Phenotyping Chronic Obstructive Pulmonary Disease Exacerbations

by

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Abstract

Rationale: Acute exacerbations of chronic obstructive pulmonary disease(AECOPD) are caused by a variety of different etiologic agents. Our aim was to phenotype COPD exacerbations using imaging(chest x-ray[CXR] and computed tomography[CT]), blood tests(C-reactive protein [CRP] and the N-terminal of the prohormone brain natriuretic peptide [NT-proBNP]), and a molecular pathogen detection method.

Methods: Subjects who were hospitalized with a primary diagnosis of AECOPD were enrolled in the Rapid Transition Program(RTP). We examined a subset of subjects who had had CXRs, CT scans, and blood collected for CRP and NT-proBNP. A radiologist blinded to the clinical and laboratory characteristics of the subjects interpreted the CXRs and CT images. Logistic regression models were used to assess the performance of these biomarkers in predicting the radiological parameters. Sputum samples in a subset of subjects were tested by a molecular pathogen detection method to phenotype AECOPD into non-infectious, bacterial, and virallyassociated phenotypes. Differences between the phenotypes in terms of clinical features, CRP and NT-proBNP concentrations, complete blood counts, and 1-year mortality rate were examined.

Results: NT-proBNP was associated with cardiac enlargement, pulmonary edema, and pleural effusion on CXR, whereas on CT images, NT-proBNP was associated with pleural effusion. CRP, on the other hand, was associated with consolidation, ground glass opacities, and pleural effusion on CT images. A CRP sensitivity-oriented cut-point of 11.5 mg/L was reached by setting a minimum sensitivity of 90% and applying the Youden index, for the presence of

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consolidation on CT images in subjects admitted as cases of AECOPD, which had a sensitivity of 91% and a specificity of 53% (P<0.001). Subjects who had a negative result on the molecular pathogen detection array had higher NT-proBNP, lower hemoglobin, and higher RDW compared to the subjects who had a positive result.

Conclusions: In summary, this thesis demonstrated that elevated CRP may indicate pneumonia, while elevated NT-proBNP may indicate cardiac dysfunction, and having a negative result on the respiratory pathogen array may indicate a non-infectious causation of AECOPD. These readily available tests may provide more accurate phenotyping of AECOPD, and may lead to better treatment strategies and resource utilization in subjects admitted with AECOPD.

Lay Summary

Chronic Obstructive Pulmonary Disease (COPD) patients go through times in which they are very sick and out of breath, and they are currently being treated for that as if a single agent causes this, while in reality, this is caused by a variety of different agents. Our goal was to examine the use of two different blood tests and a sputum test to better identify the patients who need to have a different kind of treatment, and we show that these tests are very good in achieving this goal. If these tests are used in patients who have these flare-ups, this will help doctors in treating these patients in a better way.

Preface

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This dissertation is original, unpublished, independent work by the author Nawaf Alotaibi.

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List of Abbreviations

- AECOPD: acute exacerbation of chronic obstructive pulmonary disease
- AHF: acute heart failure
- AUC: area under the curve
- BMI: body mass index
- BNP: brain natriuretic peptide
- CAP: community-acquired pneumonia
- CI: confidence interval
- COPD: chronic obstructive pulmonary disease
- CRP: C-reactive protein
- CT: computed tomography
- CXR: chest X-ray
- EDTA: ethylenediaminetetraacetic acid
- eGFR: estimated glomerular filtration rate
- FEV₁: forced expiratory volume in 1 second
- FVC: forced vital capacity
- GERD: gastroesophageal reflux disease
- GOLD: global initiative for chronic obstructive lung disease
- L: liter
- LVD: left ventricular dysfunction
- MCH: mean corpuscular hemoglobin
- MCHC: mean corpuscular hemoglobin concentration

mg: milligram

ng: nanogram

NT-proBNP: amino-terminal of the prohormone brain natriuretic peptide

PE: pulmonary embolism

RBC: red blood cell

RDW: red blood cell distribution width

ROC: receiver-operating characteristics

RTP: rapid transition program

SD: standard deviation

uL: microliter

WBC: white blood cells

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Dedication

To my wonderful family who has provided me with unconditional love and support. I am eternally grateful for all the words of wisdom, encouragement, and guidance that you have given me in life.

Chapter 1: Introduction

In this thesis, we explore the potential use of blood-based biomarkers and molecular methods of pathogen detection in phenotyping chronic obstructive pulmonary disease (COPD) exacerbations (AECOPD). Currently, the treatment for exacerbations that require hospitalization is the same regardless of etiology. We hypothesized that certain blood-based biomarkers and molecular pathogen detection methods could be useful in phenotyping AECOPD and could lead to better disease management strategies. More details will be provided in later chapters but here are brief descriptions of the chapters to follow. In chapter 2, we determine the use of the two biomarkers C-reactive protein (CRP) and the amino-terminal of the prohormone brain natriuretic peptide (NT-proBNP), as discriminators between infectious and cardiac causes based on radiological evidence. In chapter 3, we determine the potential use of a panel that utilizes molecular methods for pathogen detection in phenotyping AECOPD. We assess the differences in the phenotypes of AECOPD based on the array results with regards to the blood-based biomarkers, peripheral blood counts, radiological data, and clinical outcomes including length of hospitalization and 1year mortality. In the final chapter, we summarize the main findings from each chapter and discuss how this thesis in its entirety adds to the management of AECOPD.

Chapter 2: Phenotyping COPD Exacerbations Using Imaging and Bloodbased Biomarkers

Chronic obstructive pulmonary disease (COPD) remains a major public health problem. It is estimated to affect 384 million people worldwide, with a global prevalence of 11.7%(1), and is forecasted to be the fourth leading cause of death by 2030(2). Acute exacerbations of COPD (AECOPD) are amongst the leading causes of hospitalization in the United States (US), and are the major cost driver for the illness, accounting for 50-70% of total direct costs(3). Currently, the most serious AECOPD events are treated with antibiotics and/or systemic corticosteroids regardless of etiology(4).

Brain natriuretic peptides (BNP and NT-proBNP) are considered to be relevant biomarkers of acute coronary syndrome (ACS)(5), heart failure (HF)(6, 7), and show potential utility in COPD. In COPD, for example, an elevated NT-proBNP is a strong predictor of early mortality among patients admitted to hospital with AECOPD independent of other known prognostic indicators(8). Brain natriuretic peptides have been found to correlate with several radiologically relevant endpoints including right ventricular enlargement detected on chest computed tomography (CT) in patients with acute pulmonary embolism(9, 10), and coronary atherosclerosis on angiography(11). C-reactive protein (CRP) is also an important biomarker in AECOPD. In a systematic review for AECOPD biomarkers(12), CRP was one of the most commonly studied biomarkers in AECOPD, and elevated concentrations have been found in the AECOPD state compared to the convalescence state. We have previously demonstrated that NTproBNP and CRP are both elevated in AECOPD as compared to convalescence, and together

they can reasonably discriminate between AECOPD requiring hospitalization compared to clinical stability with a cross-validated area under the curve (AUC) of 0.80, nonetheless, their concentrations were only weakly related to each other(13), which may indicate that they might represent different phenotypes of AECOPD.

The aim of this study was to determine whether blood CRP and NT-proBNP represent different disease processes as determined on thoracic imaging for AECOPD, which could be potentially used for phenotyping AECOPD and lead to more tailored therapeutic decisions.

2.1 Methods

2.1.1 Study Subjects

The Rapid Transition Program (RTP) study population details have been described previously(13). For this sub-study, we assessed subjects who were enrolled between July 2012 and October 2015. All subjects included in this study had a confirmed primary diagnosis of AECOPD as deemed by board-certified general internists or pulmonologists who cared for these subjects. All diagnoses were validated by chart review by at least one additional pulmonologist based on the criteria recommended by the Global initiative for chronic Obstructive Lung Disease (GOLD) Committee(4). All the subjects included in this study received standard antiexacerbation treatment during their hospitalization, including short-acting bronchodilators, prednisone, and antibiotics.

We retrospectively examined the associations between radiological parameters and the blood biomarkers NT-proBNP and CRP, in subjects who had had either chest x-rays (CXRs) or CT scans during their hospitalization, and biomarkers were measured no more than 10 days before or after their CXRs and CT scans (Figure 1). More details are provided in Appendix A.

The study is registered on ClinicalTrials.gov website with Identifier: NCT02050022 (registered January 28, 2014). The study was approved by the University of British Columbia Clinical Research Ethics Board (certificate number H11-00786). Written informed consent was provided by each participant in accordance with the Ethics Board.



Figure 1: Study Flow Diagram.

All subjects had radiological imaging and blood-based biomarker measurements within 10 days of each other. CXR= chest X-ray; CT scan= chest computed tomography; NT-proBNP= N-terminal pro b-type natriuretic peptide;

CRP= C-reactive protein.

2.1.2 Specimens and Measurement Technique

Following informed consent from subjects, blood samples were collected in PAXgene[®], EDTA, and serum tubes on day 1 and 3 of hospitalization, at discharge, and on day 30 and day 90 post-admission date. Blood components were processed as per standardized protocol and stored at - 80°C until analysis. Serum CRP was measured via a high-sensitivity assay on the Advia® 1800 Chemistry System (Siemens Healthcare GmbH, Erlangen, Germany), in the Clinical Laboratory of St Paul's Hospital (Department of Pathology and Laboratory Medicine, Vancouver, BC) following standard operating procedures. NT-proBNP was measured from EDTA whole blood specimens on the RAMP® 200 (Response Biomedical Corp, Vancouver, Canada), which has a measurement range of 18 to 35,000 ng/L.

Baseline lung function measurements were performed at the time of convalescence (i.e., at day 30 or day 90) for AECOPD patients. Spirometry was used to obtain lung function parameters after bronchodilator administration according to recommendations from ATS/ERS guidelines(14).

2.1.3 Review of Imaging and Statistical Analysis

To ensure uniformity of the reads, CXRs and CT images were read by a single blinded reviewer and assessed according to the Fleischner Society glossary of terms(15). The standard for CXRs was the posteroanterior projection. In subjects unable to cooperate or stand upright, only the anteroposterior projection was obtained. Cardiac size, pulmonary edema, and pleural effusion were the three parameters that were evaluated on CXRs. CT scans were ordered based upon the attending physician's discretion, and the methods of CT acquisition were pulmonary angiographic studies utilizing intra-venous contrast with a slice thickness of 2.5 mm, non-contrast exams with a slice thickness of 2.5 mm, or non-contrast exams with a slice thickness of 1.25 mm. The parameters that were assessed on CT scans are listed in Table 3. For categorization of emphysema burden each lung was divided into 3 zones (Appendix B), and the degree of emphysema was scored on a 6-point scale (0 to 5) as a percentage of total lung volume involved with emphysema in each zone (0: 0%, 1: <5%, 2: 6-24%, 3: 25-49%, 4: 50-74%, 5: 75-100%). The total emphysema score per subject was a sum of the scores in each zone divided by the total number of zones. Bronchiolitis was scored using a quartile system according to Fleischner guidelines and the following scale: none (0), trivial (1), mild/moderate (2), or moderate/severe (3). Bronchiolitis was defined as centrilobular micronodules and patchy ground-glass opacity using Fleischner Society Glossary of terms(15).

ANOVA and Spearman's correlation were performed to test for associations between the imaging parameters and blood-based biomarkers. Receiver-operating characteristic (ROC) curves were constructed based on logistic regression models for predicting the radiological parameters according to log-transformed NT-proBNP or CRP concentrations, with and without the adjustment for age and sex. Additional details regarding the methods of measurement of the pulmonary artery to aorta (PA/A) ratio are provided in Appendix B.

2.2 Results

2.2.1 Subject Characteristics

A total of 309 subjects with either a CXR or CT scan were examined for this study. Subjects had a mean age of 65.6 ± 11.1 years, 66.7% of them were males, 85.8% were Caucasian, and 62.4%were current smokers. All subjects had airflow limitation, with a mean forced expiratory volume in one second (FEV₁) $54.4\pm21.5\%$ of predicted (Table 1). Comorbid conditions included congestive heart failure (16.2%), coronary artery disease (21%), hypertension (46%), diabetes (16.5%), arrhythmia (13.9%), and asthma (24.6%). 36.9% of our subjects underwent thoracic CT scan during admission; of which 62.4% were CT pulmonary angiography.

Characteristic	All (N=309)
Age in years	65.6 ± 11.1
Male sex	66.7%
BMI, kg/m ²	27 ± 7.1
Caucasian	85.8%
Current smokers	62.4%
Former smokers	21.9%
Never smokers	15.6%
Unknown smoking status	23.3%
Pack-Years tobacco	51.3 ± 31.6
smoking	
$FEV_1(L)$	1.57 ± 0.68
FEV ₁ , percent predicted	54.4 ± 21.5
FVC (L)	2.89 ± 0.92
FVC, percent predicted	78.1 ± 19.9
FEV1/FVC ratio (%)	54.8 ± 16.1
Creatinine (umol/L)	85.2 ± 69.3
NT-proBNP (ng/L)	413 (174, 1243)
CRP (mg/L)	26.6 (5.5, 70.7)
CHF	16.2%
CAD	21.0%
Hypertension	46.0%

Table 1: Subject Characteristics

Characteristic	All (N=309)	_
Diabetes mellitus	16.5%	
Arrhythmia	13.9%	
Congenital heart disease	0.32%	
Asthma	24.6%	
History of MI	17.5%	

Data are represented as mean \pm SD or median and interquartile range(IQR) or (%).

CHF= congestive heart failure; CAD= coronary artery disease; MI= myocardial infarction.

2.2.2 Radiological Parameters

The parameters that were measured on CXR are listed in Table 2. In our study, 15.5% had radiographic evidence of pulmonary edema, 11.6% had pleural effusion, and 16.2% had abnormal cardiac size. On CT scans, 32.5% had evidence of consolidation. The mean PA/A ratio was 1.24 ± 0.21 (Table 3).

Variable	Category	Value
Number		304
	None	84.5%
	Mild (cephalized flow)	9.2%
Pulmonary edema	Moderate (septal lines)	5.6%
	Severe (alveolar edema)	0.7%
	Unknown	0.3%
Pleural effusion	Absent	88.4%
	Present	11.6%
	Unknown	0.3%
	Normal	83.8%
	Mild enlargement	11.6%
Cardiac size	Moderately enlargement	4.3%
	Severe enlargement	0.3%
	Unknown	0.3%

Table 2: Chest X-ray Parameters

Data are represented as %. For statistical analysis, moderate and severe categories for

pulmonary edema and cardiac size were combined.

Table 3: Chest CT Parameters

Variable	Category	Value
Number		117
NT-proBNP		411 (169, 1272)
CRP		20.9 (5.3, 76.4)
Paraseptal emphysema		65.8%
Centrilobular emphysema		77.8%
Panacinar emphysema		9.4%
Emphysema score RUL		1.86 ± 1.36
Emphysema score RML		1.41 ± 1.21
Emphysema score RLL		1.15 ± 1.19
Emphysema score LUL		1.79 ± 1.30
Emphysema score LML		1.33 ± 1.18
Emphysema score LLL		1.19 ± 1.20
Emphysema average score		1.14 ± 0.86
Respiratory bronchiolitis score		0.84 ± 1.02
DLP (mGy-cm)		332 (244, 487)
mSv		5.60 (4.13, 8.3)
Airway thickening		67.5%
Mucous plugging		49.6%
Bronchiectasis		23.1%
Reticulation		4.27%
Mosaic attenuation		10.3%
Consolidation		32.5%
Ground glass opacities		24.8%
Presence of nodules		46.2%
	0(absent)	94%
Pulmonary edema	1(moderate)	4.3%
	2(severe)	1.7%
Aortic diameter (mm)		34.1 ± 3.8
PA/A ratio		1.24 ± 0.21
Pulmonary artery diameter (mm)		28 ± 4.7
Pleural effusion		22.2%
Pericardial effusion		0.85%
	IV contrast 2.5 mm	62.4%
Chest CT scan type	Noncontrast 2.5 mm	29.1%
	Noncontrast 1.25 mm	8.5%

Data are represented as mean ± SD or median and interquartile range(IQR) or (%). mm= millimetre; RUL= right

upper lung zone; RML= right middle lung zone; RLL= right lower lung zone; LUL= left upper lung zone; LML=

left middle lung zone; LLL= left lower lung zone; DLP= dose length product; mSv= millisievert; PA/A=pulmonary artery to aorta ratio.

2.2.3 Associations between Radiological Parameters and Blood-based Biomarkers

NT-proBNP concentrations were significantly associated with pulmonary edema (P=0.008), pleural effusion (P=0.006), and cardiac size (P<0.001) on CXR. NT-proBNP concentrations were significantly associated with pleural effusion (P<0.001), pulmonary artery diameter (r=0.22; P=0.020), and aortic diameter (r=0.21; P=0.027) on CT imaging. CRP concentrations were associated with consolidation (P<0.001), ground glass opacities (P=0.027), pleural effusion (P<0.001), and pulmonary artery diameter (r=0.23; P=0.018) on CT imaging. Additional details are provided in Appendix C.

2.2.4 ROC Curves

ROC curves were constructed to determine the relationships of CT findings of clinical interest with biomarker concentrations, with and without adjustments for age and sex (Figure 2). The unadjusted AUC of NT-proBNP to predict pleural effusion on CT was 0.71 (P=0.002, 95% CI 0.58-0.83). On CXR, the AUCs of NT-proBNP to predict cardiac size, pulmonary edema, and pleural effusion were 0.72 (P<0.001, 95% CI 0.62-0.81), 0.63 (P=0.009, 95% CI 0.53-0.73), and 0.64 (P=0.01, 95% CI 0.53-0.75), respectively. For using CRP to predict consolidation, the AUC was 0.75 (P<0.001, 95% CI 0.64-0.86), for pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), for pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), for pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), for pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), for pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), for pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86), to pleural effusion the AUC was 0.72 (P<0.001, 95% CI 0.64-0.86)

applied the Youden Index(16). The cut-point for consolidation was a CRP level of 11.5 mg/L which possessed a sensitivity of 91% and a specificity of 53%. Additional details regarding the ROC curves are provided in Appendix D.



Figure 2: ROC Curves for The CT Parameters of Clinical Interest.

A, CRP and consolidation. B, CRP and pleural effusion. C, NT-proBNP and pleural effusion.

2.3 Discussion

To the best of our knowledge, this is the first study that has evaluated the relationships of CRP and NT-proBNP with radiological parameters in AECOPD simultaneously. We found significant associations between elevated NT-proBNP concentrations and the presence of pulmonary edema, pleural effusion and cardiac size on CXRs in patients who were diagnosed with AECOPD. We also found a significant relationship between elevated serum CRP concentrations and the presence of consolidation, ground glass opacities, and pleural effusion on CT scans but not CXR, consistent with the improved resolution of CT scans in detecting these abnormalities in COPD patients. To our knowledge, our study is the first to examine the capability of CRP in predicting consolidation present on CT scans in subjects who were admitted as cases of AECOPD and not community-acquired pneumonia (CAP). In this cohort, a CRP concentration less than 11.5 mg/L was a sensitive marker to rule out consolidation present on CT scans, suggesting potential utility in AECOPD.

NT-proBNP is a polypeptide secreted predominantly from the cardiac ventricles in response to multiple stimuli (for example, volume overload/endotoxemia/ischemia/hypoxia)(17). It exerts its actions through multiple pathways, which contributes to its diuretic and hypotensive properties(18). Measurement of NT-proBNP may be indicated for individuals who present to the emergency department with acute dyspnea in order to differentiate acute heart failure (AHF) from non-cardiac causes(19), and is currently being investigated for its potential use in AECOPD, with two systematic reviews demonstrating that elevated NT-proBNP concentrations have a strong association with the presence of left ventricular dysfunction (LVD) in AECOPD(20, 21). Of note, the association between NT-proBNP concentrations and LVD in

AECOPD appears to be sustained even in the presence of renal dysfunction, albeit with higher NT-proBNP levels (22).

Our findings are consistent with those of Høiseth et al., who applied a standardized assessment of pulmonary congestion present on CXR in 99 patients admitted for AECOPD, and found that this standardized (but not routine) assessment of pulmonary congestion correlated with NTproBNP concentrations(23). We extend this finding by evaluating 269 subjects who had CXRs at the time of hospitalization, demonstrating that NT-proBNP was a good indicator of radiological parameters related to cardiac dysfunction and/or volume overload including cardiac size, pulmonary edema, and pleural effusion with AUCs of 0.72, 0.63, and 0.64, respectively. Of note, the AUC for using NT-proBNP to predict pleural effusion as detected on CXR was 0.64, while the AUC for detecting pleural effusion based on CT was 0.71.

There is uncertainty in the diagnosis of CAP among patients with COPD, and this may be due in part to the high rate of misdiagnosis based on the presence of pulmonary infiltrates on CXR(24), which has an overall inter-observer agreement Kappa of 0.53. In patients who have COPD, our population of interest, the Kappa is considerably lower at 0.20. In a study that systematically performed thoracic CT scans in a population of suspected CAP patients visiting the emergency department(25), CT scan revealed pulmonary infiltrates consistent with CAP in 33% of patients in whom an opacity had been absent on CXR. Thus, the inclusion of CT results led to increased physician confidence in the CAP diagnosis. The same group also sought to improve CAP diagnosis by measuring CRP in these patients (26), finding an AUC of 0.78. A cut-point of 50 mg/L (which was associated with a sensitivity of 84.7% and a specificity of 63.4%) was

proposed for detecting CAP. Of note, the study was limited in the evaluation of COPD patients as only 28.5% of the studied population had chronic respiratory diseases, and the study excluded patients who were in the highest CURB-65 categories, making it hard to evaluate those 65 years of age and older. Another study that examined the diagnostic capability of CRP in CAP in an outpatient setting(27), proposed a CRP cut-point of 20 mg/L (which was associated with a sensitivity of 73% and a specificity of 65%) to predict a pulmonary infiltrate on CXR. Huerta et al.(28) proposed a CRP cut-point of 129 mg/L, which had an AUC of 0.71, with a sensitivity of 62% and a specificity of 63% in discriminating between AECOPD and CAP+COPD (in which CAP+COPD was diagnosed based on the presence of consolidation on CXR). We extend these findings by demonstrating that CRP is also useful in predicting consolidation present on CT scan that could not otherwise be detected on CXR in AECOPD, and propose a cut-point value of 11.5 mg/L which had a sensitivity of 91% and a specificity of 53% in ruling out the presence of consolidation on CT scan. We believe that CRP has clinical utility in predicting pulmonary infiltrates on CT scans and may help to reduce the number of CT scans ordered to spare COPD patients additional radiation risk. By using a CRP cutoff of 11.5 mg/L, most patients with AECOPD who have pulmonary opacities will be identified.

Our study has several limitations. First, it was a retrospective analysis and thus is affected by the common shortcomings of these types of studies. Second, we have taken the closest blood-based biomarker measurement to the radiological test, and the temporal relationship between changes in radiological findings and changes in the concentrations of measured biomarkers is not known. In addition, only 36.9% of our subjects required CT scans during admission, resulting in a selection bias. Thus, our findings should be prospectively validated.

Our study adds to the existing literature on the utility of NT-proBNP in predicting cardiac causes and/or complications in AECOPD, and we propose here that NT-proBNP and CRP should be utilized in patients admitted as AECOPD, in order to improve the identification and management of cardiac involvement in AECOPD. Moreover, in cases where CRP is less than 11.5 mg/L, it is highly unlikely that such individuals have pulmonary infiltrates; therefore, additional imaging such as a CT scan could be averted. In such cases, other etiologies of AECOPD aside from infection should be considered, and the empiric administration of antibiotics needs careful consideration and should be taken in the context of other features suggestive of an infectious etiology.

In conclusion, CRP less than 11.5 mg/L indicates that the presence of pneumonia is unlikely, while elevated NT-proBNP concentrations in the blood may indicate cardiac dysfunction and pulmonary edema. These readily available blood biomarkers, along with imaging modalities, may provide more accurate phenotyping of AECOPD which in turn may enable discovery of more precise therapies to treat AECOPD. We propose a CRP cut-point that will aid in averting the need for CT scans if there is a suspicion of an infectious lung process in subjects admitted with AECOPD.

Chapter 3: Utilizing Molecular Methods of Pathogen Detection to Phenotype COPD Exacerbations

COPD exacerbations (AECOPD) are caused by a variety of etiological factors(29). In AECOPD, the major driver is respiratory tract infections; however, in roughly 30% of the cases, no clear inciting factor is found(30). An autopsy study of COPD patients who died within 24 hours of hospital admission due to AECOPD demonstrated that acute heart failure and pulmonary embolism were the primary causes of death, which highlights the importance of phenotyping AECOPD to target and treat underlying causes that drive AECOPD(31). According to the latest GOLD document(4), sputum cultures are generally not useful for guiding initial antibiotic choice, or in phenotyping AECOPDs. This is because sputum cultures have relatively poor sensitivity in identifying respiratory pathogens and in determining therapeutic responsiveness to antimicrobials(32). Sputum culture is indicated only in a subset of patients in whom detection of antimicrobial resistance pattern of potential pathogens is required. The role of molecular diagnostics in detecting sputum pathogens in AECOPD is largely unknown, though it is widely accepted that nucleic acid based technologies offer superior sensitivities to those of traditional cultures in detecting potential respiratory tract pathogens.

In this chapter, our aim was to phenotype severe AECOPD by using a molecular pathogen detection method. Our hypothesis was that a nucleic acid-based assay of sputum will identify AECOPDs associated with viral, bacterial, or non-infectious etiologies and that these etiologies will confer different prognoses to patients.

3.1 Methods

3.1.1 Study Subjects

This study consisted of subjects who were able to provide adequate sputum samples in the COPD Rapid Transition Program (RTP). Samples were classified as being adequate sputum samples based on color, transparency and viscosity. The cohort has been described in detail in the previous chapter. In brief, the cohort consisted of COPD patients who were hospitalized with a physician diagnosis of AECOPD. All patients received standard anti-exacerbation therapy including prednisone and antibiotics. The study is registered with ClinicalTrials.gov with an Identifier: NCT02050022 (registered January 28, 2014).

3.1.2 Specimens and Measurement Technique

The collection and storage conditions of blood samples and radiological data have been described in the previous chapter. NT-proBNP was measured using the RAMP® diagnostic rapid kit (Response Biomedical Corp, Vancouver, BC, Canada) in whole blood specimens collected in EDTA tubes. RAMP assay uses quantitative immunochromatography and has a measurement range of 18 to 35,000 ng/L.

Serum CRP was measured via a high-sensitivity assay on the Advia® 1800 Chemistry System analyzer (Siemens Healthcare GmbH, Erlangen, Germany), located in the Clinical Laboratory of St Paul's Hospital (Department of Pathology and Laboratory Medicine, Vancouver, BC). The analytical range of the assay is 0.2 to 200.0 mg/L including an auto-dilution capability on board the analyzer. In cases where the samples were over the analytical range, they were manually diluted.

Complete blood count and differential was measured on whole blood specimens collected in EDTA tubes using the ADVIA [®] 2120i Hematology System (Siemens Healthcare GmbH, Erlangen, Germany).

Sputum samples were collected in OMNIgene®•ORAL (OM-505) tubes and stored in -80 °C freezers until measurement. The tubes were thawed in a hot bath at 50 °C for 1 hour and then placed in an air incubator at 24 °C for 30 minutes. Next, sputolysin was added in 1:1 ratio to liquefy the samples; after which the samples were sub-aliquoted into 500 uL volumes and stored in -80 °C freezers.

For quantifying bacterial load, DNA was extracted from a 500 uL aliquot using the DNeasy Tissue and Blood extraction kit (Qiagen). The aliquot was centrifuged for 10 minutes at 7500 RPM to form a pellet. After the supernatant was discarded, the pellet was resuspended in 180 uL buffer ATL, according to manufacturer's instructions (DNeasy, Qiagen). DNA concentration was measured using Nanodrop. Droplet digital PCR (ddPCR) (Bio-Rad QX200) was used to quantify the bacterial load, which uses an EvaGreen qPCR assay with primers specifying the 293bp amplicon of the 16S rRNA gene(33). Briefly, the following protocol was used: 1 cycle at 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, 1 cycle at 4°C for 5 minutes, and 1 cycle at 90°C for 5 minutes all at a ramp rate of 2°C/second. Bio-Rad's T100 thermal cycler was used for the PCR step. A threshold cutoff of 10.000 and a 1/10 dilution of the samples were chosen based on preliminary experiments. Negative controls that comprised of DNase and RNase free water were used and ran alongside the samples. Finally, a correction

factor using the formula ([average number of 16S copies per sample – average number of 16S copies in the negative control] / DNA concentration) was applied.

For detecting pathogenic microorganisms, the Randox Respiratory Multiplex Array II was used, which allows the simultaneous detection of 22 bacterial and viral pathogens in nucleic acids extracted from sputum samples (a detailed description of the pathogens covered and the method of detection is provided in Appendix E). In brief, the assay is a combination of multiplex PCR and biochip array hybridization. In order to extract DNA and RNA simultaneously from the samples, QIAamp MinElute Virus Spin Kit (Qiagen) was used according to manufacturer's instructions. The nucleic acid concentration was then measured using Nanodrop. The extracted nucleic acid samples were sent in 50 uL volumes to Randox Laboratories (Crumlin, UK) for molecular pathogen detection.

3.1.3 Statistical Analysis

Continous variables that were normally distributed are reported as means ±SDs, non-normally distributed variables as medians and interquartile ranges, and categorical variables as percentages. Continuous variables that were not normally distributed were log10 transformed prior to application of a parametric test where appropriate. Student's t-test and Mann-Whitney-Wilcoxon Tests were used to determine differences between the infectious and non-infectious groups where appropriate, while ANOVA and Kruskal-Wallis tests were used to determine differences between the negative, virus, and bacterial infection groups. Fisher's exact test was used to test for differences in the categorical variables between the groups. Comparisons of 1-year mortality rate and 1-year combined endpoint of mortality or rehospitalization across groups

were analyzed by Kaplan-Meier survival curves with a log-rank test, and Cox proportional hazards models adjusting for age and sex. Statistical tests were two-sided, and significance was assigned to results with P-values <0.05.

3.2 Results

3.2.1 Subject Characteristics

Demographic and clinical data for the 72 subjects studied are displayed in Table 4. The subjects had a mean age of 65.8 ± 11.5 years, 63.9% were male, 80.6% were Caucasian, and 61.1% were current smokers; all subjects had airflow limitation, with a mean forced expiratory volume in one second (FEV₁) of 46.6 ± 16.7 % of predicted, and 38.9% had a history of cardiac comorbidities (heart failure, coronary artery disease, myocardial infarction, and arrhythmia).

Characteristic	All subjects (N=72)
Age (years)	65.8 ± 11.5
Male sex	63.9%
BMI, kg/m2	24.6 ± 7
Caucasian	80.6%
Current smokers	61.1%
Cardiac comorbidities	38.9%
Home oxygen use	19.4%
ICS use	69.4%
eGFR	80.8 ± 25.4
(mL/min/1.73 m2)	
FEV ₁ % of predicted	46.6 ± 16.7
NT-proBNP*	463 (217-1295)
(ng/L)	

Table 4: Demographic and Clinical Characteristics of The Study Subjects

Characteristic	All subjects (N=72)
CRP**	48.1 (16.6-116)
(mg/L)	
Length of hospital stay	6 (3.25-9)
(Days)	

ICS=inhaled corticosteroids; eGFR = estimated glomerular filtration rate.

*41 subjects had NT-proBNP measured at admission day.

** 44 subjects had CRP measured at admission day.

3.2.2 Pathogens Detected

The pathogens that were detected in our 72-subject cohort are presented in table 5. The most common pathogen was *Haemophilus influenzae*, accounting for 33.7% of all pathogens detected; *Rhinovirus* was the most common virus detected, accounting for 13.3% of all pathogens detected. Details pertaining to the pathogens detected for each subject are provided in Appendix F.

Pathogen	Total count	%	Independent count	%
Haemonhilus influenzae	20	22 70/	14	45 20/
Haemophilus injiuenzae	20	55.770	14	43.270
Streptococcus pneumoniae	22	26.5%	6	19.4%
Rhinovirus	11	13.3%	6	19.4%
Influenza A virus	6	7.2%	1	3.2%
Influenza B virus	3	3.6%		
Respiratory syncytial virus A	3	3.6%		

Table 5: Pathogens Detected on The Randox Array.

Pathogen	Total count	%	Independent count	%
Moraxella catarrhalis	2	2.4%		
Parainfluenza virus 4	2	2.4%	2	6.5%
Coronavirus	2	2.4%		
Adenovirus	1	1.2%		
Metapneumovirus	1	1.2%		
Parainfluenza virus 2	1	1.2%	1	3.2%
Parainfluenza virus 3	1	1.2%	1	3.2%
Total	83	100 %	31	100%

Total count shows the number of times the pathogen was detected in our 72-subject cohort. Independent count shows the number of times the pathogen was the only pathogen detected in the sample.

3.2.3 Phenotyping COPD Exacerbations

20 out of 72 subjects (27.8%) had a negative result on the Randox array, while 52 out of the 72 subjects (72.2%) had a positive result. The subjects with a negative result did not have lower nucleic acid concentrations than the ones who had a positive result (P=0.71, Appendix G). We considered the subjects with a negative result on the Randox array to have had a non-infectious exacerbation and the ones who were positive on the array to have had an infectious exacerbation. We further subdivided the subjects with an infectious exacerbation into either a bacterial or a virally-associated exacerbation based on the results. If the subjects demonstrated positivity to a
virus exclusively or to a virus and bacteria, they were classified into the virally-associated exacerbation group (Figure 3).



Figure 3: AECOPD Phenotypes Pie Charts.

Bacteria= subjects who had a positive result for bacterial organisms; Virus= subjects who had a positive result for viral organisms either independently or with bacterial organisms.

3.2.4 Demographics and Clinical Data for AECOPD Phenotypes

Demographic data for the groups are listed in Appendix H. There were no statistically significant differences between the non-infectious (negative) and infectious (positive) groups in terms of age, sex, cardiac comorbidities, inhaled corticosteroid use, and FEV₁ % of predicted. Of note, the positive group had fewer subjects who were on home oxygen therapy (35% in the negative group and 14% in the positive group), but this comparison did not reach statistical significance (P=0.094). All hospitalizations were right censored at 30 days, as hospitalizations beyond this timeframe were likely driven by factors other than AECOPD. There were no significant differences in the length of stay in hospital between the negative and the positive groups

(P=0.096). However, a subgroup analysis revealed that there were significant differences in the length of hospital stay across the bacterial, viral, and microbial negative groups (P=0.046 on the overall ANOVA). These differences were largely driven by the comparison between the bacteria associated AECOPD group and the microbial negative group (P=0.02 on post hoc analysis with Fisher's least significant difference test [LSD]). Consistent with this analysis, there was a significant trend in the length of hospitalization across the three groups, with the microbial negative group having the longest length of stay and the bacterial group having the shortest (P=0.017) (Table 6, Figure 4).

Group	Number	Mean (SD)	F (<i>df</i>)	P-value	P-value Trend
		(log10)			
Negative	20	0.9 (0.33)	3.21 (2,69)	0.046	0.017
Virus	26	0.8 (0.35)			
Bacteria	26	0.7 (0.28)			

Table 6: Length of Hospital Stay ANOVA Statistical Analysis Results.

ANOVA statistical analysis results for the length of hospital stay between the three groups (log10 transformed).



Figure 4: box Plots Depicting The Length of Hospital Stay in The Three Groups.

The negative group had a longer length of hospitalization than the bacteria group (P=0.02), and a significant linear trend was present demonstrating that the length of hospital stay decreases from the negative group to the bacteria group (P=0.017).

3.2.5 NT-proBNP, CRP, and Complete Blood Counts

Appendix H contains the complete data on the concentrations of NT-proBNP, CRP, and the complete blood counts for each of the groups. We examined NT-proBNP, CRP, and complete blood counts at the date of hospital admission for all the subjects. The microbial negative group had significantly higher NT-proBNP concentrations (P=0.042), lower concentrations of hemoglobin (P=0.031), and higher red blood cell distribution width (RDW) values (P=0.025) compared with the microbial positive group (Figure 5). Subgroup analyses demonstrated a statistically significant difference between the microbial negative and the virus group for RDW

values (P=0.046 on the overall Kruskal-Wallis test, and a Bonferroni adjusted P value of 0.04 on post hoc pairwise comparison between the negative and virus group). There was no statistically significant difference between the three groups for NT-proBNP concentrations (P=0.081), but there was a significant trend in NT-proBNP concentrations across the three groups (P=0.029 on Jonckheere-Terpstra test for trend) with the negative group having the highest concentrations, and the bacteria group having the lowest (Figure 6).



Figure 5: Box Plots Depicting The Significantly Different Variables Between Groups.

Significant differences were present in NT-proBNP (P=0.042), hemoglobin (P=0.031), and RDW (P=0.025) between the negative and positive groups.



Figure 6: Box Plots Depicting NT-proBNP Concentrations in The Three Groups.

A statistically significant trend (P=0.029) in NT-proBNP concentrations was observed, with the negative group having the highest concentrations, and the bacteria group having the lowest.

3.2.6 Bacterial Load

Bacterial load measured by ddPCR is shown in Figure 7. There was no statistically significant difference between the microbial negative and positive groups (P=0.503).



Figure 7: Bacterial Load Comparisons Between Groups.

Box plots depicting the bacterial load in different groups. A) Negative (non-infectious) and positive (infectious) groups. B) The positive (infectious) group subdivided by viral detection. There were no statistically significant differences between groups in bacterial load.

3.2.7 Radiology

A subset of subjects had CXRs and CT images taken during the admission. 14.3% of the subjects who had CXRs in the microbial negative group had evidence of pulmonary edema, while 12.1% in the positive group had pulmonary edema. 24.2% in the positive group had evidence of cardiac enlargement, while 7.1% of the negative group had evidence of cardiac enlargement. 3 out of 5 subjects who had CT scans taken had evidence of bronchiectasis in the negative group, while 2 out of 11 in the positive group had bronchiectasis. Interestingly, 3 out of 5 subjects in the negative group had consolidation on CT scan, while 3 out of 11 on the positive group had evidences in the parameters measured on CXR and CT between groups (Table 7).

CXR Parameters	Negative Group (n=14)	Positive Group (n=33)	P-value
Pulmonary edema	2 (14.3%)	4 (12.1%)	1
Pleural effusion	0 (0%)	1 (3%)	1
Cardiac enlargement	1 (7.1%)	8 (24.2%)	0.244
CT Parameters	Negative Group (n=5)	Positive Group (n=11)	P-value
Bronchiectasis	3 (60%)	2 (18.2%)	0.245
Mucous plugging	3 (60%)	6 (54.5%)	1
Airway thickening	5 (100%)	7 (63.6%)	0.245
Emphysema average score	1.46 ± 1.22	1.06 ± 0.823	0.446
Pulmonary edema	2 (40%)	0 (0%)	0.083
Pleural effusion	0 (0%)	3 (27.3%)	0.509
Ground glass opacities	2 (40%)	6 (54.5%)	1
Consolidation	3 (60%)	3 (27.3%)	0.299

 Table 7: Radiological Parameters Measured in The Study.

Data are represented as counts and percentage. CXR= chest X-ray; CT= chest computed tomography.

3.2.8 1-year Mortality

Of the 72 subjects included in our study, 12 of them died within 1 year of follow-up (Table 8). On Kaplan Meier survival analysis, there were no statistically significant differences between the microbial negative and positive groups (P=0.065, Figure 8-A). There were no statistically significant differences in survival between the virus and microbial negative groups (P=0.262), nor between the virus and bacteria groups (P=0.376). However, there was a trend towards a

difference in survival between the microbial negative and bacteria (P=0.053, Figure 8-B). Across the three groups, the trend towards increased mortality in the microbial negative group compared with the bacterial group showed a similar result (P=0.052). Findings were similar in the age- and sex-adjusted Cox proportional hazards models (Tables 9 and 10).

Group	Number of	Number of	Number	Mean survival time in days
	cases	events	censored	(95% CI)
Negative	20	6	14 (70%)	292 (236-348)
Positive	52	6	46 (88.5%)	329 (303-356)
Group	Number of	Number of	Number	Mean survival time in days
	cases	events	censored	(95% CI)
Negative	20	6	14 (70%)	292 (236-348)
Virus	26	4	22 (84.6%)	317 (273-360)
Bacteria	26	2	24 (92.3%)	342 (311-372)

 Table 8: Survival Characteristics of AECOPD Phenotypes.



Figure 8: Kaplan-Meier Survival Analysis Curves for 1-year Mortality According to Randox Groups.

A) According to negative or positive result. B) The positive group subdivided by viral detection.

-2 Log Likelihood	Chi- square	df	P-value				
96.962	3.738	3	0.291				
Variables	В	SE	Wald	df	P-value	HR	95% CI
Age	-0.009	0.028	1.01	1	0.751	0.991	0.939-1.046
Sex (male)	0.376	0.651	0.333	1	0.564	1.456	0.407-5.211
Negative group	1.102	0.595	3.428	1	0.064	3.001	0.938-9.668

Table 9: Cox Proportional Hazards Model Comparing The negative and Positive Groups.

B= regression coefficient; SE= standard error; HR= hazard ratio.

-2 Log Likelihood	Chi- square	df	P-value				
96.129	4.336	4			0.3	62	
Variables	В	SE	Wald	df	P-value	HR	95% CI
Age	-0.007	0.027	0.074	1	0.785	0.993	0.942-1.046
Sex (male)	0.401	0.642	0.390	1	0.553	1.493	0.424-5.250
Bacteria group			3.811	2	0.149		
Negative group	1.545	0.829	3.471	1	0.062	4.69	0.923-23.831
Virus group	0.768	0.869	0.782	1	0.377	2.156	0.393-11.834

Table 10: Cox Proportional Hazards Model Comparing The negative, Virus, and Bacteria Groups.

B= regression coefficient; SE= standard error; HR= hazard ratio.

3.2.9 1-year Combined Endpoint of Mortality or Rehospitalization

Out of the 72 subjects included in our study, 37 experienced the combined endpoint of death or rehospitalization within 1-year of follow up (Table 11). In Kaplan Meier analysis, there were no statistically significant differences in this combined endpoint between the microbial negative and the positive groups (P=0.097, Figure 9-A). Between the three groups, there were no statistically significant differences between microbial negative and bacteria (P=0.314), virus and bacteria (P=0.312). However, there was a trend towards statistical significance in the occurrence of the combined endpoint between the microbial negative and virus groups (P=0.073, Figure 9-B). Across the three groups, a trend towards an increase in the combined endpoint in the microbial negative group compared with the virus group showed a similar result (P=0.059). Findings were similar in the age- and sex-adjusted Cox proportional hazards models (Tables 12 and 13).

Group	Number of cases	Number of events	Number censored	Mean combined endpoint time in days (95% CI)
Negative	20	13	7 (35%)	197 (133-260)
Positive	52	24	28 (53.8%)	259 (222-297)
Group	Number of cases	Number of events	Number censored	Mean combined endpoint time in days (95% CI)
Negative	20	13	7 (35%)	197 (133-260)
Virus	26	10	16 (61.5%)	267 (212-322)
Bacteria	26	14	12 (46.2%)	250 (200-300)

Table 11: Combined Endpoint Characteristics of AECOPD Phenotypes.





A) According to negative or positive result. B) The positive group subdivided by viral detection.

-2 Log Likelihood	Chi- square	df	P-value				
286.604	4.434	3	0.218				
Variable	В	SE	Wald	df	P-value	HR	95% CI
Age	-0.016	0.016	1.064	1	0.302	0.984	0.954-1.015
Sex (male)	0.442	0.380	1.356	1	0.244	1.556	0.739-3.27
Negative group	0.711	0.366	3.767	1	0.052	2.037	0.993-4.177

Table 12: Cox Proportional Hazards Model Comparing The negative and Positive Groups.

B= regression coefficient; SE= standard error; HR= hazard ratio.

Table 1	13: Co	x Prope	rtional	Hazards	Model	Comparing	The negative,	Virus,	and Bacteria	Groups.
						- · · ·		,		

-2 Log Likelihood	Chi- square	df	P-value				
285.671	5.109	4			0.2	276	
Variable	В	SE	Wald	df	P-value	HR	95% CI
Age	-0.017	0.016	1.138	1	0.286	0.983	0.952-1.015
Sex (male)	0.439	0.385	1.298	1	0.255	1.551	0.729-3.299
Virus group			4.463	2	0.107		
Negative group	0.931	0.444	4.393	1	0.036	2.537	1.062-6.058
Bacteria group	0.399	0.416	0.919	1	0.338	1.491	0.659-3.372

B= regression coefficient; SE= standard error; HR= hazard ratio.

3.3 Discussion

To our knowledge this is the first study that has examined the utility of a molecular method for pathogen detection in AECOPD for the purpose of phenotyping some of the exacerbations as being non-infectious, and we demonstrate that subjects who had a negative result on the array had significantly higher NT-proBNP concentrations, lower hemoglobin concentrations, and higher RDW values. Also, we demonstrated that subjects who had only bacteria detected on the array had the shortest length of hospitalization.

Viral etiologies of AECOPD have previously been studied in an experimental *Rhinovirus* human model in which COPD subjects were infected with Rhinovirus(34), and in a community-based, time-matched, case-control study of respiratory viruses and AECOPD (35), both studies confirming viral causation of AECOPD. In this study, we demonstrate the feasibility of using molecular pathogen detection methods that have been proven to be more sensitive in detecting viral pathogens than culture and serology methods (36). In addition, we demonstrate their utility in analyzing sputum samples, which are considered to be more representative of lower airway infection than nasopharyngeal (NP) samples(32). Also, the detection of viral pathogens is higher in sputum samples compared to NP samples when both sites are simultaneously assayed(37, 38). Bacteria are also considered to be a major causative agent of AECOPD(39), and multiple studies have shown that molecular methods for detecting bacteria have higher detection rates compared to culture(40-42), making it a more suitable method to detect bacterial pathogens. The ability to simultaneously detect and identify multiple microorganisms through nucleic acid amplification platforms from a single clinical specimen is especially useful for patient care. This approach, known as multiplexing, is increasingly being utilized for the diagnosis of a variety of different

infectious diseases, and currently, there are multiple FDA approved panels designed to aid in the diagnosis of respiratory, gastrointestinal, and central nervous system infections(43). A strength of our study is that we chose a clinically approved panel that detected common respiratory pathogens. This enabled us to use a single clinical specimen, while other studies have used different tests and different sampling sites (i.e., NP for viruses, blood and urine for atypical bacteria, and sputum for typical bacteria) (44-47). The pathogens detected in our study are consistent with the published literature on AECOPD in terms of type and prevalence(48).

The current paradigm on the etiologies of AECOPD is that roughly 80% are infectious in origin(39, 45), with a third being caused by viruses(49), while the remainder being attributed to multiple non-infectious etiologies. Non-infectious exacerbations in COPD are frequently attributed to various causes, including heart failure(50), atrial fibrillation(51), gastroesophageal reflux disease (GERD)(52), and acute pulmonary embolism(53). We demonstrate here that subjects with a negative result on the array had higher NT-proBNP concentrations, and an elevation in this biomarker is associated with acute cardiac dysfunction in AECOPD(20, 21). This strongly suggests that at least a subset of subjects in the microbial negative group had a non-infectious exacerbation which was most likely driven by cardiac dysfunction.

We did not observe any significant differences between the groups in CRP concentrations. We postulate that this is due to several reasons. First, we had a relatively small sample size that may have limited our ability to detect statistically significant differences in CRP concentrations. Second, we did not exclude subjects who had evidence of bronchiectasis on CT scan, and not all subjects underwent CT scan in our study; but we report that 3 out of the 5 subjects that had

undergone CT scans in the microbial negative group had radiographic evidence of bronchiectasis, and it has been shown that subjects who suffer from COPD and also have bronchiectasis as a comorbidity have higher CRP concentrations(54). Third, the array does not cover all the bacterial organisms that are associated with AECOPD. Most notably *Staphylococcus aureus* and *Pseudomonas aeruginosa* are not in the array, therefore patients with these infectious organisms may have been overlooked and included in the microbial negative group. We also did not find any significant differences in CRP concentrations between viral and bacterial groups, which is comparable to what has been published in the literature(55, 56).

We demonstrate that subjects who had a negative result on the array also had lower hemoglobin concentrations compared to the ones with a positive result, and they were at levels consistent with anemia(57). No differences between the groups were observed in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values, both of which were within the normal range(58). This pattern is most consistent with normocytic normochromic anemia, and the differential diagnosis for this pattern of anemia is quite wide(58), and encompasses several etiologies, amongst them is anemia of chronic disease (ACD), which is the most common cause of this pattern of anemia in patients admitted with AECOPD has been estimated to be between 19.3% and 33%, and many etiological mechanisms, which are not mutually exclusive, are implicated(60). COPD patients that are anemic are significantly more short of breath as measured by the Medical Research Council Dyspnea scale (MRC), have lower exercise capacity as measured by the six-minute walking distance test (6MWD), and have worse health-related quality of life (HRQL) as measured by the Saint George Respiratory Questionnaire(61). It

has been observed that anemic COPD patients experience more frequent COPD exacerbations and have a higher risk of death than their non-anemic counterparts(62-65). It is plausible that the subjects in the microbial negative group had clinically apparent anemia which may have worsened their symptom burden and lowered their threshold for an exacerbation event.

We show here that RDW values were also higher in the microbial negative group, along with the low hemoglobin concentrations. RDW has been used almost exclusively for the differential diagnosis of anemia for many years; but recently, increasing and convincing evidence shows that it is associated with a variety of diseases and their complications(66). There are very few studies that have examined RDW in COPD, however the studies that have been performed have shown that an increase in RDW is associated with an increased mortality risk in COPD patients and is also associated with cardiac dysfunction(67, 68). It is not currently known if elevated RDW values are an indicator of cardiac dysfunction in COPD or merely a marker of an increased comorbidity burden. Further studies investigating iron levels and peripheral blood smears in COPD patients may help clarify the role of red blood cell changes in COPD.

In AECOPD, there are specific treatments for bacterial infections that have very high cure rates, while in the case of viral infections, influenza is the only respiratory virus that has an available treatment(69). We show here that subjects who only had bacteria detected in their sputum had the shortest length of hospitalization, while interestingly, subjects who had a negative result on the array had the longest length of hospitalization. It has been demonstrated that subjects who have a high burden of comorbidities like anemia have longer hospitalization for AECOPD independent of age, sex, and $FEV_1(70)$, and elevated NT-proBNP concentrations on admission

are also associated with a prolonged length of hospitalization in AECOPD(13). The microbial negative group in our study had elevated NT-proBNP and lower hemoglobin concentrations, which are very plausible explanations for the prolonged length of hospitalization in this group.

Interestingly, we observed a trend towards a higher 1-year mortality rate in patients in the microbial negative group compared to the bacteria group. The most common cause of death in COPD patients according to death certificate data is cardiac disease(71), and elevated NT proBNP concentrations are strongly associated with mortality in AECOPD(8, 13), which might explain the increased mortality rate observed in subjects who had a negative result on the array. When examining the 1-year occurrence of death or rehospitalization, the subjects with viruses detected in their sputum had the lowest rate compared to the microbial negative group (P=0.073).

There are several limitations in our study and in studies utilizing molecular methods of pathogen detection in general. First, we used a qualitative diagnostic method, and colonization could lead to false positive results, especially since the rate of bacterial colonization in COPD is estimated to be around 29% (72). Colonization rates may also be increased in those with concomitant bronchiectasis, which appears to affect just over half of patients with COPD(54). If quantitative methods were to be used to separate colonization from infection, establishing a cut-point in which the pathogen detected would be considered causative may be problematic. In viruses, for example, respiratory syncytial virus (RSV) quantification in respiratory secretions in children with acute respiratory infections and adults with AECOPD varies by 2000-fold(32, 73). For bacteria, there are several groups that have proposed a cutoff of 10^4 – 10^5 gene copies/mL for *Streptococcus pneumoniae* in CAP(74, 75), and it is generally accepted that 10^5 - 10^6 CFU/mL is

considered for culture positivity in most bacteria(76, 77). Gadsby et al.(78) have applied the same cutoff value for culture on quantitative molecular diagnostics, and this did not significantly decrease overall pathogen detection. Of note, the group has found that positive culture specimens had a higher bacterial load by PCR in comparison to culture negative specimens, but the culture negative group was more frequently exposed to antibiotics prior to sample collection. Given the fact that PCR is able to detect viable as well as dead bacteria, it is not known for how long bacterial loads by PCR might be detectable after initiation of antibiotics, and applying quantitative cutoffs for molecular diagnostic methods might rule some patients negative for a respiratory infection who have been partially treated with antibiotics. Molecular pathogen detection methods are relatively new, and there is a clear need for determining thresholds that delineate colonization from infection, but for the time being, the issue of separating between the two is still unresolved. Second, we have used sputum samples as surrogates for phenotyping exacerbations. A sputum sample does not necessarily represent the whole lung, as sputum is collected after traveling through the upper respiratory tract. Moreover, there are regional differences in detection rates of bacterial pathogens within the same lung(79, 80), adding more difficulty to the use of qualitative and quantitative methods for phenotyping exacerbations. Third, our study was a retrospective study in which stored sputum samples in -80 °C were tested for respiratory pathogens, and the effects of prolonged storage conditions at low temperatures on microbial pathogen detection have not been systematically studied(81). However, in a study that examined the ability of the Xpert MTB/RIF assay to detect Mycobacterium tuberculosis in sputum samples that have been stored in -80 °C freezers for up to 4 years(82), the assay showed a sensitivity of 95.7%, which was within the range reported in fresh samples. Another study that examined the microbial communities in stored BAL samples for cystic fibrosis subjects in -80 °C

for more than five years(83) showed results that were consistent with historical culturing results. Fourth, a limitation of the array is that it does not cover all pathogenic organisms implicated in AECOPD, and of these, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most notable. For staphylococcus, as with other organisms that possess thick cell walls, specialized DNA extraction methods are required(84-87). If these methods were to be used, it has been shown that the increased detection of Staphylococcus comes at the expense of microbial organisms with a fragile cell wall and viruses (88, 89). Currently, there is no commercial nucleic acid extraction kit that can simultaneously extract gram positive, gram negative, and viruses from the same sample. Uyehara et al.(89) have proposed a method for achieving this goal through methods that prevent the loss of smaller viral particles while still extracting grampositive bacteria. This has been successfully used on 300 archived clinical samples of respiratory origin. This method appears promising, and if externally and prospectively validated, it could become the gold standard for nucleic acid extraction for molecular pathogen detection. Lastly, we had limited radiological data on the subjects in the study, given its retrospective nature, and we did not have echocardiographic data to confirm with the elevated NT-proBNP concentrations the evidence of cardiac dysfunction in the microbial negative group.

In conclusion, comprehensive molecular methods of pathogen detection are soon to be considered a cornerstone for diagnosing respiratory infectious diseases. Molecular diagnostic methods are significantly faster and more sensitive than culture methods, and not affected by prior antibiotic use to the same extent as culture methods. One important aspect is that these methods are not only capable of simultaneously detecting a wide gamut of different pathogens (bacteria, viruses, and fungi), but also are capable of simultaneously and accurately detecting

antibiotic resistance genes within 4-6 hours(90). We show here that a commercially available respiratory multiplex array could aid in phenotyping AECOPD into infectious or non-infectious exacerbations. These results are encouraging to further explore and develop better molecular pathogen panels that possess broader pathogen coverage and address the issue of colonization. Prospectively examining respiratory multiplex arrays in AECOPD that requires hospital admission and assess the point of care advantages that these panels might possess would be the logical next step to validate their value in the utilization of hospital resources and clinical outcomes.

Chapter 4: Conclusion

In this thesis, we addressed an issue that is commonly encountered by physicians and healthcare professionals, which is phenotyping COPD exacerbations to enable a more tailored therapeutic approach. Most exacerbations that require hospitalization are treated currently with the same therapeutic approach regardless of the etiology, and we investigated the possibility of improving this therapeutic approach by utilizing blood-based biomarkers and molecular pathogen detection methods.

We explored the associations between NT-proBNP and CRP and radiological findings consistent with either an infectious or a cardiac cause of the COPD exacerbation. We showed that NTproBNP was strongly associated with radiological findings consistent with cardiac causes, while CRP was strongly associated with radiological findings consistent with infectious causes of COPD exacerbations. These findings should lead physicians to utilize these two biomarkers in COPD exacerbations, and this could lead to better therapeutic approaches especially in patients who have cardiac involvement. We proposed a sensitivity-oriented cut-point for CRP, which has the potential to guide the administration of antibiotics and make informed decisions about the use of advanced radiological tests [namely thoracic computed tomography (CT) scan].

In addition, we interrogated the potential use of a molecular pathogen detection Array in COPD exacerbations. We demonstrated that the results from the array with regards to the infectious organisms and their incidence is consistent with the published literature on COPD exacerbations. We also showed that subjects who had a negative result on the array had higher NT-proBNP concentrations, lower hemoglobin concentrations, and higher RDW values as compared to the

individuals who were positive, which is strongly suggestive of a non-infectious cause of AECOPD. Most importantly, the non-infectious group had the worst prognosis as indicated by the increased length of hospitalization and a trend towards increased 1-year mortality.

Overall, we demonstrated that the blood-based biomarkers NT-proBNP and CRP, and the Randox respiratory molecular pathogen detection array, can be used to phenotype COPD exacerbations objectively, and we propose instituting these already existing tests in order to phenotype COPD exacerbations. Collectively as presented in this thesis, utilizing these tests will aid in phenotyping of COPD exacerbations better, alert physicians to cardiac involvement, and implement more specific tests aimed at diagnosing the heterogeneous causes of non-infectious exacerbations of COPD.

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Appendices



Appendix A Days Between Radiological Tests and Blood-based Biomarkers

Difference in days between radiological study acquisition and blood biomarker test. Differences are calculated as the radiological study date minus the blood biomarker test date. Positive values indicate that the radiological study was done after the blood biomarker test.

Appendix B Measurement of Radiological Parameters



B.1 Craniocaudal Division of The Lungs

Non-contrast transaxial CT image on lung windows of a 65 year old female subject with mild centrilobular emphysema. A, black arrow denotes the carina (the boundary between the upper and mid lung zones). B, black arrow denotes the cranial most inferior pulmonary vein ostia (the boundary between mid and lower lung zones).

B.2 Pulmonary Artery to Aorta Ratio Measurement



Intra-venous contrast enhanced CT image of a 61 year old male subject with measurements of the ascending aorta(green) and main pulmonary artery(yellow) at the level of the right pulmonary artery ostia. PA/A ratio: 1.28.

Appendix C List of Associations Between Radiological Parameters and Blood-based

Biomarkers

Source	Variable 1	Variable 2	N	Analysis	Correlation Coefficient	P-value
СТ	Airway thickoning	Log10 NT-proBNP	108	ANOVA		0.893
CI	All way unckenning	Log10 CRP	110	ANOVA		0.381
СТ	A ortic diameter (mm)	Log10 NT-proBNP	108	Correlation	0.213	0.027
CI	Aortic diameter (mm)	Log10 CRP	110	Correlation	0.056	0.565
СТ	Bronchioctasis	Log10 NT-proBNP	108	ANOVA		0.723
CI	Dionemeetasis	Log10 CRP	110	ANOVA		0.668
CYR	Cardiac size	Log10 NT-proBNP	269	ANOVA		<0.001
CAR	Cardiac Size	Log10 CRP	297	ANOVA		0.179
СТ	Centrilohular emphysema	Log10 NT-proBNP	108	ANOVA		0.320
CI	Centritobular empirysema	Log10 CRP	110	ANOVA		0.685
СТ	Consolidation	Log10 NT-proBNP	108	ANOVA		0.050
	Consolidation	Log10 CRP	110	ANOVA		<0.001
СТ	Emphysema average	Log10 NT-proBNP	108	Correlation	0.034	0.727
CI	Emphyseina average	Log10 CRP	110	Correlation	-0.11	0.241
СТ	Emphysema score LLL	Log10 NT-proBNP	108	Correlation	-0.03	0.777
CI	Emphysema score EEE	Log10 CRP	110	Correlation	-0.15	0.116
СТ	Emphysema score I MI	Log10 NT-proBNP	108	Correlation	0.05	0.595
	Emphyseina score EME	Log10 CRP	110	Correlation	-0.10	0.274
СТ	Emphysema score I III	Log10 NT-proBNP	108	Correlation	0.06	0.563
	Emphyseina score LOL	Log10 CRP	110	Correlation	-0.04	0.670
СТ	Emphysema score RLI	Log10 NT-proBNP	108	Correlation	0.03	0.784
		Log10 CRP	110	Correlation	-0.15	0.119
СТ	Emphysema score RMI	Log10 NT-proBNP	108	Correlation	0.06	0.562
	Empirysenia score Rivie	Log10 CRP	110	Correlation	-0.13	0.182
СТ	Emphysema score RIII	Log10 NT-proBNP	108	Correlation	0.05	0.624
	Emphyseina seore ROL	Log10 CRP	110	Correlation	-0.08	0.415
		Log10 NT-proBNP	108	Correlation	0.09	0.344

Source	Variable 1	Variable 2	N	Analysis	Correlation Coefficient	P-value
СТ	Pulmonary edema	Log10 CRP	110	Correlation	-0.06	0.562
CT		Log10 NT-proBNP	108	ANOVA		0.549
CI	Ground glass opachies	Log10 CRP	110	ANOVA		0.027
СТ	Mosaic attenuation	Log10 NT-proBNP	108	ANOVA		0.111
CI	Wosale allentation	Log10 CRP	110	ANOVA		0.239
СТ	Mucous plugging	Log10 NT-proBNP	108	ANOVA		0.943
C1	Mucous plugging	Log10 CRP	110	ANOVA		0.158
СТ	Presence of nodules	Log10 NT-proBNP	108	ANOVA		0.197
01	Tresence of notaties	Log10 CRP	110	ANOVA		0.094
СТ	PA/A ratio	Log10 NT-proBNP	108	Correlation	-0.03	0.748
01	111111110	Log10 CRP	110	Correlation	-0.14	0.151
СТ	Panacinar emphysema	Log10 NT-proBNP	108	ANOVA		0.414
01		Log10 CRP	110	ANOVA		0.195
СТ	Parasental emphysema	Log10 NT-proBNP	108	ANOVA		0.918
01	i uruseptur empirysemu	Log10 CRP	110	ANOVA		0.234
СТ	Pericardial effusion	Log10 NT-proBNP	108	ANOVA		0.723
01		Log10 CRP	110	ANOVA		0.101
СТ	Pleural effusion	Log10 NT-proBNP	108	ANOVA		<0.001
01		Log10 CRP	110	ANOVA		<0.001
CXR	Pleural effusion	Log10 NT-proBNP	269	ANOVA		0.006
		Log10 CRP	297	ANOVA		0.247
CXR	Presence of pulmonary edema	Log10 NT-proBNP	269	ANOVA		0.008
		Log10 CRP	297	ANOVA		0.154
СТ	Pulmonary artery diameter	Log10 NT-proBNP	108	Correlation	0.22	0.020
	(mm)	Log10 CRP	110	Correlation	0.23	0.018
СТ	Respiratory bronchiolitis score	Log10 NT-proBNP	108	Correlation	-0.17	0.076
CI	Respiratory oronemonus score	Log10 CRP	110	Correlation	0.13	0.160
СТ	Reticulation	Log10 NT-proBNP	108	ANOVA		0.750
	CI Reticulation	Log10 CRP	110	ANOVA		0.602

List of associations between radiological parameters and blood-based biomarkers. ANOVA and Spearman's correlation were the statistical methods used. CXR= chest X-ray; CT= chest CT scan; RUL= right upper lung zone; RML= right middle lung zone; RLL= right lower lung zone; LUL= left upper lung zone; LML= left middle lung zone; LLL= left lower lung zone; PA/A=pulmonary artery to aorta ratio.



Appendix D ROC Curves for Radiological Parameters and Blood-based Biomarkers

ROC curves for blood-based biomarkers and Radiological parameters. The first row depicts ROC curves for using NT-proBNP to predict radiological parameters obtained on chest X-rays, with (A) showing cardiac size, (B) pulmonary edema, and (C) pleural effusion. (D) depicts the ROC curve for using CRP to predict ground glass opacities on CT.

Appendix E Pathogens Covered in The Randox Respiratory Multiplex Array and The Principles and Procedure of The Array.

Bacteria	Viruses
Legionella pneumophila	Influenza A virus
Chlamydophila pneumoniae	Influenza B virus
Mycoplasma pneumoniae	Human respiratory syncytial virus A
Moraxella catarrhalis	Human respiratory syncytial virus B
Streptococcus pneumoniae	Human parainfluenza virus 1
Bordetella pertussis	Human parainfluenza virus 2
Haemophilus influenzae	Human parainfluenza virus 3
	Human parainfluenza virus 4
	Human coronavirus 229E/NL63
	Human coronavirus OC43/HKU1
	Human adenovirus A/B/C/D/E
	Human rhinovirus A/B/C
	Human enterovirus A/B/C
	Human bocavirus 1/2/3
	Human Metapneumovirus

E.1 Pathogens Covered by The Randox Array

E.2 Principles and Procedure of The Array

The Respiratory Multiplex Array II is designed to rapidly screen for the presence of 22 different respiratory pathogens simultaneously from one patient sample. The array is based on a combination of multiplex PCR, target hybridization and chemiluminescence to allow qualitative
detection of respiratory pathogens within specimens. The array is approved for nasal swab, bronchoalveolar lavage or sputum specimens. The first step is nucleic acid extraction, and the recommended kit is QIAamp MinElute Virus Spin Kit (Qiagen). After that, a one-step process of reverse transcription combined with multiplex PCR amplification is performed to allow detection of both viral and bacterial nucleic acids within the specimen. If respiratory pathogens are present within the sample, target genes will be amplified to detectable levels. Amplified samples are then added to the Biochip permitting target gene sequences to hybridize to complementary probes spotted on specific regions of the Biochip surface. The Biochip is then imaged on the Evidence Investigator[™] where onboard software will identify the presence of respiratory pathogens within the sample. An extraction control (EC) is incorporated into the array which confirms successful sample nucleic acid extraction and PCR amplification.

Subject Number	Pathogen 1	Pathogen 2	Pathogen 3	Pathogen 4
1	Negative			
2	HI	FLU B		
3	Negative			
4	HRV	HI	FLU B	
5	HRV			
6	HI			
7	Negative			
8	Negative			
9	HRV			
10	SP			
11	Negative			
12	HRV			
13	HI	SP	FLU B	
14	Negative			
15	SP			
16	Negative			
17	Negative			
18	Negative			
19	HI			
20	FLU A	HI	SP	
21	SP			
22	HRV			
23	Negative			
24	SP			
25	HI			

Appendix F List of The Pathogens Detected for Each Subject in The Cohort

Subject Number	Pathogen 1	Pathogen 2	Pathogen 3	Pathogen 4
26	Negative			
27	RSV A	HI		
28	RSV A	HI	MCAT	
29	PIV 3			
30	HI			
31	HI			
32	HI			
33	FLU A	HI	SP	
34	FLU A	HRV	SP	CORO
35	Negative			
36	FLUA	HRV	SP	
37	PIV 4			
38	SP			
39	FLU A	HI	SP	
40	HI	MPV	SP	
41	Negative			
42	HI	SP		
43	HRV			
44	SP			
45	HI	SP		
46	HI	SP		
47	HI			
48	Negative			
49	HAV	RSV A		
50	Negative			
51	HI	SP		
52	Negative			

Subject Number	Pathogen 1	Pathogen 2	Pathogen 3	Pathogen 4
53	MCAT	SP		
54	Negative			
55	HRV	SP		
56	HRV	SP		
57	PIV 4			
58	HI			
59	HI			
60	PIV 2			
61	SP	CORO		
62	Negative			
63	HI			
64	HRV			
65	Negative			
66	FLU A			
67	HI	SP		
68	HI			
69	HI			
70	HI			
71	HI			
72	Negative			

HI= haemophilus influenzae; SP= streptococcus pneumoniae; HRV= human rhinovirus; FLU= influenza virus;

RSV= respiratory syncytial virus; MCAT= moraxella catarrhalis; PIV= parainfluenza virus; HAV= human

adenovirus; MPV= metapneumovirus; CORO= coronavirus.



Array



Box plots representing the nucleic acid concentration in the subjects in which no pathogens were detected and the ones with positive pathogen detection. There was no statistically significant difference between the groups (P=0.71).

Appendix H Demographic and Clinical Data of Study Subjects According to The Results

of The Randox Array

	Negative Group	Positive Group	P-value
Age (years)	68.2 ± 11	64.8 ± 11.6	0.273
Male sex	55%	67.3%	0.414
BMI, kg/m2	22.7 ± 4.7	25.2 ± 7.5	0.404
Caucasian	85%	78.8%	0.744
Current smokers	45%	68.6%	0.102
Cardiac comorbidities	35%	40.4%	0.790
Home O2 use	35%	14%	0.094
ICS use	75%	68.6%	0.774
16S copies (copies/ng/ul)	20.53 (6.31-101.59)	35.9 (8.95-83.76)	0.624
eGFR (mL/min/1.73 m ²)	78.6 ± 25.9	81.7 ± 25.3	0.652
FEV1, percent predicted	44.6 ± 14.5	47.2 ± 17.4	0.613
FEV/FVC ratio (%)	74.4 ± 18	69.8 ± 16.2	0.447
NT-proBNP* (ng/L)	1216.5 (311-1920)	369 (183-843)	0.042
CRP** (mg/L)	25.7 (8.27-98.46)	56.49 (21.56-150.33)	0.382
Length of hospital stay (Days)	6.5 (5-17)	6 (3-8)	0.096
WBC (10 ³ cells/uL)	11.1 (5.6-12.75)	8.74 (5.8-13.8)	0.851
Neutrophils (10 ³ cells/uL)	8.7 (4.2-11.8)	6.3 (4.3-11.9)	1
Neutrophil%	84.6 (74.5-93.5)	85.1 (79-90.4)	0.939
Lymphocytes (10 ³ cells/uL)	$0.81 \pm .49$	$0.83 \pm .49$	0.891
Lymphocyte%	11.3 ± 8.4	9.3 ± 5.5	0.377
Eosinophils (10 ³ cells/uL)	0.007 (0-0.06)	0.025 (0.01-0.06)	0.139

H.1 Negative and Positive Groups

	Negative Group	Positive Group	P-value
Eosinophil %	0.13	0.24	0.246
	(0-1.2)	(0.156)	
Monocytes (10 ³ cells/uL)	0.227	0.44	0.513
	(0.10-0.72)	(0.24-0.58)	
Monocyte%	4.7 ± 4.1	4.8 ± 3.06	0.984
Basophils (10 ³ cells/uL)	0.01	0.02	0.695
	(0.004-0.06)	(0.01-0.04)	
Basophil%	0.21	0.22	0.988
-	(0.08-0.74)	(0.1-0.4)	
RBC (10 ⁶ cells/uL)	3.8	4.4	0.029
	(3.1-4.4)	(4-4.8)	
Hematocrit	32.5	39	0.029
	(29.8-39)	(35.6-42.9)	
Hemoglobin (g/L)	114	128	0.031
	(95-133)	(111-138)	
MCV	89.4 ± 8.6	88.3 ± 5.2	0.714
МСН	29.8	28.7	0.432
	(27.9-31.5)	(26.9-31.4)	
МСНС	335	330	0.590
	(32.1-341.2)	(31.1-342.7)	
Platelets (10 ³ cells/uL)	229	213.5	0.761
	(182-269)	(162.2-308.2)	
RDW%	16.2	14.6	0.025
	(14.4-20)	(13.9-16.1)	

Data are represented as mean ±SD or median and interquartile ranges. ICS= inhaled corticosteroid; eGFR= estimated glomerular filtration rate; MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration; RDW= red blood cell distribution width.

* 12 and 29 subjects had NT-proBNP measured at admission in the negative and positive group, respectively.

** 12 and 32 subjects had CRP measured at admission in the negative and positive group, respectively.

	Negative Group	Virus Group	Bacteria Group	P-value
Age (years)	68.2 ± 11	64.5 ± 12.6	65.3 ± 10.8	0.533
Male sex	55%	61.5%	73.1%	0.459
BMI, kg/m2	22.7 ± 4.7	26.8 ± 9.8	23.9 ± 4.8	0.513
Caucasian	85%	80.8%	76.9%	0.930
Current smokers	45%	72%	65.4%	0.180
Cardiac comorbidities	35%	42.3%	38.5%	0.953
Home O2 use	35%	16.7%	11.5%	0.161
ICS use	75%	64%	73.1%	0.683
16S copies	20.53	35.9	34.2	0.789
(copies/ng/ul)	(6.31-101.59)	(9.9-73.9)	(6.8-86.1)	
eGFR	78.6 ± 25.9	85.80 ± 27.04	77.77 ± 23.49	0.483
$(mL/min/1.73 m^2)$				
FEV ₁ , percent predicted	44.6 ± 14.5	51 ± 18.4	43.8 ± 16.2	0.340
FEV/FVC ratio (%)	74.4 ± 18	70.7 ± 16.8	69.1 ± 16.1	0.721
NT-proBNP*	1216.5	370	310	0.081
(ng/L)	(311-1920)	(209-1358)	(165-713)	
CRP**	25.7	40.1	76.8	0.618
(mg/L)	(8.27-98.46)	(26-71.1)	(15.4-169.3)	
Length of hospital stay	6.5	6	5	0.046
(Days)	(5-17)	(3-11)	(3-7)	
WBC	11.1	6.47	10.4	0.745
(10 ³ cells/uL)	(5.6-12.75)	(5.4-12.2)	(6.9-13.8)	
Neutrophils	8.7	5.52	6.53	0.812
(10 ³ cells/uL)	(4.2-11.8)	(4.32-10.85)	(4.77-12.90)	
Neutrophil%	84.6	85.20	85	0.966
	(74.5-93.5)	(82.20-90.40)	(80.10-89.30)	
Lymphocytes	0.81 ± 0.49	0.75 ± 0.37	0.91 ± 0.57	0.666
	113 + 84	9 56 + 6 09	9.14 + 5.19	0.669
Ensinonhils	0.007	0.03	0.02	0.326
(10 ³ cells/nL)	(0-0.06)	(0.03)	(0.02)	0.520
Eosinonhil %	0.13	0.30	0.20	0.460
	(0-1,2)	(0.10-0.70)	(0.12-0.40)	0.700
Monocytes	0.227	0.31	0.47	0.671
(10^3 cells/uL)	(0.10-0.72)	(0.26-0.52)	(0.25-0.59)	

H.2 Subjects Divided into Negative, Virus and Bacteria

	Negative Group	Virus Group	Bacteria Group	P-value
Monocyte%	4.7 ± 4.1	4.60 (3.08-5.60)	3.50 (2.09-7.13)	0.889
Basophils (10 ³ cells/nL)	0.01	0.01	0.02	0.152
Basophil%	0.21 (0.08-0.74)	0.18 (0.10040)	0.27 (0.20-0.69)	0.155
RBC (10 ⁶ cells/uL)	3.8 (3.1-4.4)	4.43 (4.06-4.56)	4.55 (3.93-4.93)	0.086
Hematocrit	32.5 (29.8-39)	38.9 (36.50-39.90)	39.4 (35.10-43.50)	0.089
Hemoglobin (g/L)	114 (95-133)	128 (112-135)	129 (118-144)	0.163
MCV	89.4 ± 8.6	88.36 ± 5.92	88.39 ± 4.90	0.898
МСН	29.8 (27.9-31.5)	29.2 (27.80-29.30)	28.2 (26.90-31.40)	0.719
МСНС	335 (32.1-341.2)	32.6 (31.50-335)	334 (31.30-342)	0.640
Platelets (10 ³ cells/uL)	229 (182-269)	201 (190-294)	217 (139-337)	0.942
RDW%	16.2 (14.4-20)	14 (14-15)	15 (14-16)	0.046

Data are represented as mean ±SD or median and interquartile ranges. ICS= inhaled corticosteroid; eGFR=

estimated glomerular filtration rate; MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin;

MCHC= mean corpuscular hemoglobin concentration; RDW= red blood cell distribution width.

* 12, 14, and 15 subjects had NT-proBNP measured at admission in the negative, virus, and bacteria groups

respectively.

** 12, 15, and 17 subjects had CRP measured at admission in the negative, virus, and bacteria groups respectively.