well. The top is then covered with clingfilm, and the plate placed in the fridge for incubation overnight at 4 degrees Celsius. The next day the plate is washed twice with 220µl of PBS, and then blocked with 150µl of PBS with 1% milk for 2 hours.

2.5.3 Incubation and Development of Plates

2.5.3.1 Incubation of Plates

50µl of each already diluted sample is added to the pre-prepared plate in the corresponding wells. This can be done using a multi-channel pipette, but tips need to be discarded between each row. The plate is covered in cling film and incubated overnight at room temperature on a shaker (this does not have to be in the dark).

2.5.3.2 Development of Plates

The next day, the plate is washed 4 times with 220µl of washing buffer. 50µl of detection antibody solution is then added to each well, the plate is covered in cling film and returned to a shaker, at room temperature for 2 hours.

After 2 hours of incubation, the plate is washed 4 times with the washing solution.

50µl of TMB (at room temperature) is added into each well and incubated in the dark for 15 minutes. This gives enough time for the colour change to occur. 50µl of 2N H₂SO₄ (stopping agent) is then added to each well and the plates are read on the reader at 450nm within 15 minutes.

2.5.4 Plate Data Analysis

It is best to keep to a maximum of 2 patients samples per plate, as only a maximum of 7 serum samples can be analysed each plate. A standard serum can be added to the bottom row to be able to compare readings across plates.

The last dilution (most diluted) that produces an optical density (OD) reading 2 times the background reading ($OD_{450} \geq 2 \times$ plate background) is considered the first positive. Serial dilutions were analysed during the development of this ELISA to find the most optimal dilution to get the desired results.

2.5.5 Example of processed ELISA plate with raw results

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.774	0.726	0.743	0.606	0.504	0.424	0.351	0.255	0.173	0.133	0.089	0.083
B	0.713	0.675	0.757	0.574	0.488	0.434	0.336	0.239	0.198	0.134	0.09	0.086
C	0.778	0.782	0.69	0.565	0.482	0.376	0.316	0.244	0.168	0.138	0.095	0.088
D	0.762	0.774	0.695	0.577	0.463	0.384	0.278	0.213	0.146	0.097	0.086	0.079
E	0.816	0.877	0.748	0.663	0.555	0.407	0.365	0.263	0.162	0.129	0.096	0.087
F	0.545	0.568	0.622	0.595	0.532	0.446	0.408	0.288	0.217	0.169	0.09	0.087
G	0.651	0.604	0.515	0.508	0.447	0.403	0.313	0.209	0.136	0.125	0.082	0.083
H	0.096	0.364	0.267	0.095	0.108	0.091	0.098	0.09	0.088	0.088	0.08	0.071

Figure 9 - Example of processed ELISA plate with raw results

2.6 Janssen ELISA

This ELISA was developed and internally validated by Janssen. The samples were analysed for Pre-F and Post-F RSV protein specific IgG. The results were sent back for analysis in this thesis. The Janssen ELISA process is proprietary.

2.7 Multiplex PCR and RSV qPCR (sent to GSK)

The multiplex PCR and RSV qPCR were sent to GSK's laboratories where they were analysed using validated proprietary techniques. The multiplex PCR analysed the samples for RSV A, RSV B, Rhinovirus, Influenza (Flu) A, Flu B, Human Coronavirus (HCV)-NL63, HCV-229E, HCV-OC43, Human bocavirus, Human Enterovirus, Human Adenovirus, Parainfluenza Virus (PIV)-1, PIV-2, PIV-3, PIV-4 and Human Metapneumovirus (HMPV). The qPCR analysed the samples for RSV A and RSV B.

2.8 In-House qPCR (used in Pilot data)

2.8.1 Sputum Preparation and RNA Extraction

The Sputum samples to be analysed are collected from the -80°C freezer and taken to the laboratory. After defrosting the sample is mixed using a vortex and 100ul of sample was transferred to a 96 well RNeasy kit (Qiagen). Manufacturer's guidelines are followed.

2.8.2 cDNA Conversion

The extracted RNA was converted into Complementary DNA (cDNA) using a Fluidigm reverse transcription mastermix (Fluidigm, San Francisco, USA) as per manufacturers guidelines.

2.8.3 qPCR Analysis

The converted cDNA was transferred onto reading plates and analysed on a Fluidigm Biomark HD as per manufacturers guidelines.

2.9 Statistical analysis

Both STATA and GraphPad Prism were used for the statistical analysis of this study. STATA IC version 16 and Graph Pad prism version 9. Details of which statistical tests are given in the results section. STATA was used for all statistical analysis other than the preliminary data chapter, where GraphPad Prism was used.

2.10 Groningen Protocols

The protocols involving participant recruitment, sample collection, sample storage and sample processing were all identical in Groningen as described in this methods chapter. The samples were sent to the same partners and the same protocols were used for analysis at these sites, in the same batches as the Imperial samples.

2.11 Summary:

All of the methods for the study are explained in this chapter. They include the recruitment methods used, visit schedule information, sample collection and sample processing details.

This chapter also includes the methods to an in-house designed ELISA. This was not utilised on all of the serum samples in this study because the Janssen ELISA and the RIVM Pentaplex became available at a later date. The main reason for choosing these over the in-house ELISA was that they measure antibodies to specific RSV proteins rather than the in-house ELISA that measures antibody titres to RSV in general. The pentaplex was also much faster than the in-house ELISA, with 70 samples able to be prepared and analysed within one lab-day. This included time for preparing the samples, incubating the reagents with the samples and reading. The in-house ELISA would take 3 lab-days to analyse 21 samples (3 plates), mainly due to the much longer incubation periods. The in-house ELISA does have the advantage of being low-cost, with each run of 7 samples costing around £5, and using 'off the shelf' disposables.

3 Literature Search

I received assistance from: Ryan Thwaites, Sophie Janet and Simon Drysdale with the literature searches. Professor Openshaw, Professor Donaldson, and Professor Wedzicha aided in data analysis and discussion. The anonymous reviewers suggested extra papers not found in the original search.

This review was registered on Prospero with the #: CRD42019122496¹²²

This review has been published in the Journal of Infectious Diseases on 30/03/2020: <https://doi.org/10.1093/infdis/jiaa063>¹

3.1 Introduction

We will be discussing and investigating RSV disease severity at COPD exacerbation during this thesis. This will include examining the severity of COPD exacerbations caused by RSV as compared to COPD exacerbations caused by other respiratory pathogens (both bacterial and viral) and unknown causes. We will use various measurable factors to investigate severity, including symptom duration, patient symptom burden and biomarkers of severity. We will be measuring biomarkers of disease severity amongst the participants; therefore a thorough / systematic literature search of the previously published data is crucial.

As with most published RSV literature, the vast majority of studies and manuscripts are focusing on paediatric cases as there is a paucity of data regarding RSV in adults ¹²³. As I will highlight in this thesis, this is an important unmet research topic, especially in individuals with COPD.

We conducted a review of the peer-reviewed literature investigating RSV infections in adults, specifically those studies that focus on COPD patients (as laid out in the introduction and throughout this thesis). It would not be feasible to conduct a full systematic literature review regarding all these published data, therefore we focused on one topic, which is biomarkers of severity and susceptibility to RSV infection in the adult population. We aim to consolidate all the current literature available using our search, with a view to then be able to identify gaps

in knowledge and at a later stage and targets for future research. These data will also help when analysing the severity of RSV exacerbations once these data points from the cohort are available.

3.2 Methods

The search databases Ovid Medline, Global Health, Embase, Web of Science and Scopus were utilized for this literature search. The search terms used were “human respiratory syncytial virus,” “respiratory syncytial virus,” or “RSV”. The initial search was not carried out by myself, but my colleagues Dr Simon Drysdale and Dr Sophie Janet at Oxford University. The dates for search were between 1946 and October 2017.

The below inclusion and exclusion criteria were prospectively applied to assess the abstracts. We then evaluated the full texts of those abstracts which passed the initial criteria. Once the selected abstracts were identified we then performed the analysis, comparison and the formal discussion.

The inclusion criteria are: studies that are based on human models, studies that assess the severity of RSV infections, studies that investigate biological markers and studies that explore the relationship between the biomarker and infection severity. We excluded any studies using animal models, studies in children, studies without a clear definition of disease severity, studies with no definitive RSV diagnosis or any other literature reviews. These inclusion / exclusion criteria are summarised in table below. (Table 2– a version of which was previously published¹)

Inclusion	Exclusion
• Human respiratory syncytial virus studies in adults • Severity of RSV infection assessed • Biological marker investigated • Studies written in English, French, Spanish, Italian or Portuguese	• Studies in animal models • <i>In vitro</i> studies • Studies exclusively in children • Studies of treatment, diagnostics or epidemiology of RSV infection • Absent definition of disease severity • Studies without a definitive RSV diagnosis • Studies focusing on viral characteristics. • Literature reviews

Table 4– Eligibility and Exclusion Criteria – adapted from Wiseman, D.J. et al¹

3.3 Results

The initial search terms yielded 24,152 abstracts. Of those 24,152, 13,332 were duplicates and therefore removed. Of the 10,820 unique abstracts, 27 met the inclusion criteria and exclusion criteria and warranted full review. We then reviewed the list of papers attached to those 27 abstracts comparing them to the aforementioned inclusion and exclusion criteria.

Of these 27 fully reviewed papers, 12 of these met the criteria for inclusion into the literature review. An additional 9 articles were added that were either known to me, Dr Thwaites, Professor Openshaw, published after October 2017, or suggested during the external peer review process prior to publication. Each included study was subsequently reviewed by at least 2 researchers using the Cochrane tool for quality assessment¹²⁴. The search results are summarised in figure 5 below.

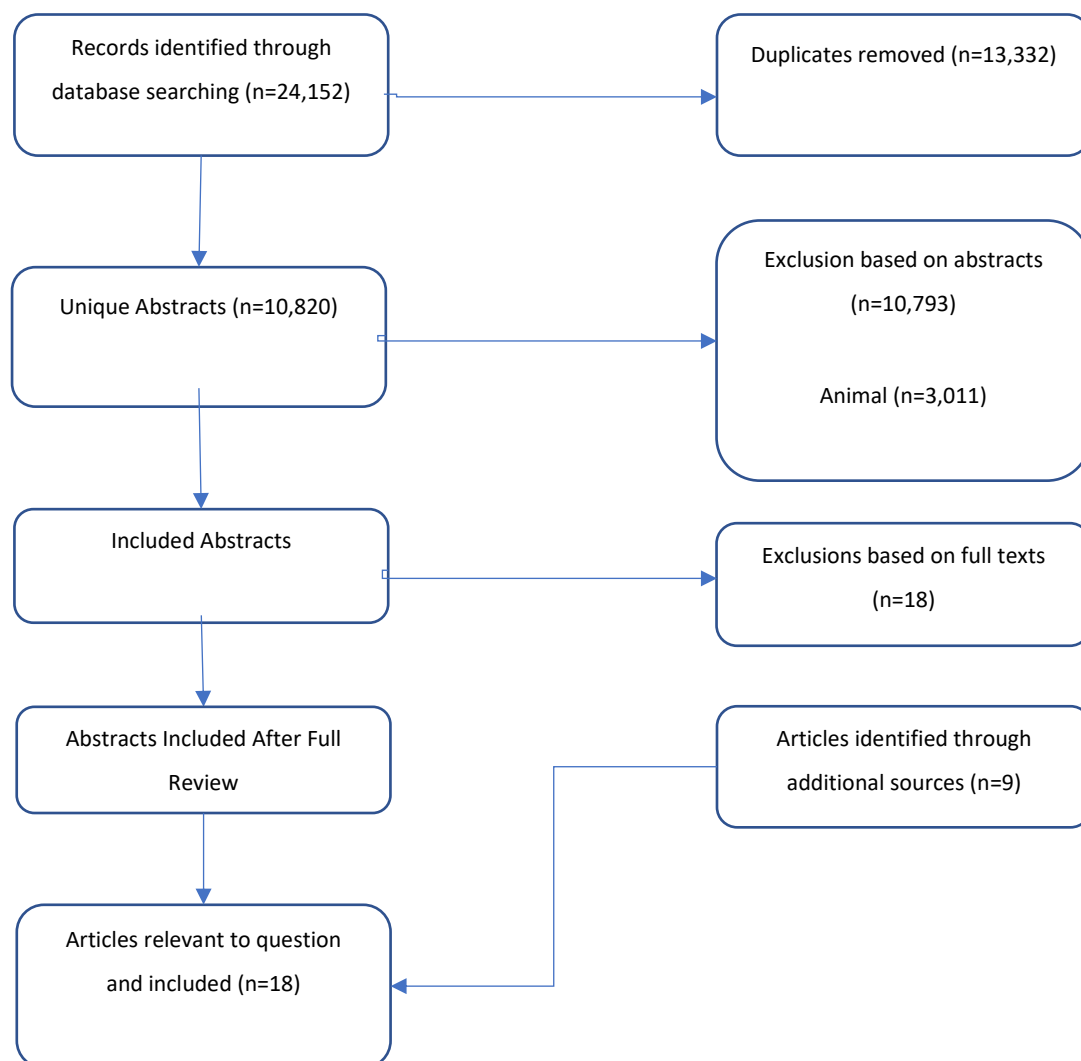


Figure 10 – Search Results - adapted from Wiseman, D.J. et al¹

3.3.1 Humoral Immunity:

Antibodies are central to our protection from an array of respiratory pathogens and other, non-respiratory infections. As elderly and comorbid populations have a higher tendency to severe disease from RSV, particular attention has been shown to their antibody response.

Levels of anti-RSV serum IgG are known to increase following an RSV infection. In one study, in older adults (>65 years) 79% had a greater than 4-fold increase in RSV-Specific IgG when compared to 64% of younger adults (<50 years)¹²⁵. As the cut-off of a 4-fold change is more frequently met in older adults infected with RSV, this would suggest the humoral response to RSV is not deficient in the elderly. However, such analysis is confounded by disease severity, as older patients may experience more severe infections, typically associated with a more robust antibody response. This will also help with diagnosing an RSV related exacerbation in this study as outlined in later chapters. There is evidence that neutralising antibody is protective in adults, as those in the lowest third of pre-infection levels had an increased susceptibility to symptomatic infection¹²⁵.

In patients with underlying respiratory conditions, Walsh et al demonstrated a strong association between low serum neutralizing antibody titres and RSV infection (odds ratio 5.89 CI 1.69-20.57, $P=0.006$)¹²⁶. In this study, COPD was identified on univariate analysis as a significant risk factor for RSV infection (independent of other factors – OR 3.97)¹²⁶. As discussed in the introduction, RSV has a strong association with acute exacerbations of COPD and hospital admissions¹²⁷.

Some reports have demonstrated an inverse relationship between RSV associated hospitalisation and serum anti-RSV neutralizing antibodies in elderly populations^{128 108 129}. A human-experimental RSV challenge study also backed up these assertions as lower serum neutralizing antibody (MNA) titres were found in those participants that would become infected ($9.5\log_2$ versus $10.6\log_2$, $P = .04$)¹³⁰. A post-hoc analysis of these data demonstrated that increased susceptibility to RSV infection occurred with an MNA titre of $\leq 10.36\log_2$. This study also found that lower serum RSV F-Protein IgG titres were present in those with confirmed RSV infection compared to those who did not have evidence of confirmed RSV infection ($15.2\log_2$ versus $16.4\log_2$, $P = 0.02$)¹³⁰. Luchsinger et al calculated antibody titre

amongst adults hospitalized with community-acquired pneumonia (CAP) and discovered that a lower RSV serum neutralising titre was more likely to be associated with an RSV associated CAP (RSV B 9.3 vs 10.4 and RSV A 8.1 vs 8.9 $P < .02$ for both)¹³¹.

Both neutralising antibody (MNA) and antibody titres increase rapidly post RSV infection¹³²¹³³. This response diminishes over time and one study by Falsey et al following 30 participants over a four year period demonstrated that every 6 months there is an approximately two-fold decrease in measurable levels¹³³. One very interesting conclusion from this study was that a stable baseline antibody level was maintained in those participants who did not develop an RSV infection. This adds extra evidence to the claim that if the pre-infection titres are known, a rise in these levels might represent a stable biomarker to help diagnose RSV infection¹³³. This is another useful finding that we can use to add weight to our methods for diagnosing RSV associated exacerbations later in this thesis.

There is a known phenomenon of 'immune amnesia' after RSV infections, where patients have been re-infected by antigenically and genetically similar RSV viruses and this might be caused by an antibody titre drop following an infection¹³⁴¹³⁵. It has however been demonstrated in young adults using human challenge studies that once an infection occurs disease severity is not impacted by antibody levels¹³²¹³⁶.

Antibodies do play a role in RSV prevention, and RSV IgA in the nasal passage has been found to be protective against RSV infection in challenge studies¹²⁸. Cherukuri et al also reported that those elderly subjects with high titres of RSV F-specific nasal IgA were protected from severe RSV illness¹²⁸. Lower MNA neutralisation assays have been found in older age in some studies however this was not the case for total RSV antibody levels, which were equivalent¹²⁵¹³⁷. Cells secreting RSV F-specific antibodies were found to be at higher concentrations in younger adults compared to older adults¹³⁸. Cherukuri et al also demonstrated RSV specific IgG and RSV F-specific IgA was equivalent in young and older adults¹²⁸.

A further study examined 1,284 participants, split into 4 groups and aimed to investigate the mucosal and humoral protection from natural RSV infection. The 4 groups were 1) healthy adults 19-40 years old, 2) healthy adults >65 years old, 3) nursing home residents, and 4)

adults with symptomatic cardiopulmonary conditions. Only 67 participants out of the 1,284 prospectively enrolled participants developed a symptomatic RSV infection, highlighting the challenge posed by observational studies designed to examine naturally acquired RSV infection in adults¹⁰⁹. When levels of serum IgG against Ga, Gb and RSV-F, and nasal levels of IgA were evaluated, those infected with RSV, across all groups, had lower levels of antibodies to start with. They also had lower levels of serum neutralizing antibodies¹⁰⁹. The authors were not able to determine a level of an antibody that inferred protection from RSV but did note that participants with antibody titres in the highest quartile were 3 times less likely to develop a symptomatic RSV infection¹⁰⁹. Lee et al conducted a challenge study of 12 participants where the 67% that became infected with RSV had a four-fold rise in anti-F antibodies and 58% had a four-fold rise in anti-G antibodies¹³⁸. This again demonstrates the usefulness in antibody titre rise to diagnose an RSV infection if the pre-infection serum level, and post-infection serum level are known. It also highlights that it is possible to become infected with RSV and not see this four-fold rise.

Regarding humoral protection from RSV, these studies indicate that IgA in respiratory secretions can provide some protection but do little to affect disease severity. A rise in antibody levels post-infection is a useful way of diagnosing RSV infections where pre-infection serology is available. This rise does appear to be short lived, and this is something that will be explored further with the laboratory work in later chapters, as we will be investigating immunoglobulin levels at multiple timepoints.

3.3.2 Cellular Immunity:

Cellular immunity is another means of providing important protection against RSV infection, which has also been investigated previously. Antigens presented from most component proteins of RSV have been shown to drive T-Cell cytotoxicity from in vitro studies. The immunodominant peptides are considered to be transcription antitermination protein (M2) and SH^{139 140}.

Younger adults T-cells and PBMCs produce more Interferon-gamma (IFN- γ) when exposed to RSV F-Protein compared to the PBMC's isolated from older adults¹²⁸. There were also fewer RSV-Specific CD8⁺ T-cells isolated from the blood of the older participants when compared to younger participants^{128 141 142}.

In a study investigating pre and post-RSV infection bronchoalveolar lavage (BAL) samples, both infection severity and viral load were related to pre-infection BAL levels of CD8⁺ RSV specific T-cells¹⁴³. This indicates a combination of both decreased frequency and function of RSV-specific T-cells may contribute to the rate of viral clearance and disease severity in the elderly. There was no association between peripheral (blood) CD8⁺ RSV specific T-cell levels and infection severity or viral load¹⁴³. There was also no correlation between pre-RSV infection BAL CD8⁺ RSV specific T-cells and susceptibility to infection. This suggests the airway CD8⁺ RSV specific T-cells are not involved with preventing infection but do limit the severity of the disease. Airway inflammation reported on the serial bronchoscopies remained after the symptoms had resolved, even with the milder infections¹⁴³. These patients had otherwise healthy airways, so it is feasible this phenomenon may be even more pronounced in COPD patients with abnormally inflamed airways. RSV-specific CD8⁺ T-cells were found to be undetectable in the peripheral blood of 9 COPD patients in De Bree et al's study when compared to 31 healthy controls¹⁴².

'Inflammaging' refers to the diminished specific immune response that can be seen in some elderly individuals, along with chronic inflammation¹⁴⁴. The anergy seen of T-cells and PBMC's to epitopes and proteins of RSV may be a feature of this. As a patient ages, their T-cells demonstrate increased T-reg frequency and increased transition to a Th-2 phenotype¹⁴⁵. These factors combined may contribute to the severity of infection in the elderly by impeding the anti-viral immune response. COPD is also known to compromise both the innate and adaptive functional immune response and cause chronic inflammation^{146 147}. This could compound the effect of severity of response to RSV in COPD patients and will be discussed in more detail in the discussion chapter.

3.3.3 Viral Load:

Data from human challenge studies documented infection rates between 50 and 80%. There are strong associations between upper respiratory tract viral load and disease severity^{136 143 148 149 150}. Nearly all of the challenge studies have been conducted in younger adults. We are awaiting the publication of hVIVO's RSV challenge study in adults 60-75 years old, demonstrating that this type of challenge study can be conducted safely in older participants¹⁵¹. It is unclear which of these findings from the younger adult challenges can be extended to older adults and at-risk groups, as the immune response is markedly different in older adults compared to younger adults, as discussed earlier in this chapter. Studies recruiting at-risk patients, such as COPD patients, are needed to evaluate the immune response to naturally occurring infections, though such studies would raise ethical questions regarding safety. There may also be differences between 'wild-type' RSV and the RSV used in challenge studies, which may also affect the immune response and study results.

When investigating natural infection in adults it was discovered that when comparing hospitalized to non-hospitalized RSV illnesses, there was no significant difference in nasal viral load^{152 153}. There was a significant difference when looking at the duration of viral shedding between the two groups (9.8 vs 13.1 days $P=.003$)¹⁵². Investigating hospitalized subjects, it was found that there were significantly higher peak viral loads amongst those requiring mechanical ventilation compared to those that did not. In a multivariate analysis, high viral load was found to be an independent risk factor for respiratory failure^{153 154}.

3.3.4 Innate Inflammatory Response:

There is evidence that the innate immune response can contribute to viral clearance and protect against infection, as discussed in this section. There is also evidence that the inflammatory response might also contribute to disease severity itself¹³⁴. In a study by Wilkinson et al, 32.8% of COPD patients were found to test positive for RSV at some point in the sputum (by PCR) when serially collected over 2 years¹⁸. 18 of these patients tested positive for RSV in >50% of their sputum samples – these patients had elevated levels of the

inflammatory cytokines IL-8, IL-6 and myeloperoxidase (MPO) in their sputum. These patients also had a faster decline in respiratory function (measured by forced expiratory volume in 1 second - FEV1)¹⁸. Due to the repeated finding of RSV in sputum in the same patients, this raises the theory of chronic RSV infection (or diminished RSV clearance) in patients with chronic airways disease, such as COPD. This theory was also discussed by Sikkel et al¹⁵⁵ and Seemungal et al¹⁷.

3.4 Biomarkers of RSV Severity and Susceptibility in Adults - Discussion

The papers summary and study characteristics are detailed the table below (Table 5):

Author, year	Prospective Study	Natural Infection	Healthy Controls / Younger Adults Included	High Risk / Co-Morbid Adults Included	Older Adults Included
Falsey AR, 1999 ¹²⁵	Yes	Yes	Yes	Yes (nursing home eligible but living in the community)	Yes
Cherukuri A, 2013 ¹²⁸	Yes	No	Yes	No	Yes
Falsey AR, 1998 ¹⁰⁸	Yes	Yes	Yes	Yes (nursing home eligible but living in the community)	Yes
Walsh EE, 2004 ¹²⁶	Yes	Yes	Yes	Yes (Underlying symptomatic cardiopulmonary conditions)	Yes
Lee FE-H, 2004 ¹³⁰	Yes	No	Yes	No	No

Habibi MS, 2015 ¹³²	Yes	No	No	No	No
Falsey AR, 1992 ¹²⁹	Yes	Yes	No	Yes (Nursing Home Residents)	Yes
Bagga B, 2015 ¹³⁶	Yes	No	Yes	No	No
Walsh EE, 2013 ¹⁵²	Yes	Yes	Yes	Yes	Yes
Jozwik A, 2015 ¹⁴³	Yes	No	Yes	No	No
Duncan CB, 2009 ¹⁵³	Yes	Yes	Yes	Yes	Yes
Lee N, 2015 ¹⁵⁴	Yes	Yes	Yes	Yes	Yes
Wilkinson TM a, 2006 ¹⁸	Yes	Yes	No	Yes (all patients had COPD)	Yes

Table 5 – adapted from Wiseman DJ et al¹ : Study Characteristics

The summary of findings are detailed the table below (Table 6):

Marker of Infection Severity		Marker of Susceptibility for Infection	
Systemic Markers	Airway Markers	Systemic Markers	Airway Markers
Low RSV neutralizing antibody titer in serum ¹²⁹ (Trend P=0.07)	Low levels of RSV CD8 ⁺ T cells in BAL ¹⁴³ (P=0.0142 r= -0.691)	Low RSV neutralizing antibody titer in serum ¹³¹ (P≤ .028) ¹⁰⁸ (P = 0.008 and P = 0.01 for RSV/A and RSV/B respectively ¹²⁶ (P=0.018) ¹³⁰ (P = 0.04)	Low IgA to RSV F, Ga + Gb proteins in nasal secretions ¹⁰⁹ (r = .58–.76; P = .0001)
	High levels of IL-6 in airway ¹⁸ (P < 0.001)	Low IgG to F, Ga + Gb RSV protein levels in serum ¹⁰⁹ (r = .54–.80; P = .0001) ¹³⁰ (only F-Protein P = 0.02)	Low IgA to RSV in nasal mucosa ¹³² (P < 0.05) ¹³⁶ (P=0.292)
	High levels of IL-8 in airway ¹⁸ (P= < 0.001)		Low IgA to RSV F-Protein in nasal mucosa ¹³² (P < 0.05)
	High levels of MPO in airway ¹⁸ (P= < 0.001)		

	High Viral Load ¹⁴³ (P=0.003) ¹⁴⁸ (P=<0.05) ¹⁴⁹ 150 (P=0.0340) ¹⁵⁴ (P =0.011)		
	Viral shedding in nasal secretions for longer ¹⁵² (13.1 vs 9.8 days; P = 0.003)		
	Low levels of IgA to RSV Ga + Gb in nasal mucosa ¹⁵² (Ga P=0.003 Gb P<0.0001) ¹⁴⁹ (P=0.03)		
	Higher levels of nasal IL-6 ¹⁵² (OR 2.2 (1.2-4.2) P=0.01) and MIP-1 α ¹⁵² (OR 9.1 (0.95 – 87.6) P= 0.06)		

Table 6 – adapted from Wiseman DJ et al¹ : Summary of RSV Biomarkers in Adults

Study characteristics are described in detail in the table (Table 5 - a version of which has been previously published¹). As shown in the table, there are a lot of high-quality studies published investigating RSV infection in patients, but there are several gaps in the literature. Diagnostics are often not focused on, and the papers that are published looking at diagnostics are now quite dated with newer more accurate techniques not being included¹⁵⁶. There is also

evidence that RSV related exacerbations or infections in adults is under-recognized with diagnosis often delayed^{157 158 159} , limiting the availability of systematic reports from healthcare records.

Reviewing the summary table (Table 6) there is evidence that IgA in the mucosa is linked to prevention of RSV. High levels of viral load also appear to be associated with disease severity in a number of studies. This may be further complicated by such factors as cardiovascular disease and lung disease, as they independently influence severity¹²⁶ . As COPD patients often have other underlying medical conditions as well, they may be even more severely affected. The evidence also indicates elevated inflammatory mediators (MPO, IL-6, IL-8) in the sputum during RSV infection as being correlated with faster lung function decline¹⁸ .

There are limitations with this review, as the initial search missed some seminal papers discussing this topic. The reason for this is unclear, but the authors did their best to include all relevant studies missed by the initial search.

In conclusion, additional studies are needed focusing on at-risk populations, such as those with COPD. This will help our understanding of RSV infection in at-risk individuals, kinetics of viral clearance and antibody responses. This may help development of anti-RSV therapies and identify those at-risk populations that should be offered prevention or treatment in the future. The subsequent table (Table 7 – a version of which has been previously published) identifies key limitations in our current knowledge and ideas for future research as highlighted by this review.

Limitations With Current Research	Possible Future Research Options
Challenge studies have previously been only in young healthy adults	Challenge studies in older adults and comorbid populations
Mainly upper airway studies have been performed in the observational groups	Lower airway sampling in infected adults is needed with comparison between upper and lower airway biomarkers
Virus has been found at multiple timepoints in the same patient. It is not known if this is RSV recurrence or chronic infection	Genotyping of RSV found in the same patient at multiple timepoints may confirm if infection with a new strain is occurring or if the virus is not being cleared
Viral clearance is poorly understood	Investigate which host or virologic factors influence the rate of viral clearance and disease severity, eg, the kinetics of viral clearance and antiviral immune responses
	Investigate the role of prefusion F antibodies in disease susceptibility in adults
	Investigate why is infection severe in some populations and mild in others and are the kinetics of viral clearance distinct between these groups

Table 7 – Limitations With Current Research and Future Research Options

4 Preliminary Data – RSV in the Non-Exacerbating State

This section has been previously presented by myself as a short poster presentation at the ATS in 2019⁸ at which it won a National Emphysema Foundation Abstract Scholarship.

This is preliminary investigative work undertaken prior to starting any data analysis on the main aspect of the thesis and aimed to help in forming a part of the research plan. There will be discussion on how these results compare to those in the main results chapter.

Permission was given to access the blood and patient data from Dr Faisal Kamal's research work. Dr Kamal investigated exhaled hydrocarbons at exacerbation to determine novel biomarkers for COPD exacerbation diagnosis. The participants were seen and sampled as per his protocol and the participants were recruited from the London EXCEL COPD Cohort and overlapped with some of the participants recruited into the main RSV study of this thesis. Dr Kamal's main findings have now been published⁴.

4.1 Methods:

As discussed in detail in his thesis, for Dr Kamal's research he screened COPD participants both at exacerbation and baseline. The cohort of participants used for Dr Kamal's research were recruited from the London EXCEL COPD cohort, many of whom were later recruited as participants of the main RSV study of this thesis. The inclusion and exclusion criteria are identical (See Methods section). Dr Kamal sought to identify bacteria and viruses using sensitive diagnostic methods as described in detail in his thesis¹⁶⁰. Dr Kamal and those involved gave me permission to use their data for this analysis. Dr Kamal has already completed his data analysis and published⁴. Neither his thesis nor the paper published include these data, investigating RSV at baseline in COPD, as presented here.

151 sputum samples were tested for RSV using viral PCR analysis (see methods), as per Dr Kamal's thesis¹⁶⁰. These sputum samples were then split into 2 groups for analysis depending on whether they were exacerbation or baseline samples. Baseline samples were coded as those collected either at least 5 weeks post an exacerbation recovery date, or at least 2 weeks

prior to an exacerbation start date. Those samples that were taken at exacerbation, or within 7 days of the exacerbation starting were marked as exacerbation samples. Exacerbations were defined as at least two consecutive days of increased respiratory symptoms as per diary card criteria (see Methods). All exacerbations were treated with antibiotics and / or oral steroids.

The biomarkers of interest that were evaluated were Fibrinogen and CRP. These readings were analyzed in the Royal Brompton NHS biochemistry laboratory. A sub-set of serum samples were also analyzed to investigate the levels of IL-6 and MPO. These serum samples were taken on the same visit as the corresponding sputum. The analysis was conducted using commercial ELISA kits at the NHLI Brompton campus COPD laboratory.

4.2 Results:

151 sputum samples were tested for RSV, obtained from 75 individual patients (for demographics see table 16 in Appendix). 86 met the above criteria to be described as baseline samples, and 65 were coded as exacerbation samples.

For the baseline samples: RSV was found in 17 (19.8%), Picornaviruses in 3 (3.5%) and Influenzae in 0 (0%). For the exacerbation samples: RSV was found in 6 (9.2%), Picornavirus in 14 (21.5%) and Influenzae in 1 (1.5%).

When comparing viruses found in exacerbation sputum samples to viruses found in baseline sputum samples (as per above definitions), Picornaviruses were found to be more common at exacerbation compared to baseline (3/86 vs 14/65 $P=0.0003$ using Chi squared test). Conversely RSV was found to be more common at baseline compared to exacerbation in this sample set (17/65 vs 6/86 $P=0.0011$ using Chi squared test). There were not enough influenzae positive samples to make a statistical comparison.

Next CRP and Fibrinogen levels (measured by the Royal Brompton hospital laboratory) from serum samples taken at baseline visits were investigated. There was a significantly higher fibrinogen and CRP level from the baseline visits where RSV was identified in the sputum compared to the baseline visits where RSV was not identified in the sputum. For CRP the

difference was statistically significant (4.34 RSV +ve / 3.74 RSV -ve $P=0.0383$ using an unpaired T-Test; Figure 12). For Fibrinogen this difference was also statistically significant (0.863 RSV +ve / 0.589 RSV -ve $P=0.0332$ using an unpaired T-Test; Figure 11).

An ELISA was conducted for a subset of the serum samples calculating the MPO and IL-8 levels in the RSV +ve and RSV -ve serum. This was conducted using the previously validated 'off the shelf' Quantikine ELISA from R&D Systems (Catalog # D6050 IL-6¹⁶¹ / # DMYE00B MPO¹⁶²). For IL-6 in the RSV +ve group, the reading = median 0.11375 pg/ml (IQR 0.098 – 0.151). For IL-6 in the RSV -ve group, the reading = median 0.1195 pg/ml (IQR 0.10275 – 0.1365). For MPO in the RSV +ve group, the reading = median 0.87525 ng/ml (IQR 0.76475 – 2.19325). For MPO in the RSV -ve group, the reading = median 1.78875 ng/ml (IQR 1.14125 – 2.26275). After statistical analysis there was no statistical difference using an unpaired T-Test ($P=0.7764$ for IL-6 in the RSV +ve vs RSV -ve group. $P=0.1328$ for MPO in the RSV +ve vs RSV -ve group).

Serum Fibrinogen at Baseline Visit

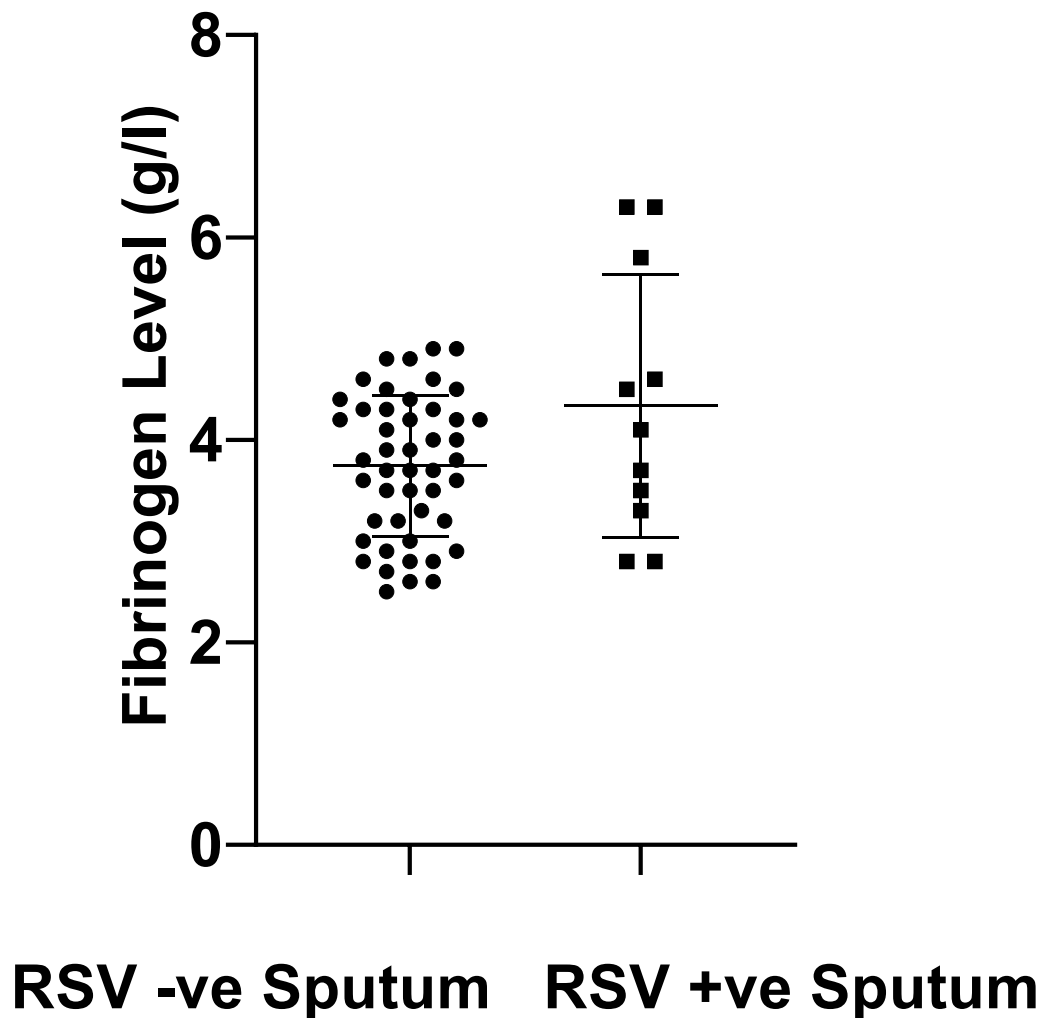
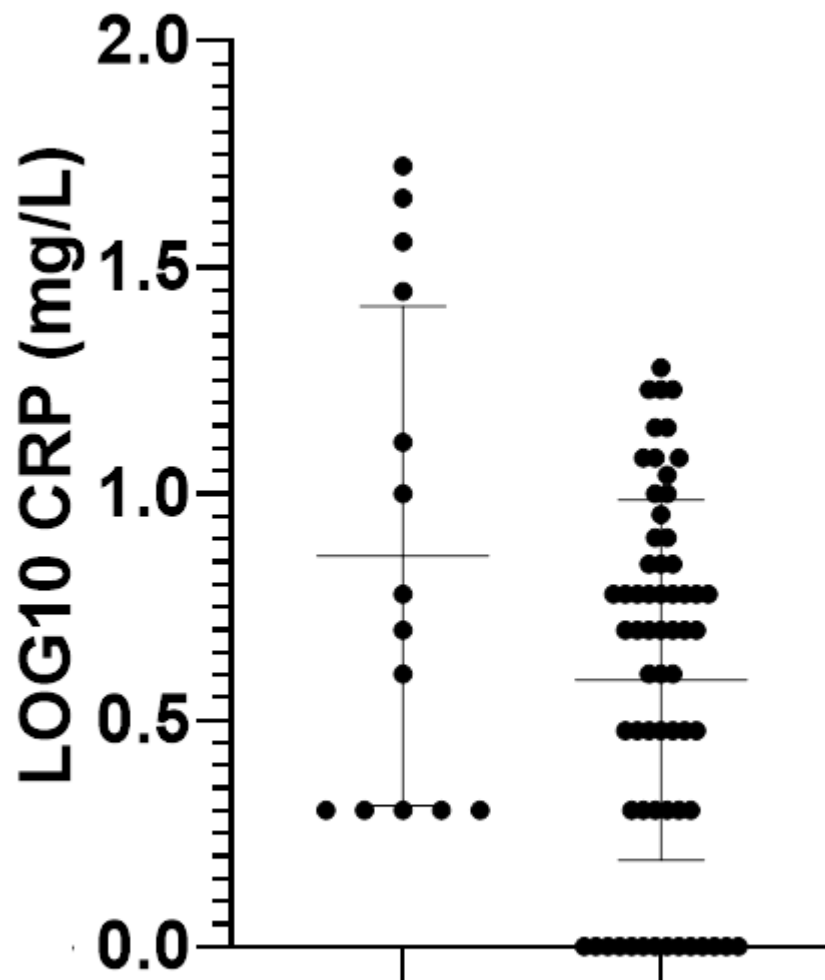


Figure 11 – Serum fibrinogen level at RSV -ve (n=48) vs RSV +ve (n=17) baseline visits. There are three outlying levels in the sputum positive group (P=0.0332 using an unpaired T-Test)

Serum CRP at Baseline Visit



RSV+ve Sputum	RSV -ve Sputum
1. Normal 2. Abnormal	1. Normal 2. Abnormal

Figure 12 – Log10 Serum CRP at RSV -ve (n=48) vs RSV +ve (n=17) baseline visits. There are four outlying levels in the sputum positive group. (P=0.0383 using an unpaired T-Test)

4.3 Summary:

This chapter describes some preliminary data collected before the main study was undertaken. The aim of this chapter was to investigate RSV prevalence in the non-exacerbating state, building on prior data indicating that chronic RSV infection of the lower airway may occur. This chapter also aimed to study the host inflammatory state when RSV was present in sputum. This built on work previously published by Professor Wedzicha's research group. An increase in systemic CRP and Fibrinogen was discovered when RSV was found to be present in the sputum. This indicates that not only may RSV be present in a baseline (non-exacerbating) state, but the presence of RSV is also associated with markers of systemic inflammation.

RSV prevalence in baseline sputum samples will be investigated later on in this study. No evidence of RSV persistence was discovered at the baseline state from the sputum samples analysed in the main results chapter of this thesis. This was however using a different method of PCR based RSV detection (as compared to gel-based in this analysis) and might not be directly comparable. More studies are needed in this area as the current literature remains conflicting as to the presence of RSV in COPD outside of acute exacerbations.

5 Results

RSV is a disease found world-wide that affects all age groups. Amongst adults, those with chronic heart or lung conditions, such as COPD appear to be most adversely affected by RSV. This study aims to fill gaps in our understanding of RSV in COPD patients by investigating the prevalence and severity of RSV illness along with differences in severity between RSV and other COPD exacerbation causes.

5.1 Recruitment:

5.1.1 Recruitment Introduction:

There were multiple methods used to recruit participants into the study and the success rates for each method are described below. Four methods were used to generate a pool of participants to contact and subsequently recruit into this study. Each of these methods is described in detail below. I have listed the methods from least successful to most successful in terms of recruitment success. These methods were not conducted sequentially but were performed simultaneously. More time and effort were focused on the more successful methods. Most of the participants were recruited directly from the London EXCEL COPD Cohort as described below. We did not have access to the exact details of the recruitment methods used to recruit participants into the Groningen cohort.

5.1.2 Recruitment Methods and Success Rates:

5.1.2.1 Advert:

The first, and least successful method was to place an advert in the local newspaper (Kensington and Chelsea Times)¹⁶³. The advert was written by myself, and I sent the advert for both ethical approval (as discussed in the methods), and approval by The Royal Brompton Foundation Trust prior to publication (Advert in Addendum). Out of 15 individuals that contacted us by telephone, only 3 met the telephone screening criteria (as per methods) and were invited to a screening visit. Of those 3 potential participants, only one met the inclusion criteria and was eventually recruited into the study as a participant (1/15 6.67%). We did not

try any other local newspapers, as the recruitment from this method was so poor on the initial attempt. Despite the circulation advertised as being ~200,000 and the paper being out three monthly, there was little interest shown for this advert.

5.1.2.2 Discover Register

The second method utilised the newly created 'Discover' register¹⁶⁴. This is a private register of patients in North-West London who have agreed to be contacted for medical research purposes. They have each been coded (by the system) with any medical conditions or diagnoses they have. We asked to see a list of those coded as COPD. Of the 38 individuals contacted, 18 passed the telephone screening and were sent a copy of the PIS. Out of those 18 individuals, 9 attended for a recruitment visit and of those, 3 participants were recruited into the study (figure 14).

Method 2 – Discover Register

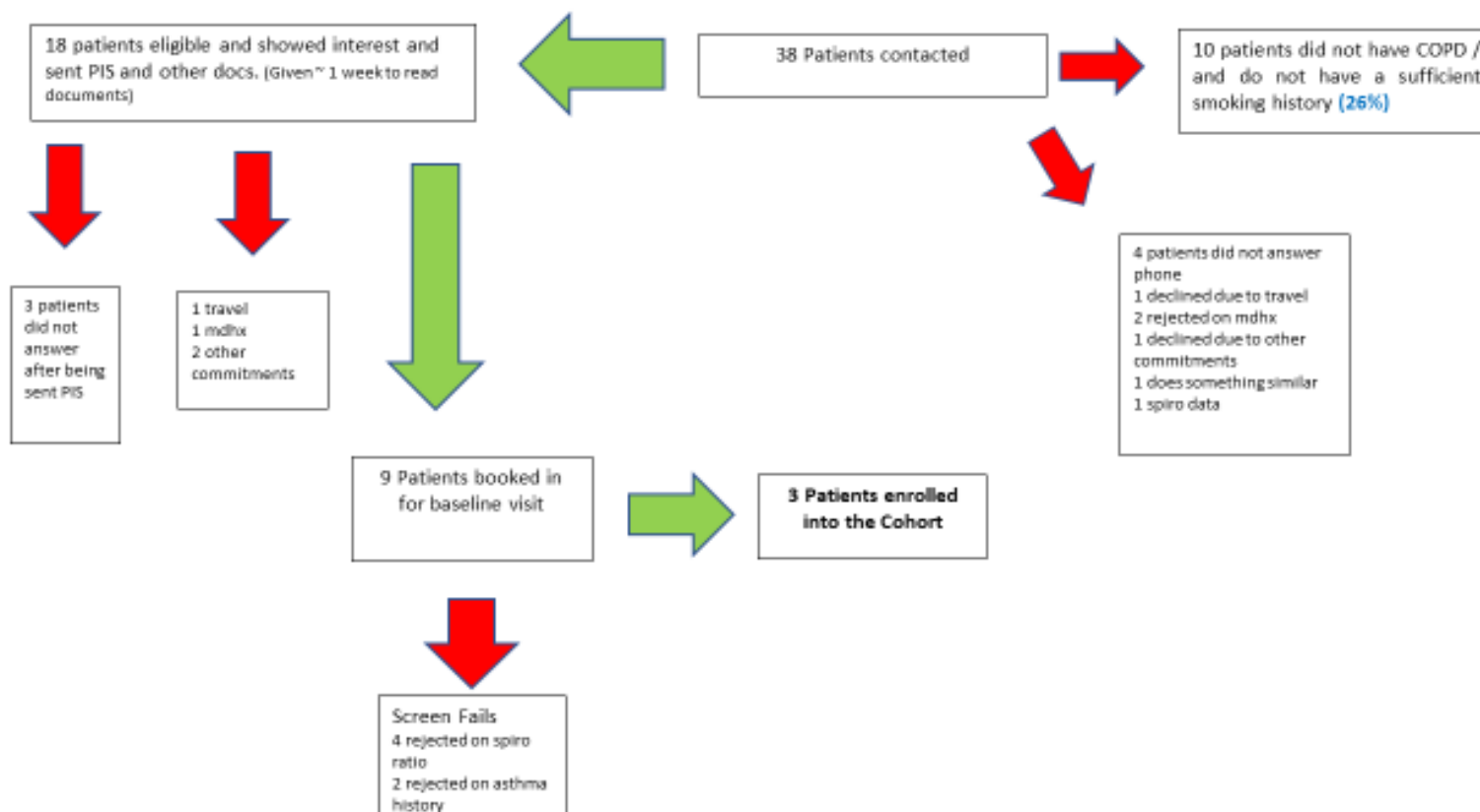


Figure 13 – Discover Register Recruitment

5.1.2.3 West London Lung Cancer Screening Study

The third method of recruitment used was to contact participants enrolled in the West London Lung Cancer Screening Study¹⁶⁵. These patients were pre-screened for respiratory obstruction, with spirometry in the community, before entering the cancer screening study. They had also signed a form consenting to contact for recruitment to other studies. From an initial list of 195 patients, 169 participants were electronically screened on EPR (electronic patient records). Of those 169, 111 appeared to meet the inclusion criteria and were contacted. Of those 111 contacted, 47 showed an interest and were sent a PIS. This resulted in 28 individuals being booked in for a screening visit. Out of those 28 individuals, 26 met the inclusion criteria and were recruited into the study as participants.

5.1.2.4 London EXCEL COPD Cohort

The majority of participants were recruited directly from London EXCEL COPD Cohort¹⁶⁶. This is a well-established, precisely defined cohort of COPD patients based at the Royal Brompton hospital. Under the leadership of Professor Jadwiga Wedzicha, it has been active since 2002. Multiple highly cited papers have already been generated by this cohort and visits for this study could run alongside the usual visits the participants would be having as members of the London EXCEL COPD Cohort. The inclusion/exclusion criteria for the COPD RSV study (this study) were created with the EXCEL COPD Cohort in mind, and therefore identical. This means that all of the patients in the EXCEL COPD Cohort should theoretically meet the inclusion criteria for the COPD RSV study. Out of the 149 individuals contacted and sent the PIS, 147 agreed to join and were recruited into the COPD RSV study as participants (figure 15).

5.1.3 Recruitment Method Success Overview:

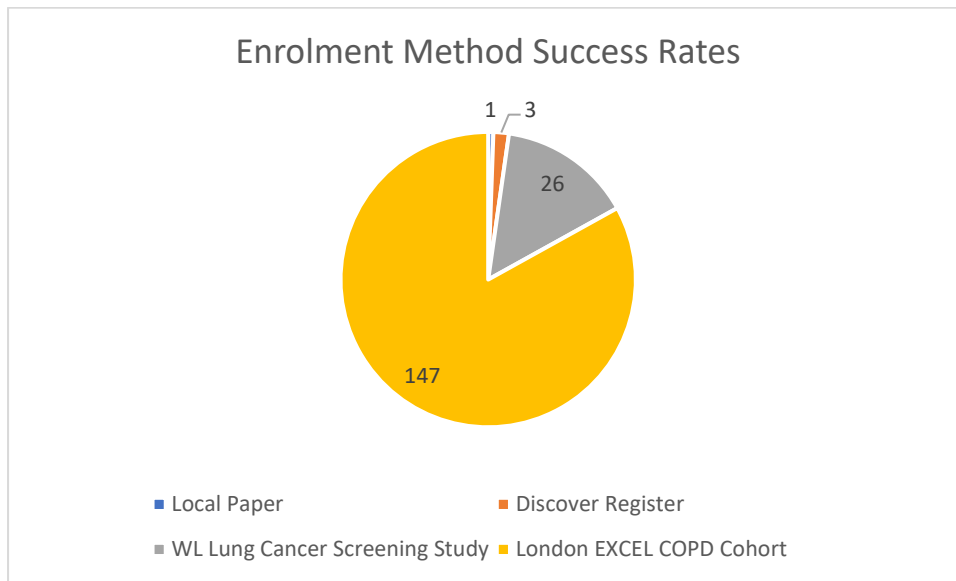


Figure 14 – Overall Recruitment Success Rates by Method

5.2 Recruitment results:

Using the three above methods, and recruiting directly from the London EXCEL COPD Cohort, a total of 177 participants were recruited into the study between 13/11/2017 and 13/12/2019 (for demographics see table 8). The mean demographic data included: age 72.1 years, height 1.69m, FEV1% predicted 65.2%, FEV1/FVC ratio 0.53, average pack years 49.5 years. 34% are current smokers and 63% male, 37% female.

	Mean	St Dev	Min	Max
Age (years)	72.11	7.40	43.98	90.85
Height (m)	1.69	0.09	1.44	1.89
Predicted FEV1 (L)	2.56	0.60	1.10	3.98
FEV1 percentage of predicted	65.21%	20.73%	20.63%	132%
FEV1/FVC	53.28%	10.83%	19.04%	69.95%
Pack Years	49.53	31.15	10.00	180.00
Current Smoker	60 (34%)			
Male Sex	112 (63%)			
Female Sex	65 (37%)			

Table 8– Cohort Demographics

155 of the 177 patients recruited completed the study from recruitment till the end date (87.57%). Of those that did not complete the study, 8 participants died before the end date (4.52%), while 14 participants requested to withdraw before the study end date (7.19%) (figure 16). The total time in the study ranged from 49 to 850 days. The median number of days in the study per participant was 748 (Inter Quartile Range (IQR) 424-814). The total follow-up days were 108,557 with total follow-up years of 296.8 years.

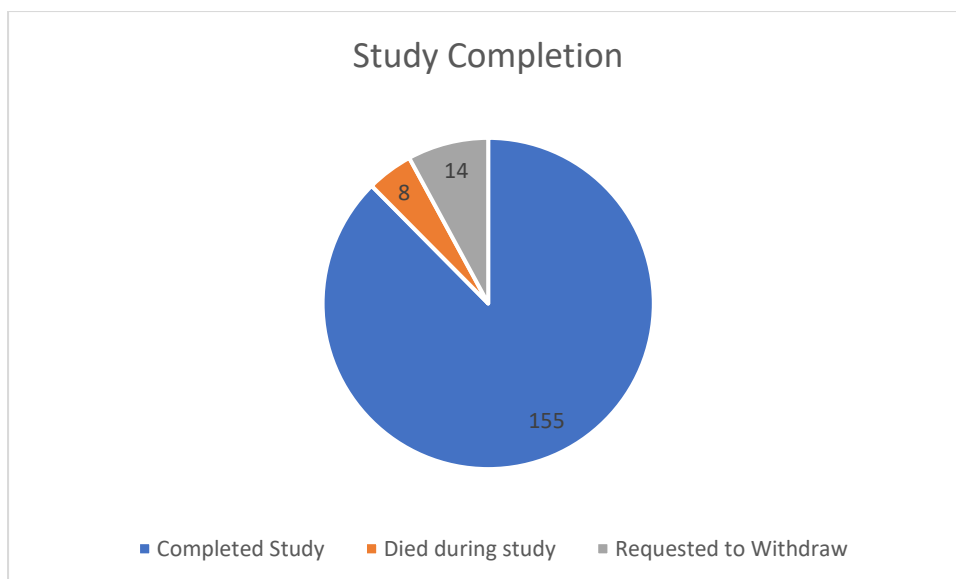


Figure 15 – Study Completion Numbers

5.3 Visit Statistics:

1,175 individual visits were conducted consisting of 188 exacerbation visits, 222 exacerbation follow-up visits and 765 baseline (3 or 6 monthly) visits. These visits all followed the protocol outlined in the methods section regarding recruitment, baseline, exacerbation, and exacerbation follow-up visits. There were 248 ‘unreported’ exacerbations, where diary card criteria were met, however the participant did not attend hospital for an exacerbation visit. There are multiple reasons why a participant may not be able to be seen in the cohort at exacerbation, as described in the methods, but the main reason was that they did not contact the cohort clinical team as they felt the symptoms were not severe enough.

5.4 NP swab and Sputum Results

5.4.1 Samples Tested for viral PCR:

During 175 of the 188 (93.01%) exacerbation visits the participant was sampled using NP swabs for multiplex PCR and RSV qPCR. 5 participants declined the NP swab at exacerbation visit, and for 8 of the visits there was temporarily not enough reagent (M4RT) available to take and store the swab. 195 sputum samples were also tested using the same multiplex PCR

and qPCR for RSV, 93 sputum samples from exacerbation visits, 20 sputum samples from exacerbation follow-up visits and 82 sputum samples from baseline visits. 60 NP swabs were sent for testing at exacerbation using the in-house multiplex PCR at the Royal Brompton Hospital NHS labs. 35 of these swabs were overlapping (from the same patient visit) and were able to validate the swabs sent for external PCR.

5.4.2 PCR Results (GSK):

These samples were collected and stored as per protocol which has been described in detail in the methods chapter. These samples were sent to GSK for analysis of the multiplex PCR panel and qPCR for both RSV A and B. The multiplex PCR included testing for Respiratory Syncytial Virus (RSV) A, RSV B, Rhinovirus, Flu A, Flu B, Flu A (California/7/2009 H1N1), Flu A (H3), Human Coronavirus (HCV)-NL63, HCV-229E, HCV-OC43, Human bocavirus, Human Enterovirus, Human Adenovirus, Parainfluenza Virus (PIV)-1, PIV-2, PIV-3, PIV-4 and Human Metapneumovirus (HMPV).

3/175 (1.71%) of the NP exacerbation swabs were positive for RSV (2 RSV B, 1 RSV A). The viral count numbers ranged from 6,853 to 86,503,569. 5/93 (5.38%) of the sputum samples at exacerbation were positive for RSV (1 RSV B, 4 RSV A). The viral count numbers ranged from 691 to 32,516,780. Comparing the NP swabs vs sputum there was a trend to a higher positivity rate for RSV from the sputum analysed compared to the NP swabs, but this did not meet statistical significance (using a chi-squared test, $P=0.0936$).

60 sputum samples from baseline, or non-exacerbating visits were also investigated for RSV using PCR. None of these baseline sputum samples were found to be positive for RSV in this cohort.

Full GSK PCR NP swab results in figure 17 and the GSK PCR sputum results are shown in figure 18.

PCR Data from GSK NP Exacerbation Swabs

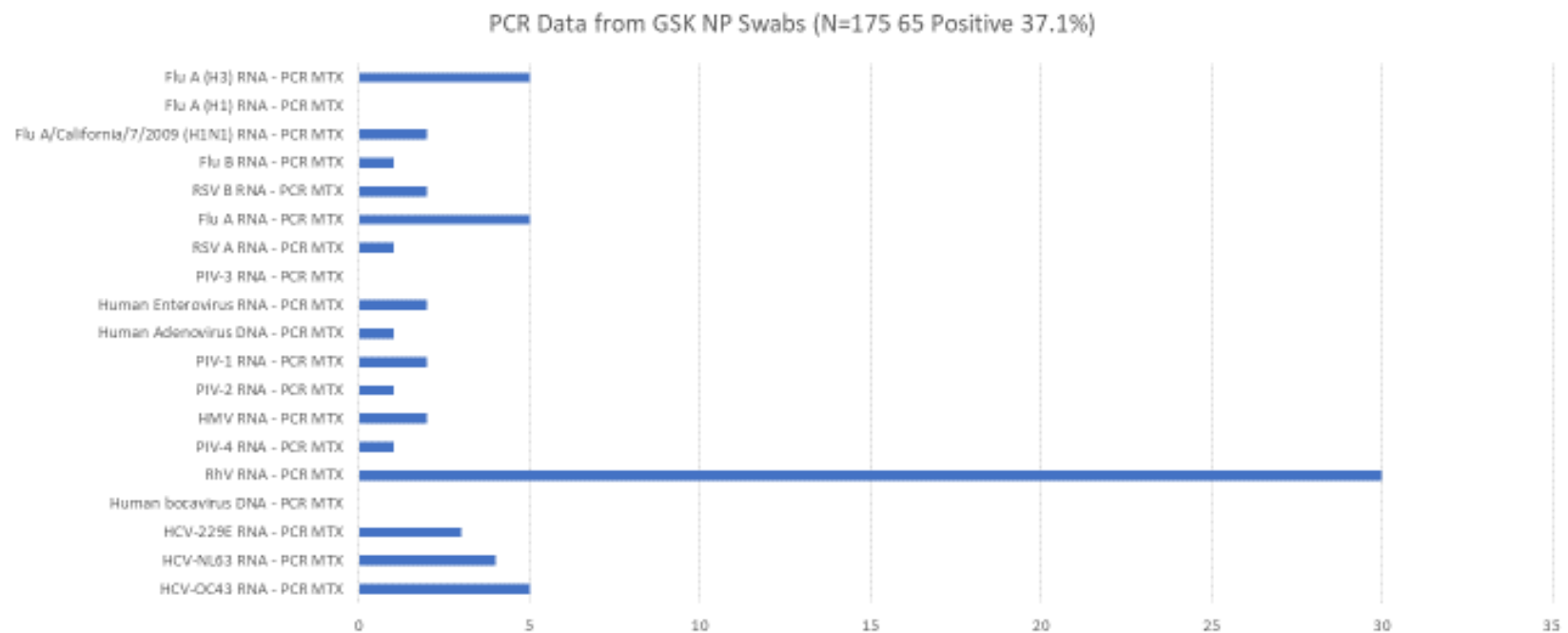


Figure 16 – NP Exacerbation Swabs Viral PCR Results (GSK)

PCR Data from GSK Exacerbation Sputum

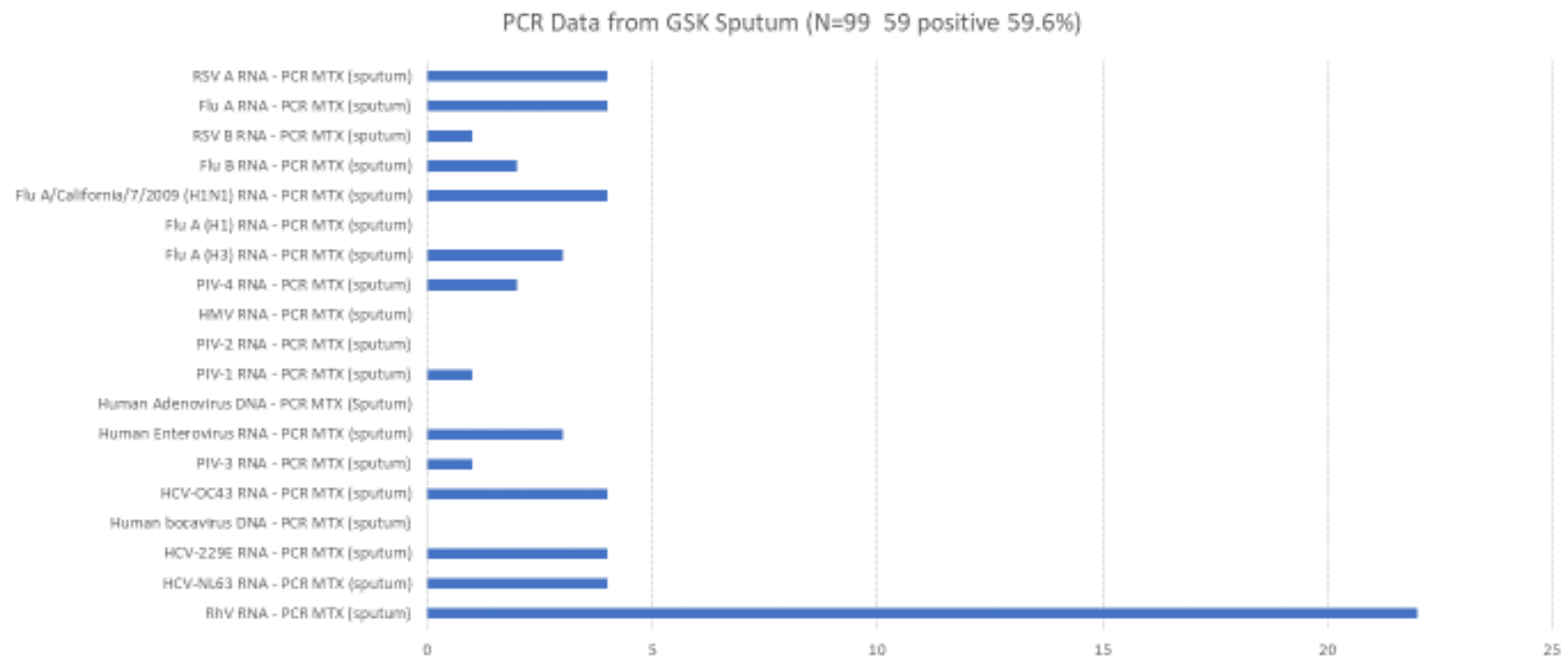


Figure 17 – Exacerbation Sputum Viral PCR Result (GSK)

5.4.3 PCR RSV Results (Royal Brompton PCR):

The GSK swabs were being sent off and tested in large batches, therefore the results would not be ready for answering clinical questions or guiding treatment. Because of this NP swabs from our participants were also often sent to the Royal Brompton Hospital (RBH) NHS laboratory for PCR analysis based on clinical indication.

2/60 (3.33%) RBH NP swabs were positive for RSV at exacerbation. One of those RSV positive results over-lapped with the externally tested NP swabs. No RBH RSV swabs were negative when the external swabs were positive. One RBH NP RSV PCR positive swab was taken when we were out of stock of the GSK swab reagent, therefore no comparison swab was sent (full RBH PCR NP swab results in the figure 19).

PCR Data from Brompton NP Exacerbation Swabs

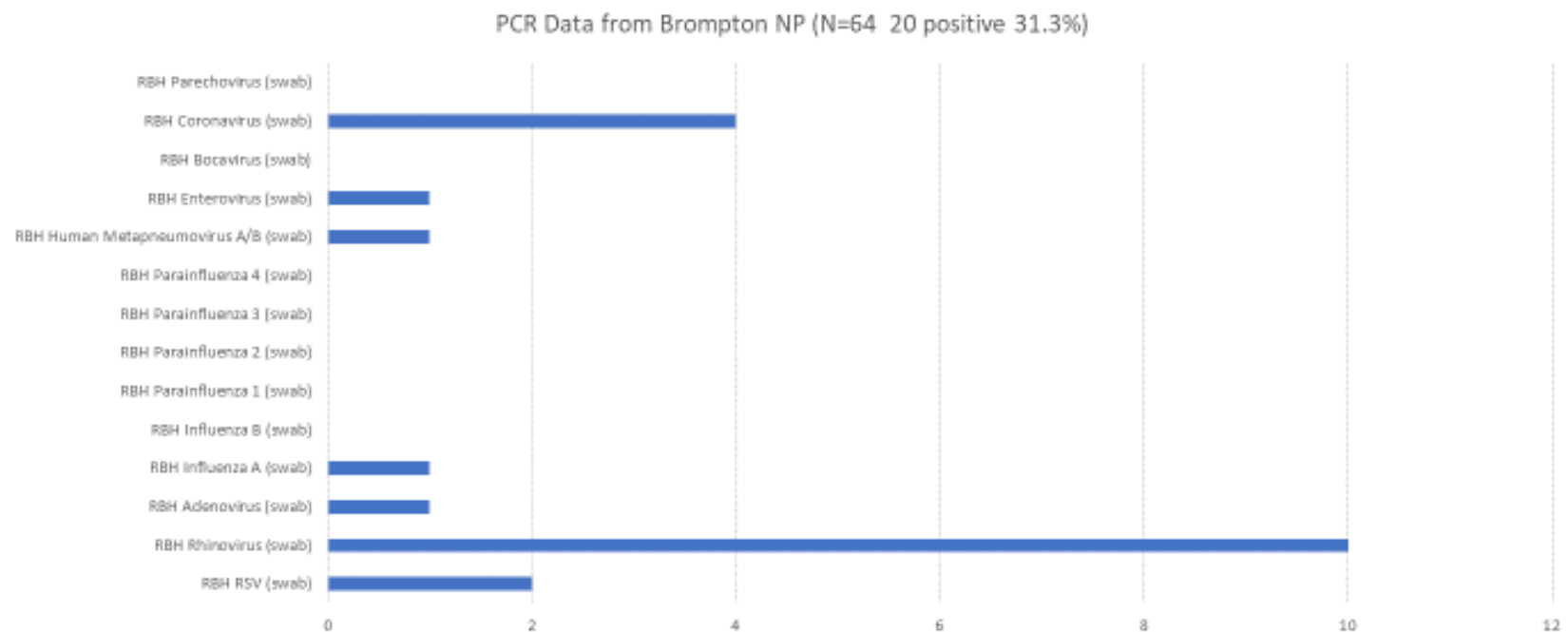


Figure 18 – NP Exacerbation Swabs Viral PCR Results (RBH)

5.4.4 RBH Sputum Bacteriology Results:

Sputum was sent as per protocol to the Royal Brompton hospital laboratories for bacterial analysis of sputum culture. The results are graphed (figure 20). These results were used to guide clinical decisions such as antibiotic treatment. Sensitivities are available on the Royal Brompton hospital electronic patient records system.

RBH Sputum Bacteriology at Exacerbation

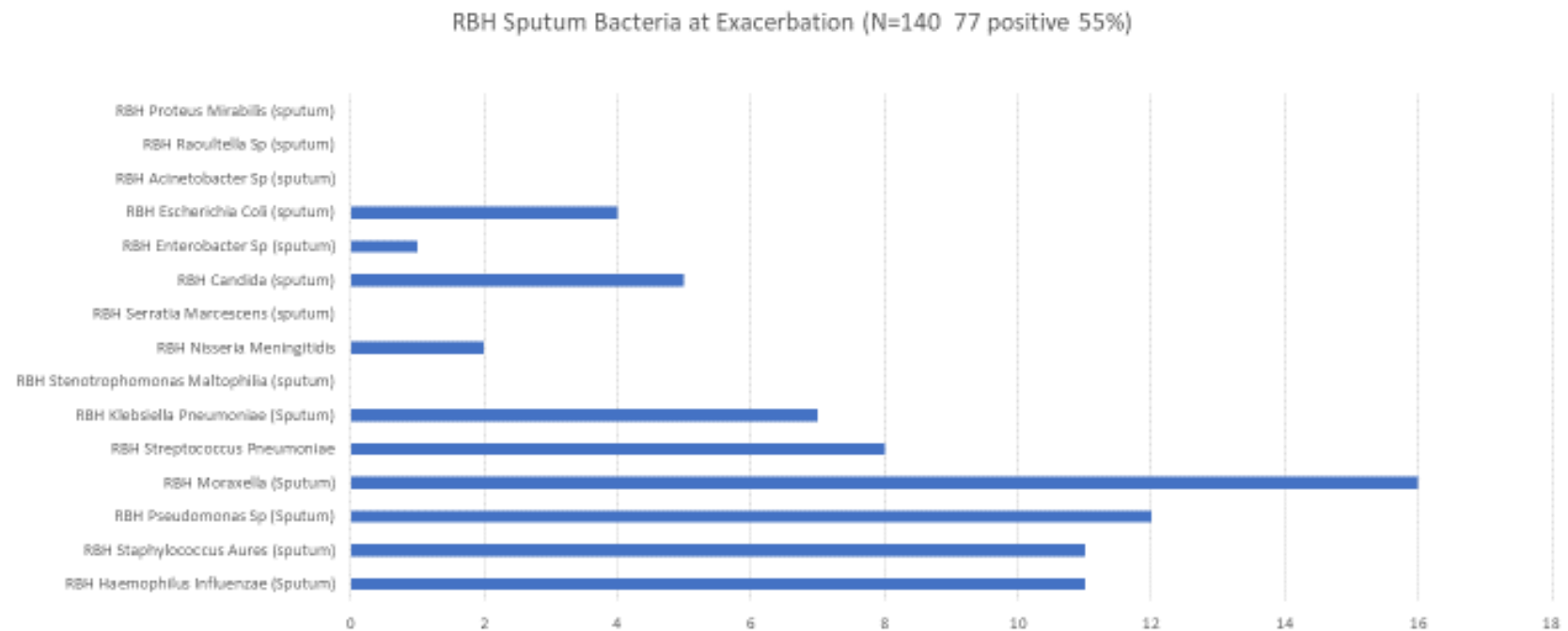


Figure 19 – Sputum Microbiology Results

5.5 RSV Antibody measurements:

PCR remains the most specific test for RSV virus related exacerbation, but any positive diagnosis relies on the timing of sample collection. Late sampled exacerbations may miss an RSV positive exacerbation. In addition, patients who fail to report exacerbations will also be unsampled. Blood tests that detect antibodies due to RSV will overcome some of these problems and may be a useful way to gain a more accurate estimation of the true prevalence of RSV infection.

30 RSV associated exacerbations were diagnosed using a 4-fold rise in antibody measured in the serology (see methods chapter for details). This was using the novel RIVM Pentaplex method measuring Ga, Gb, N protein, Pre-F and Post-F RSV specific IgG and the Janssen ELISA method for measuring Pre-F and Post-F RSV specific IgG. Seven of these 4-fold rises overlapped with the RSV PCR positive results. This resulted in 32 individual RSV-related exacerbations being diagnosed in total over the 3-year study period (figure 49). These included both the attended exacerbations and those unattended exacerbations identified through review of diary cards and applying the criteria for diagnosing an exacerbation as described in the methods section. As described in the methods, to detect a 4-fold rise in antibody, there needs to be a serum sample pre and post the event available for analysis. This was not the case for every exacerbation, both attended or unattended. If more than one exacerbation occurred between the serum sample collections, these were excluded from severity analysis (as described later in this chapter) as it would not be possible to accurately determine which of the multiple exacerbations during that time point were RSV related. These 4-fold rises were however included in the total RSV associated exacerbation numbers.

5.5.1 Pentaplex Specific Antibody measurements:

Six full 96 well plates were analysed using the pentaplex method, as described in detail in the methods chapter. This enabled the analysis of serum from 89 patients (most covering multiple exacerbations and recovery points). As previously stated, we discovered 30 RSV related events, when counting a 4-fold rise in RSV specific IgG as a positive result. A four-fold change

in any three of the RSV specific IgG antibody types (N, Pre-F, Post-F) was considered a positive RSV result (meaning the exacerbation would be designated as RSV related). Using the pentaplex method we analysed 178 events, of which 30 were positive (16.85%). Not all serum samples were analysed, as there was only enough reagent available for a selection of the samples. Note 4 cases only showed a 4-fold rise in either Ga or Gb and were discounted from the RSV totals, as discussed later in the results section.

If one of the five RSV specific IgG antibodies met the four-fold rise cut-off, in 85% of cases at least one other also reached the 4-fold cut-off (figure 21). Taken across multiple timepoints often more RSV specific IgG antibodies would eventually meet the four-fold rise. Some of the RSV protein specific IgG antibodies (especially the Ga, Gb and Pre-F), rise slower than the N + Post-F. This does not alter the number of positive cases.

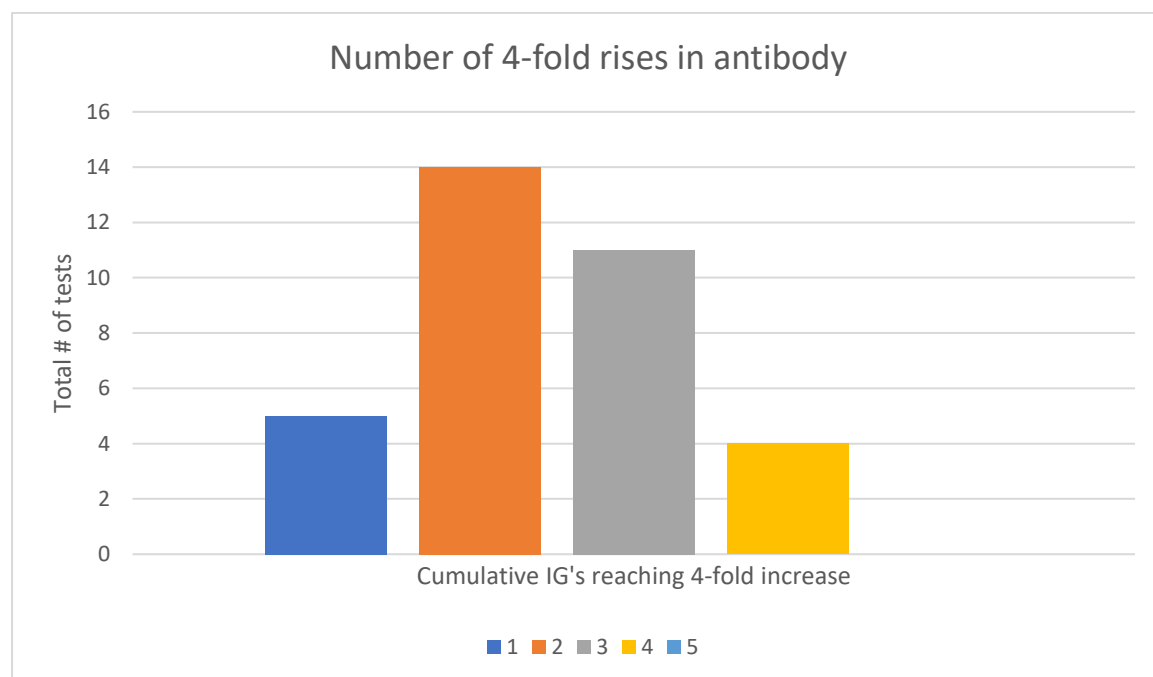


Figure 20 – 4-fold cumulative antibody rises for 'positive' results

5.5.2 PCR Positive Cases Pentaplex results:

Below are 3 representative charts showing 3 RSV positive exacerbations where we also analysed serology using the pentaplex method across multiple timepoints. Time in days is displayed along the X-axis and fold change in antibody titre on the Y-axis. These are exacerbations where RSV was diagnosed on PCR at the time of exacerbation. Day 0 does not

indicate the day the exacerbation started, but the day that the exacerbation NP swab was taken (which subsequently was found to be positive for RSV), therefore this was the day the participant visited the research centre. What is immediately evident from the charts is that regarding these three RSV associated exacerbation events the IgG specific for RSV N protein demonstrates a greater fold change as compared to IgG specific for RSV Pre-F, Post-F Ga and Gb protein (figures 22-24).

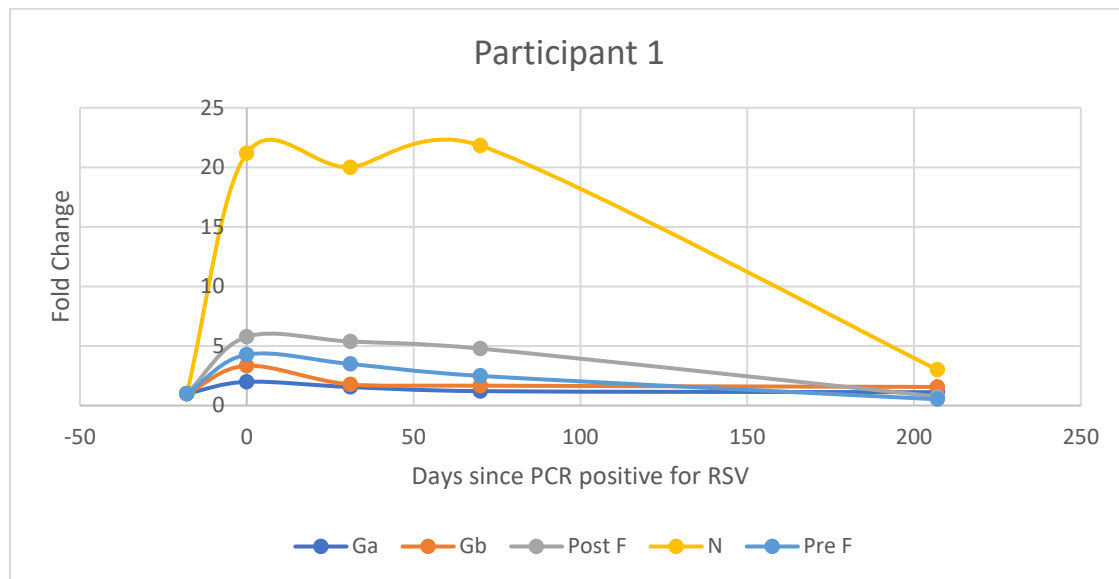


Figure 21 – Fold change (x-axis) vs time in days (y-axis) for participant 1. Day 0 is when PCR was positive for RSV

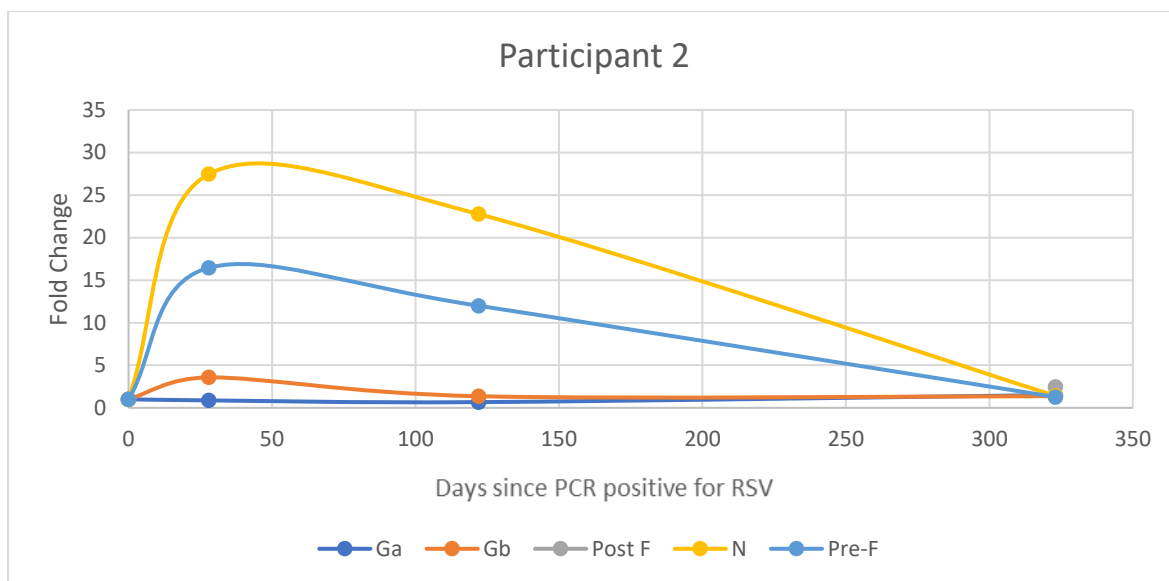


Figure 22 – Fold change (x-axis) vs time in days (y-axis) for participant 2. Day 0 is when PCR was positive for RSV

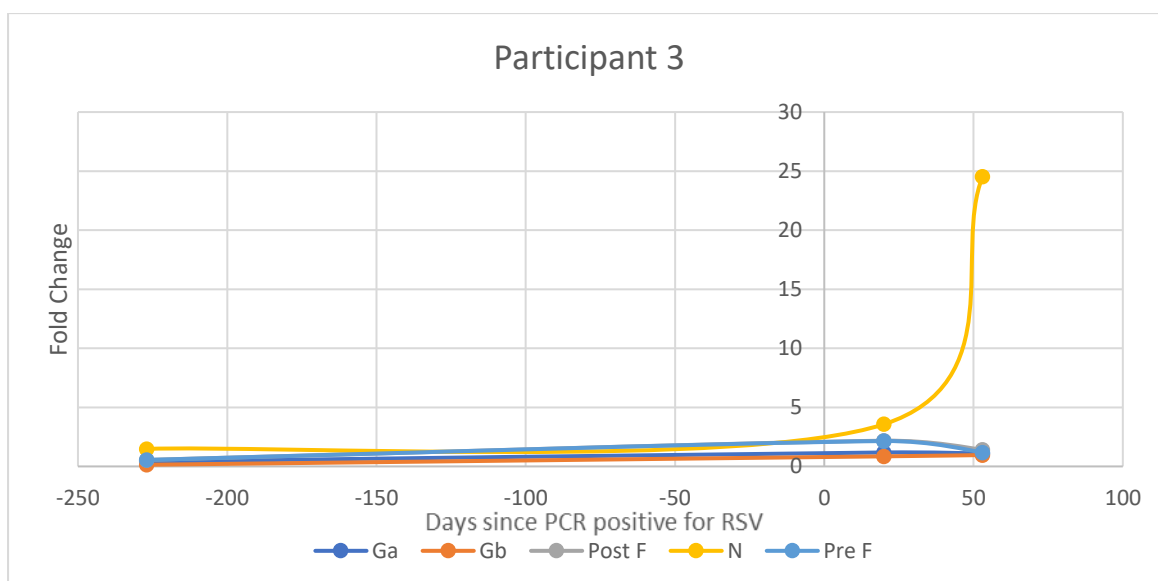


Figure 23 – Fold change (x-axis) vs time in days (y-axis) for participant 3. Day 0 is when PCR was positive for RSV

5.5.3 N Antibody rises:

Figure 25 shows 5 RSV positive exacerbations where serology was also analysed post PCR positivity using the RIVM pentaplex method. Time in days is displayed along the X-axis and fold change for RSV N protein specific IgG antibodies on the Y-axis. These are exacerbations

where RSV was diagnosed on PCR at the time of exacerbation. Again, day 0 does not indicate the day the exacerbation started, but the day that the exacerbation NP swab was taken which subsequently was found to be positive for RSV, therefore this was the day the participant visited the research centre. These charts together indicate that RSV associated N antibody might have a significant rise during RSV associated exacerbations. We will investigate and explore this further in the next section of this chapter where we aim to validate antibody rise for RSV specific IgG to N protein as a diagnostic method for RSV related events.

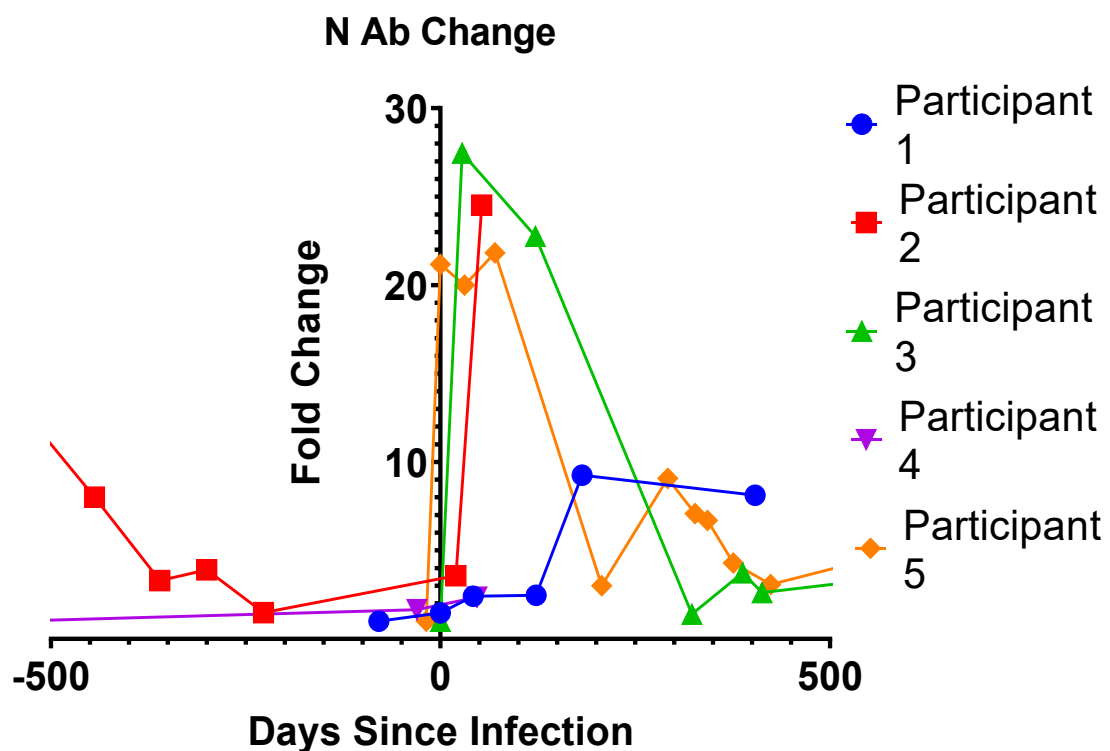


Figure 24 – Fold change for RSV specific protein N Ab for 5 participants with positive PCR for RSV. Day 0 is day of positive PCR test.

5.6 Pentaplex Results Validation:

As this is the first study where the novel RIVM pentaplex has been used for diagnostics, here we will aim to validate this method.

5.6.1 RSV vs Rhinovirus associated Exacerbations

5.6.1.1 Plan of analysis and selection of exacerbations for comparison

The previous analysis indicated that there are increases in serum anti-RSV antibody titres shortly after an RSV-associated exacerbation, and that titre rises in anti-N antibodies are particularly apparent in these 5 cases where sequential samples were collected shortly before and after an exacerbation. We next sought to determine the potential use of anti-RSV antibody titre rises as a diagnostic method for identifying RSV cases that may have been missed by PCR testing.

Exacerbations can have many triggers, the best comparator to examine whether antibody changes could identify RSV associated exacerbation, was to compare to those exacerbations triggered by another respiratory viruses. Rhinovirus is known to cause COPD exacerbations¹⁶⁷. There are also a number of rhinovirus associated exacerbations observed (n=14 with concurrent serology analysed) in this cohort. Comparing the RSV related exacerbations with the Rhinovirus related exacerbations can therefore take place. These exacerbations occurred with Rhinovirus being found at the exacerbation visit, either through NP PCR sampling or sputum PCR sampling. The reason for choosing rhinovirus positive exacerbations is to use this group as a surrogate for RSV negative exacerbations. This is not a perfect solution, as RSV and Rhinovirus exacerbations have been found to occur together in previous literature¹⁶⁸. There were no cases observed in this study of co-infection between these two viruses. There is a possibility of one or more viruses being undetected by the sampling protocol, so co-infections cannot definitely be ruled out.

Any events where evidence of any other exacerbation causing pathogen (as a co-infection), either bacterial or viral, were excluded from this analysis. Any exacerbations where the participant was not seen and sampled again by our team before having a second exacerbation

were also excluded. The diary cards were then carefully reviewed, and we excluded any participants that had exacerbated by diary card criteria between visits, and therefore between serological sampling. The aim of this process was to, as best as possible, filter out any exacerbations that might have been caused by other pathogens, and to ensure, as best as possible, that the participant had not exacerbated again in-between visits. If they had exacerbated again in-between visits we may be measuring a rise in RSV specific IgG related to a different exacerbation event.

The above criteria were employed with the aim of being able to compare clinical and serological data between these two groups and have the minimal chance of these comparisons being influenced by secondary or concomitant infections.

After the above criteria was employed to the exacerbation data, 5 RSV related exacerbations and 14 Rhinovirus related exacerbations were left to compare and analyse. These would form the first comparator group for the initial analysis of serological changes during known RSV exacerbations and presumed non-RSV related exacerbations.

5.6.1.2 RSV Specific IgG Antibody Change – RSV vs Rhinovirus Related Events (PCR Diagnosed):

To evaluate the sensitivity and specificity of the RIVM pentaplex measuring RSV specific IgG antibodies (Ga, Gb, N, Pre-F, Post-F) these selected RSV associated exacerbations (as per PCR diagnosis, n=5) were compared to rhinovirus associated exacerbations (as per PCR diagnosis, n=14). All of the RSV and rhinovirus associated exacerbations had a serum sample analysed on the pentaplex at / before and after the exacerbation with no more than a maximum of 180 days in-between sampling, and no more than 90 days post the exacerbation episode. 180 days was chosen as ‘across one season’ is the standard maximal duration between measurements as per previous literature¹⁶⁹. Looking at the charts of RSV N protein specific IgG above (Figure 25), 90 days post event should include most of the four-fold rises, as after 90 days post-exacerbation, RSV-N antibody titres started to decline.

These fold changes were compared to for all 5 RSV protein specific IgG antibody types evaluated by the RIVM pentaplex, namely Pre-F, Post-F, N, Ga and Gb (see methods). There was a statistically significant increase in the fold-change for Pre-F, Post-F and N after an RSV exacerbation when compared to a Rhinovirus exacerbation (using a Mann Whitney U-Test (fold change averages given here Rhinovirus / RSV) 2.03 / 7.05 $P=0.011$, 0.92 / 8.41 $P=0.011$, 1.42 / 13.44 $P=0.006$ respectively) (see figures 26 – 28). There was no statistical significance in fold change for Ga or Gb antibodies when comparing rhinovirus exacerbations to RSV exacerbations. RSV A was not separated from RSV B when conducting this part of the analysis, as the number of RSV positive exacerbations that met the above criteria was too small. This is likely to be why the IgG to RSV specific Ga and Gb did not meet the sensitivity level (see figures 29+30).

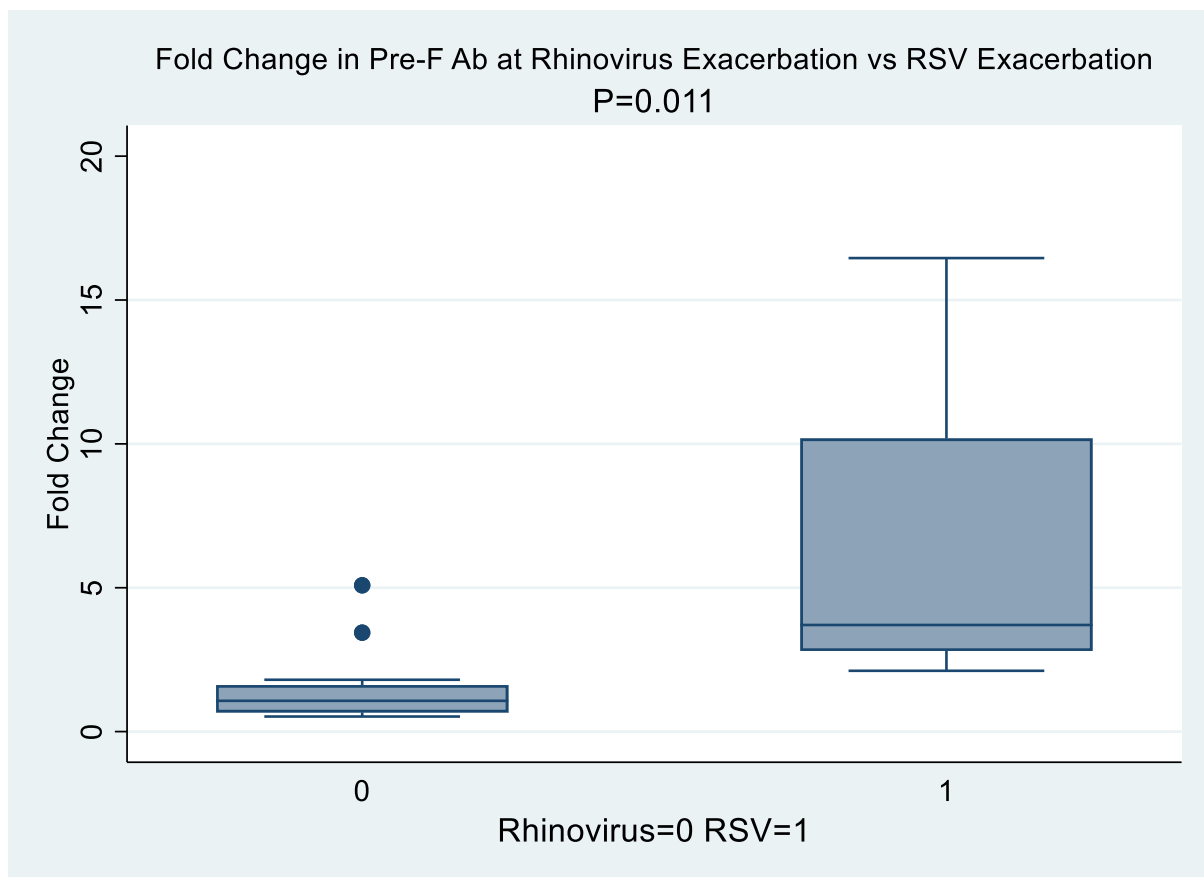


Figure 25 – Fold change in RSV specific Pre-F protein Ab in RSV vs Rhinovirus associated exacerbations. $P=0.011$ using Mann Whitney U-Test (RSV $n=5$ Rhino $n=14$)

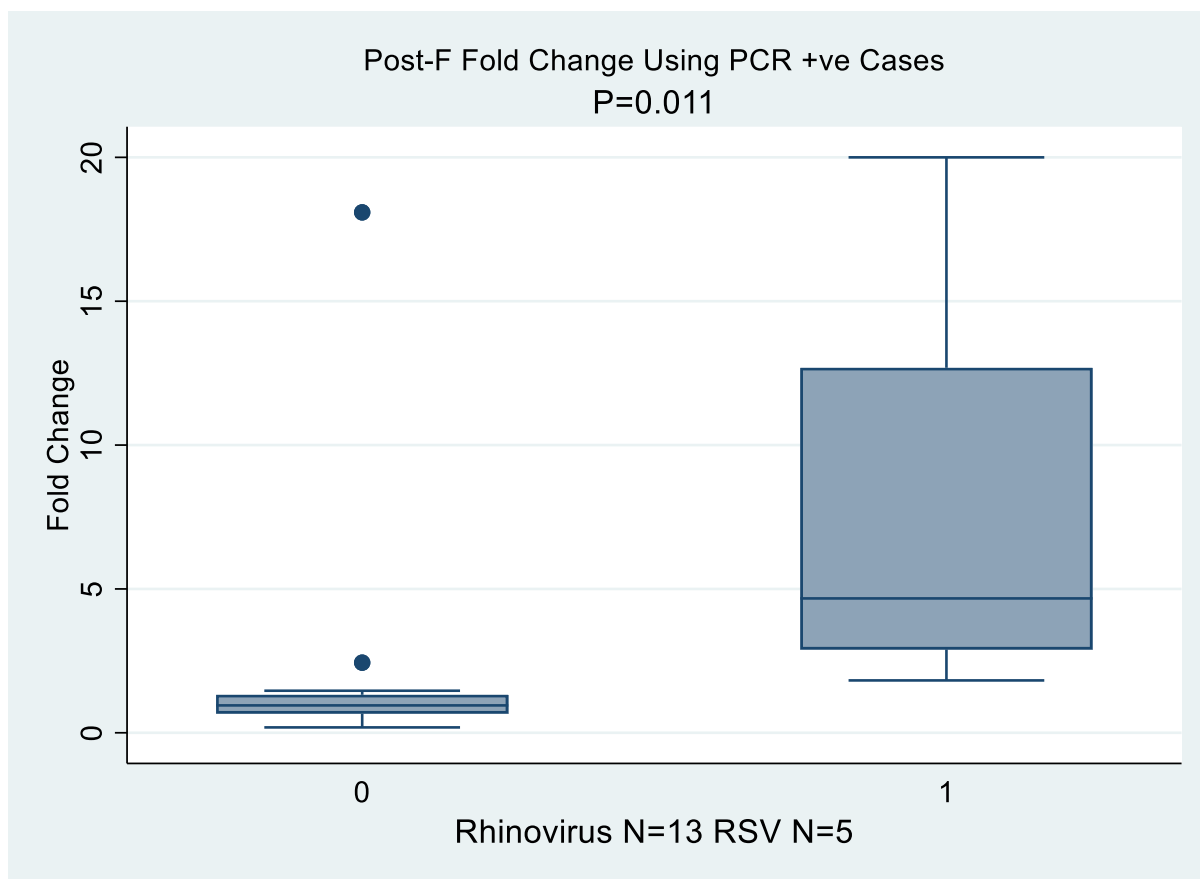


Figure 26 – Fold change in RSV specific Post-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.011 using Mann Whitney U-Test. 0=Rhinovirus associated Exacerbations, 1=RSV associated Exacerbations. (RSV n=5 Rhino n=14)

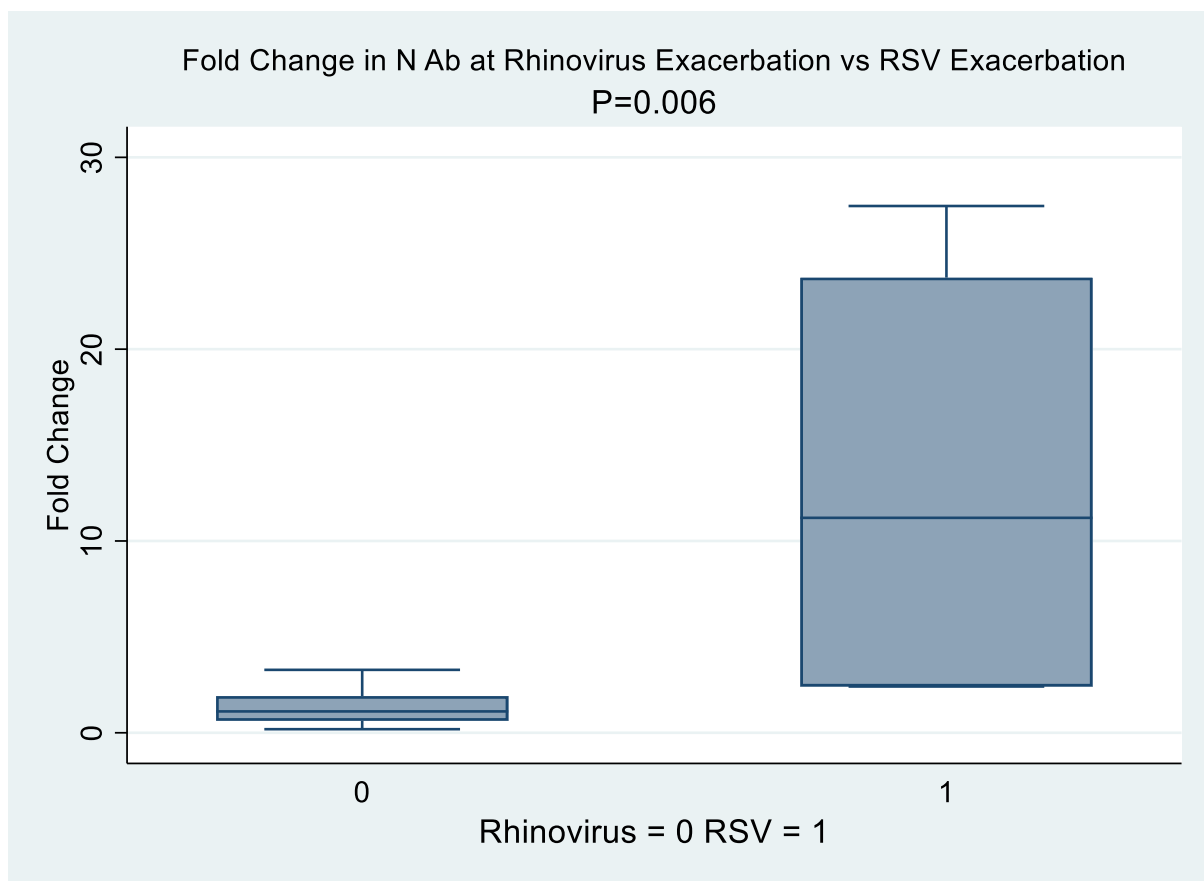


Figure 27 – Fold change in RSV specific N protein Ab in RSV vs Rhinovirus associated exacerbations. $P=0.006$ using Mann Whitney U-Test (RSV $n=5$ Rhino $n=14$)

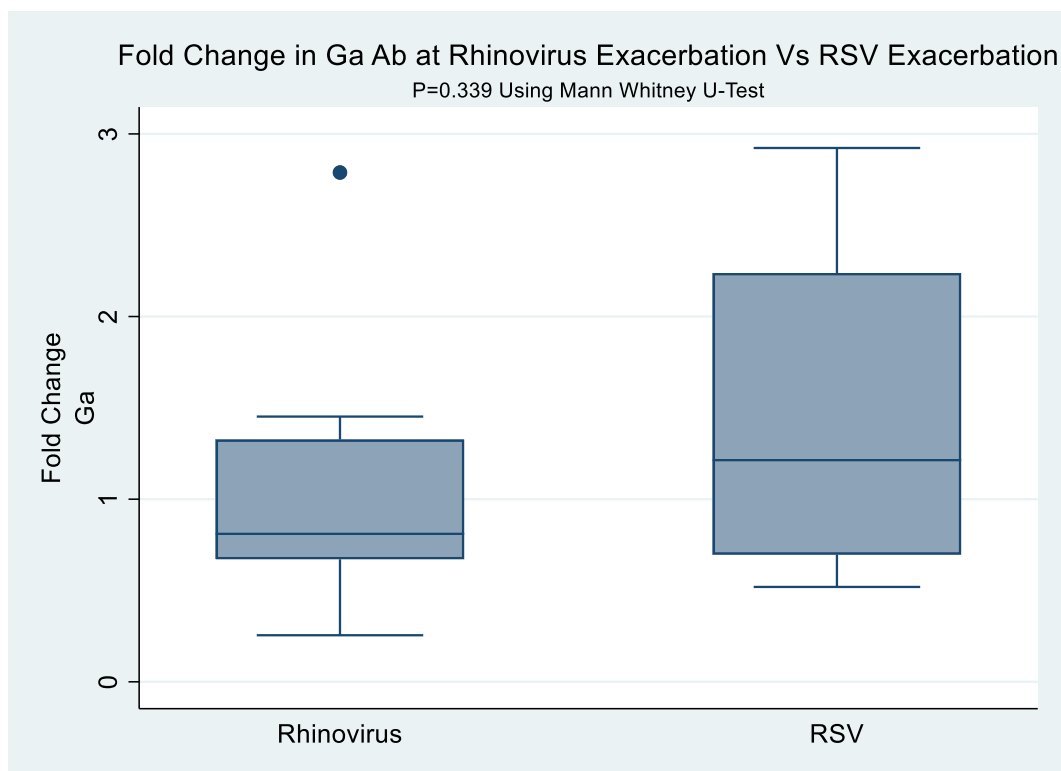


Figure 28 – Fold change in RSV specific Pre-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.339 using Mann Whitney U-Test (RSV n=5 Rhino n=14)

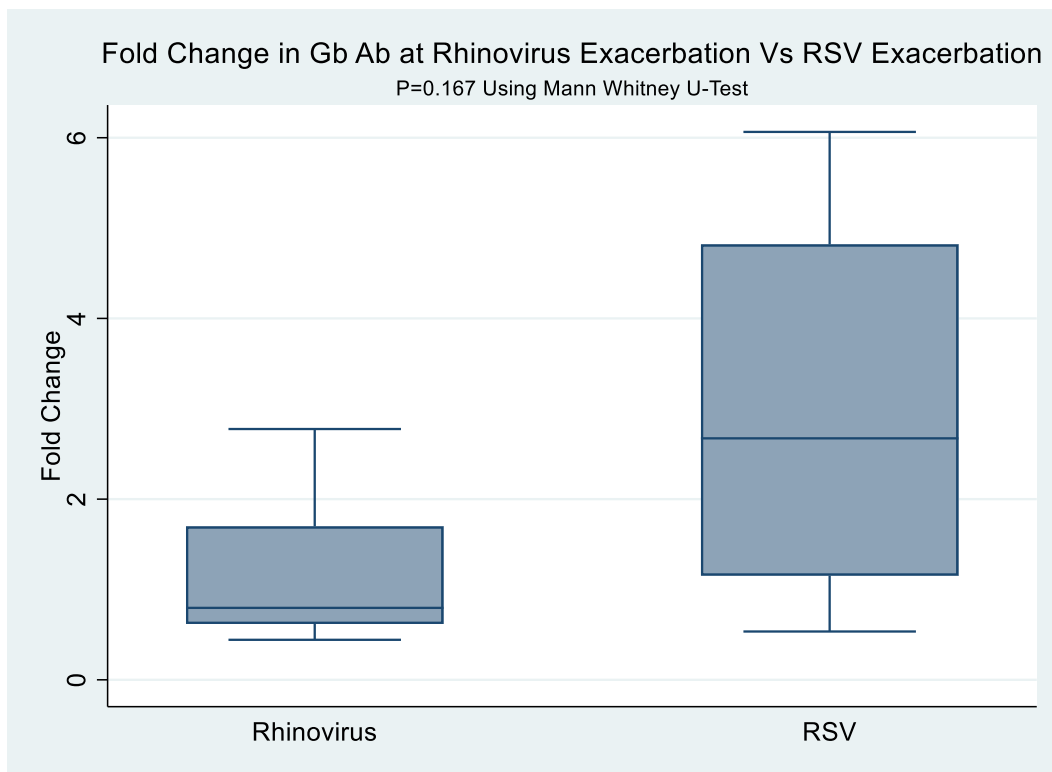


Figure 29 – Fold change in RSV specific Pre-F protein Ab in RSV vs Rhinovirus associated exacerbations. P=0.167 using Mann Whitney U-Test (RSV n=5 Rhino n=14)

5.6.1.3 Pentaplex Validation Conclusions

As the statistical significance was highest for the N antibody fold change, a four-fold rise in RSV specific N-antibody was therefore included in the criteria for RSV related exacerbation diagnosis along with RSV protein specific IgG antibody to Pre-F and Post-F.

5.6.2 Pentaplex Results:

Using these criteria outlined above there were 18 attended exacerbations in total that can now be evaluated against Rhinovirus associated exacerbations and coded as RSV associated. This includes the serological diagnosis and the PCR diagnosis. Again, only exacerbations where the study participant has visited the clinic within 6 months prior of the exacerbation, attended for the exacerbation visit (within 2 weeks of onset) and has not exacerbated again until they were seen by us (between 5 weeks and 90 days) were included. These included self-reported exacerbations and those identified using the diary card criteria. If there was more than one exacerbation, attended or otherwise, between serological readings these could not be

counted in the analysis, as it would not be possible to know for sure which exacerbation event could have caused the fold-rise.

5.7 PCR vs Serological diagnosis of RSV associated events:

Using serology to diagnose RSV associated exacerbations and compare them to the other causes of exacerbations is one of the aims of this study. Therefore the markers of exacerbation severity, namely time to recovery and FEV1% change, between PCR diagnosed RSV associated exacerbations and serologically diagnosed RSV associated exacerbations were compared. These data have been graphed and presented below (figure 31 and 32). The outcomes graphed show no significant difference between the two diagnostic methods in the severity of their exacerbations (FEV1 % change -14.3 / -18.1 $P=0.965$ Time to recovery 7.67 / 7.20 days $P=0.947$). It was therefore felt to be clinically acceptable to combine these methods of diagnosis in the analysis. There is precedent in previous literature for doing this ^{169 170}.

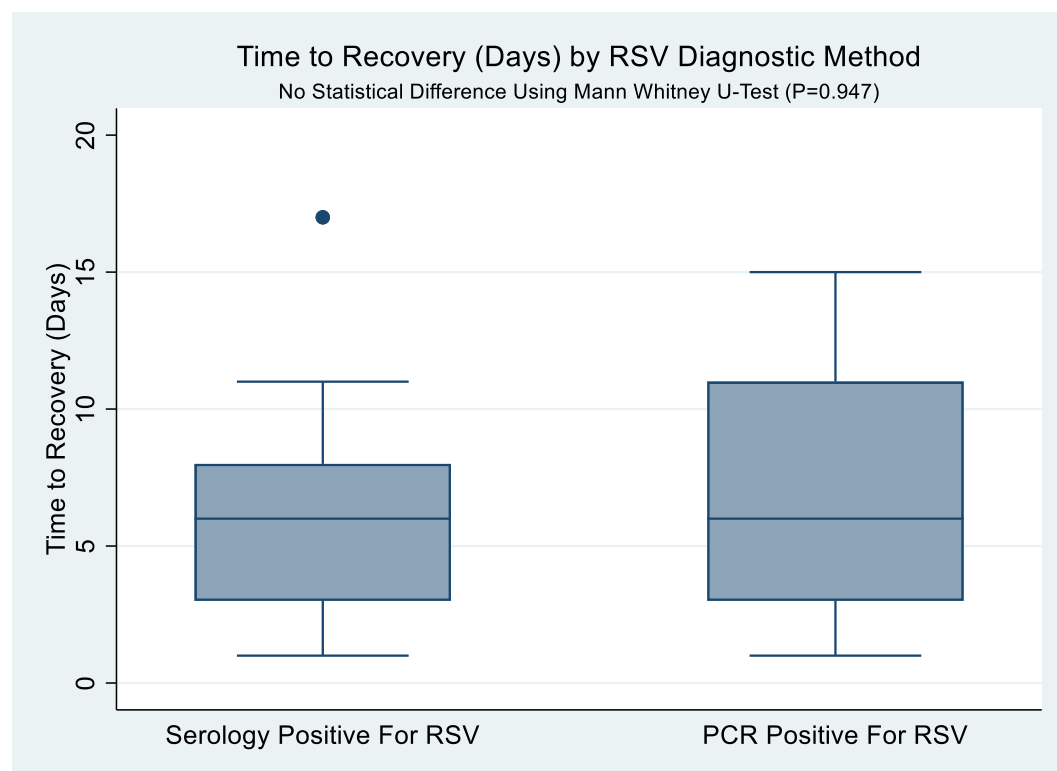


Figure 30 – Time to recovery (days) in RSV associated exacerbations by diagnostic method. $P=0.947$ using Mann Whitney U-Test (serology diagnosis $n=13$ PCR diagnosis $n=5$)

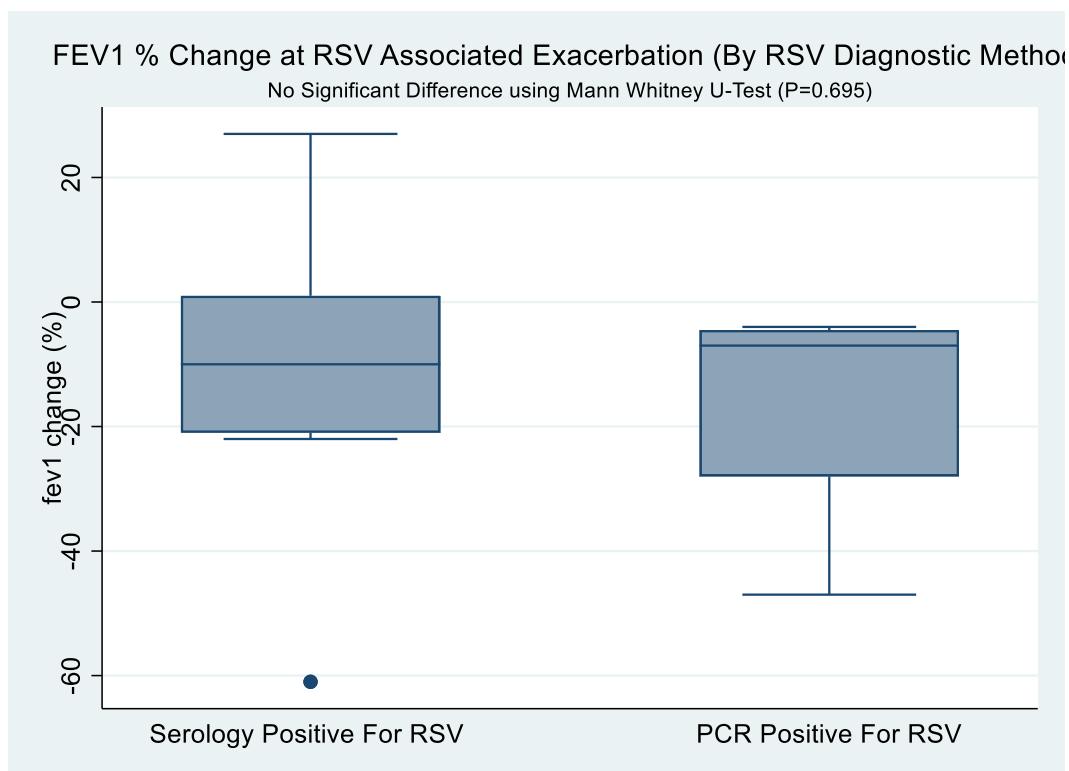


Figure 31 – FEV1 (%) Change in RSV associated exacerbations by diagnostic method. $P=0.947$ using Mann Whitney U-Test (serology diagnosis $n=13$ PCR diagnosis $n=5$)

5.8 RIVM Pentaplex and Janssen ELISA:

To validate the RIVM pentaplex results, the correlation between the pentaplex data and the serology data from the internally validated Janssen ELISA was reviewed. Initially any cases where serology (pre and post F RSV specific IgG antibodies) was analysed using both methods were compared. There were 274 separate serological samples analysed by both methods, resulting in 166 comparative readings which can be calculated for a fold change (having both a pre- and post-exacerbation analysed serum sample). A result was considered positive if either Pre-F or Post-F RSV specific IgG antibody reached the four-fold cut-off, and it was considered negative if it did not. The reason that 274 separate samples can result in 166 readings, is that samples have to be paired, but 3 samples taken at 3-6 months apart can result in 2 separate fold-change readings.

For Pre-F and Post-F antibodies the different methods agreed with each other in 153/166 readings (92.2%). A Cohen's kappa agreement (using STATA) was used to evaluate this

similarity and the agreement was above the 85.77% or expected agreement, therefore was seen as statistically in agreement (table 9).

Agreement	Expected Agreement	Kappa	Standard Error	P value
92.17%	85.77%	0.450	0.065	0.000

Table 9 – Cohen's Kappa agreement of Pentaplex vs Janssen methods for assessing fold change in Pre-f and Post-F RSV specific protein antibodies

The above statistics were calculated with the four-fold cut off as either positive or negative (categorical). To get a more detailed view of the correlation, the raw data was log transformed and then a Bland Altman plot was constructed (figure 33 and 34). This was comparing the raw numbers of all 275 serum samples that had been analysed using both methods. When evaluating the Bland Altman plots, they do indicate the presence of proportion bias. The more extreme (high) readings from the Janssen ELISA do not show such high readings from the RIVM pentaplex. This should not affect the results of the study however, as we are not measuring raw, or absolute numbers, but fold change.

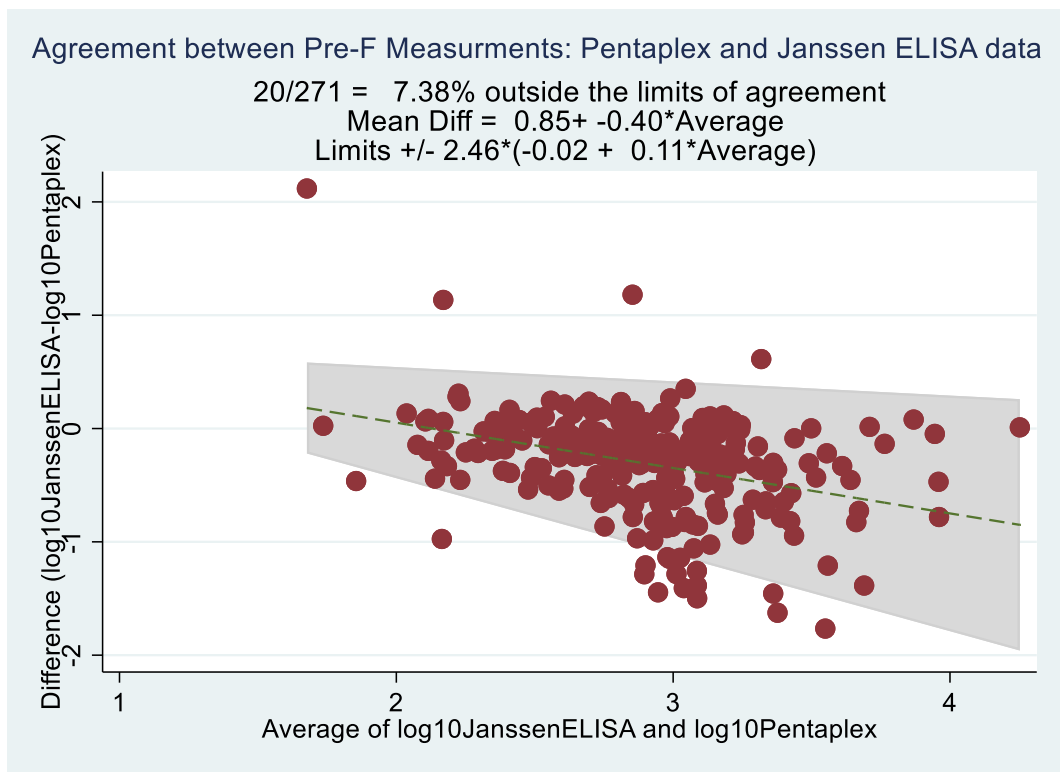


Figure 32 – Bland Altman Plot showing the agreement of Pre-F measurements between the Janssen and Pentaplex methods (data log transformed before analysis). $\log_{10}\text{janssenelisa}$ = Janssen data $\log_{10}\text{pentaplex}$ = pentaplex data

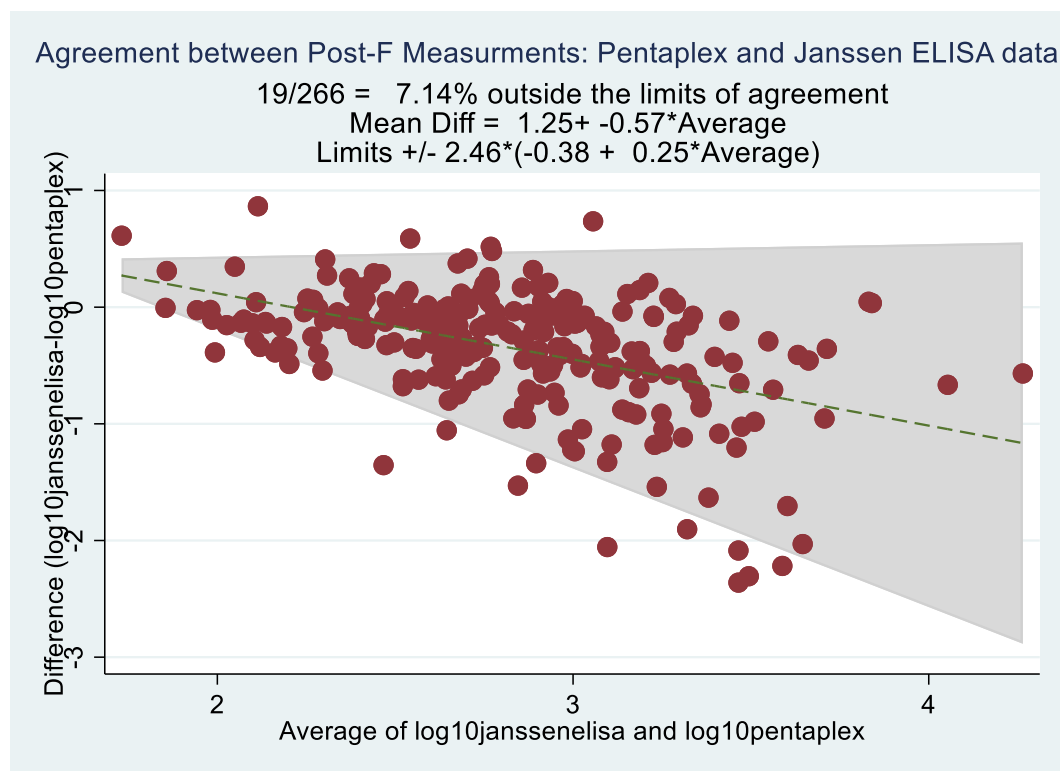


Figure 33 – Bland Altman Plot showing the agreement of Post-F measurements between the Janssen and Pentaplex methods (data log transformed before analysis). $\log_{10}\text{janssenelisa}$ = Janssen data $\log_{10}\text{pentaplex}$ = pentaplex data Severity Analysis - Rhinovirus Vs RSV

5.8.1 Time to Recovery – RSV vs Rhinovirus

Initially time to recovery, as a marker of disease severity, for these 18 RSV exacerbations and the 14 Rhinovirus exacerbations that also met the above criteria was evaluated. Note that the rhinovirus related exacerbations were all diagnosed by PCR, as no Rhinovirus specific antibodies were ever tested for in this study. Time to recovery was evaluated by examining the participants diary-cards and counting symptomatic days. Once the participant has had two symptom free days, and not suffered from another exacerbation by diary card criteria within five days, the exacerbation was considered to be over. If they exacerbated again by diary card criteria before the visit, they were excluded from this analysis as the serological changes could not be definitively linked to a specific exacerbation.

The time to recovery analysis revealed that there was a significant difference between days to recovery in the RSV and rhinovirus groups (figure 35). The average days till recovery in the 18 RSV exacerbations were 10.33 (SD 7.98). The average days till recovery in the 14 Rhinovirus

exacerbations were 17.77 (SD 9.22). The difference between these two groups was found to be statistically significant ($P=0.021$ using a Mann Whitney U-Test).

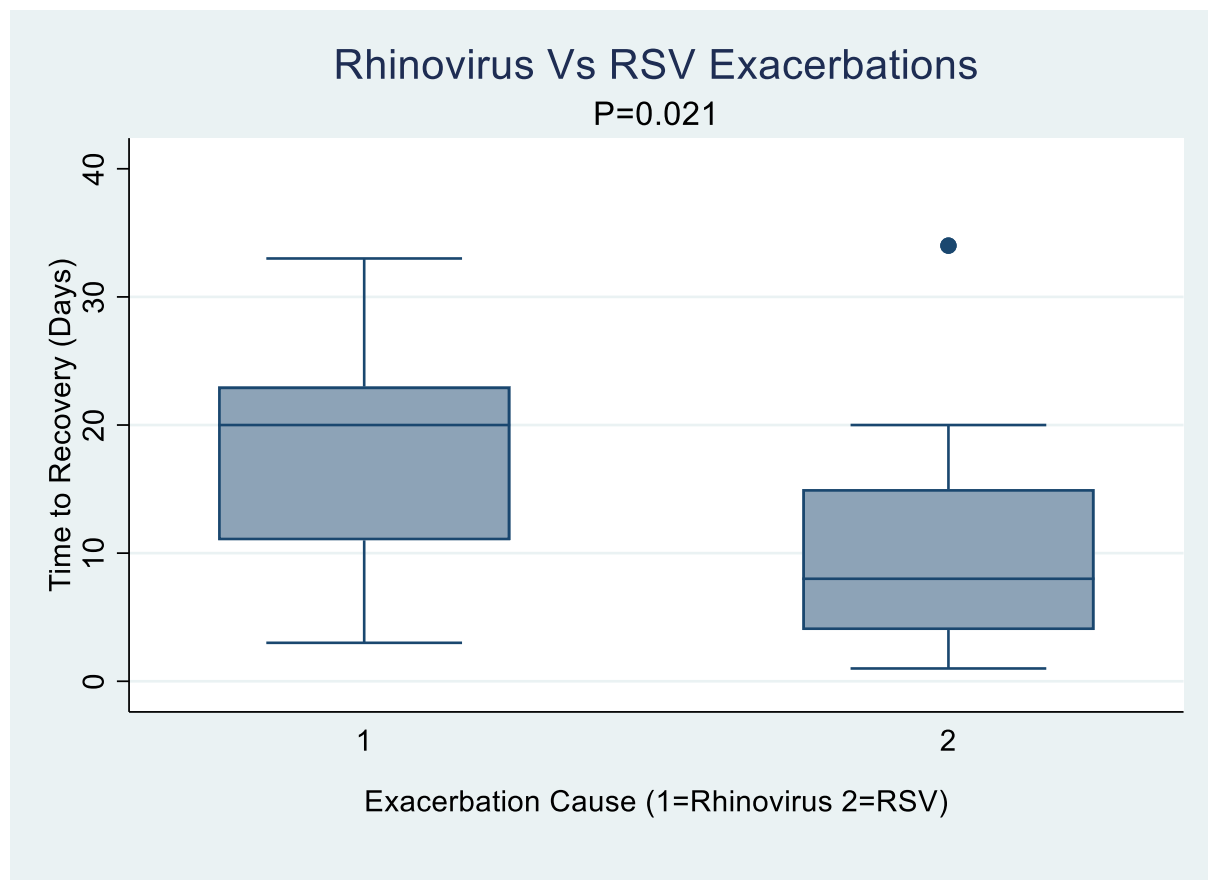


Figure 34 – Time to Recovery (days) RSV associated exacerbation Vs Rhinovirus associated Exacerbation. $P=0.021$ using Mann Whitney U-Test (RSV $n=18$ Rhinovirus $n=14$)

5.8.2 FEV1% change Analysis – RSV vs Rhinovirus

Next, we analysed the FEV1 change at exacerbation (by percentage) between the RSV associated exacerbations and Rhinovirus associated exacerbations. We excluded any exacerbations where we did not have a baseline FEV1 reading from at least five days before the exacerbation starting (not the date of the visit) as calculated from the information on the diary cards. We also needed to have undertaken an FEV1 reading at the exacerbation visit. We included both Rhinovirus positive exacerbations (as from PCR) and RSV positive exacerbations (from PCR or serology as discussed above). This resulted in 14 Rhinovirus exacerbation and 15 RSV exacerbations being analysed as having met these criteria. There

The average change in FEV1 for a rhinovirus exacerbation was -10.56% (SD 11.51 min -31.43%, max 5.13%). The average change for an RSV exacerbation was -4.54% (SD 11.43 min -35.21%, max 7.30%). There is no statistically significant difference between the FEV1 change at exacerbation in RSV associated exacerbations compared to Rhinovirus associated in this sample ($P=0.138$ using Mann Whitney U-Test) (figure 36). There was however no statistically significant difference between pre and post exacerbation FEV1 (or change in FEV1) at an RSV exacerbation. There was a statistically significant difference between pre and post exacerbation FEV1 (or change in FEV1) at a Rhinovirus exacerbation.



There was no statistically significant difference of CRP at RSV associated exacerbation when compared to Rhinovirus associated exacerbation (41.78 / 47.21 mg/L $P=0.41$ using Mann

Whitney U-Test) (figure 37). There were 18 RSV associated exacerbations with a CRP reading taken and 14 Rhinovirus exacerbations with CRP readings available for comparison.

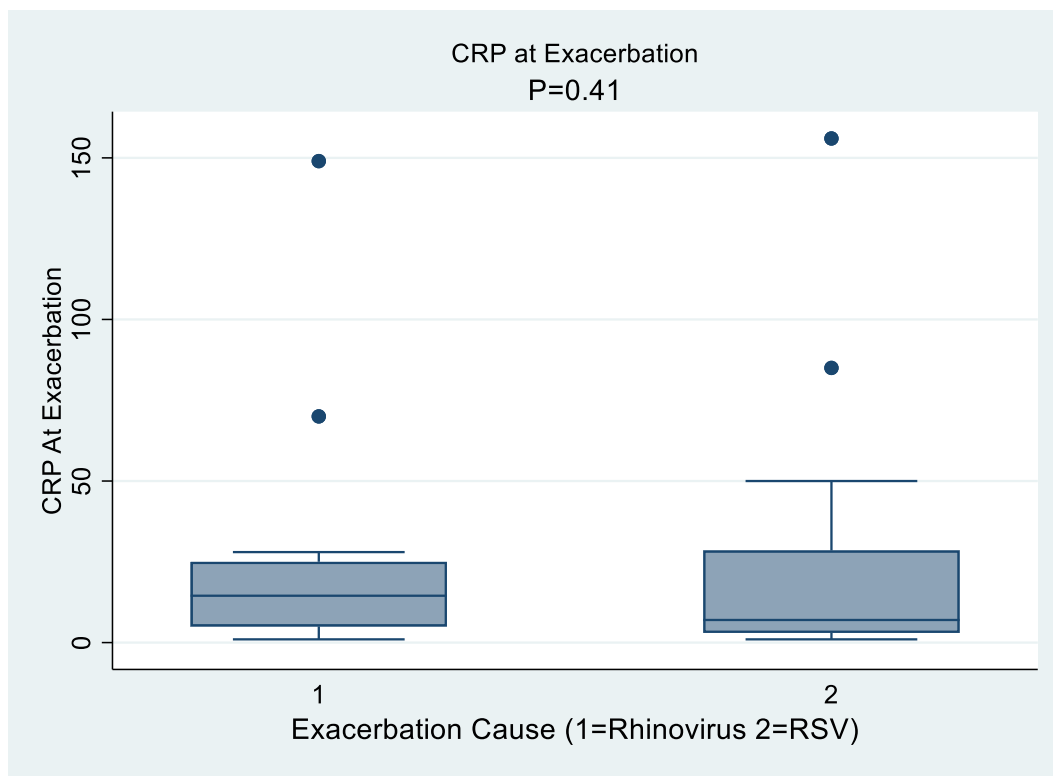


Figure 36 – CRP (mg/L) in RSV associated exacerbation Vs Rhinovirus associated Exacerbation. $P=0.41$ using Mann Whitney U-Test (RSV $n=18$ Rhinovirus $n=14$)

There was no statistically significant difference in Fibrinogen levels during RSV associated exacerbations when compared to Rhinovirus associated exacerbations (4.45 / 4.63 g/L $P=0.64$ using Mann Whitney U-Test) (figure 38). There were 18 RSV associated exacerbations with a Fibrinogen reading taken and 14 Rhinovirus exacerbations with Fibrinogen readings available for comparison.

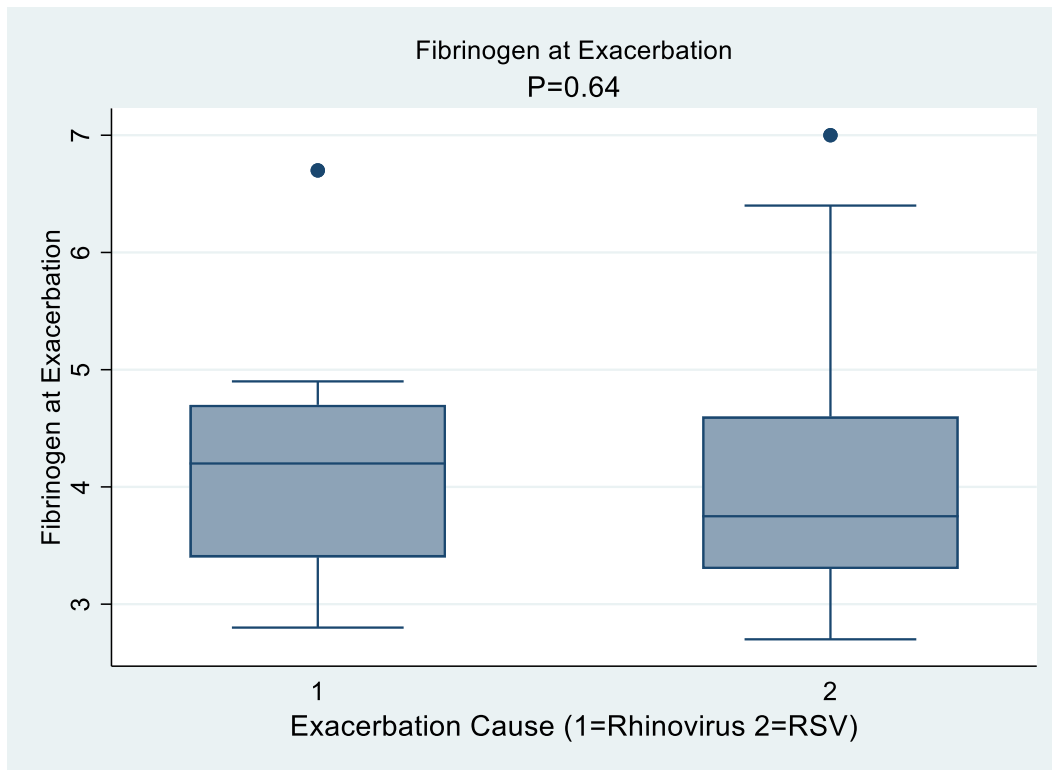


Figure 37 – Fibrinogen (g/L) RSV associated exacerbation Vs Rhinovirus associated Exacerbation. $P=0.64$ using Mann Whitney U-Test (RSV $n=18$ Rhinovirus $n=14$)

There was no statistically significant difference in CAT score change at RSV associated exacerbation when compared to Rhinovirus associated exacerbation ($9.92 / 4.70$ $P=0.391$ using Mann Whitney U-Test) (figure 39), though RSV associated exacerbations trended higher. There were 18 RSV associated exacerbations with a CAT score taken and 14 Rhinovirus exacerbations with CAT score available for comparison.

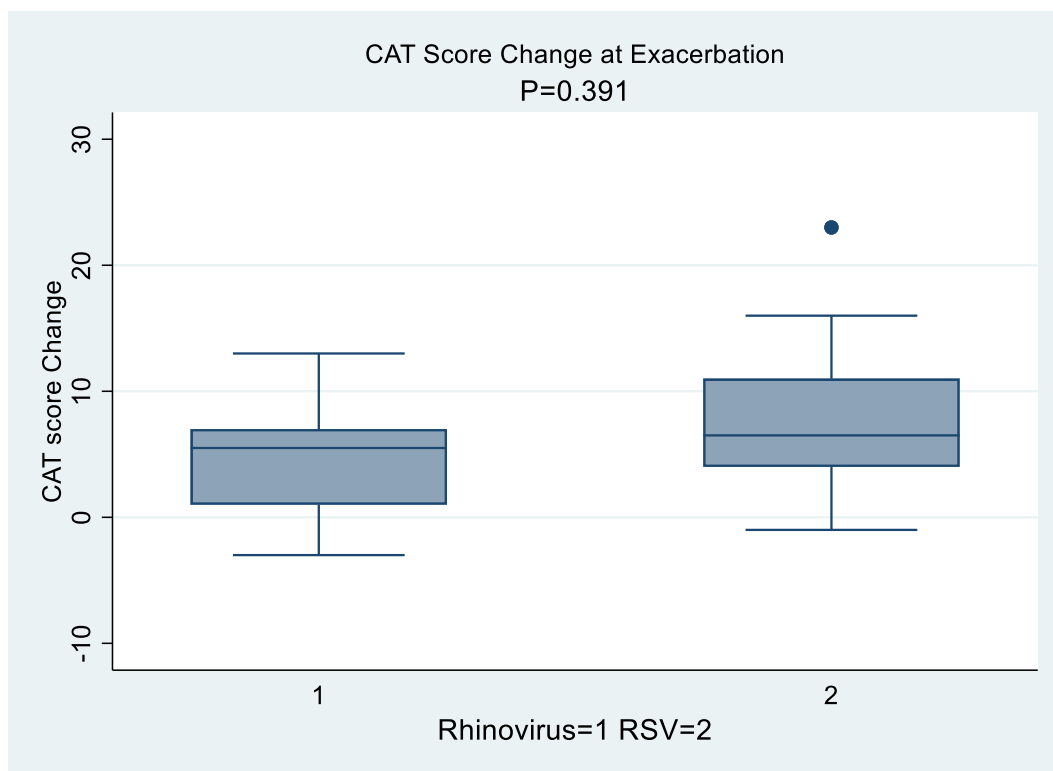


Figure 38 – CAT Score Change in RSV associated exacerbation Vs Rhinovirus associated Exacerbation. P=0.391 using Mann Whitney U-Test (RSV n=18 Rhinovirus n=14)

5.9 RSV vs All Other Exacerbation Causes:

5.9.1 Plan of analysis

The next step was to investigate whether the apparent difference in severity of Rhinovirus exacerbations, relative to RSV (as evidenced by differences in time to recovery), were the result of RSV being unusually short, or whether Rhinovirus exacerbations of COPD were of longer duration than other respiratory viruses. Therefore, FEV1 percentage change at exacerbation, change in CAT score, Fibrinogen, CRP and time to recovery for all the causes of exacerbation were calculated. These were calculated in the same way those outcomes for RSV and Rhinovirus related exacerbations were calculated previously. There are more Rhinovirus associated exacerbations in this analysis as we included those with a PCR positive diagnosis and did not exclude those without serology pre and post exacerbation as the serological methodology has already been validated.

The exacerbations were categorised as;

- Unknown: which included all the exacerbations where no cause was found, including where sputum and NP swabs were analysed, but came back negative, where no sputum or NP swab was analysed for whatever reason, or where colonizing bacteria but no pathogenic bacteria were found in the sputum.
- RSV associated: where RSV was found on NP swab PCR, sputum PCR or RSV was positive through serology as described previously in this chapter. Note RSV was the only virus being diagnosed using serological techniques along with PCR.
- Rhinovirus associated: where Rhinovirus was found on NP swab PCR or sputum PCR (this included those without the need for pre + post exacerbation serum to exclude RSV).
- Flu associated: where Flu (Influenza (Flu) A, Flu B, Flu A (California/7/2009 H1N1), Flu A (H3)) were found on NP swab PCR or sputum PCR.
- Other: where other viruses (Including Human Coronavirus (HCV)-NL63, HCV-229E, HCV-OC43, Human bocavirus, Human Enterovirus, Human Adenovirus, Parainfluenza Virus (PIV)-1, PIV-2, PIV-3, PIV-4 and Human Metapneumovirus (HMPV)) were found on NP swab PCR or sputum PCR.
- Bacterial associated: where non-colonising bacteria were found on sputum microbiology at the Royal Brompton microbiology Laboratory. Any colonising bacteria previously seen at baseline visits for the participant, not previously causing an exacerbation were excluded.

5.9.2 Time to Recovery Analysis – RSV vs All Causes:

Time to recovery was calculated as described previously in this chapter.

Firstly, time to recovery for each of the exacerbation causes was calculated (table 11). Summary statistics shown below (table 10), the figure 40 represents this graphically. There was a statistically significant difference in time to recovery between RSV related

exacerbations and exacerbations related to all other causes (assuming a significance of $p < 0.05$), using a Kruskal-Wallis test, then Dunn's Test to correct for multiplicity testing.

RSV related vs Unknown	$P=0.0131$ using Dunn's Test
RSV related vs Rhinovirus related	$P=0.0003$ using Dunn's Test
RSV related vs Flu related	$P=0.0259$ using Dunn's Test
RSV related vs other virus related	$P=0.0157$ using Dunn's Test
RSV related vs Bacteria related	$P=0.0320$ using Dunn's Test
RSV related vs Bacterial and Viral Co-Infection	$P=0.0210$ using Dunn's Test

Table 10 - Kruskal-Wallis and Dunn's test to give significance of time to recovery (days) in RSV associated exacerbations vs other exacerbation causes.

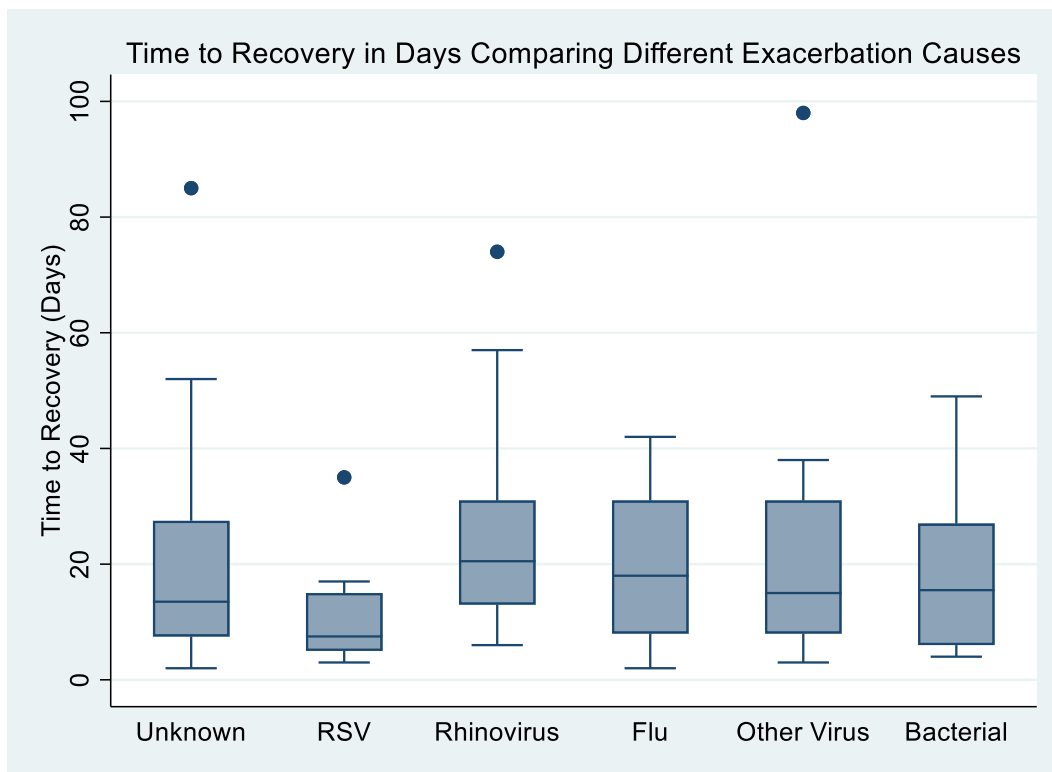


Figure 39 – Time to Recovery (days) for different exacerbation causes

Exacerbation Cause	Mean days to recovery	Standard Deviation	Min Value	Max Value
Unknown (n=64)	18.95	15.58	2	85
RSV (n=18)	10.29	8.57	3	35
Rhinovirus (n=26)	24.27	15.42	6	74
Flu (n=9)	19.78	13.23	2	42
Other Virus (n=16)	22.63	23.04	3	98
Bacteria (n=24)	17.96	12.93	4	49
Mixed Viral + Bacterial (n=15)	24.63	21.79	5	69

Table 11 -- Mean days to recovery for different exacerbation causes

This confirmed that it was RSV that was the outlier when it came to time to recovery when compared with rhinovirus associated exacerbations, as all other causes of exacerbation were associated with longer times to recovery. RSV associated exacerbations demonstrated a statistically shorter time to recovery compared to all other exacerbation types using Dunn's Test (Table 10 + 11).

Investigating the difference in time to recovery between viral associated exacerbations compared to bacterial associated exacerbations, viral associated exacerbations took statistically longer to recover ($P=0.016$ using Mann Whitney U-Test). There was no statistically significant difference between the joint viral / bacterial co-infection associated exacerbations,

or the unknown group (no exacerbation causing pathogen found) (figure 41). The viral group here includes RSV associated exacerbations.

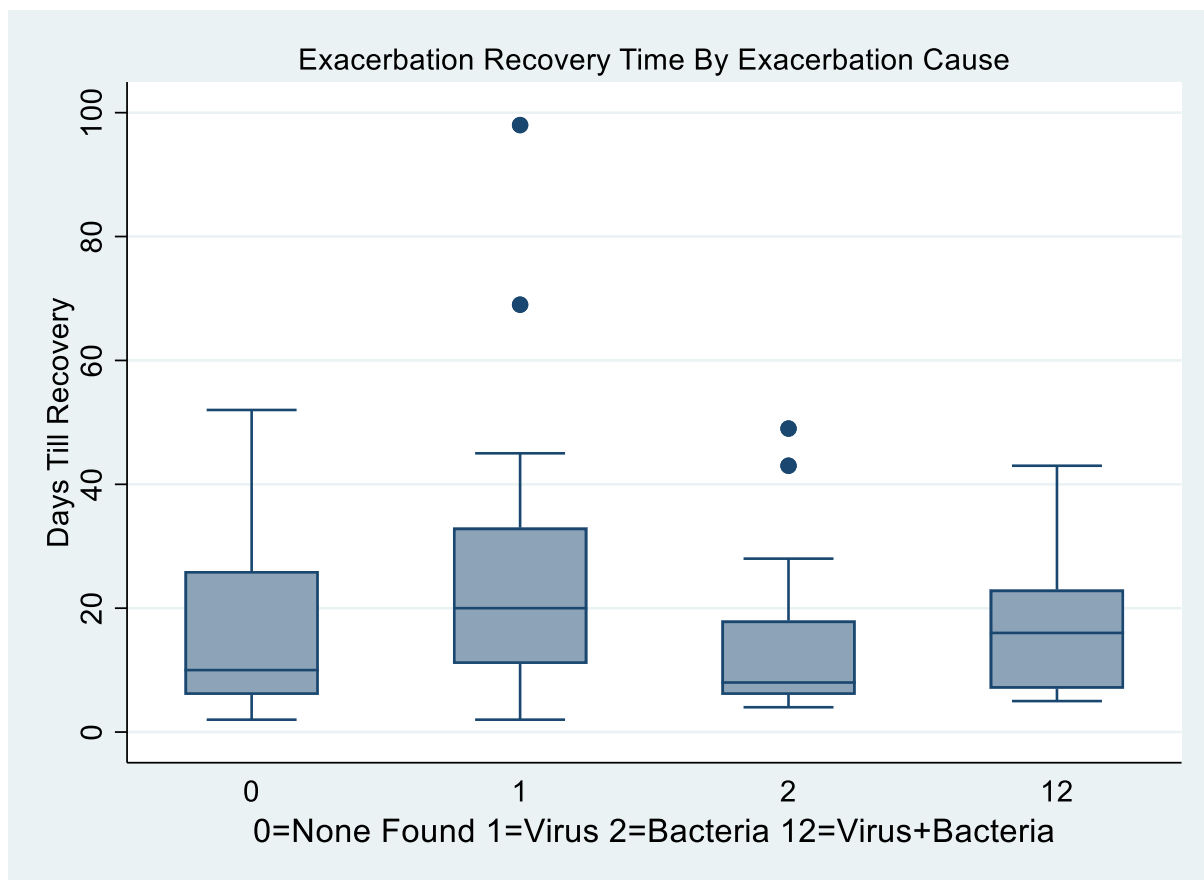


Figure 40 -- Days to recovery for exacerbation groupings (none n=64 virus n=69 bacteria n=24 virus + bacteria n=15)

5.9.3 FEV1 Change Analysis – RSV vs All Causes:

Next, FEV1 percentage change at exacerbation for each of the exacerbation causes was calculated using the method previously discussed in this chapter. Summary data and statistics are shown in the tables 12 and 13, figure 42 represents this graphically. Again, there was no difference seen between FEV1 percentage change between any of the exacerbation causes using a Kruskal-Wallis and then Dunn's test (table 12).

RSV related vs Unknown	$P=0.271$ using Dunn's Test
RSV related vs Rhinovirus Related	$P=0.478$ using Dunn's Test
RSV related vs Flu Related	$P=0.376$ using Dunn's Test
RSV related vs other virus related	$P=0.207$ using Dunn's Test
RSV related vs Bacteria related	$P=0.431$ using Dunn's Test

Table 12 - - Kruskal-Wallis and Dunn's test to give significance of FEV1 (%) Change in RSV associated exacerbations vs other exacerbation causes

Exacerbation Cause	Mean FEV1 % Change	Standard Deviation	Min Value (% change)	Max Value (% change)
Unknown (n=62)	-8.65	16.62	-51	39
RSV (n=13)	-7.69	17.06	-47	27
Rhinovirus (n=26)	-7.62	10.23	-31	10
Flu (n=5)	-2	19.85	-24	29
Other Virus (n=16)	-11.5	14.32	-47	14
Bacteria (n=19)	-6.37	12.83	-31	22

Table 13 - FEV1 (%) Change in RSV associated exacerbations vs other exacerbation causes

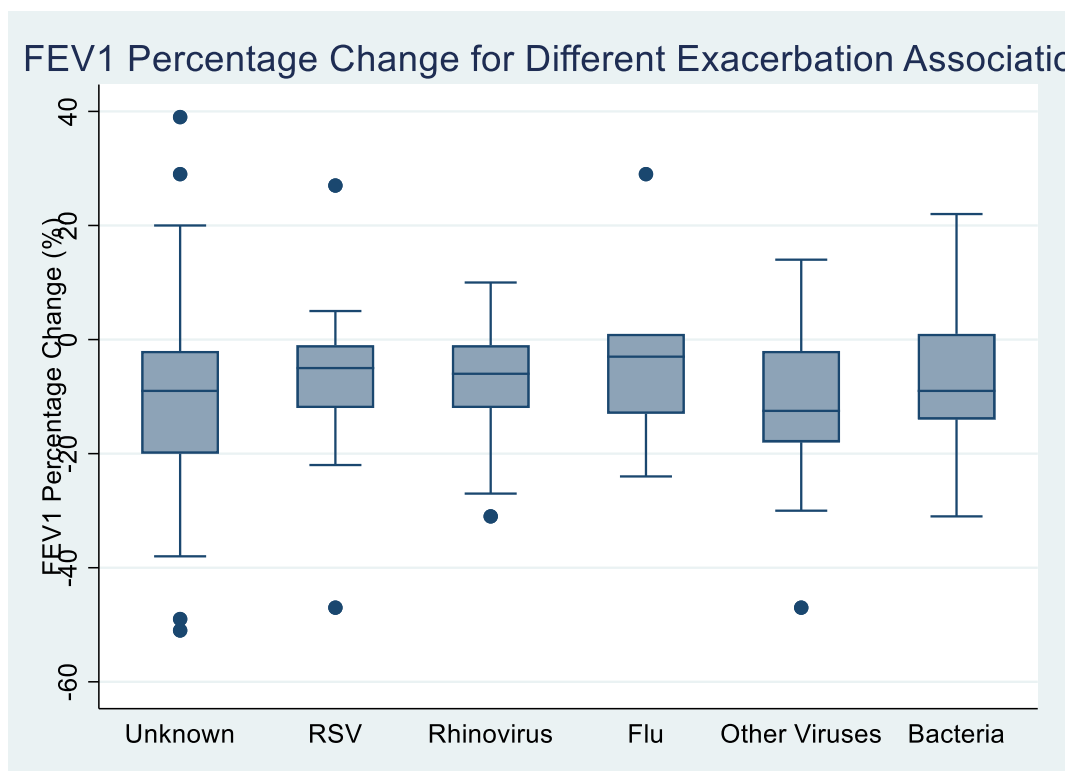


Figure 41 - FEV1 (%) Change during RSV associated exacerbations vs other exacerbation causes

When investigating FEV1 percentage change at exacerbation individually (using one-sample t-tests), unknown exacerbation cause, Rhinovirus associated exacerbations, other virus associated exacerbations and bacterial associated exacerbations all demonstrated significant decreases in FEV1 during exacerbations. RSV associated exacerbations and Flu associated exacerbations both trended down but did not meet statistical significance (meaning no statistically significant drop in FEV1 from baseline – although they both trend downwards (table 13, figure 42)).

5.9.4 Other Markers of Severity Analysis – RSV vs All Causes:

CRP at exacerbation was the next to be investigated. Again there was no difference in CRP at exacerbation between the different exacerbation associations using Mann Whitney U-Tests (figure 43). There was also no statistically significant difference with measured fibrinogen at different exacerbation associations.

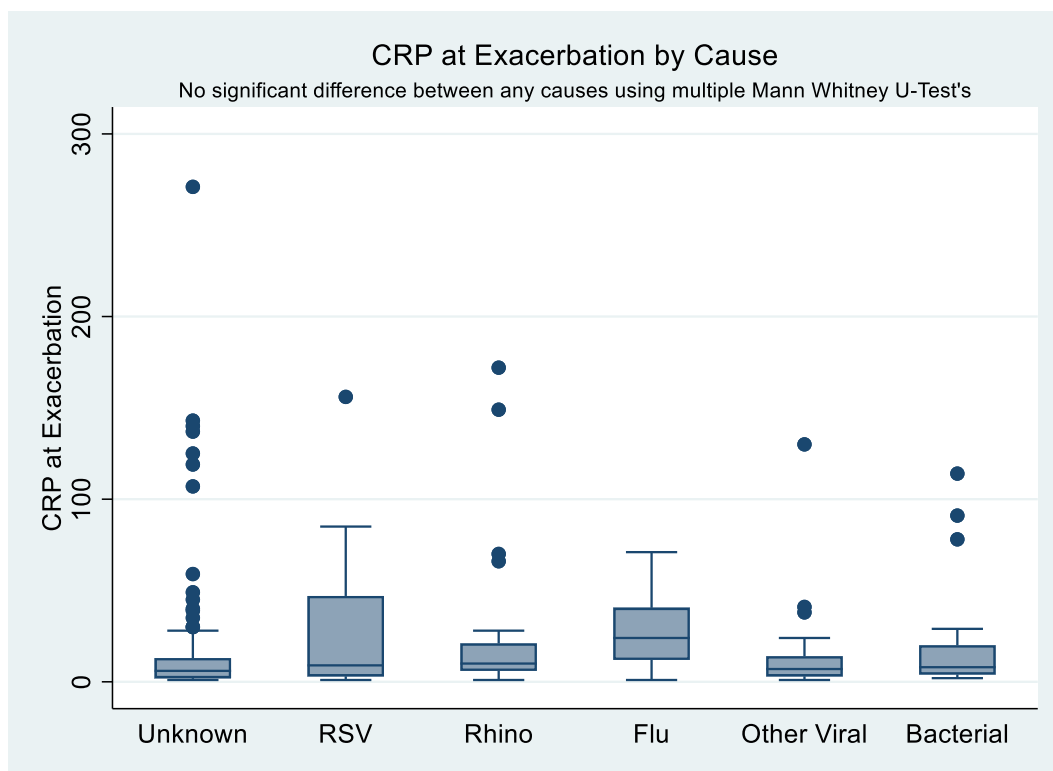


Figure 42 – CRP (mg/L) during RSV associated exacerbations vs other exacerbation causes

Change in CAT score for each exacerbation associated pathogen group was also analysed. There was a trend seen for higher scores in RSV and Flu related exacerbations, but this was not statistically significant (figure 44). There was a statistically significant difference between CAT score at RSV associated exacerbation and CAT score at exacerbation associated with the 'other viruses' group ($P=0.019$ using Mann Whitney U-Test). There was also a statistically significant difference between the CAT score in Flu associated exacerbations and those associated to the 'other viruses' group ($P=0.016$ using Mann Whitney U-Test) (table 14).

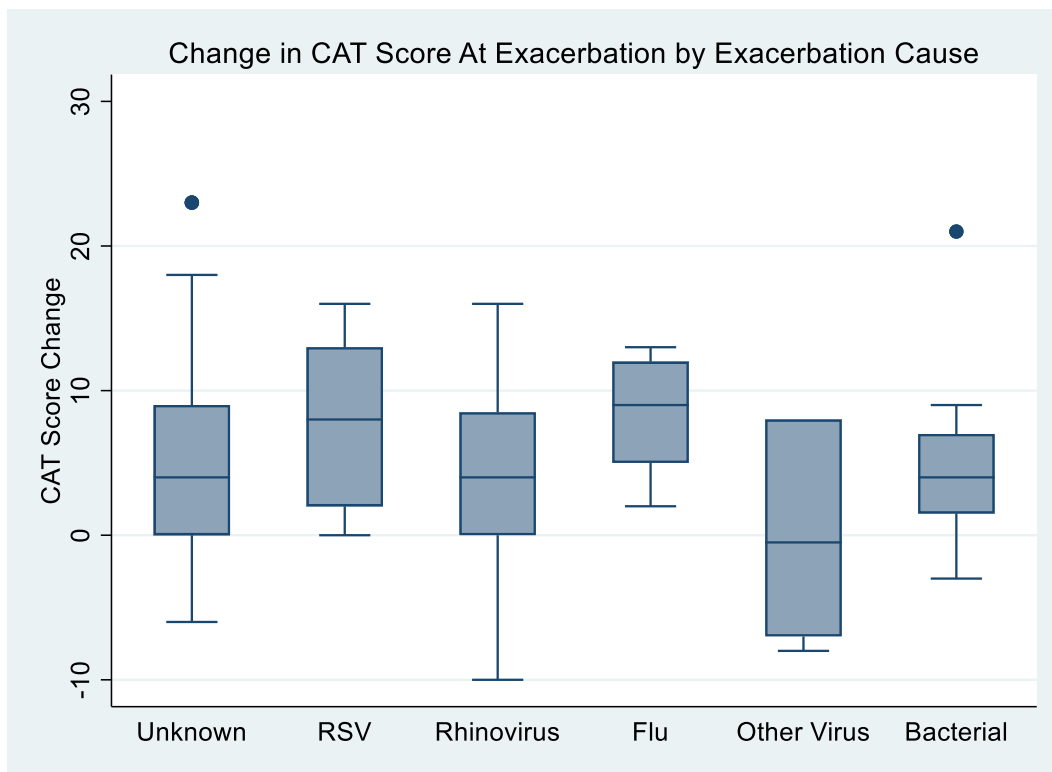


Figure 43 - CAT score change during RSV associated exacerbations vs other exacerbation causes

Summary table of exacerbation associated pathogen:

	RSV	Rhinovirus	Flu	Other Viruses	Bacterial	Viral + Bacterial	Unknown
Days to Recovery	6.5 (3.5 – 12.5)	20 (11.5 – 30.5)	18 (8 – 31)	13 (7 – 29)	15 (6 – 26)	15.5 (10 – 36)	11 (5 – 25)
Fibrinogen g/L	4 (3.6 – 4.9)	4.2 (3.5 – 4.7)	3.9 (3.4 – 4.4)	4.2 (3.4 – 5.3)	3.8 (3.4 – 4.4)	4.2 (2.7 – 5.8)	3.8 (3 – 4.5)
CRP mg/L	9 (3 – 47)	10 (6 – 21)	24 (12 – 41)	7 (3 – 14)	8 (4 – 20)	24 (2 – 64)	6 (2 – 13)
WBC *10 ⁹ /L	8.2 (7.5 – 9.7)	8.3 (6.9 – 9.1)	8.5 (7 – 10.1)	6.5 (5.7 – 9.9)	9.4 (7.7 – 10.9)	10.5 (8.5 – 12.4)	8 (6.9 – 9.6)
FEV1 % Change	-5 (-1 – -12)	-6 (-1 – -12)	-2.9 (-1 – -13)	-12.5 (-2 – -18)	-8.9 (1 – -14)	-4 (-2 – -8)	-8.9 (-2 – -20)
CAT Score Change	8 (2 – 13)	4 (0 – 8.5)	9 (5 – 12)	-0.5 (-7 – 8)	4 (1.5 – 7)	6 (-1 – 8)	4 (0 – 9)

Table 14 - Markers of Exacerbation severity by Exacerbation Cause Kruskal-Wallis and Dunn's test to give significance. If significance when compared to RSV related exacerbations, these are highlighted in bold. Data shown as Median + IQR

5.10 RSV Cases and Exacerbation Frequency:

5.10.1 Why is COPD Exacerbation Frequency important in RSV related exacerbations?

As previously discussed in the preliminary data chapter of this thesis, previous work has indicated that RSV may be present in the lower airways of some COPD patients chronically -

potentially causing regular exacerbations, or not clearing after an exacerbation. These regular exacerbations may either be caused through the virus lying dormant and then becoming active occasionally, or by promoting a lower airway inflammation, as discussed in the literature review chapter.

5.10.2 Plan of analysis

To investigate the association between RSV infection, Rhinovirus infection and frequent exacerbators, two approaches were used. Firstly those that ever tested positive for RSV (either at an exacerbation visit, or subsequently from serological analysis looking for a 4-fold rise in RSV antibodies to Pre-F, Post-F or N protein) were compared to those patients that never tested positive for RSV by the aforementioned means.

5.10.3 Results – RSV related Exacerbations vs Other exacerbation Causes

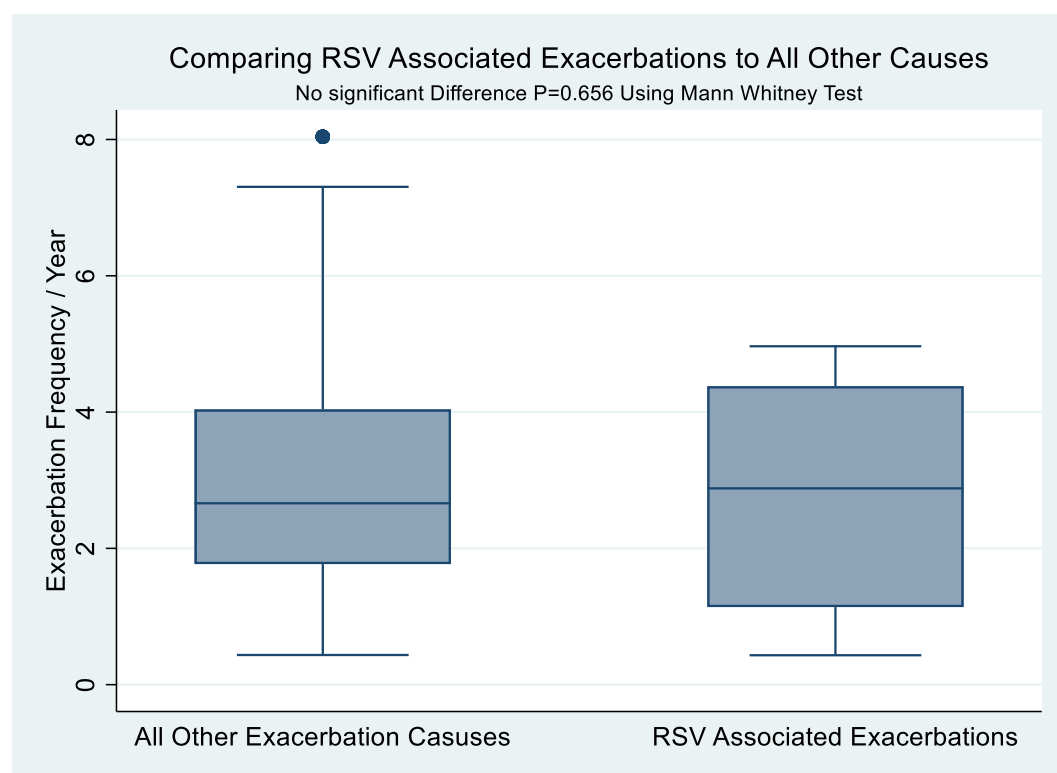


Figure 44 - Exacerbation Frequency over the course of the study in participants who had an RSV associated exacerbation vs those that had an exacerbation by another pathogen or unknown pathogen. $P=0.656$ using Mann Whitney Test (all other $n=146$ RSV $n=32$)

Initially the exacerbation frequency for the participants that attended with RSV associated exacerbations vs those that attended with other exacerbation causes was calculated (2.76 / 4.04 days $p=0.656$ figure 45).

Next, anyone from the analysis that was in the study for less than 1 year and therefore was not a participant in the study for a full RSV season was excluded. 111 participants that had never tested positive for RSV were compared to 31 participants that had ever tested positive for RSV during the study timepoints. There was a statistical difference between exacerbation frequency of those that ever tested positive for RSV compared to those that never tested positive for RSV (4.22 / 2.33 per year $P=0.003$ using a Mann Whitney Test) (figure 46).

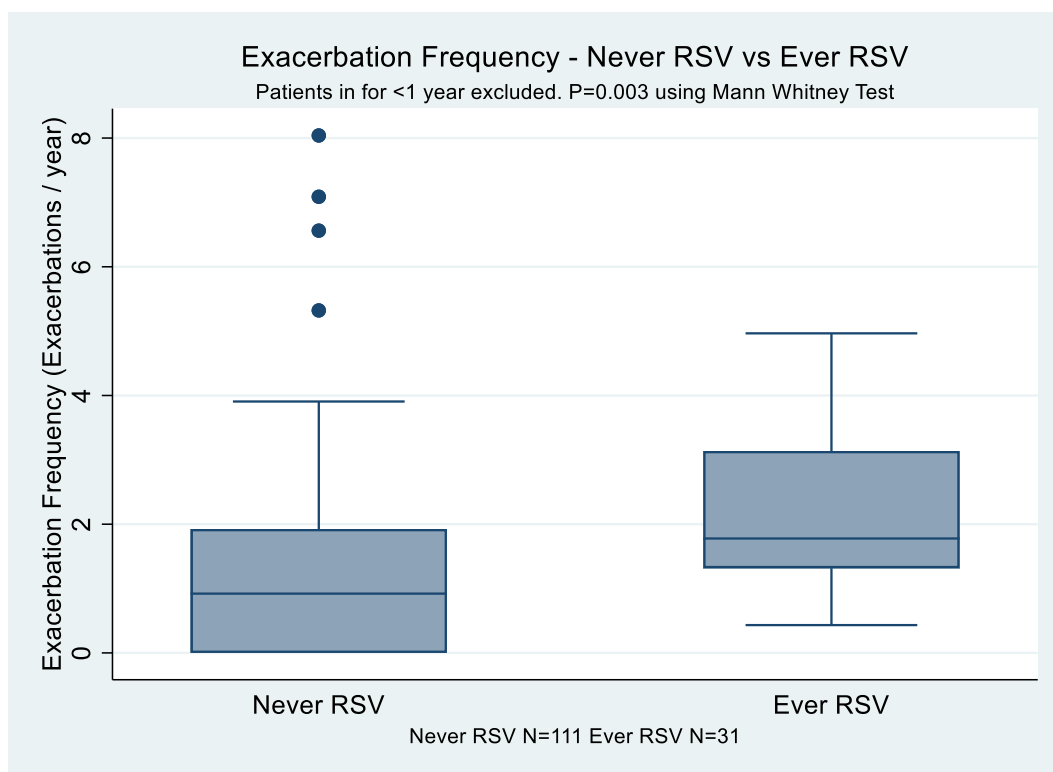


Figure 45 - Exacerbation Frequency over the course of the study in participants who had RSV diagnosed vs those that never did. $P=0.003$ using Mann Whitney Test (all other $n=111$ RSV $n=31$)

5.10.4 Rhinovirus Cases Vs Exacerbation Frequency:

The same analysis for those that ever tested positive for Rhinovirus vs never positive for Rhinovirus was then calculated. There was also a statistically significant difference between those two groups. This showed the same statistical significance as those that had ever tested positive for RSV. (3.33 / 3.62 per year $P=0.003$ using a Mann Whitney Test) (figure 47).

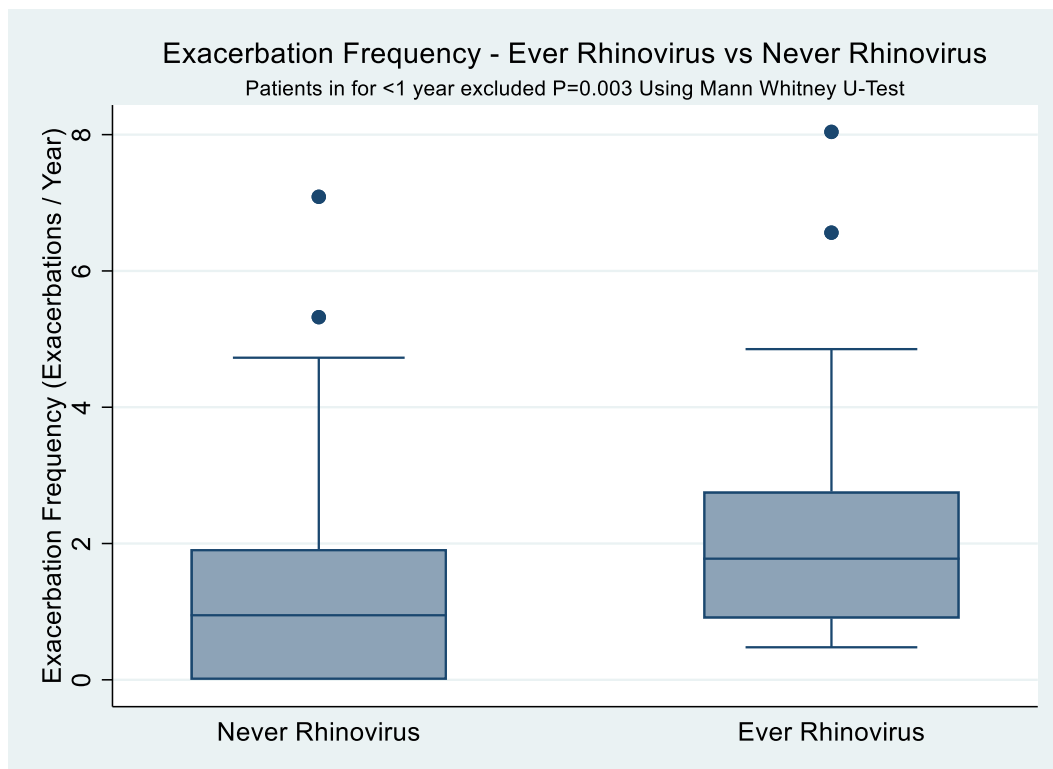


Figure 46 - Exacerbation Frequency over the course of the study in participants who had Rhinovirus diagnosed vs those that never did. P=0.003 using Mann Whitney Test (ever rhinovirus n=24 never rhinovirus n=118)

5.10.5 Difference in Exacerbation Frequency – RSV and Rhinovirus

When comparing the two graphs, there is no obvious difference in the effect of ever having an RSV associated exacerbation on exacerbation frequency when compared to ever having a Rhinovirus associated exacerbation. There is a statistical problem with this type of analysis, as the ever RSV or ever Rhinovirus associated exacerbation group are not going to include any participants that have never exacerbated, hence always swinging the significance towards the group containing the known exacerbators.

The above analysis was repeated, although this time excluding the participants who have never exacerbated - this was done to attempt to remove the potential bias that including patients without any exacerbations in the non-RSV or non-Rhinovirus groups would introduce. This shows a trend towards participants that have ever been diagnosed with RSV having more frequent exacerbations than those that have never had RSV, but statistical significance was not met (4.01 / 2.33 per year $P=0.174$ using Mann Whitney U-Test) (figure 48).

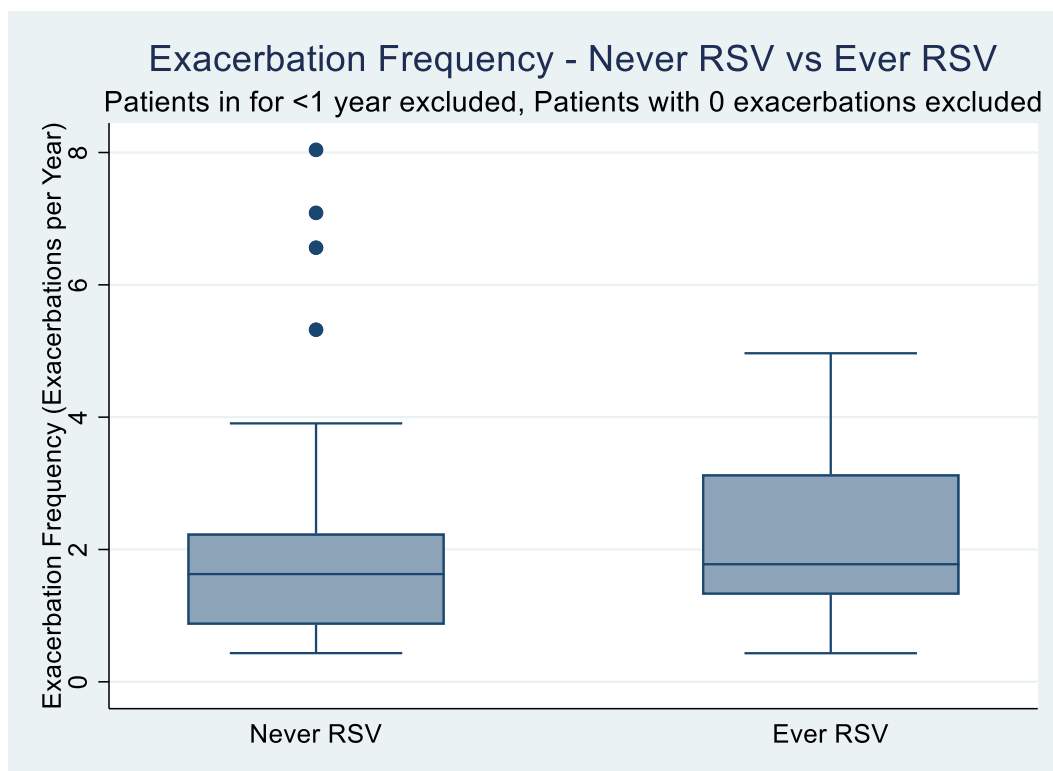


Figure 47 - Exacerbation Frequency over the course of the study in participants who had RSV diagnosed vs those that never did (those participants that never exacerbated excluded). $P=0.174$ using Mann Whitney U-Test

5.11 Infrequent / Frequent Exacerbators - RSV and Rhinovirus:

The next method used to evaluate RSV and Rhinovirus infection related to exacerbation frequency was to group the patients into frequent or infrequent exacerbators. Frequent exacerbators were those with 2 or more treated exacerbations per year on average, and infrequent exacerbators were those with less than 2 treated exacerbations per year on average. Again, any participants that were in the study for less than 1 year were excluded. There was a statistically significant association between frequent exacerbators and those ever testing positive for Rhinovirus ($P=0.019$ using Chi Squared test). There was a trend with those participants ever testing positive for RSV, but it did not meet statistical significance ($P=0.074$ using Chi squared test).

There was a significant association between those participants that had ever had RSV exacerbations, and those that had Rhinovirus ($P=0.020$ using Spearman's correlation). This indicated if participants are likely to have an RSV associated exacerbation, they are also likely

to have a Rhinovirus associated exacerbation. More research could be undertaken with the serology samples to investigate for markers of viral exacerbation susceptibility.

5.12 Other Associations Between RSV and Rhinovirus Related Exacerbations

5.12.1 Smoking History Vs RSV and Rhinovirus:

A Spearman Correlation was used to check for associations between clinical parameters and those that have ever had RSV or ever had Rhinovirus related exacerbations. Initially any association between smoking history and those that have ever had RSV or Rhinovirus associated exacerbations was tested for.

Looking at pack years, smoking status, there was an association between ever having a Rhinovirus exacerbation and pack-years ($P=0.011$ using Spearman's correlation). This association was not present with RSV ($P=0.565$ using Spearman's correlation). There were no other statistically significant associations here apart from between smoking status, age started smoking and pack years which were all statistically significant between each other using Spearman's correlation, as would be expected.

5.12.2 Lung Function Vs RSV and Rhinovirus:

When comparing those that had ever had RSV or Rhinovirus associated exacerbations to FEV1% predicted, FEV1/FVC ratio or age, there were no statistically significant associations using Spearman's correlation.

5.13 Overall RSV numbers at Imperial:

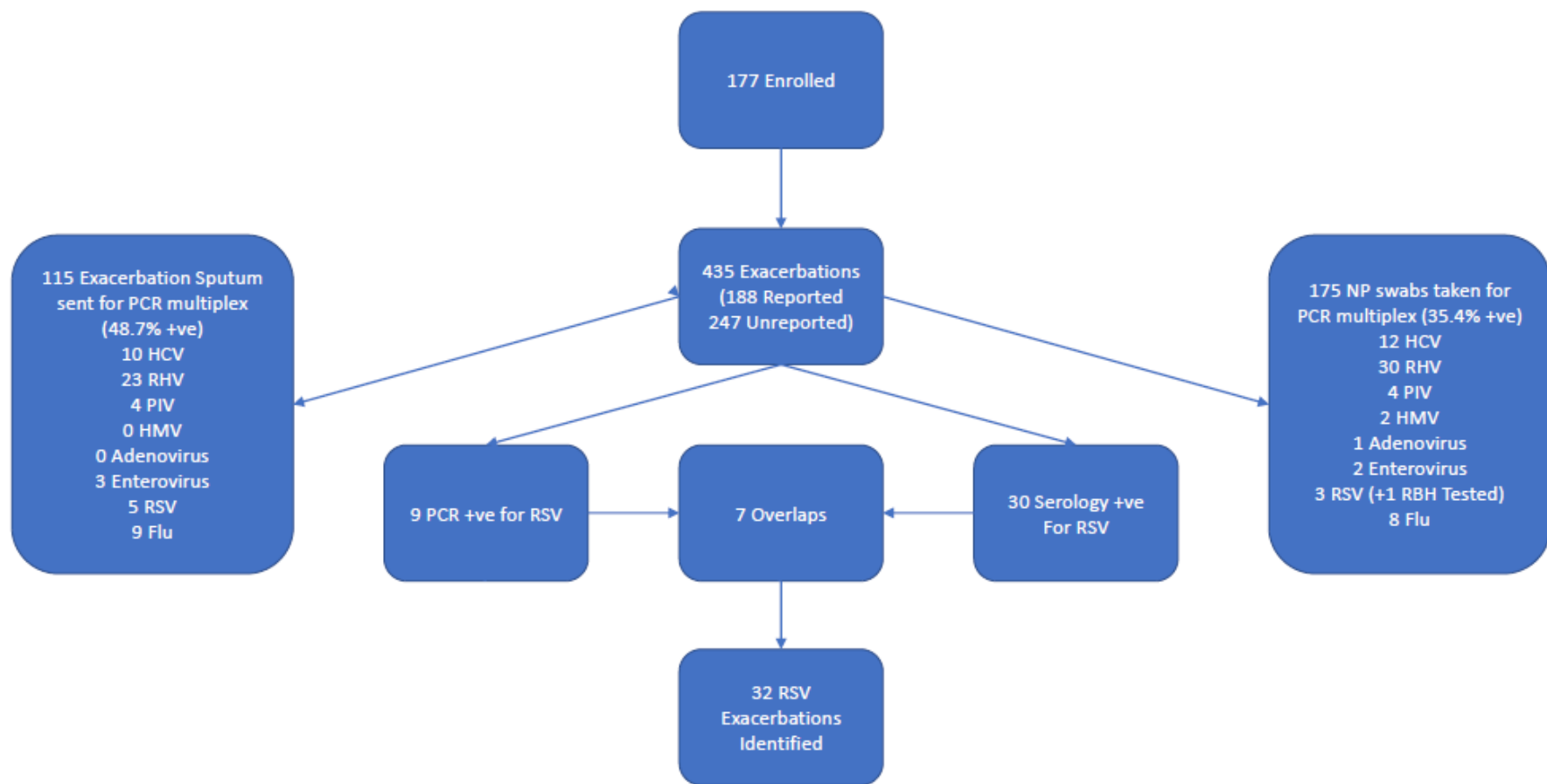


Figure 48 – Total RSV associated exacerbation events diagnosed during the study

5.14 Summary

One hundred and seventy-seven patients were recruited into the study at the Imperial site and followed for an average of 748 days, giving a total of 296.8 patient-years of data.

RSV infection has been identified in 32 exacerbations, 7.4% of the total (figure 49). Thirty of these RSV-associated exacerbations were detected using serology ; 3 were discovered by PCR of 175 nasopharyngeal specimens and 5 by PCR of 93 sputum samples taken at exacerbation. In total, only 2 additional RSV-associated exacerbations were found by PCR, calling into question the validity and justification for the direct viral detection in studies of this kind.

26 attended exacerbations were identified as being Rhinovirus related, 18 RSV related, 9 flu related, 24 bacterial related, 16 related to other viruses and 15 having both viruses and bacteria present. 64 attended exacerbations were without any viruses or bacteria detected.

When investigating the exacerbation severity, RSV associated exacerbations had high CRP and CAT score changes, but a shorter duration of illness, of 10.3 days. The duration of illness difference was statistically significant to all other exacerbation.

The results from this chapter will be also discussed in the discussion chapter, with conclusions and discussions split between different subheadings. There will also be discussions bringing together the data from the results section, the Groningen data, the literature search and introduction chapters to formulate conclusions and further questions for future research.

6 Results from Groningen and Imperial Cohorts

6.1 Introduction

As discussed in the introduction describing the thesis plan, a separate, mirrored cohort was set up in Groningen using the same protocols and inclusion / exclusion criteria. The study team at the Groningen site have very kindly given me access to their data and permission to discuss these data in my thesis.

6.2 Demographics

Presented are the demographics for the Groningen patients next to the demographics for the Imperial (London) patients (table 15). P values are stated as to any statistically significant difference between the two groups.

	Imperial (N=177)	Groningen (N=209)	P=
Age (years) (median IQR)	72.11 (71.0-73.2)	67.6 (66.6-68.5)	<0.005
BMI (median IQR)	27.03 (26.30 – 27.77)	27.04 (26.19 – 27.87)	0.991
FEV1 percentage of predicted	65.21%	64.28%	0.265
Gold Stage 3 or 4 (spirometry)	25%	23%	0.425
FEV1/FVC	55.4% (46.9-62.3)	52.5% (41.2-62.7)	0.274
Pack Years	50.8 (46.2-55.4)	45.6 (41.1-50.1)	0.117
Current Smoker	60 (34%)	42 (21%)	=0.005
Male Sex	112 (63%)	138 (69%)	0.597

Table 15 – Demographics of Imperial participants against Groningen participants with statistical difference measured

As is evident above (table 15), the Groningen participants were slightly younger statistically than the Imperial participants. There was also a higher propensity for the Imperial participants to be smokers as compared to the Groningen participants.

6.3 RSV Positivity Data

For the Groningen mirror study, RSV positivity was investigated by PCR of nasopharyngeal swabs at exacerbation, sputum PCR analysis at exacerbation and the Jansen ELISA Pre-F and Post-F antibody serology. All protocols for taking samples, storage and analysis were identical to those discussed in the methods chapter for the Imperial cohort.

3/122 (2.5%) NP swabs taken at exacerbation from Groningen participants were positive for RSV (all RSV B). 1/22 (4.5%) sputum samples were positive for RSV (RSV B). These samples did not overlap.

Using the Janssen ELISA serology method, there were 8 four-fold rises equating to a 'positive' result. One of these overlapped with the PCR methods.

This gave a total of 11 RSV associated exacerbations discovered by any of the methods listed above from the Groningen cohort.

Note that fewer sputum samples were sent at exacerbation from the Groningen cohort compared to the Imperial Cohort (22/122=18.03% from Groningen 93/175=53.14% from Imperial). There was access to the RIVM pentaplex for the Imperial samples, but this was not available for the Groningen samples. These differences in diagnostics mean that there are caveats when direct comparison are made between the two sites. This is also why the Groningen data was not included in the RSV prevalence, severity or time-to-recovery analysis.

6.4 Totals Across Both Cohorts

From GSK NP swabs 6/297 were positive at exacerbation for RSV (5 RSV B, 1 RSV A). The viral count numbers ranged from (6,853 to 86,503,569).

8/104 (7.69%) of the sputum samples at exacerbation were positive for RSV (3 RSV B, 5 RSV A). The viral count numbers in sputum ranged from 691 to 142,438,576.

Comparing the NP swabs vs sputum there was a higher positivity rate for RSV from the sputum analysed compared to the NP swabs (using a chi-squared test, $P=0.0061$). There was also a trend towards a higher viral load for RSV in sputum samples compared to NP swabs.

Using the Janssen ELISA serology technique, for Groningen (6th monthly samples sent), 8 positive (1 overlap with PCR) and Imperial (6 week and 6th monthly samples sent), 12 positive (3 overlaps with PCR). This equates to 11 PCR positives (excluding overlaps) and 16 Serology positives (excluding overlaps), so 27 RSV cases in total across both cohorts using both these methods.

6.5 Other Virus Positivity Data (From Multiplex PCR)

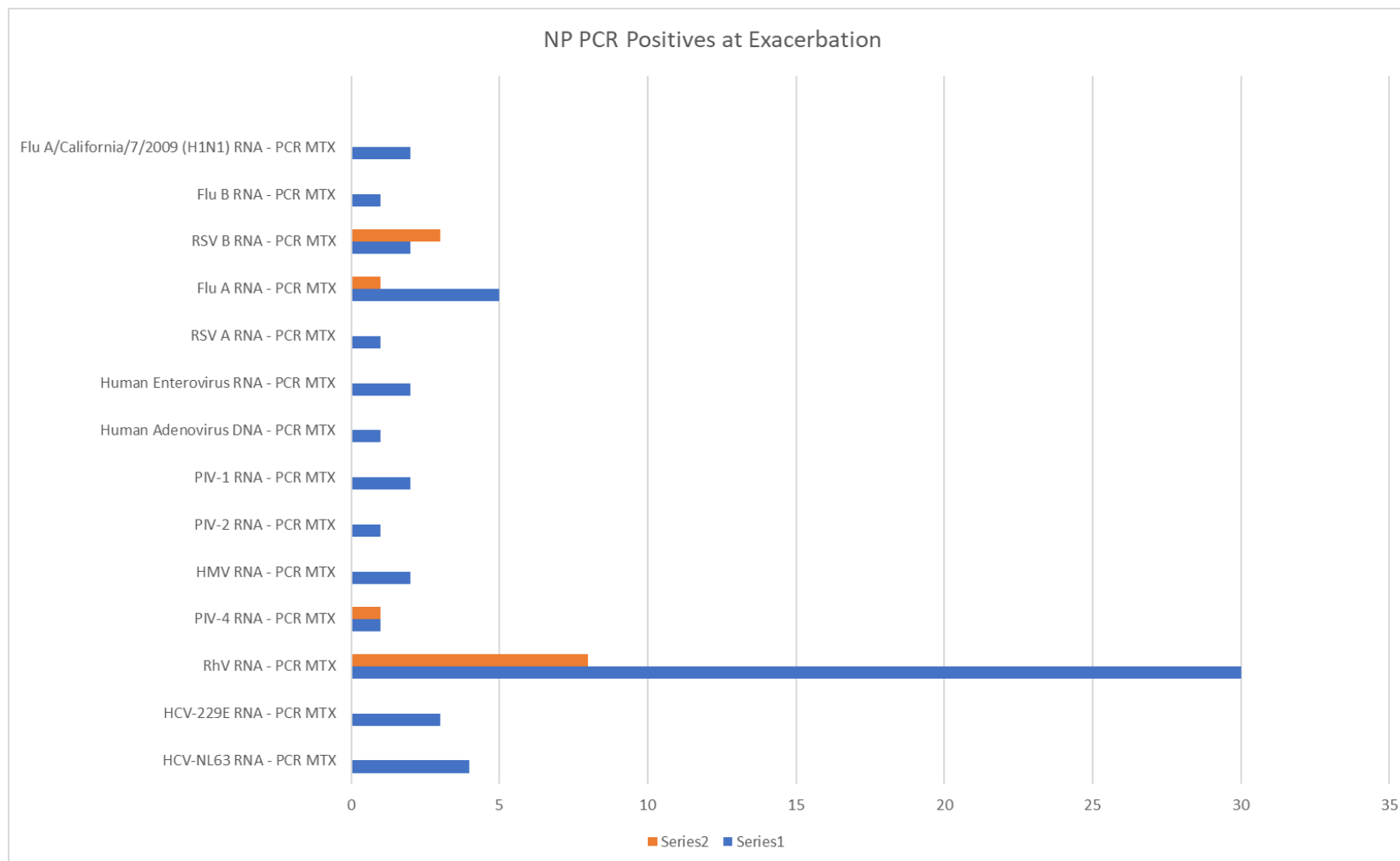


Figure 49 - Graph showing PCR positivity from NP swabs at exacerbation, Groningen in orange, Imperial in blue. Imperial (N=175) Groningen (N=122)

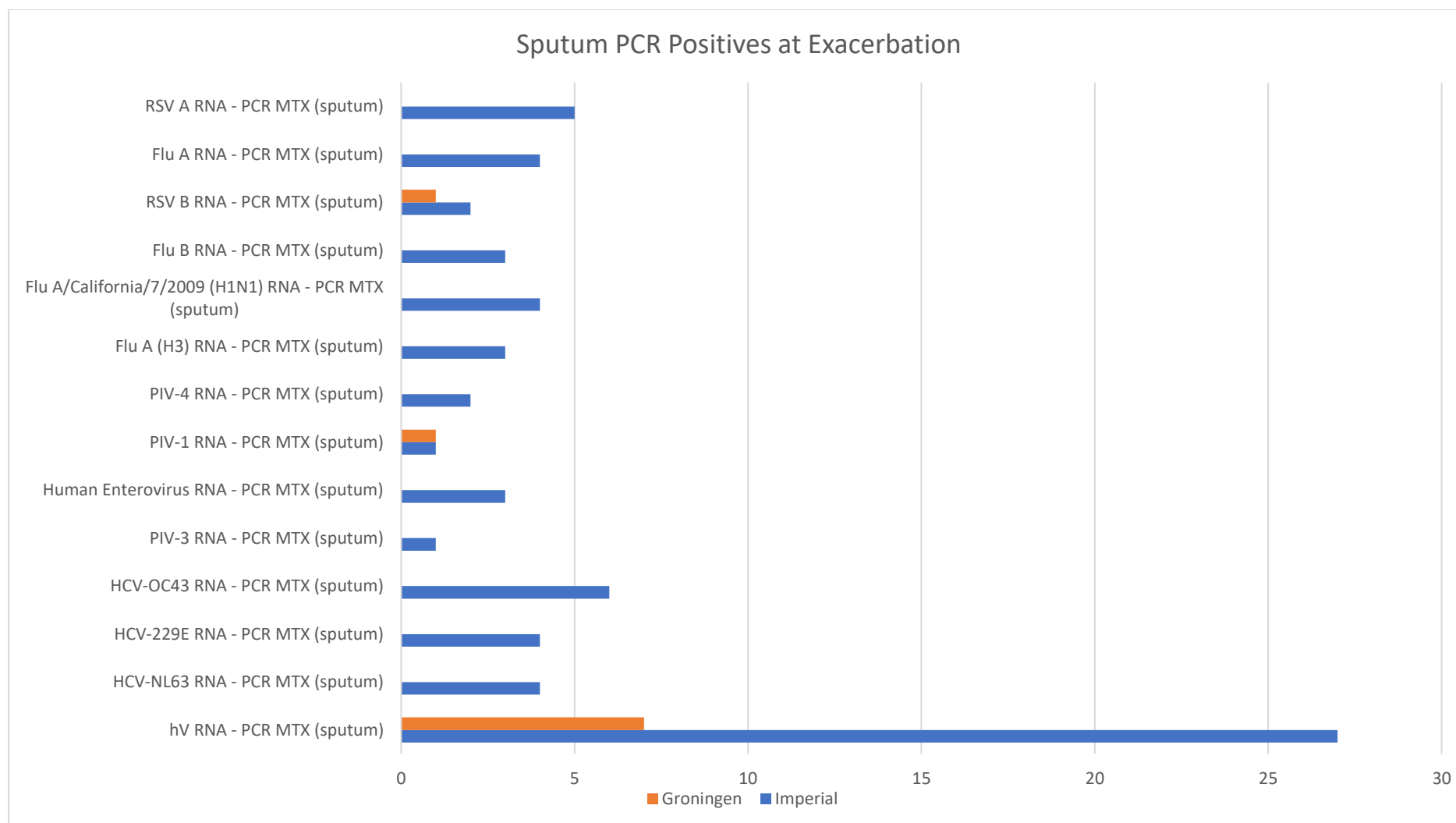


Figure 50 - Graph showing PCR positivity from Sputum Samples at exacerbation, Groningen in orange, Imperial in blue. Imperial (N=93) Groningen (N=22)

The two graphs (figure 50 and 51) show the other viruses present from PCR analysis of exacerbation visit NP swabs and sputum. There was a greater variety of viruses present from the Imperial samples, with 14 different viral pathogens present from the exacerbation NP samples, and 14 from the exacerbation sputum samples. There were in contrast 4 different viral pathogens present in the Groningen NP exacerbation samples and 3 in the Groningen sputum samples.

6.6 Conclusions

The lack of variety in viral pathogens from the Groningen samples (both sputum and NP) stands out. One theory as to the reason for this is that London is a larger city, with a population including lots of international travelers and tourists. This may mean that the citizens of London are exposed to a greater variety of exacerbation causing pathogens. There is some, but markedly limited evidence for greater viral diversity in areas of high population density¹⁷¹.

What was also noted is that there was a higher rate of viral positivity on the multiplex PCR amongst the Imperial participants compared to the Groningen participants. (22/144 or 15.3% amongst Groningen participants and 127/268 or 47.4% amongst Imperial $p < 0.001$ using Chi Squared test). There was no sputum bacteriology data collected from the Groningen participants, so direct comparisons of exacerbation cause across the two cohorts is not possible. It may be that more of the exacerbations amongst the Groningen cohort had a bacterial cause compared to the Imperial cohort. It is not possible to make any further conclusions about the cause of this apparent difference without the bacteriology data. It became evident when investigating exacerbation severity by cause how essential sputum bacteriology and PCR is in diagnosing exacerbation cause. It could also be argued that further phenotyping of the exacerbations where no cause was found would be needed. This will be discussed later, but unfortunately this analysis was unfeasible due to the time constraints of this research project.

The proportion of current smokers amongst the Groningen cohort was also less than amongst the Imperial cohort (60/177 34% vs 42/209 21% $p = 0.005$ using Chi Squared test). There has

been a recent increase in tobacco cessation funding in the Netherlands¹⁷² whereas in the UK there has been a steady decrease in funding¹⁷³. This could potentially explain the difference, but more research would be needed to investigate this in more detail and was not the focus of this study.

6.7 Summary

There were a total of 11 RSV associated exacerbations discovered by any of the methods listed above from the Groningen cohort.

Total numbers of RSV associated COPD exacerbations across both the Imperial and Groningen cohorts equates to 11 PCR positive cases (excluding overlaps) and 16 Serology positive cases (excluding overlaps), so 27 RSV cases in total across both cohorts using both the PCR and Janssen ELISA methods (figure 51). Note this does not include the positivity data from the Pentaplex used at the imperial site, as this method was not mirrored at the Groningen cohort.

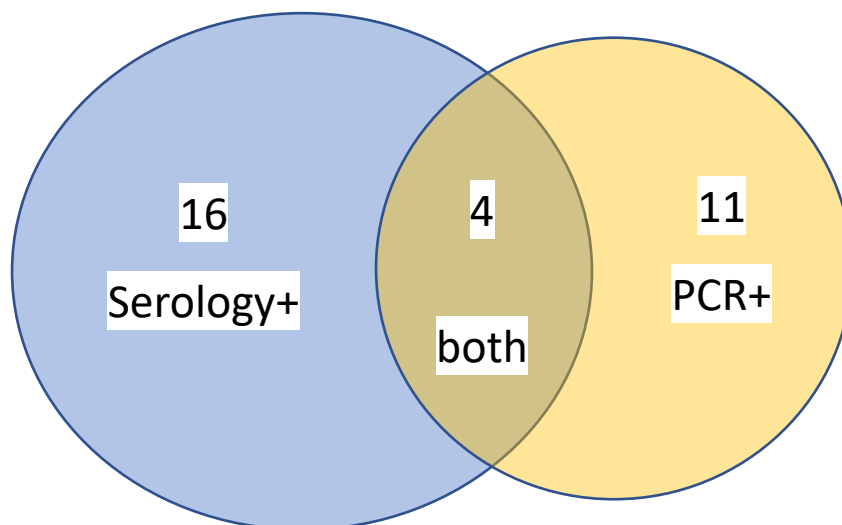


Figure 51- RSV Totals Across Both Cohorts by Diagnostic Method

7 Discussion

7.1 Why RSV matters in older adults and why Diagnosis is Important

Through the introduction chapter of this thesis evidence has been presented, from multiple sources, outlining the prevalence of RSV in adults and the potential severity of RSV infection in adults. As will be discussed in more detail later, RSV vaccines for older adults are on the horizon and their potential benefit is probably understated when evaluating existing literature. This impacts the cost / benefit analysis that influences the likelihood of future vaccines being offered to older adults or those with comorbidities.

Diagnosing RSV is currently not a priority for physicians treating adult or elderly patients, as there are no RSV focused treatments currently available for medical use and the severity and prevalence of RSV is under-recognised amongst hospital physicians currently⁹⁹. This may well change if RSV specific treatments emerge, and it may only be then that the true burden of RSV amongst the older adults is realised.

RSV is highly infectious and there are cases of outbreaks in long term care facilities in the US^{174 175}. If there was more emphasis on diagnosis of RSV in older adults, it may well result in the initiation of barrier nursing when a case is discovered. This could potentially reduce transmission to other residents if implemented well. Barrier nursing is already standard practice in most western countries neonatal and paediatric wards when RSV is suspected¹⁷⁶.

One study published in 2000 calculated the estimated cost savings for the US if an effective vaccine for RSV was produced and used. The study estimated that the cost effectiveness would be similar to the flu vaccination programme for older adults¹⁷⁷. It could be argued that adults of any age with COPD should be amongst the first in line for receiving an RSV vaccine⁶. However, the relative importance of vaccinating groups of adults with comorbidities is largely unknown, given the limitations in existing data about the burden of RSV in these groups.

Growing data is also emerging comparing RSV to Influenza in older adults. Amongst those that contract the illness and end up hospitalized, RSV is at least as severe as Influenza. One paper comparing 20 day all-cause mortality for RSV related admissions (in those over the age of 18) to influenza associated admissions found that mortality was greater in the RSV compared to the influenza group (18.4% vs 6.7%).¹⁷⁸ A paper from 2019 by Ackerson et al investigated the 1 year all-cause mortality for individuals aged over 60 who tested positive for either RSV or influenza during a hospital admission. They discovered that RSV was associated with a greater length of stay, frequency of pneumonia, and ICU admissions. When investigating the all-cause mortality, RSV was significantly higher, with 74.2% survival compared to 81.2% for influenza (p=0.019)¹⁷⁹.

7.2 Diagnosis of RSV in adults – an impossible task? Lessons learned from this study

Diagnosing RSV in an adult of any age, with or without underlying comorbidities, is not a simple process. Older laboratory techniques such as viral culture have been overtaken by more specific methods such as PCR and were therefore not used in this study¹⁸⁰. PCR based diagnostic techniques are however not close to being 100% sensitive for detecting RSV associated COPD exacerbations. Viral load in adults is lower than when infants have an RSV associated infection, which can be one of the reasons PCR appears to be less sensitive in adults¹⁸¹. There is also a propensity for RSV to replicate in the lower airways, therefore nasal swabbing alone might not be sufficient to obtain a diagnosis¹⁸². Using a combination of viral PCR along with serological methods will increase the rate of RSV diagnosis and allow for retrospective diagnosis of cases when respiratory swab samples could not be collected within the window of the acute infection. COPD exacerbations can last for weeks, and if the sample is not taken within the first few days of symptoms occurring, the RSV viral load may already have started to decline.

As discussed in the introduction, and again found to be true in this study, diagnosis of RSV from a symptomatic perspective is not possible. Analysing the symptoms of different COPD exacerbation causes in this study, there was no observable difference in symptoms from diary

card or CAT score perspective for any exacerbation cause. This fits with the previous literature, as previously discussed.

In a study from 2019 by Falesy et al, a combination of serology and PCR was used for diagnostics, where 33% of RSV associated illnesses were negative on PCR diagnosis but positive with serology¹⁸³. This is very similar to the findings of this thesis, where out of the 188 attended exacerbations, 18 were RSV positive by any means, 9 by PCR (64%) and 16 by serology (with 7 overlaps). This means that 11% were negative on PCR despite a swab being taken within 5 days of symptom onset as per diary cards. This supports the value of serological assessments as a tool for understanding the burden of RSV infection.

As mentioned earlier, sampling of lower airways gives a better yield for RSV diagnostics than only using upper airway samples such as nasal swabs^{184 185}. It was not feasible to perform bronchoscopy at exacerbation amongst the COPD participants in this study, and it would not have been without potential risk. The next-best method for sampling the lower airways is to analyse the sputum produced. Again it was not feasible to perform induced sputum collection in these participants, but we asked participants to either bring sputum with them to the visit (produced that morning) or spontaneously produce sputum during the visit (see methods). This also enabled the additional analysis of sputum culture to analyse for concomitant bacteria, or bacterial causes of exacerbation. In this study 93 out of 188 (49.1%) Imperial exacerbation visits produced sputum samples suitable for analysis with both PCR and bacterial culture. 5/93 (5.4%) sputum samples were positive for RSV compared to 3/175 (1.7%) NP swab results. This indicated a trend towards sputum having a higher yield for RSV diagnostics, however did not meet statistical significance ($P=0.094$ using Chi Squared test). When adding in the data from Groningen for comparison however the relative yield from sputum increased and statistical significance was met.

It could be argued that as RSV shows a propensity for replicating in the lower airways the ability to produce sputum might be more common during RSV related exacerbations than exacerbations caused by other viral pathogens. There is no published evidence for this amongst COPD patients. The only way to investigate this accurately would be to examine induced sputum or bronchoscopy samples collected at exacerbation for all participants and

compare sputum load amongst RSV related exacerbations to other viral causes. Unfortunately neither such approach was feasible in this study.

The RIVM Pentaplex serological assay, specifically the N, Pre-F and Post-F antibodies were found to be as sensitive as the Janssen ELISA serological method for diagnosing RSV and was therefore used in the diagnostics of this study. In the results section there are depicted Bland-Altman plots detailing the agreement. Ga and Gb antibodies were not sensitive for RSV related exacerbations when compared to other established antibodies. As detailed in the results they did not meet statistical significance when comparing the PCR diagnosed RSV associated exacerbations to rhinovirus associated. The Ga and Gb antibody rises were therefore not used in diagnostics and in future analysis I would not include them in the pentaplex to save on costs. RSV-A induces Ga antibodies and RSV-B induces Gb antibodies, so the value of either antibody will be dependent on which subtype circulates during any study period. Rather than using these to define RSV infections, it is perhaps more useful for understanding the serotype of those RSV infections that did occur.

One of the potential future benefits of measuring antibody rises to RSV N specific protein is that these levels would likely not be affected by RSV vaccines (most candidates of which (those currently in phase 3 trials) target the F-protein) and could still be used for RSV diagnostics in a vaccinated population. Monitoring RSV specific N antibody levels every 3 months, during vaccine studies, could be an effective way of investigating for RSV infection and could potentially replace PCR based diagnostics. There is no reason to think this would be any different in adults without COPD.

The in-house ELISA was designed for measuring RSV specific immunoglobulins, but not to specific RSV proteins. Once the RIVM pentaplex became available for this study, the in-house ELISA was no longer used, as it was more time consuming and less specific. N protein antibodies are not generally reported as being used in diagnostics in any older adult studies. There appears to be a higher titre rise for N Glycoprotein specific antibodies than Pre-F and Post-F glycoprotein specific antibodies, when an RSV associated exacerbation has occurred. Anti-N antibodies may therefore be a useful tool for serological diagnosis of RSV for clinical

trials in the future. If found to be effective and sensitive, this approach could save money and manpower during large multi-centre studies.

Using a 2-fold rise in antibody would result in considerably more positive cases being diagnosed and therefore classified as RSV related exacerbations. It was also found that there would be many cases of two-fold rises in antibody titer where there were no documented exacerbations, either self-reported or on diary cards. All of the published literature I am aware of uses a four-fold rise as a diagnostic cut-off. There is discussion in Korsten et al's¹⁶⁹ paper supplements regarding using a two-fold rise, but in the end they reverted to using four-fold rise for their analysis. A four-fold rise has its history in dilutional ELISA's, where in adjacent wells the serum concentrations were halved, and the investigator would look for a two well gap between positive cases, therefore a four-fold change. This does seem to be a reliable cut off, and is now the cut-off used in the majority of literature and studies, including to diagnose RSV in COPD^{169 170 186}. While the 4-fold rise is therefore rooted in historical convenience, the alternative of using a 2-fold rise presents the possibility of increasing the sensitivity for RSV detections, at the potential cost of decreasing specificity.

7.3 RSV and development of COPD

There is mounting evidence that childhood chest infections, pneumonia and wheeze are strongly associated with airways disease and COPD in later life^{187 188}. RSV is the most common cause of childhood wheeze, bronchiolitis and lower respiratory tract infection in the United States. It is also the most common cause of hospitalization for infants <1¹⁸⁹.

There is an increased basal inflammatory state in COPD¹⁹⁰ and RSV is known to cause inflammation. RSV in the presence of COPD may well cause a 'perfect storm' of inflammation and unhelpful pro-inflammatory host responses, which would help explain the high degree of morbidity and mortality RSV is accountable for amongst COPD patients.

It appears to be the case therefore that RSV not only causes significant morbidity and mortality in COPD patients by way of triggering an exacerbation but may also be partially

responsible for the development of COPD in the first instance. More research is needed in this area and is actively underway.

7.4 Comparing RSV Related COPD Exacerbations to Rhinovirus Related

Rhinovirus and RSV are two of the most common viral causes of COPD exacerbations¹⁹¹. In this thesis these two viruses have been compared in detail in the results section. In the results section 18 RSV associated exacerbations were compared to 14 rhinovirus associated exacerbations.

The spine of this thesis focuses on RSV associated COPD exacerbations, investigating prevalence and severity, therefore more methods were used to diagnose RSV than other viruses, including RSV specific antibody serology. These methods were not used for diagnosis of rhinovirus infections, therefore, when discussing prevalence of the two viruses at COPD exacerbation, this must be considered. The outcomes for RSV associated exacerbations diagnosed by different means have been compared, and no statistical difference has been found between the two methods. This indicates that serological based diagnosis of RSV is comparable to a PCR based diagnosis in terms of severity of exacerbation, supporting the validity of comparing infection types diagnosed using different diagnostic methods. There is precedent for using multiple diagnostic methods in previous literature^{169 192 193}.

When investigating time to recovery for the exacerbation, RSV associated exacerbations were statistically significantly shorter than rhinovirus associated exacerbations. The average time to recovery was 10.33 days for the 18 RSV associated exacerbation vs 17.77 for the 14 rhinovirus associated exacerbations. As far as I am aware, this is the first occasion that time to recovery have been investigated for RSV associated exacerbations vs other causes of COPD exacerbation in the outpatient setting.

There was no statistically significant difference in change in FEV1 percentage at exacerbation between RSV and Rhinovirus associated exacerbations. RSV associated exacerbations were not statistically significantly different from baseline in terms of FEV1 percentage change,

whereas Rhinovirus associated exacerbations were. As RSV is an airways predominant virus, it is surprising that the rhinovirus associated exacerbations had a larger drop in FEV1 percentage at exacerbation.

CRP, Fibrinogen and CAT scores at exacerbation between the two groups showed no statistically significant difference.

Rhinovirus was by far the most common viral cause of COPD exacerbation found on PCR in this cohort, which also fits with the previously published literature¹⁹⁴. This was also the case in the mirrored Groningen cohort.

7.5 RSV severity compared to other exacerbation causes: Is RSV or Rhinovirus the Outlier?

An interesting finding to come out of this thesis is that, amongst the exacerbation causes investigated, RSV related exacerbations resolved faster than the exacerbations related to the other pathogens tested for (10.28 days recovery for RSV related exacerbations compared to 24.27 days for Rhinovirus and 17.96 days for bacterial associated exacerbations). It was beyond the scope of this work to thoroughly investigate the reasons for this. I am also not aware of any papers or manuscripts that have investigated this finding or even described it in the past. What must also be noted is that the diagnostic methods for RSV also included serology, which was not used to diagnose any other viral or bacterial pathogens.

The results section displays all the markers of infection severity that were measured in this study: Time to recovery, fibrinogen, CRP, WCC, FEV1% change and CAT score change. There was a higher CAT score seen in flu related and RSV related exacerbations (although not statistically significant). CRP was higher with both RSV related exacerbations and mixed bacterial and viral associated exacerbations (again not statistically significant). A similar pattern was seen with WCC.

These data suggested that RSV appears to cause exacerbations of a similar severity to other viral causes of exacerbation. The inflammatory nature of these exacerbations when compared

to other viral or bacterial causes of COPD exacerbations (as measured by fibrinogen, CRP and FEV1 % change) are no less, however these RSV related exacerbations are of shorter duration.

7.6 Exacerbation Frequency and viral susceptibility

There was an increase in exacerbation frequency amongst those participants that ever had an RSV, or rhinovirus infection compared to those participants that did not at any time during the study. There are factors involved with COPD and older adults generally such as inflammaging and immunosenescence that predispose to viral infections and prevent effective immune responses from being mounted¹³⁴. These results speak to the frequent-exacerbator phenotype of some COPD patients and indicate that one positive viral PCR test may be a useful indicator of this frequent-exacerbator phenotype. Identifying this group is particularly important as decline in lung function is typically more rapid, and greater healthcare intervention is commonly required. It was out of the scope of this study, however there is scope for future research to investigate biomarkers for viral susceptibility by comparing the two groups.

7.7 Biomarkers

As discussed in both the introduction, and in the literature search chapter there are known biomarkers of susceptibility and severity in adult RSV infections. In this study we investigated CRP during exacerbations. The CRP was higher amongst RSV exacerbations, but it did not reach statistical significance. There was no statistical significance or observed trend between fibrinogen readings amongst different causes of COPD exacerbation.

It was out of the scope of this study to investigate further biomarkers of susceptibility to RSV infection or severity amongst COPD participants. We have serum put aside for possible future investigations involving novel biomarkers, or to validate the biomarkers discussed in the literature review chapter amongst those with COPD.

As previously discussed, using antibody rises to RSV specific N protein can be used in diagnostics. As previously argued, this may prove to be very useful in a vaccinated population.

7.8 Other findings from the Results

7.8.1 Recruitment

As described in detail in the results section, there were four recruitment methods utilized, and the majority of participants were recruited directly from Professor Wedzicha's London EXCEL COPD Cohort.

Other than recruiting from the EXCEL COPD Cohort, utilizing the West London lung cancer screening study patients was by far the most successful method used. Nearly half of those participants contacted from this pool showed interest in joining the RSV study (47/111 42%). 28 out of the 47 agreed to come for a screening visit, and only 2 failed the screening, meaning that 93% of screening visits were successful. As a screening visit can take between 1.5 and 3 hours, so this was also very time efficient. Moving forward I would try to utilize patient cohorts already within the local hospital system if possible, as these methods were by far the most effective at recruitment. It also saved a lot of admin time as the participants were already registered on the local hospital system. The participants from the West London lung cancer screening study were recruited from the community, agreed to be contacted for other research projects, had a smoking history taken and had their spirometry measured. Because of this we could focus on those participants we deemed likely to meet the inclusion, and not the exclusion criteria of the study.

7.8.2 Baseline (non-exacerbating) Samples

The theory of RSV being present during baseline COPD, or in other words outside of a symptomatic exacerbation is controversial¹⁹⁵. The preliminary data chapter describes some work undertaken investigating RSV detection at the stable state and the inflammatory marker profile when RSV was detected. As described in detail in that chapter, RSV was found to be present in nearly 20% of the baseline samples.

There was no RSV found using PCR amongst the 60 tested baseline sputum samples in this study. The multiplex PCR and RSV specific PCR by GSK were used for this analysis using the same protocol, and in the same batch as the exacerbation samples. The cut-off for positivity used in this study was 67 viral copies. It is difficult therefore to compare this to the results regarding stable-state RSV discussed in the preliminary data chapter. I have not undertaken a direct comparison between the two methods to investigate sensitivity differences. It is possible that the gel PCR method used in the preliminary data is more sensitive for picking up low-levels of RSV present in baseline samples as compared to the GSK multiplex PCR.

Contained in this thesis are therefore conflicting data for the hypothesis of RSV persistence and this question remains open as in the published literature. A large-scale study of COPD patients at baseline would be needed to investigate this fully, ideally including lower airway sampling. As discussed, bronchoscopy for sampling in patients with COPD at exacerbation is not always practical for multiple reasons. There have been studies examining COPD participants at baseline with bronchoscopy and sampling for microbial analysis¹⁹⁶. Having a baseline lower airway sample from every participant, through either bronchoscopy or induced sputum, ideally at more than one timepoint, would have aided in this research. Modern genetic analysis techniques allow for detailed interrogation of the viral genome¹⁹⁷. Sequential RSV positive samples from the same participant analyzed this way could indicate if the virus was persisting. Differences have been noted however within host RSV populations from the same sampling date, so even this method may be flawed¹⁹⁷.

If there is RSV present at baseline in some COPD patients it could be possible that their immune system is therefore constantly primed to tackle the infection. This is one hypothesis as to why the RSV related exacerbations were found to be of shorter duration in this thesis. The question of RSV prevalence at baseline remains unclear.

7.9 Limitations of the Study

Smoking is not the only cause of COPD¹⁹⁸ and this study excluded any participants that had COPD caused by other means. I am not aware of any studies looking at the difference in RSV related exacerbation symptoms and severity amongst COPD patients focusing on differences based on COPD causes. Non-smoking related COPD is more common in parts of the

developing world (LMIC's), for example triggered by chronic exposure to bio fuels, and the results of this study do not necessarily translate to these differing geographical regions.

As discussed in this chapter, analyzing lower airway samples for RSV is important in diagnosing RSV and gives a higher yield of PCR positivity. This study did not collect lower airway samples at every exacerbation visit, just those where the participant was spontaneously expectorating. It would be interesting to perform induced sputum in a future study, to see if this changes the RSV prevalence.

A number of the exacerbations (n=64 – 37% of total) from the Imperial cohort were found to have no associated viral or bacterial pathogen and therefore labelled as 'unknown'. It was unfortunately beyond the scope of the time-frame of this thesis to investigate these exacerbations in more detail – for example by examining sputum for cytokines or eosinophils. Detailed description of these non-infective exacerbations could form potential future work based on the findings from this thesis.

There is always the risk of studies being underpowered – initially there were very few RSV related exacerbations found on PCR analysis, however with the use of the various serological techniques more were identified. Future studies using only PCR based diagnostics would need to take this into account.

There were a few hospital admissions (7 at Imperial) during this study. Unfortunately, as the participants presented at various hospitals around London, it was not possible to obtain samples from these admissions, or always find the cause of these admissions. There were also 8 participants that passed away during the study period. Some of the participants that passed away would also have presented to hospital prior to this. Therefore for both of these events RSV cases may have been missed. Although this is a limitation of the study, there are already various studies investigating RSV on admission to hospital, or cause of in-hospital mortality¹⁹⁹. This study was focusing on the outpatient setting, which is very much under-studied when it comes to RSV.

7.10 RSV Vaccines – is this the future?

Most of the major world vaccine developers are working towards an RSV vaccine for either pediatrics patients, maternal patients or older adults. As of January 2023, there were two vaccine candidates in phase 2 trials and five in phase 3 aimed at older adults. See Figure 52 for a snapshot of all of the vaccine candidates currently in development. This figure has been put together by PATH²⁰⁰.

At a vaccine meeting (IDWeek 2022) Professor Falsey presented the results of a phase 3 vaccine trial (CYPRESS Ad26.RSV.preF–RSV preF protein vaccine) for older adults indicating safety and good humoral and T-cell response. The preliminary results of this study has recently been published and peer reviewed in a medical journal, and look very promising²⁰¹. Out of 5,782 participants that have so far received the vaccine in the trial, vaccine efficacy has been reported as between 69.8% and 80.0% depending on the definition of RSV related disease²⁰¹. The frequency of severe adverse events was reported as similar in the vaccine and placebo groups²⁰¹.

A second vaccine trial has also reported the provisional phase 3 results (RSVPreF3 OA vaccine). Out of 12,467 participants that have so far received the vaccine in the trial, vaccine efficacy has been reported as between 71.7% and 94.1% depending on the definition of RSV related disease²⁰². The frequency of severe adverse events was reported as similar in the vaccine and placebo groups²⁰².

All of the vaccines in phase 3 development are currently designed to create an immune response to the RSV specific F-protein. As I have demonstrated in this thesis, human immunoglobulin rise to RSV specific N protein can be used in diagnostics in older adults. This may prove to be very useful amongst a vaccinated group of patients, as antibodies to the N protein will not rise post vaccination (although antibodies to F protein may) and a rise can therefore be used to diagnose RSV infection post vaccination.

As indicated in the introductory chapter to this thesis, there is growing evidence that there is a considerable burden of disease caused by RSV in older adults. I have shown in the results chapter of this thesis that amongst the COPD cohort studied 7.4% of the exacerbations were

associated with RSV. A safe and effective vaccine against RSV in adults would be beneficial in COPD in particular, and I would argue that COPD patients should be amongst the first cohorts inoculated with a new vaccine.

RSV Vaccine and mAb Snapshot

TARGET INDICATION: P = PEDIATRIC M = MATERNAL E = ELDERLY

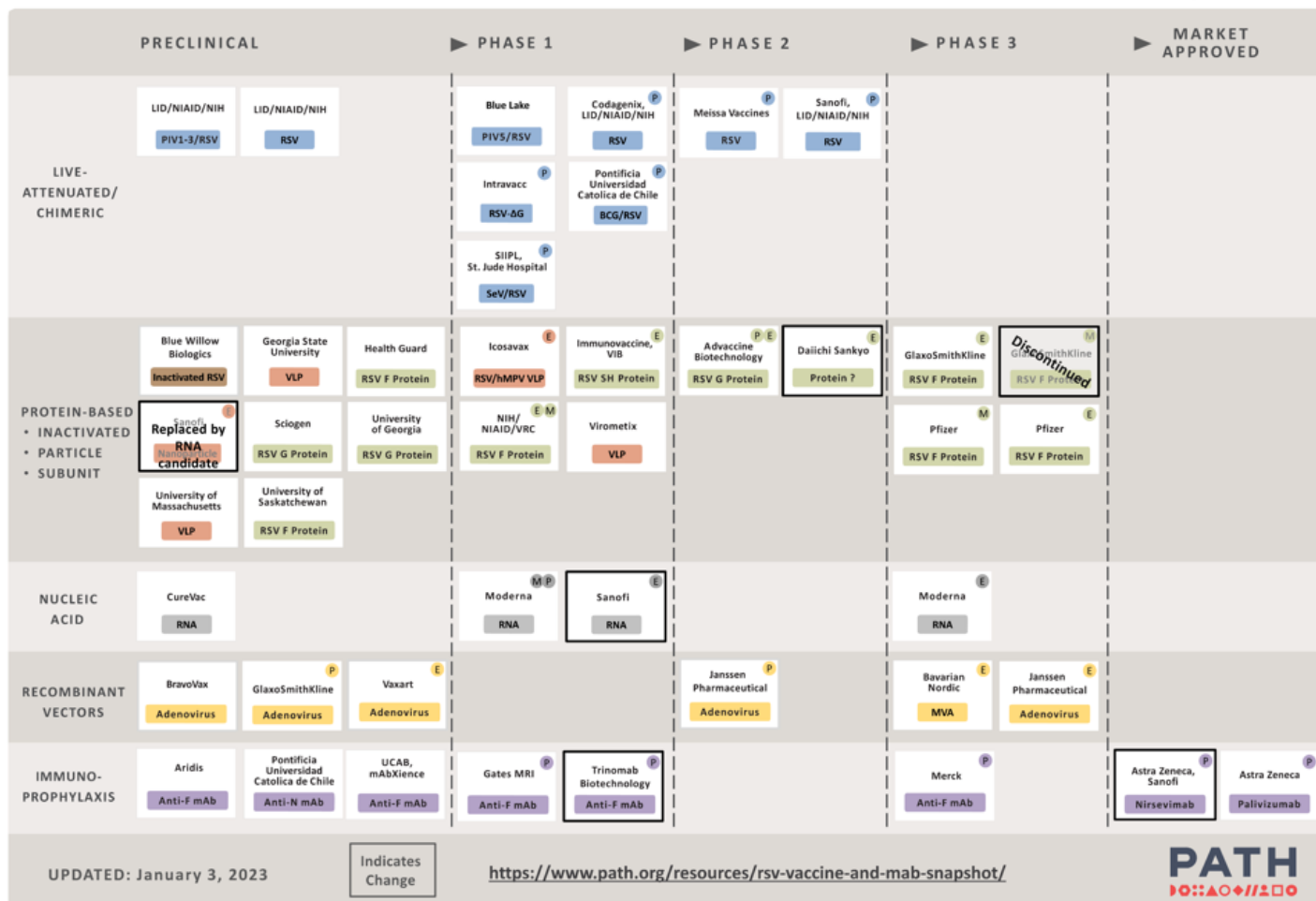


Figure 52 Vaccine and mAb snapshot indicating progress of vaccine and mAb candidates. Published online by PATH (as of January 2023) – permission to include sought

7.11 Extrapolation of Imperial Results

RSV was associated with 7.4% of COPD exacerbations during this study period. Up to 50% of COPD patients are estimated to have at least one exacerbation every year²⁰³. There are an estimated 1.4 million patients with COPD in England²⁰⁴. This would equate to ~ 700,000 exacerbations per year. If 7.4% of those are RSV related, then this would equate to ~ 50,000 RSV associated COPD exacerbations per year.

As the rates of RSV were similar in the Groningen cohort when comparing the same methods, it could be postulated that these rates of RSV associated COPD exacerbations would be similar across Europe.

There are a number of vaccines in development for older adults, one of which is an adenovirus based vaccine (Ad26.RSV.preF). The results of the phase 2 trial for this vaccine were presented at ID week in 2021. The preliminary results indicate it may be 80% effective in preventing lower respiratory tract disease in older adults²⁰⁵. This study was not looking at COPD patients specifically, but if the efficacy was similar, then this vaccine could potentially prevent a significant number of COPD exacerbations per year.

7.12 RSV in a Post Covid-19 world

The clinical phase of this study was conducted before Covid-19 became a pandemic in the United Kingdom, and to my knowledge none of the participants developed Covid-19 during the study period. It is clear that the Covid-19 epidemic and the public health measures used to mitigate the epidemic have had a substantial impact on RSV infections, seasonality and RSV genetic diversity²⁰⁶. However, as the clinical phase of this study was conducted prior to the start of the pandemic, this will not have influenced the rate or nature of COPD exacerbations in this study.

There is no data to suggest that RSV will not continue to cause substantial morbidity and mortality amongst adults after the Covid-19 pandemic has subsided. There was a noticeable

drop in infections during lockdowns in western Europe²⁰⁷ and the US²⁰⁸ – but this process is now reversing. Australia noticed a decrease in RSV genetic diversity, but there has been a surge post lockdown in many states and territories, and the seasonality of RSV is not following the usual pattern this last couple of winters²⁰⁹.

One possible benefit from the pandemic is that vaccine development is now much better understood by the general population, with some studies indicating that vaccine hesitancy has decreased²¹⁰. RSV confers significant morbidity and mortality amongst the frail, comorbid and elderly population. A vaccine will be available for older adults in the next year which, if effective will mitigate the burden of disease. COPD patients should, in my opinion, be amongst the first group to be offered such a vaccine.

7.13 Ideas For Future Research Arising from this Thesis

- Investigate subsequent RSV infections in the same cohort – are there a sub-set of patients that become frequently re-infected by RSV?
- Look further into bacterial exacerbations post viral infections. Were the participants that acquired a viral infection at more risk for subsequent bacterial infection and if so, were particular viruses more predisposing?
- Further work is needed to re-evaluate the RSV burden after the Covid-19 pandemic.
- Research needed to investigate in detail the prevalence of RSV in the lower airways of baseline (non-exacerbating) COPD patients. Ideally this would be bronchoscopy samples.

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9 Appendix

9.1 Demographics from Chapter 3

Supplementary Table 2: Demographic and clinical characteristics of London COPD cohort patients	
Characteristic	N (%)
Male Sex	91 (65.5%)
Age	71.8 (67.4-75.9)
FEV1	1.41 (1.02-1.91)
GOLD Stage	
I	21 (15.1%)
II	67 (48.2%)
III	45 (32.4%)
IV	6 (4.3%)
Smoking History	
Current Smoker	43 (30.9%)
Ex smoker	96 (69.1%)
Pack years	44.6 (29.1-57.3)
Comorbidities	
Cardiac disease	13 (9.4%)
Hypertension	85 (61.2%)
Diabetes Mellitus	17 (12.2%)
Chronic liver disease	1 (0.7%)
Cerebrovascular disease	3 (2.2%)
Inhaled therapies	
Inhaled corticosteroid use	96 (69.1%)
Long acting Beta-2 agonist	98 (70.5%)
Long acting muscarinic agent	99 (71.2%)
<i>Data shown as n(%) or median (IQR)</i>	

Table 16 - Demographics for the patients in Chapter 3 – taken from F. Kamal et al 2021⁴

9.2 Example Diary Card

NAME		ENUC										
Study Number		2659										

December 2018		NEXT APPOINTMENT 01/02/18 14.00m	
WORSENING SYMPTOMS? CALL US			

DATE	1 Sat	2 Sun	3 Mon	4 Tues	5 Wed	6 Thurs	7 Fri	8 Sat	9 Sun	10 Mon	11 Tues
Peak Flow	210	190	190	240	250	220	230	250	230	230	230
CHANGE in Symptoms	22.6	22.0	22.61	22.81	22.14	22.62	22.62	22.82	22.84		
CHANGE in Treatment			15	65	65	65	65	65	65		
Hours out of the home	7:00	3:30	7:15	6:15	8:30	8:00	8:15	8:15	5:00	7:45	5:45

DATE	12 Wed	13 Thur	14 Fri	15 Sat	16 Sun	17 Mon	18 Tues	19 Wed	20 Thur	21 Fri	22 Sat
Peak Flow	250	240	230	240	240	240	240	230	240	250	240
CHANGE in Symptoms											
CHANGE in Treatment											
Hours out of the home	8:00	6:15	8:00	3:00	5:30	7:30	5:30	7:30	9:15	2:30	8:00

DATE	23 Sun	24 Mon	25 Tues	26 Wed	27 Thur	28 Fri	29 Sat	30 Sun	31 Mon
Peak Flow	230	230	240	230	220	230	230	220	220
CHANGE in Symptoms									
CHANGE in Treatment									
Hours out of the home	8:30	6:15	4:30	4:15	5:45	6:15	4:15	9:00	7:00

Next appointment 17th December 11:00

Figure 53 - Front of Diary Card giving an example of an exacerbation

Instructions for filling in the DIARY CARDS

EVERY DAY...

1. After taking morning medications record the best of 3 attempts at the PEAK FLOW blowing test in the box on the sheet.
2. Please record any **WORSENING** of symptoms **ABOVE YOUR USUAL** daily level. The symptoms we are interested in are listed below, just put the appropriate letter in the box on the sheet. Continue recording until the symptom has gone away or got back to the level you consider 'normal'.

Letter	Symptom
A	increased BREATHLESSNESS.
B1	increased SPUTUM COLOUR.
B2	increased SPUTUM AMOUNT.
C	a COLD (such as a runny or blocked nose).
D	increased WHEEZE or CHEST TIGHTNESS.
E1	SORE THROAT.
E2	increased COUGH.
F	FEVER.

If you experience a worsening in any of these symptoms please phone us to arrange an assessment visit, and do this BEFORE starting any antibiotic or steroid tablets. The phone number is

07762 038662.

Lydia, Dexter or Andrew will have the phone and we can usually arrange to see you later the same day or the following morning.

Please phone if you are not sure what to write down or you have any questions.

3. Please record any **CHANGE** to your usual treatment for as many days as it applies. Again, just put the appropriate letter in the box on the sheet.

Letter	Treatment
H	I am in Hospital.
I	I am taking more than usual INHALED STEROID (red / brown/purple)
R	I needed to take extra RELIEVER (blue / green / grey / nebuliser). HOW MANY PUFFS? Write, eg 'R3' for 3 puffs, 'R2' for 2 etc
S	I am taking STEROID (Prednisolone) TABLETS. HOW MANY TABLETS? Write, eg 'S6' for 6 tablets, 'S5' for 5 etc
X	I am taking ANTIBIOTIC TABLETS. PLEASE RECORD WHICH (write the name on the diary card).

4. Finally, please estimate the time that you were out of your own home on the previous day. Please record this in the column on the day before.

Figure 54 – Rear of Diary Card

9.3 Annual Review Form

Annual review (V1) THE LONDON COPD COHORT/ RESCEU

Dyspnoea and wheeze

1. When are you short of breath? (please tick)					
All day		Only in the mornings		1-2x a week	
Only on exercise		Only with chest infections		Never	
2. When do you wheeze? (please tick)					
All day		Only in the mornings		1-2x a week	
Only on exercise		Only with chest infections		Never	

Chronic bronchitis and cough

3. Does your cough wake you up at night?			Yes		No	
4. Do you bring up phlegm (sputum) for more than 3 months during the year?			Yes		No	
5. On average how much sputum do you bring up in a day?						
None		< egg cup (<30 ml)		>egg cup (>30 ml)		
6. On an average day what is the colour of your sputum?						
White		Yellow		Green		
Brown						

Exacerbations

7. How many exacerbations have you had over the last 12 months					
Total exacerbations		Treated exacerbations		Hospital admissions	
Steroid courses		Antibiotic courses		Bone protection?	

Smoking

8. Do you currently smoke?			Yes		No	
----------------------------	--	--	-----	--	----	--

9. Are you an intermittent quitter?				Yes		No	
Age started smoking		Age quit smoking		Cigarettes/rollups/day			
10. If still smoking what/ how may are you smoking (number/day for all that apply)							
Cigarettes		Roll ups		e-cigarettes			
11. If still smoking would you like to quit				Yes		No	
12. Advice/ information offered?				Yes		No	

Atopy

13. History of atopy – please tick all that apply							
Do you have asthma?		Do you have hay fever?		Do you have eczema?			
Have you had skin prick tests?	Yes		No		Results		
Other medical problems							

Pulmonary rehabilitation

14. Have you completed a pulmonary rehabilitation course?				Yes		No	
Date started		Date finished		Still exercising?			

15. Immunisation

Have you had a flu vaccine?	Yes		No		Dates	
Pneumonia vaccine?	Yes		No		Dates	

16. Nasal symptoms

Nasal symptoms over 3 months(0= no symptoms, 1= v.mild, 2=mild, 3=mod, 4=severe, 5=bad as can be)					
Runny		PND		Blocked	
Sneezing		Anosmia		Sum	

17. Medication history

Medication	Dose	units	freq	route	Indication	start	stop
Allergies							
Social History							
18. Marital status							
Married/ civil partner		Widow/ed				Divorced/ separated	
Single		Other					
19. Number of children					Age youngest child		
20. Number of times out of home/ week					No visitors to home/week		
Times contact with children < 16 years last week	>2		1-2		<1		none
21. Accommodation and pets							
House		Flat				Maisonette	
Bungalow		Other					
Number of occupants in home							
Partner/spouse		Children				Siblings	
Parents		Others				Lives alone	
Family pets							
Cats		Dogs				Birds	
None		Others					
22. Clinical findings							
SaO2		FiO2				Heart rate bpm	

BP mmHg		Steroid bruising		Peripheral oedema	
Finger clubbing		Height cm		Weight kg	

Spirometry

FEV1				% predicted
FVC				% predicted

Questionnaires

SGRQ		Depression scale		Fatigue score	
MRC scale		VAS		CAT	

Bioimpedence

%Fat		Fat kg		%FFM	
FFM kg		Total body water			
Basal metabolic rate		BMI		Impedence	

Other investigations (tick completed)

ECG		Sputum		Urine	
Blood + 1 extra red top		Book lung function		NP swab	

Table 17 – Annual Review Form

9.5 Exacerbation Form

The London COPD Cohort Exacerbation Form/ RESCEU

Exacerbation onset and symptoms							
Date exacerbation onset		Date phone call		Reported			
Time of day symptoms started		Waking	Morning	Afternoon	Evening	Night	
Time symptoms most intense		Waking	Morning	Afternoon	Evening	Night	
Respiratory Symptoms today (symptoms over last 24 hours which are increased compared to baseline)							
A Increased Breathless	B1 Sputum Colour	B2 Sputum Amount	C Cold	D Chest Tightness Wheeze	E1 Sore Throat	E2 Increased Cough	F Fever
Nasal symptoms (0=none, 1= very mild, 2=mild, 3= moderate, 4= severe, 5= as bad as can be)							
Runny nose	PND	Blocked	Sneezing	Anosmia	Total nasal score		
Health Burden		0 = no change 1 = ↓ ability to go out 2 = ↓ ability to cook 3 = Bed bound at home 4 = Hospitalised 5 = Hospitalised + ↑ social package 6 = death					
MRC dyspnoea score		1 = SOB with strenuous exercise 2 = SOB hurrying on a level surface of slight hill 3 = walks slower than peers on flat or stops walking at own pace on level 4 = Stops for breath walking approx. 100 yards 5 = Too SOB to leave house, SOB washing when dressing					
Ankle swelling		Reflux		Tremor		Palpitations	

Social activity			
Hours out of home last 24 hours		Unwell contact in last week? Y/N	
No visitors last week		No. days out of home last week	
Contact with child in last week		Age youngest child	
Recent travel outside UK? details			

Exacerbation treatment given (Include drug, start date, dose, frequency, duration)	
Bronchodilators	
Antibiotics prescribed	
Oral steroids	
Increase in inhaled steroid (Y/N)	

Date treatment started		
------------------------	--	--

Investigations							
FEV1		FEV1		FEV1		predicted	
FVC		FVC		FVC		predicted	
O2 sats		FiO2		BP		HR	
Home PEFR			CAT score			SGRQ	
Blood + 1 extra red top		Urine		Sputum		Swabs NP+OP	

Table 18 – Exacerbation Form

9.7 Sputum Processing: STANDARD OPERATING PROCEDURE.

Title: Sputum Processing

Date 24 Sep 2014

Version 2.0

Author: Ray Sapsford

Aim: To process sputum on spontaneous or induced sputum collection for measurement of biomarkers (cytokines and inflammatory markers) in sputum.

Equipment: All equipment shown below can be substituted for similar alternatives.

Glass beads 2.5 – 3.5mm size

48um nylon filter gauze

15ml polypropylene centrifuge tubes

40mm diameter glass funnels

1.5ml micro centrifuge tubes (Eppendorf)

Whirlimixer IKA – Vibrax – VXR (bench rocker)

Vortex mixer – variable speed

Bench top refrigerated centrifuge

Top pan analytical balance, 500g capacity with readability of 0.001g

-80°C freezer

Gilson type pipette 1000ul size

Test tube and micro centrifuge racks

Reagents:

Dulbecco's Phosphate buffered Saline (D-PBS)

Method – Sputum collection and processing:

Samples will be examined within 5 hours of collection. Sputum plugs will be separated from contaminating saliva by macroscopic examination. Selected portions of the sputum will be processed with PBS and glass beads for bacterial qPCR and supernatants stored for inflammatory markers as follows:

- Select sputum plugs from the saliva, aiming for 0.5-1g of plug weight sputum (if only a small amount of sputum is obtained, select as much as possible).
- Transfer sputum plug into an empty (pre-weighed) polypropylene centrifuge tubes with a screw top.
- Calculate weight of sputum to be processed (weight of tube and sputum – weight of the empty tube).
- Add 8 volumes x sputum weight (in grams) Dulbecco's phosphate buffered saline and approximately 0.5mL of glass beads.
- Vortex tube for 15 seconds and then 15 minutes on a bench rocker. Re-vortex for further 15 seconds.
- Sub-aliquot 500µl samples into separate Eppendorf tubes. Label with specimen type, participant code, date of sample and stores at -80°C for microbiology qPCR.
- Filter the remaining sputum suspension through 48 µm nylon gauze, and then centrifuge the remaining sample at 790g (2000rpm) for 10 minutes.
- Sub-aliquot 500µl samples of supernatant into separate Eppendorf tubes. Label with specimen type, participant code, date of sample and store at -80°C for subsequent airway cytokines.

9.8 CAT Questionnaire

Your name:

Today's date:



CAT
COPD Assessment Test

How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers, and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

Example: I am very happy 0 ☒ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 I am very sad

	SCORE	
I never cough	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	I cough all the time
I have no phlegm (mucus) in my chest at all	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	My chest is completely full of phlegm (mucus)
My chest does not feel tight at all	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	My chest feels very tight
When I walk up a hill or one flight of stairs I am not breathless	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	When I walk up a hill or one flight of stairs I am very breathless
I am not limited doing any activities at home	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	I am very limited doing activities at home
I am confident leaving my home despite my lung condition	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	I am not at all confident leaving my home because of my lung condition
I sleep soundly	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	I don't sleep soundly because of my lung condition
I have lots of energy	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	I have no energy at all
		TOTAL SCORE

COPD Assessment Test and the CAT logo is a trade mark of the GlaxoSmithKline group of companies.
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Last Updated: February 24, 2012

Figure 55 – CAT Score Questionnaire

9.9 Recruitment Advert



Royal Brompton & Harefield 
NHS Foundation Trust

Do you have COPD? Are you over 40 years of age?

We are a world-class lung research group based at Royal Brompton Hospital, Chelsea. We are looking for COPD patients to enter a study which aims to understand how infections cause 'flare-ups' or worsening of the condition.

You will meet our enthusiastic team of lung specialists and also receive a lung check-up.

This will not affect any of your usual COPD care in the hospital clinics or community.

To find out more, contact [Dr Dexter Wiseman](#) at Royal Brompton Hospital:

Tel: 07762 038662
Email: londoncopd@imperial.ac.uk

Figure 56 - Recruitment Advert