Proteolytic imbalance in COPD
Epidemiological and clinical aspects
The Obstructive Lung Disease in Northern Sweden (OLIN) studies, thesis XX.

Robert Linder
Dedicated to my family; past, present, and future.

THE BLIND MEN AND THE ELEPHANT
A HINDOO FABLE

I.
It was six men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind.

II.
The First approached the Elephant,
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me!—but the Elephant
Is very like a wall!"

III.
The Second, feeling of the tusk,
Cried: "Ho!—what have we here
So very round and smooth and sharp?
To me 't is mighty clear
This wonder of an Elephant
Is very like a spear!"

IV.
The Third approached the animal,
And happening to take
The squirming trunk within his hands,
Thus boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake!"

V.
The Fourth reached out his eager hand,
And felt about the knee.
"What most this wondrous beast is like
Is mighty plain," quoth he;
"'T is clear enough the Elephant
Is very like a tree!"

VI.
The Fifth, who chanced to touch the ear,
Said: "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can,
This marvel of an Elephant
Is very like a fan!"

VII.
The Sixth no sooner had begun
About the beast to grope,
Than, seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

VIII.
And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong!

MORAL.
So, oft in theologic wars
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!

The poems of John Godfrey Saxe (1872), by John Godfrey Saxe (1816–1887). Public domain. Transcribed from a scanned copy of the original text available at "openlibrary.org", Book-ID: OL7138711M.
# Table of Contents

Abstract .................................................................................................................. iii  
Abbreviations ........................................................................................................ v  
Original papers ..................................................................................................... vi  
Sammanfattning på svenska .................................................................................. vii  
Introduction .......................................................................................................... 1  
Lung function and spirometric criteria for COPD .................................................. 4  
  Lung development and decline ........................................................................ 5  
  Spirometric criteria for COPD ......................................................................... 6  
Assessment and clinical aspects ............................................................................ 8  
  Clinical features of COPD ............................................................................... 10  
  Therapy ............................................................................................................ 12  
Epidemiology ......................................................................................................... 14  
Mechanisms of pathogenesis ................................................................................ 17  
  The immune system ...................................................................................... 17  
  Airway inflammation ..................................................................................... 18  
  Oxidative stress .............................................................................................. 19  
  Proteolytic imbalance ................................................................................... 20  
  Current knowledge ......................................................................................... 22  
Aims of the thesis .................................................................................................. 26  
Materials and Methods ........................................................................................ 27  
  The OLIN studies ........................................................................................... 27  
  The OLIN COPD study ................................................................................. 27  
  The KOLIN study .......................................................................................... 27  
  Interviews and measurements ........................................................................ 32  
  Study definitions ............................................................................................. 32  
  Lung function tests and spirometric criteria ..................................................... 33  
  Lung function decline .................................................................................... 33  
  Peripheral blood .............................................................................................. 34  
  Bronchoscopy .................................................................................................. 34  
  Statistical methods ......................................................................................... 36  
  Subjects ............................................................................................................ 36  
Ethics ...................................................................................................................... 38  
Results .................................................................................................................... 39  
  Paper I ........................................................................................................... 39  
  Paper II .......................................................................................................... 40  
  Paper III .......................................................................................................... 42  
  Paper IV .......................................................................................................... 43  
Discussion of methodology .................................................................................. 49  
  OLIN COPD study ......................................................................................... 51  
  The KOLIN study .......................................................................................... 53
Discussion of main results ................................................................. 55
From epidemiology to clinical aspects .................................................. 62
Conclusions ................................................................................. 63
Future directions for research ............................................................. 64
Acknowledgement ........................................................................ 65
References ...................................................................................... 68
Abstract

Background: The complete pathologic mechanism behind the development of chronic obstructive pulmonary disease (COPD) remains unclear, but several risk factors have been identified, of which smoking is the most common. Proteolytic imbalance contributes to lung tissue degradation and is related to both smoking and COPD symptoms. Spirometry and symptomatic assessments are the standard diagnostics, but COPD has varying clinical features, that hamper clinical management and research assessment. Evaluating proteolytic markers' relationship to COPD and its clinical presentation could reveal proteolytic imbalance as an important disease mechanism.

Aims: 1) To evaluate proteolytic markers in COPD and non-COPD. 2) To study the relationship between proteolytic markers and both lung function decline and prognosis. 3) To recruit subjects from a longitudinal study to a clinical study of disease mechanisms. 4) To study proteolytic markers in airways and serum and their relation to rate of decline in lung function.

Methods: Spirometry, serum matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of metalloproteinase-1 (TIMP-1) were evaluated in a population-based study comprising 993 COPD subjects and 993 age- and sex-matched non-COPD referents. In addition, data from 2005 to 2010 were surveyed comprising longitudinal spirometry data and mortality records. For a clinical study, we described the recruitment process of COPD subjects with a FEV₁ decline of ≥60 or ≤30 mL/year, along with ever- and never-smoking controls with normal lung function. MMP-9, MMP-12, and TIMP-1 data from bronchial wash (BW), bronchoalveolar lavage (BAL) and serum (collected from 2012 to 2014) were assessed in the clinical study.

Results: COPD subjects presented higher serum concentrations of MMP-9 compared to non-COPD subjects (p = 0.017). MMP-9 and MMP-9/TIMP-1 ratio had a negative linear association with the forced expiratory volume in one second (FEV₁) percentage predicted in COPD. Associating the 2005 levels of MMP-9 and MMP-9/TIMP-1 ratio to decline in FEV₁ and FEV₁% predicted, revealed a similar negative association pattern in both non-COPD and COPD, however, this was only significant for non-COPD.
A non-response analysis comparing proteolytic marker values from 2005 between participating and non-participating subjects at follow-up in 2010 (excluding deceased individuals) demonstrated significantly higher MMP-9 and MMP-9/TIMP-1 ratios in both non-COPD and COPD, and significantly lower TIMP-1 concentration in non-participants compared to participants. Among the deceased, MMP-9 levels and MMP-9/TIMP-1 ratios were higher in COPD compared to non-COPD. In the longitudinal study, all-cause mortality was higher in the COPD group (16%), than in the non-COPD (10%) (p = 0.008).

For the clinical study, 15 subjects were recruited to the two normal lung function groups, while this goal was unachieved for the two COPD groups. The most prevalent reasons for exclusion in the COPD groups were comorbidities. BW- and BAL-MMP-12 concentrations were higher in the COPD group comprising current- and ex-smokers, compared to both ever-smokers (BW: p = 0.001, BAL: p = 0.001) and non-smokers with normal lung function (BW: p = 0.001, BAL: p = 0.001). To evaluate the impact of smoking, COPD ex-smokers were compared to COPD current smokers, with no significant difference in BW- and BAL-MMP-12. In contrast COPD-ex smokers had higher BW- and BAL-MMP-12 compared to ex-smokers with normal lung function, thus suggesting increased BW- and BAL-MMP-12 as markers of COPD rather than of smoking. MMP-12 concentrations in serum were higher for COPD current smokers compared to COPD ex-smokers (p = 0.028), but there was no significant difference between COPD ex-smokers and ex-smokers with normal lung function. BAL-MMP-12 in COPD was associated with annual decline in FEV₁ (r = 0.61, p = 0.005).

**Conclusion:** Extrapolating the data on MMP-9 and MMP-9/TIMP-1 ratio suggests increased proteolytic activity is related to airflow limitation and consequently to COPD severity. Considering the population-based nature of the study, the association of both MMP-9 and MMP-9/TIMP-1-ratio in COPD to mortality risk could be translated to the general population. Identifying COPD subjects with specific phenotypes proved difficult despite the large number of available individuals. Increased airway levels of MMP-12 indicated a state of increased proteolytic activity and were associated with rapid lung function decline in COPD. These findings imply that proteolytic imbalance is related to symptoms, lung function decline and prognosis, suggesting it represents a relevant disease mechanism in COPD.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AATD</td>
<td>Alpha-1 antitrypsin deficiency</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BOLD</td>
<td>Burden of Obstructive Lung Disease</td>
</tr>
<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
</tr>
<tr>
<td>BW</td>
<td>Bronchial wash</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
</tr>
<tr>
<td>ETS</td>
<td>Environmental Tobacco Smoke</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced Expiratory Volume in one second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GLI</td>
<td>Global Lung Initiative</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative for Obstructive Lung Disease</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>KOLIN</td>
<td>Respiratory and Cardiovascular Effects in COPD</td>
</tr>
<tr>
<td>LLN</td>
<td>Lower Limit of Normal</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>mMRC</td>
<td>Modified Medical Research Council Dyspnoea Scale</td>
</tr>
<tr>
<td>NLF</td>
<td>Normal Lung Function</td>
</tr>
<tr>
<td>OLIN</td>
<td>Obstructive Lung disease In Northern Sweden</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SVC</td>
<td>Slow Vital Capacity</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
</tr>
<tr>
<td>VC</td>
<td>Vital Capacity</td>
</tr>
</tbody>
</table>
Original papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.


IV. **R. Linder**, J. Pourazar, A. Lindberg, A. Blomberg, A. F. Behndig. Proteolytic imbalance is related to FEV$_1$ decline in COPD. *In manuscript.*

* Open access, authors retain copyright.
Sammanfattning på svenska

Bakgrund
Kroniskt obstruktiv lungsjukdom (KOL) är en folksjukdom som blir vanligare med stigande ålder. Trots att kunskapen om KOL har ökat hos både allmänhet och sjukvård är underdiagnostiken fortfarande betydande. Detta innebär att åtgärder för att bromsa sjukdomsutvecklingen kommer i ett sent skede. Rökning är den viktigaste orsaken till att KOL utvecklas, och det beräknas att cirka hälften av alla rökare drabbas av sjukdomen. Att inte fler rökare drabbas gör det tydligt att det inte bara är yttre faktorer som avgör om sjukdom utvecklas.

Syfte
Studier har försökt klargöra vilka mekanismer som orsakar sjukdomens utveckling. Forskning har visat att det förutom rökning finns ett flertal riskfaktorer för att utveckla KOL. Däribland genetiska faktorer, rökning under graviditet, luftföroreningar, förbränning av biobränslen inomhus samt yrkesexponering för damm och luftburna partiklar. Enzymer som utsändras av immunförsvaret kan bidra till nedbrytning av lungvävnaden och därigenom orsaka KOL. I denna avhandling studeras enzymerna matrixmetalloproteinlas 9 (MMP-9) och 12 (MMP-12) samt deras hämmare TIMP-1 och balansen dem emellan (MMP-9/TIMP-1-kvot).

Metod
Avhandlingen baseras på OLIN KOL-studien i Norrbotten, där 993 vuxna med KOL samt motsvarande antal köns- och åldersmatchade referenspersoner studerades. I denna grupp av 1 986 försökspersoner undersöktes lungfunktion, symtom och blodprover för att mäta ovanstående enzymer. I en senare delstudie (KOLIN) analyserades lungsköljvätska för att däri mäta dessa enzymer. KOL-patienter med olika sjukdomskarakter undersöktes, däribland individer med snabb förlust av lungfunktion och de med stabil lungfunktion.

Resultat
I den första delstudien sågs att koncentrationer av MMP-9 i serum var signifikant högre vid KOL jämfört med icke-KOL.

Den andra delstudien var en uppföljning av studie 1 mellan åren 2005 och 2010 där målet var att utvärdera det prognostiska värdet av MMP-9 och TIMP-1 i relation till sjukdomskarakteristika, dödlighet och
lungfunktionsförlust. Det noterades att fler individer med KOL dog (16 %) jämfört med individer utan KOL (10 %). En koppling mellan höga värden av MMP-9 och lungfunktionsförlust sågs också men den observationen var bara statistisk signifikant i icke-KOL-gruppen. Noterbart var att de som avlidit under observationstiden hade signifikant högre MMP-9 värde och MMP-9/TIMP-1-kvot jämfört med deltagande personer.

Den tredje delstudien beskriver rekryteringsförfarandet från stor befolkningsbaserad studie till en tvärsnittsstudie med förbestämda grupper vad gäller förlust av lungfunktion över tid. Hypotesen var att det fanns skillnader mellan KOL-patienter med snabb förlust av lungfunktion jämfört med KOL-patienter med stabil lungfunktion och jämfört med kontroller. Målet att rekrytera 15 studiepersoner till fyra grupper uppnåddes med undantag för de två KOL-grupperna. Största orsaken till att inte delta var andra samtidiga sjukdomar, vilket försvårade och ibland omöjliggjorde kliniska undersökningar såsom bronkoskopi.

Den fjärde delstudien beskriver att nivåer av MMP-12 i lungsköljvätska var högre i gruppen med KOL- rökare och KOL-ex-rökare jämfört med både lungfriska tidigare rökare och icke-rökare. Koncentrationerna av MMP-12 i blodet var också högre hos nuvarande rökare med KOL jämfört med ex-rökare med KOL. Höga värden av MMP-12 i luftvägarna var associerat till en större årlig förlust av lungfunktion.

**Sammanfattning**

De ökade MMP-9-koncentrationerna vid KOL jämfört med icke-KOL, tyder på att MMP-9 är ett i blod mätbart tecken på lungsjukdom. Högre nivåer av MMP-9 och MMP-9/TIMP-1-kvot i blod är förknippade med sämre lungfunktion, vilket antyder att enzymerna påverkar sjukdomens allvarlighetsgrad. Högre MMP-9 och MMP-9/TIMP-1-kvot i blod är associerade med ökad dödlighet i KOL, vilket påtalar en roll för enzymerna i sjukdomens prognos. Den kliniska studien med grund i en stor befolkningsstudie ger goda förutsättningar för att utvärdera proteolytiska markörer och deras relation till sjukdomsmerkaperativa i KOL. Högre MMP-12-koncentrationer i luftvägarna korrelerar till minskad lungfunktion i KOL, vilket innebär att MMP-12 kan inverka vid sjukdomsprosessen.
Introduction

‘Biology is more like history than it is like physics. You have to know the past to understand the present.’
—Carl Sagan (1934–1996) in ‘Cosmos’

Obstructive pulmonary disease: A historical overview

The Greek verb *aazein* means ‘hard breath’. Aretaeus of Cappadocia, the Greek physician who is thought to have practiced in Rome circa 200 CE, is credited as the first to describe this symptom in humans (1). Through his vivid descriptions of symptoms, Aretaeus is portrayed as a clinician imbued with the observational skills that have allowed clinical medicine to advance throughout history.

One-thousand years ago, the Persian physician Ibn Sina (known in the West as Avicenna) described the importance of epidemiology, causality, and categorising illnesses. In his seminal *Canon of Medicine*, Ibn Sina defined signs and symptoms through classification, division, and subdivision (2). He also regarded food, drink, air, residence, occupation, habits, age, and sex as important determinants of health (3). According to Ibn Sina, the science of medicine advances through an understanding of health, as well as of ill health and its causes (3). This approach of interpreting signs and symptoms and then categorising them makes the transfer and revision of knowledge easier.

Beginning in the 16th century, the scientific community showed great interest in what could be learned from autopsies. In 1679, the early pathologist Théophile Bonet correlated dyspnoea to lungs distended by air (4). By connecting pathological findings to patient symptoms, Bonet attempted to understand underlying disease mechanisms—a promising approach—and theories on signs, symptoms, and causes of disease evolved over time.
In 1808, Charles Badham published a monograph with case reports on bronchitis, the first to mention bronchitis as a separate lung disease (5). Badham theorised that the mechanism behind these symptoms was an inflammation of the mucous membrane of the bronchi (6). These theories were built on his clinical experience and earlier findings by medical researchers.

During the 19\textsuperscript{th} century, the assessment of pulmonary diseases greatly advanced. In 1816, René-Théophile-Hyacinthe Laennec introduced a new instrument, the stethoscope (7), that addressed the clear need for better patient assessment since doctors’ ears alone were insufficient and autopsies declared causes of disease much too late for intervention. Stethoscopes proved greatly beneficial for evaluating rales and wheezes, although there remained no appropriate method to evaluate lung function.

In 1846, John Hutchinson invented an apparatus for measuring lung function named the ‘spirometer’ He also prepared reference tables associating lung volume with the height, weight, and age of 2,130 subjects—essential to determining abnormal lung volume. Regrettably, sex differences could not reliably be evaluated since only 26 female subjects were included. This invention brought new prospects to the field of pulmonary medicine through the standardisation of function testing and the potential to evaluate lung volume.

Defining obstruction as a limitation to expiratory flow was another step towards our modern understanding of obstructive pulmonary disease. Willem Einthoven developed this concept in 1892 (8), with his definition of obstruction similar to present-day descriptions. In Einthoven’s time, spirometry was considered a useful tool to evaluate pulmonary disease but was still not widely used in the clinical setting.

Robert Tiffeneau and André Pinelli advanced spirometry as a clinical tool, proposing in 1947 a measurement of the maximal volume of air that can be exhaled during a normal expiratory phase while exercising. They called this ‘pulmonary capacity usable on exercise’, which later evolved into forced expiratory volume in one second, FEV\textsubscript{1} (9). The procedure could be used in daily practice and considered the dynamic aspects of ventilation, which was lacking in contemporary understanding.
An event that impacted public interest in obstructive airway diseases occurred in England’s most populous city in 1952. During a freezing December week in London, increased coal burning created a thick, black fog. In those four foggy days and those that followed, hospitals received more complaints of wheezing and breathing difficulties, and about 4,000 deaths were recorded in this period (10). While previous reports of adverse health effects in conjunction with air pollution existed, the extent of this catastrophe motivated increased research in the field of respiratory medicine.

Greater research interest combined with a lack of consensus incited attempts to standardise definitions and nomenclature surrounding pulmonary diseases. This first occurred at a CIBA (Gesellschaft für Chemische Industrie Basel) symposium in 1959, where the terms ‘chronic bronchitis’, ‘emphysema’, and ‘asthma’ were formally defined (11). Together with the American Thoracic Society Committee on Diagnostic Standards in 1962, these meetings formulated definitions for components of pulmonary diseases.

The nomenclature began to take form, with the term chronic obstructive bronchopulmonary disease used in a paper by Roger Mitchell and Giles Filley in 1964 (12). Over time, this disease was known by a variety of names: chronic obstructive lung disease (COLD), chronic obstructive airway disease (COAD), chronic obstructive respiratory disease (CORD), chronic airway obstruction (CAO), and chronic airflow limitation (CAL). In 1965, at the ninth Aspen Emphysema Conference, William Briscoe introduced the term chronic obstructive pulmonary disease (COPD) (13). This term became established, and COPD is now the common designation for this growing problem of lung health.
Lung function and spirometric criteria for COPD

‘Spiro’ comes from the Latin *spirare*, which means ‘to breathe’. Accordingly, spirometry is the act of measuring lung volume and airflow during breathing. This chapter introduces the criteria used for defining chronic obstructive pulmonary disease through spirometry and the introduction begins with an overview of the nomenclature.

**Lung function parameters**

Forced vital capacity (FVC) is the volume of forcibly exhaled air after a maximal inspiration. Slow vital capacity (SVC) is the volume of slowly exhaled air after a maximal inspiration. The slow manoeuvre used when measuring SVC can avoid a component of dynamic airway compression, often seen among subjects with airway obstruction during a forced exhalation. Vital capacity (VC) is a term sometimes used—including in this thesis—to express the highest value of FVC or SVC. Forced expiratory volume in one second (FEV$_1$) is the volume of air exhaled by force in the first second of expiration. This measurement focuses on the early, effort-dependent portion of the expiration.

**Reference values for spirometry**

To decide if a spirometric value is normal or abnormal, one must determine reference values. It is therefore essential that individual measurements are evaluated using the distribution of measurements in a healthy reference population similar in age, height, sex, and ethnicity (14). This thesis considers a select few of the many published reference values for spirometry available.
Historically, the European Respiratory Society recommended the European Coal and Steel Community reference values (15) for European countries. In Sweden, the domestic reference values developed by Berglund in the 1960s (16) and by Hedenström in the 1980s (17,18) have also often been used (19). The Global Lung Function Initiative reference values, published in 2012, are derived from a group of individuals of diverse ethnicities aged 3 to 95 (20). These reference values were meant to be used globally and are endorsed by the European Respiratory Society and American Thoracic Society (20). The OLIN reference values published in 2015 by Backman and colleagues, are based on a sample of 501 healthy non-smokers aged 22–91 years from northern Sweden and preferrable in this population (21).

**Lung development and decline**

Lifetime lung function is related to quality of life and longevity. Over the lifespan, individuals follow different lung function trajectories, as illustrated in Figure 1. Maturation of the airways and lungs begins in utero and continues through childhood into adolescence. Events and exposures during gestation, birth, childhood, and adolescence can affect lung growth (22,23); however, COPD typically does not develop, as shown by trajectory 2 in Figure 1. If the maximal attained lung function (as measured by spirometry) is low, the risk of developing COPD increases (24), shown by trajectory 4 in Figure 1. Normally, the maximum lung volume is reached at 20 to 30 years old. Notable sex differences in lung development and airway behaviour (25) must be accounted for, as they can have implications for lung volume later in life.

During childhood and adolescence, boys typically have larger lungs (26). this is also seen in adulthood even when accounting for differences in age and height (27). There is a normal decline in lung function with age, as represented by trajectory 1 in Figure 1. In normal middle-aged adults, the rate of decline in FEV$_1$ is approximately 30 mL per year in men and 25 mL per year in women (28). There are individuals with both slower and faster decline in lung function, however, and no generally accepted definition exists of slow or rapid decline. A normal maximal attained lung volume with a rapid decline in lung function can result in COPD, as displayed by trajectory 3 in Figure 1.
The first clinical guidelines for the definition and management of COPD were created at the end of the 20th century, with documents from the American Thoracic Society (29), European Respiratory Society (30), British Thoracic Society (31) and Global Initiative for Obstructive Lung Disease (GOLD) (32) published within six years of each other. These guidelines detail the threshold for significant airflow limitation (COPD) and the method to assess the severity of the airflow limitation.
Both the American Thoracic Society and British Thoracic Society papers recommended using the fixed ratio FEV₁/(F)V CO to determine significant airflow limitation. The American Thoracic Society implemented a ratio of <0.75, while the British Thoracic Society applied <0.70. The output value used in the calculation also differed, with the American Thoracic Society using FVC as the denominator and British Thoracic Society and European Respiratory Society mentioning both FVC and VC (meaning SVC or the highest of either FVC or SVC).

**GOLD**

The first GOLD consensus document in 2001 advocated using the fixed ratio of post-bronchodilator FEV₁/FVC <0.70 as a spirometric criterion for obstructive airflow limitation in COPD and using FEV₁% predicted to grade its severity (32). These criteria have remained unchanged throughout all revisions of the document (33), and are today the most commonly used. Simplicity is the main argument for the fixed ratio criterion advocated by GOLD (33,34), while arguments against using it are underdiagnosis among younger individuals and overdiagnosis among the elderly (14,35).

**Lower limit of normal**

In defining COPD, the lower limit of normal (LLN) is sometimes used as a spirometric criterion instead of the threshold of 0.70. The threshold value is set at the fifth percentile of the normal distribution of the FEV₁/FVC ratio. There are advantages to using LLN rather than the fixed ratio, such as reducing misclassification of airway obstruction (36) and better reflecting clinically significant airflow limitation (37). As a consequence, LLN has been recommend for use in epidemiological studies (38).
Assessment and clinical aspects

The Swedish national treatment guidelines on COPD (39) builds upon the GOLD report. The assessment of COPD proposed by GOLD is based on spirometry, the patient’s severity of symptoms and history of exacerbation, the severity of the spirometric abnormality, and the identification of co-morbidities.

Evaluation of COPD

As a part of the constant refinement occurring over time, the 2011 GOLD update’s ‘ABCD’ assessment tool was seen as an advance from earlier simple spirometric grading system, as it incorporated multimodality assessment and symptom burden and emphasised the importance of exacerbation prevention in COPD management (33). However, the ABCD assessment tool performed worse than the spirometric grades for mortality prediction or other important health outcomes (33). To improve assessment, the 2017 GOLD report proposed a refinement of the ABCD tool separating spirometric grades from the ‘ABCD’ group (33), as shown in Figure 2. The separation of airflow limitation from clinical parameters clarifies what is being evaluated and ranked. This allows for more precise treatment recommendations based on the parameters driving the patient’s symptoms at any given time. Spirometry, together with patient symptoms and history of moderate and severe exacerbation, remains vital for diagnosis, prognosis, and consideration of other important therapeutic approaches.
Symptoms
The most frequently occurring respiratory symptoms in COPD patients are chronic cough, breathlessness, sputum production, wheezing, and chest tightness. Even though these symptoms are common, patients generally vary in terms of the symptoms they experience \((33,40)\). Validated assessment tests are used to evaluate symptoms, the most common being the COPD Assessment Test (score 0–40) \((41)\) and the modified British Medical Research Council dyspnoea scale (mMRC) (score 0–4) \((42)\). In the ABCD evaluation (Figure 2), the well documented \((43)\) COPD Assessment Test is primarily used, sometimes alongside the mMRC, as it has been shown to relate well to other measures of health status \((44)\) and to predict future mortality risk \((45,46)\). Other tests not used in a clinical setting, as they are only validated at the population level and not for individual assessment, include in-depth questionnaires such as the Chronic Respiratory Questionnaire and St. George’s Respiratory Questionnaire.

Cough with sputum production is present among many patients and is often the first symptom of COPD. Symptoms may vary from day to day \((47)\) and sometimes precede the development of airflow limitation by several years. Chronic cough in COPD may be productive or unproductive.
Dyspnoea is a cardinal symptom of COPD and a major cause of the disability and anxiety associated with the disease (48). COPD patients usually describe their dyspnoea as a sense of increased effort to breathe, chest heaviness, air hunger, or gasping, with these terms varying individually and culturally (49).

COPD patients often expectorate sputum while coughing, which can be intermittent, with periods of flare-up and remission. Chronic bronchitis is defined as regular production of sputum for three or more months in two consecutive years. The presence of purulent sputum reflects an increase in inflammatory mediators (50,51) and could mean the onset of an exacerbation.

COPD exacerbations are defined as acute worsening of respiratory symptoms that results in additional therapy (52). Exacerbations can be classified as mild, moderate, or severe.

**Comorbidities**
A comorbidity is a disease that exists at the same time as the disease being studied. The most common comorbidities for COPD include cardiovascular disease, diabetes, osteoporosis, depression, and lung cancer (33). Comorbidities are frequent for COPD and many are related to increased mortality (53). Several studies have identified cardiovascular diseases as very common comorbidities for COPD (54-56) and strongly association with mortality (57-60).

**Clinical features of COPD**
A phenotype is generally defined as the physical expression of an organism’s genetic code, its genotype. The traits or characteristics displayed by individuals, such as biochemical or physiological properties, are observed to assess their phenotype.

**COPD phenotypes**
In the field of COPD studies, a phenotype is defined as ‘a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression or death)’ (61). Frequent exacerbator, rapid decliner, non-smoking, smoking, asthma COPD overlap, and emphysema are examples of COPD phenotypes.
The diversity of phenotypes reflects the heterogeneity of COPD; to facilitate study of this disease, only a few phenotypes are discussed in this thesis, selected because of their relation to clinically measurable outcomes.

**Bronchitic phenotype**
Chronic bronchitis, characterised by hypersecretion of mucus, is a common symptom among smokers (62-64). The bronchitic COPD phenotype is associated with more exacerbations (65,66), more rapid lung function decline (67), frequent hospitalization (67), and higher mortality (68,69).

**Frequent exacerbator**
Some patients appear particularly prone to develop exacerbations of COPD, while others do not. Those reporting two or more exacerbations of COPD per year are often defined as ‘frequent exacerbators’ (70), a phenotype that appears stable over time. Exacerbations cause airflow to deteriorate and symptoms to increase, often with an infection, an increase in airway inflammation, or both. COPD exacerbations diminish quality of life and accelerates decline in lung function while increasing both morbidity and mortality (71-73).

**Rapid decline**
Rapid decline in lung function (FEV\textsubscript{1}) may indicate a distinct phenotype. From extrapolated data generated by Fletcher and Peto (74), rapid decliners can be defined as losing lung function (FEV\textsubscript{1}) at a rate greater than 60 mL per year (75). Rapid decline in FEV\textsubscript{1} is predictive of morbidity, mortality and hospitalization rates (76). In 1977, Fletcher and Peto published the classic illustration of lung function decline (Figure 3) in relation to smoking habits over eight years among 792 men (74), and they discussed 60 mL/year as a cut-off for rapid decline. More recent data from the COPD Gene study showed the overall mean annual decline among subjects with GOLD 2 was 45.6 mL/year across a five-year observation period (77); in a Swedish study, the mean rate of decline among incident cases of COPD was estimated at 51 mL/year, over a 10-year period (78). However, it should be noted that there is no generally established definition of rapid decline in lung function.
Therapy

Therapy for COPD focuses on prevention and maintenance. Smoking cessation is still the most effective method to prevent further loss of lung function.

Multidisciplinary care

With disease progression and declining pulmonary function, the risk increases for hypoxia, when a low level of oxygen reaches the tissue. Hypoxia causes muscle function to deteriorate, which leads to reduced exercise capacity, lower quality of life, and eventually, increased risk of death (80). These symptoms arrive late in the development of COPD, but subjects with a mild form of the disease also can benefit from this care. By participating in multidisciplinary rehabilitation, COPD patients can improve their physical capacity, increase their well-being and decrease dyspnoea (81,82).
**Medication**
Existing pharmacotherapy for COPD can reduce symptoms, reduce the frequency and severity of exacerbations and improve exercise tolerance and quality of life (33). However, existing medication has demonstrated no impact on the long-term decline in lung function, even if some data point in that direction (83-85). Available medications for COPD include bronchodilators such as beta-agonists and anticholinergics, inhaled corticosteroids and antibiotics. Bronchodilators decrease resistance in the respiratory tract and increase airflow through relaxing smooth muscles surrounding the airways. Antibiotics are used to treat bacterial infections that are common in exacerbations of COPD. Systemic corticosteroids are often used for patients with exacerbations, as they can improve lung function and oxygenation and shorten recovery time and hospitalization duration (86-88).

**Oxygen therapy**
For severe cases of COPD (89), oxygen therapy is sometimes a possible treatment. Research has demonstrated that long-term oxygen therapy can increase exercise capacity, improve the quality of life, reduce hospitalisation and increase life expectancy in patients with COPD accompanied by severe hypoxemia (90,91).
Epidemiology

This chapter will introduce an overview of epidemiology and epidemiologic data regarding COPD.

Epidemiology is from the Greek *epi* (‘among, upon’) and *demos* (‘people, district’—literally ‘among the people’. Epidemiology is a medical science focused on the distribution of health and disease and the factors contributing to them. Epidemiologists examine populations to find patterns that can explain why certain groups or individuals develop an illness when others do not. Epidemiological studies are also considered important for public health, policy decisions, and evidence-based practice, as they identify risk factors for disease and targets for preventative care. Common epidemiological nomenclature includes risk, incidence, prevalence, remission, and mortality. Risk is the likelihood that an individual will contract a disease, and prevalence is the proportion of a population with a disease at any given time; incidence is the number of new cases of disease in a population over a given time, and when presented as a rate, indicates how fast new cases are added (92). Remission is the partial or complete disappearance of the clinical and subjective characteristics of a disease and may be spontaneous or the result of therapy.

Epidemiology of COPD

For COPD, challenges with diagnostics and varying definitions make assessing its true prevalence in different countries difficult. As a result, prevalence data vary across studies.

*Smoking*

Smoking tobacco is the main risk factor for COPD, but other environmental exposures such as burning biomass fuels and air pollution may contribute. Additionally, host factors such as genetic abnormalities, abnormal lung development, and aging predispose individuals to develop COPD. Apart from being important risk factors for COPD, smoking and environmental tobacco smoke are also risk factors for lung cancer, cardiovascular disease, and children's asthma (93-95).
Environmental tobacco smoke has also shown a dose-response to respiratory health in general (96), to COPD (97), and to mortality in never smokers (98). Smoking has diminished over time in Sweden and the prevalence of COPD may have decreased in parallel (99). The population based data indicates a smoking prevalence of 12% (99) which is in line with registry based data on daily smokers (Figure 4).

![Figure 4: Daily smokers as a percentage of the Swedish population (Source: The Public Health Agency of Sweden)](image)

Occupational exposures such as organic and inorganic dusts, chemical agents and fumes, and burning of biomass fuels and wood can cause COPD. An estimated 25% to 45% of patients with COPD have never smoked; the proportion of COPD cases that are not caused by smoking is therefore much higher than previously believed (100). Approximately 3 billion people, almost half the worldwide population, are exposed to smoke from biomass fuel compared to 1.01 billion people who smoke tobacco, suggesting that exposure to biomass smoke might be the biggest risk factor for COPD globally (100), with women and children identified as at greater risk than men.
Prevalence
Historically, COPD was more common in men. However, due to increased tobacco use among women, the disease now affects men and women almost equally, with a trend towards an increasing incidence in women. According to an estimate by the Burden of Obstructive Lung Disease group, more than 200 million people worldwide will suffer from COPD in 2020 (101). One population study found the prevalence of COPD in Sweden to be 16% (102) and another found it to be 8% to 14% (103), with the variation due to the spirometric criterion employed.

Community-based population studies conducted in North and South America, Europe, Australia, and Asia have revealed that 10% to 12% of adults aged 40 or older have evidence of persistent airflow limitation on spirometry, but only 20% to 30% of these subjects have been diagnosed with COPD. These studies collectively suggest that approximately 70% of COPD worldwide may be undiagnosed (104).

Mortality
Increasing mortality due to COPD is attributed to the smoking epidemic and the aging of the global population (105). It is estimated that by 2020, COPD will be directly responsible for the death of 4.7 million people annually (101). In Sweden, COPD mortality has increased among women but has been more constant among men over the past 20 years. Unfortunately, under-recognition and underdiagnosis of COPD reduce the precision of mortality data (106). Furthermore, the accuracy of diagnosis codes recorded in health databases is unclear (107,108). Reliance on administrative health data, particularly those only recording hospitalizations, may underestimate the burden of COPD (109). The recording of COPD-related deaths in mortality data is also problematic; COPD is often a primary cause of death, but it is more likely to be listed as a contributory cause or omitted from the death certificate (110).
Mechanisms of pathogenesis

This chapter reviews a selection of proposed mechanisms involved in COPD development with an emphasis on proteolytic imbalance, and it provides a short introduction to the immune system (111). The disease mechanisms should be considered together, as they do not operate separately but are strongly interrelated. Inflammation, oxidative stress, and proteolytic imbalance are all important in the pathogenesis of COPD (112-114).

The immune system

The immune system is the body’s defence against threats that can potentially cause disease. The production of blood cells occurs in the bone marrow and maturing blood cells reach the systemic circulation through vessels supplying the marrow. Depending on their origin, cells of the immune system are divided into lymphoid and myeloid lineages (Figure 5). The spleen is a storage site for B cells and T cells and a site for activation of the adaptive immune system. Causes of inflammation can be either infectious; including bacteria, viruses, fungi and protozoa, or non-infectious; including urate crystals, glucose, amyloid, asbestos, silica crystals and smoking.

Immune response can be separated into two functional divisions: innate immunity and adaptive immunity. Innate immunity depends on physical, physiological, and chemical barriers, and it can be activated by cell injury or pathogens; it involves rapid response and broad specificity. Adaptive immunity produces a specific response that depends on specific recognition of antigens either directly by antibodies on the surface of B cells or through the presentation of processed antigens by host cells to T cells. Adaptive immunity is slower to respond on first encounter but more rapid upon re-exposure, and it is much more specific than the innate immunity.

Inflammatory response and tissue repair

Inflammatory response to injury can be defined as acute or chronic. Localised acute inflammatory response is characterised by an initial vasoconstriction, followed by vasodilation and increased vascular permeability. Then, there is an infiltration of circulating inflammatory cells: first neutrophils, then macrophages and lymphocytes.
Later, inflammation ends and tissue repair begins, consisting of an inflow of macrophages stimulating fibroblasts and keratinocytes through the release of cytokines such as transforming growth factor beta (TGF-β). Macrophages also stimulate angiogenesis and direct the formation of new extracellular matrix using TGF-β and matrix metalloproteinases (MMPs), among others (115). Over time, the injury matures through remodelling and scarring. Chronic inflammation ensues when acute inflammation persists due to a lack of resolution of the initial cause and macrophages and T cells accumulate and continue to secrete cytokines.

Figure 5: Immune cell genealogy (figure by author)

*Lung immunity*

In case of a local infection, tissue damage, or exposure to harmful substances, cells existing in the epithelium (such as macrophages, mast cells, and dendritic cells) can engulf the invading object and present its antigen to other immune cells. When this occurs, the epithelial immune cells are activated and begin to produce mediators (cytokines, chemokines, and prostaglandins), which can attract new leukocytes to the lesion (111).

*Airway inflammation*

Activated immune cells release a wide range of pro-inflammatory and pro-fibrotic factors, which in turn modulates the behaviour of airway wall-forming cells, including epithelial cells, fibroblasts and airway smooth muscle cells (111). The pro-inflammatory factors released by activated immune cells stimulate the resident cells to produce and secrete additional pro-inflammatory factors, thereby attracting more immune cells to the inflamed site. This cycle sustains the inflammatory process. Once established, inflammation can persist even after exposure to the irritant has ceased (116).
COPD is characterised by a persistent inflammatory response to environmental stimuli, most commonly cigarette smoke. Important cells in the pathogenesis of COPD involves neutrophils, macrophages and T lymphocytes and innate lymphoid cells (116). Another important cell in COPD pathogenesis is the dendritic cell, which is the major antigen-presenting cell in the lungs and an important link between innate and adaptive immunity (117,118). Additionally, dendritic cells also promote helper T-cell differentiation and toxicity of cytotoxic T-cells.

Chronic inflammation related to COPD causes structural changes and narrowing of the small airways. Destruction of the lung parenchyma, also by inflammatory processes, leads to the loss of alveolar attachments to the small airways and decreases lung elasticity. In turn, these changes diminish airways’ ability to remain open during expiration (113). COPD-related inflammation does not exist independent of other mechanisms, but is related to both oxidative stress and proteolytic imbalance (112).

Oxidative stress
Oxygen is essential to living cells but can also be highly toxic. Its toxic properties are mediated through the formation of free radicals, which are highly reactive, short-lived and destructive to their surroundings. Other forms of reactive molecules can be formed from oxygen without being defined as ‘free radicals’; these molecules are termed ‘reactive oxygen species’. Oxidants can be generated either endogenously by metabolic reactions or activated phagocytes, or exogenously by air pollutants or cigarette smoke.

Oxidative stress can be defined as ‘a disturbance in the prooxidant-antioxidant balance in favour of the former’ (119). When an imbalance occurs between the production of reactive oxygen species and endogenous antioxidant defences, there is a risk of damage to the body’s own cells, including proteins, lipids, and DNA.
In COPD, oxidative stress may result in the activation of pro-inflammatory transcription factors, impaired antiprotease defences, DNA damage, cellular senescence, autoantibody generation, and corticosteroid resistance (116). Oxidative stress in the lungs of individuals with COPD, can also induce cell death of endothelial and epithelial cells in the alveoli (120). Furthermore, oxidative stress can contribute to the proteolytic imbalance by inactivating anti-proteinases (121,122).

**Proteolytic imbalance**

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix components both under physiological conditions and in pathological processes. In healthy subjects, MMPs and their physiological tissue inhibitors (tissue inhibitors of metalloproteinase, or TIMPs) are produced by various cell types, and their actions are essential for many physiological processes, such as wound healing and cell transport (123). During early life stages, MMPs are important factors in the branching, angiogenesis, and alveolarisation of lungs (124). Gross and Lapiere discovered the first member of the MMP family in 1961 (125), and more than 20 MMPs have been described in humans (126).

Dysregulation of the balance between MMPs and TIMPs, as well as inappropriate secretion of MMPs by structural or inflammatory cells, are determinant factors in the pathophysiology of lung diseases, particularly COPD (127).

**Proteolytic imbalance in COPD**

Emphysema, an important feature of COPD is believed to be caused by the imbalance of proteases and antiproteases and results in lung parenchymal destruction (128). Gross and colleagues showed that instillation of elastolytic proteases in the lungs of experimental animals caused anatomic derangements with characteristics of human emphysema (129). The classic example in humans is that of α1-antitrypsin deficiency (AATD); an inherited genetic disorder in which the protease neutrophil elastase has destructive effects on the lungs (130).
Smoke exposure from cigarettes or other air pollutants can inactivate endogenous antiproteases (131), as well as trigger an acute pulmonary response that activates resident alveolar macrophages and promotes an inflow of neutrophils from the bloodstream to the lung tissue. With continued, chronic exposure to cigarette smoke, macrophages, neutrophils and cytotoxic T-cells accumulate in the lung tissue (132).

Circulating inflammatory cells, together with resident alveolar macrophages and epithelial cells, release proteases, which can cause elastin degradation and emphysema; epithelial cells and macrophages also release TGF-β, which stimulates fibroblasts and thus results in fibrosis around the small airways (114) (Figure 6). The lung tissue components cleaved by proteinases result in destruction and debris in the form of peptide fragments. These fragments can act as a chemotactic for more inflammatory cells, thereby perpetuating the accumulation of inflammatory cells and lung destruction (133,134).

Figure 6: Inflammation cascade in COPD (from (114), use allowed without formal permission from publisher)
Current knowledge

Several MMPs have been studied and associated with the tissue destruction observed in human COPD, which results in emphysema (135,136). However, much is still unknown about how MMPs affects COPD characteristics. As previously discussed, exposure to cigarette smoke leads to changes in lung MMP expression: macrophages will increase MMP-9 and MMP-12 production leading to degradation of the lung extracellular matrix molecules elastin and collagen. It also leads to inhibition of endogenous antiproteinases such as alpha 1-antitrypsin, which causes emphysema development (136-139).

MMP-9

MMP-9, also known as Gelatinase B, can cleave denatured collagen (gelatines) and type IV collagen, which are major components of the basement membranes (140). This cleavage helps lymphocytes and other leukocytes enter and leave blood and lymph circulations (140). MMP-9 can also influence tissue remodelling, which affects lung structure. Elastin fibres absorb mechanical stress and provide the elasticity required for lung and blood vessel integrity (141). This critical function is lost when alveoli and surrounding capillary beds are destroyed in response to the inflammation caused by smoking. Additionally, the loss of elastic binding to the airway smooth muscles may lead to these muscles’ shortening and to airway hyper-responsiveness due to the narrowing of airway lumen (142).

Several conditions increase the production of MMP-9. Mice exposed to ambient particles displayed increased levels of MMP-9 in airways (143). Small observational studies in humans have demonstrated increased MMP-9 for COPD compared to controls in sputum (144), lung parenchyma (145), and serum (146). Increased sputum MMP-9 levels are associated with more emphysema (as measured by computed tomography) and reduced lung diffusion capacity (147). One study revealed higher MMP-9 concentrations in the airways of COPD patients with more severe disease as measured by GOLD grade (148), whereas another study found no association between higher MMP-9 concentration and COPD severity or emphysema (149). Higher levels of MMP-9 in sputum are also found in patients during COPD exacerbations (150).
MMP-9 has also been associated with certain features in COPD subtypes. In a phenotyping study with cluster analysis, MMP-9 was the only mediator associated with a COPD subcluster with moderate airflow limitation, the group was too small to further differentiate, but the findings increased interest in the role of this proteolytic enzyme in the pathophysiology of COPD phenotypes (151).

**MMP-12**
MMP-12 (macrophage elastase) appears to be not only a direct cause of matrix degradation, but also a pro-inflammatory substance. A study in which MMP-12 was instilled in the airways of mice found neutrophils in the airways increased during the first three days and macrophages increased after four days (152), indicating the pro-inflammatory effect of MMP-12.

Many animal experiments have demonstrated MMP-12 to be an influential enzyme in disease mechanisms. Production and release of MMP-12 is upregulated in experimental animals exposed to cigarette smoke (153-155). In one study, genetically altered mice deficient in MMP-12, were resistant to cigarette smoke-induced emphysema, implying MMP-12 is a prerequisite for emphysema (137).

Despite promising experimental animal data on MMP-12 and emphysema, initial human studies were few and displayed divergent results. Some studies have struggled to even identify MMP-12 in human airway macrophages and lung tissue (156,157). Conversely, an assessment of MMP-12 levels in the sputum of COPD stage 0 and asymptomatic smokers, revealed higher MMP-12 levels in stage 0 smokers compared to non-smokers, as well as that MMP-12 could be detected by immunohistochemistry in sputum macrophages (158). Increased levels of sputum MMP-12 have also been reported in COPD subjects compared to healthy smokers, ex-smokers, and never smokers (159). Using microarrays to examine alveolar macrophage gene expression in 15 smokers, 15 non-smokers, and 15 subjects with asthma, researchers found a nine-fold presence of MMP-12 expression in the smokers (160). Another study found MMP-12 positive macrophages in lavage fluid were more frequent in current smokers with COPD than in ex-smokers with COPD, healthy smokers or never-smokers (161).
Tissue inhibitor of metalloproteinase 1 (TIMP-1) is a member of a family of endogenous inhibitors of MMPs that block the proteolytic activity of MMPs by binding to and forming complexes with them (162,163). Studies on serum TIMP-1 levels in colorectal cancer patients have revealed a positive association with age (164,165). In fact, most studies on TIMP-1 have focused on its use as a prognostic marker in malignancies (166-168). In COPD, the focus has been on TIMPs’ role as inhibitors of MMPs. This role suggests that increased TIMP levels result in fibrosis, while loss of TIMPs leads to amplified proteolysis of the extracellular matrix (169). Studies on mice lacking TIMP have supported this role, although studies with TIMP-deficient mice have also demonstrated that loss of TIMPs can be associated with fibrosis (170). Together, these studies suggest that TIMPs’ roles in matrix accumulation and proteolysis (which together can be referred to as extracellular matrix turnover) depend on the TIMP, specific tissue, and local tissue environment (healthy versus injured tissue) (171).

MMP-9/TIMP-1 ratio
Some pro-inflammatory mediators induce the release of MMPs from macrophages without inducing an increase in TIMPs, leading to a possible protease-antiprotease imbalance. Studies of proteases in alveolar macrophages obtained by bronchoalveolar lavage (BAL) and studies on lung tissue indicate increased protease expression in subjects with COPD compared to subjects without COPD (172).

Evaluation of MMP-9/TIMP-1 ratio in serum of asthmatics displayed a positive association of steroid responsiveness to MMP-9/TIMP-1-ratio (173). In a study on COPD patients and patients with idiopathic pulmonary fibrosis (IPF), the MMP-9/TIMP-1 ratio was higher in COPD subjects compared to both controls and idiopathic pulmonary fibrosis subjects (139). Studies have also found that high MMP-9/TIMP-1 ratios are associated with low FEV\textsubscript{1} in COPD subjects (174).
According to a study of 80 women with COPD and 40 controls, not only smoking but also exposure to biomass combustion were related to differences in metalloproteinases, including increased plasma levels of MMP-9 and MMP-9/TIMP-1 ratio, among those with COPD (175). A recent study on smokers with airway hyperresponsiveness revealed that the MMP-9/TIMP-1 ratio in smokers was positively correlated with annual decline in several lung function measurements (176).

Regarding the impact of proteolytic imbalance on COPD and subtypes, studies are needed on proteolytic markers’ involvement in COPD pathogenesis. MMP-12, MMP-9, and TIMP-1 are widely investigated but mostly for small-scale populations and patient studies. Validation in larger population-based studies are needed to further confirm proteolytic markers as viable disease markers. The amount of proteases and antiproteases expressed in the lungs and the blood differ and evaluating proteolytic markers in both compartments could validate serum proteolytic markers as a sign of disease activity in the lungs.

Taking the knowledge presented in this section into consideration, the need becomes clear for further epidemiologic and clinical studies on proteolytic imbalance and its possible role in COPD, its symptoms, and associated phenotypes.
Aims of the thesis

The overall aim of this thesis is to study MMPs and their inhibitors in COPD. Its specific aims are to evaluate:

• Serum concentrations of MMP-9 and TIMP-1 and MMP-9/TIMP-1 ratio, and their associations with lung function and clinical symptoms in COPD and non-COPD subjects from a population-based cohort

• MMP-9, TIMP-1 and the MMP-9/TIMP-1-ratio in relation to prognosis, assessed as lung function decline and mortality, in subjects with and without COPD in a longitudinal population-based cohort

• The recruitment process from an epidemiological COPD study to a study to evaluate pathophysiological mechanisms

• Systemic and airway proteolytic markers in COPD related to smoking habits and rate of decline in lung function
Materials and Methods

This thesis is based on data from the Obstructive Lung Disease in Northern Sweden (OLIN) COPD study (papers I and II) and the Respiratory and Cardiovascular Effects in COPD (KOLIN) study, for which the study population was recruited from the OLIN COPD study (papers III and IV). These studies are discussed below, and Table 2 provides an overview of these papers.

The OLIN studies
The Obstructive Lung disease in Northern Sweden (OLIN) studies, is an epidemiological research programme founded in 1985. The OLIN studies have since evolved into a broad research activity investigating COPD, health economy and asthma and allergy in both adults and children. The OLIN studies’ national network includes researchers from the Norrbotten County Council, Umeå University, Karolinska Institutet, Uppsala University, Lund University, the University of Gothenburg and Luleå University of Technology.

The OLIN COPD study
The OLIN COPD study is a prospective longitudinal population-based case-referent study. The source population includes individuals from Norrbotten, Sweden, recruited from 2002 to 2004, when four previously identified population-based adult OLIN cohorts were invited for re-examination including a structured interview and spirometry. Of approximately 4 200 individuals, 993 subjects were identified as having COPD according to the GOLD spirometric criteria (FEV$_1$/VC <0.70). Additionally, a reference population of 993 age- and sex-matched individuals without obstructive lung function impairment were identified and included in the total study population (n=1 986). Since 2005, the study population has been invited to annual examinations with a basic program including structured interviews; spirometry, height, weight, body mass index measurements and health status questionnaires.

The KOLIN study
The KOLIN study is a mechanistic cross-sectional study comprising four different groups representing clinical phenotypes of COPD. The study population was recruited from the OLIN COPD study. Recruitment of subjects was carried out in 2010 and 2012/2013.
Criteria were set for the four groups (Table 1) and 162 subjects were identified as matching the criteria; of these, 52 individuals were included in the final study. Various COPD phenotypes have been suggested as related to underlying pathophysiological mechanisms. The KOLIN study aims to evaluate pathophysiological mechanisms in COPD in relation to one of the previously mentioned clinical phenotypes: rapid decline in lung function. The study was designed to address the hypothesis that certain proteolytic markers would differ between COPD subjects with rapid decline in lung function and those with a non-rapid decline and taking the impact of smoking status into consideration.

### Table 1: Group criteria for the KOLIN study

<table>
<thead>
<tr>
<th>Group</th>
<th>Label</th>
<th>Spirometric criteria</th>
<th>Smoking history</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>COPD rapid decline</td>
<td>COPD GOLD grade 2–3 decline in FEV₁, ≥60 mL/year</td>
<td>Ever-smokers: smoking history of &gt;10 pack-years at baseline</td>
</tr>
<tr>
<td>B</td>
<td>COPD non-rapid decline</td>
<td>COPD GOLD grade 2–3 decline in FEV₁, ≤30 mL/year</td>
<td>Ever-smokers: smoking history of &gt;10 pack-years at baseline</td>
</tr>
<tr>
<td>C</td>
<td>Ever-smokers with normal lung function</td>
<td>FEV₁/VC ≥ 0.70 and FEV₁ ≥80% of predicted*, decline in FEV₁ &lt;20 mL/year</td>
<td>Ever-smokers: smoking history of &gt;10 pack-years at baseline</td>
</tr>
<tr>
<td>D</td>
<td>Non-smokers with normal lung function</td>
<td>FEV₁/VC ≥ 0.70 and FEV₁ ≥80% of predicted*, decline in FEV₁ &lt;20 mL/year</td>
<td>Non-smoker*</td>
</tr>
</tbody>
</table>

* At baseline and at recruitment
Figure 7: Recruitment and participation of study population for papers I and II

The OLIN COPD-study
n=1986

In 2005, n=1542
complete spirometry and blood test data

non-COPD n=948
COPD n=594

Whereof eligible subjects 2010 n=1379

Examination n=1031

In 2005 & 2010, n=952
complete spirometry from 2005 and 2010 and blood test data from 2005

non-COPD n=602
COPD n=350

Non-participants: 184 whereof 71 deceased
Telephone interviewed n=164
Examined n=1638
Spirometry n=1626, Blood sampling n=1621

Whereof deceased before examinations in 2010 n=163*

Whereof eligible subjects 2010 n=1379
Deceased n=169*
from examination 2005 until 31st Dec 2010
non-COPD n=88, COPD n=81

Deceased n=169*
from examination 2005 until 31st Dec 2010
non-COPD n=88, COPD n=81
**Figure 8**: Recruitment and participation of study population for papers III and IV (two subjects added to group A in second recruitment, one added to group B in second recruitment (for pre-defined criteria see Table 1).
<table>
<thead>
<tr>
<th>Paper</th>
<th>Study design</th>
<th>Spirometric criteria</th>
<th>Data source</th>
<th>Key factors assessed</th>
</tr>
</thead>
</table>
| I     | Epidemiological cross-sectional, examinations in 2005 | Non-COPD: FEV₁/VC ≥0.70  
COPD: ¹FEV₁/VC <0.70  
Ref. value: Berglund | OLIN COPD study | Serum: MMP-9, TIMP-1  
Productive cough, pack-years, smoking status |
|       |              |                      |             |                      |
| II    | Epidemiological longitudinal, examinations in 2005; mortality data from 2005 to 31 December 2010 | Non-COPD: FEV₁/VC ≥0.70  
COPD: ¹FEV₁/VC <0.70  
Ref. value: Backman | OLIN COPD study | Serum: MMP-9, TIMP-1  
FEV₁ decline (mL/year and change in % predicted)  
Productive cough, pack-years, smoking status, heart disease |
|       |              |                      |             |                      |
| III   | Longitudinal examinations in 2002–04, follow up in 2010 or 2012/13 | NLF: FEV₁/VC ≥0.70 and FEV₁ ≥80% of predicted  
COPD: ¹FEV₁/VC <0.70  
Ref. value: Berglund | OLIN COPD study | Descriptive analyses  
FEV₁ decline, mL/year  
Productive cough, pack-years, smoking status, heart disease |
|       |              |                      |             |                      |
| IV    | Clinical cross-sectional, examinations: at time of bronchoscopy | NLF: FEV₁/VC ≥0.70 and FEV₁ ≥80% of predicted  
COPD: ¹FEV₁/VC <0.70  
Ref. value: Berglund | KOLIN study | Serum: MMP-9, MMP-12, TIMP-1  
BAL and BW: MMP-9, MMP-12, TIMP-1  
FEV₁ decline |

¹Based on best values before or after bronchodilation  
FEV₁: forced expiratory volume in one second; NLF: normal lung function; VC: vital capacity, highest of forced vital capacity (FVC) and slow vital capacity (SVC); reference values from either Berglund (16) or Backman (21).
Interviews and measurements

For these studies, the interview questionnaires were developed primarily using the British Medical Research Council questionnaire (177) and influenced by the American Thoracic Society questionnaire (178), the International Union Against Tuberculosis and Lung Disease questionnaire (179), and the questionnaire used in the Tucson studies (180); these questionnaires have been thoroughly described previously (181-183). Trained nurses and research assistants carried out the interviews. Questions were added to cover smoking habits, co-morbidities, exacerbations and health economics (184). The Modified Medical Research Council Dyspnoea Scale (mMRC), was included in the questionnaire to grade dyspnoea, on a scale from 0 to 4 (185).

Study definitions

Body mass index was calculated as: weight (kg) / ((height (m)×height (m)) (papers I and II) and was classified as underweight (<20), normal (20– < 25), overweight (25– < 30) and obese (≥30) (paper I) (186).

Smoking habits were classified as: non-smokers (less than one cigarette per day during a maximum of one year), ex-smokers (cessation for at least one year), and current smokers (current smoker or cessation within the last 12 months); ‘ever-smoker’ was defined as an ex-smoker or current smoker. Pack-years were calculated as ((number of cigarettes smoked per day) × (number of years smoked)) / 20.

Productive cough was defined as cough and phlegm on most days for at least three months during the last 12 months. Clinically significant dyspnoea was set as an mMRC score ≥2. A score of 2 on the mMRC scale is defined as: ‘walks slower than people of the same age because of dyspnoea or has to stop for breath when walking at own pace’.

Heart disease was defined as one or more of the following: angina pectoris, previous myocardial infarction, coronary artery bypass graft surgery, percutaneous coronary intervention and chronic heart failure (papers II, III and IV).
Lung function tests and spirometric criteria

Lung function tests were carried out in accordance with the American Thoracic Society guidelines (29), except that subjects were in a standing position instead of sitting down. The instruments used were a set of dry volume spirometers, the Vicatest 5 (Gebr. Mijnhardt B.V., Odijk, Hollandodijk, 3984NV, The Netherlands). Spirometry procedures followed the current ATS/ERS recommendations (187) but with a repeatability criterion of $\leq 5\%$ (188) instead of $\leq 150$ mL deviation from the second highest value, or $<100$ mL difference if the spirometric values were $<2$ L. Adequate spirometry was achieved when it complied with recommendations. Quality control of the spirometers with a 3L syringe was carried out each morning on working days. The highest value for FEV$_1$, FVC and SVC, respectively, was used after at least three and up to eight measurements in order to fulfil the repeatability criterion. Vital capacity (VC) was defined as the highest value of forced vital capacity (FVC) or slow vital capacity (SVC). If FEV$_1$ was lower than 80% of the predicted value or if FEV$_1$/VC was below 0.70, a reversibility test with Salbutamol (Ventoline discus) 4 x 0.2 mg was carried out.

The Berglund reference values (16) were used for FEV$_1$ in papers I, III and IV, and local reference values were used paper II (21). COPD was defined by the spirometric criteria FEV$_1$/VC $<0.70$, using the highest value pre- or post-bronchodilation for papers I, II, III and IV. Non-COPD was defined as FEV$_1$/VC $\geq 0.70$ in papers I and II. In papers III and IV, normal lung function was defined as FEV$_1$/VC $\geq 0.70$ and FEV$_1$ $\geq 80\%$ of the predicted at baseline and at recruitment. In COPD, spirometric grading was assessed as FEV$_1$% of predicted as a continuous variable and also categorised into GOLD 1–4 (33) for papers I, II, III and IV.

Lung function decline

In paper II, lung function change from 2005 until 2010 was assessed as change in FEV$_1$ using the highest value of FEV$_1$ either pre- or post-bronchodilation and was calculated as:

1) The mean FEV$_1$ decline (mL/year): the unadjusted difference in FEV$_1$ in mL (absolute value of FEV$_1$ 2010 − (absolute value of FEV$_1$ 2005 divided by number of years of observation time, based on person days)).

2) Annual change in units of per cent predicted normal value of FEV$_1$: (FEV$_1$% predicted 2010 − FEV$_1$% predicted 2005 divided by number of years of observation time, based on person days) (78).
In papers III and IV, decline in FEV\(_1\) (mL/year) was calculated as: \((\text{FEV}_1 \text{ at baseline} – \text{FEV}_1 \text{ at recruitment})\) (based on highest value pre- or post-bronchodilation) / number of years (based on person days) of follow-up.

For papers III and IV, decline in FEV\(_1\) (mL/year) was calculated as \((\text{FEV}_1 \text{ at baseline} – \text{FEV}_1 \text{ at recruitment})/\text{number of years (based on person-days)}\). Furthermore, in papers III and IV, non-rapid decline was defined as COPD GOLD grade 2–3 with a decline in FEV\(_1\), \(\leq 30\) mL/year, rapid decline as COPD GOLD grade 2-3 with a decline in FEV\(_1\), \(\geq 60\) mL/ year, and, normal lung function as decline in FEV\(_1\) <20 mL/year.

**Peripheral blood**

Blood samples for papers I and II were stored beginning in 2005 at \(-20^\circ\text{C}\), and they were thawed and analysed in 2012 to determine MMP-9 and TIMP-1, using enzyme-linked immunosorbent assay (also known as ELISA). Blood samples for analysis of MMP-9, MMP-12 and TIMP-1 were collected in conjunction with the bronchoscopies and then directly frozen and stored at \(-80^\circ\text{C}\) for analysis later (paper IV). Serum concentrations of MMP-9 and TIMP-1 were assayed using the same batch of a commercially available ELISA kit (DuoSet ELISA Development System, R&D Systems Europe Ltd., United Kingdom) according to the manufacturer’s instructions for papers I and II. For paper IV, serum, bronchial wash (BW) and bronchoalveolar lavage (BAL) concentrations of MMP-9, MMP-12 and TIMP-1 were assayed using one batch of an ELISA kit (DuoSet, R&D Systems) according to the manufacturer’s instructions. Briefly, recombinant human MMP-9, MMP-12, and TIMP-1 were used to construct a standard curve (range 39–2 500 pg/mL) for each set of samples assayed. Serum samples were diluted 1:150 and concentrations read from the standard curve were multiplied by the dilution factor (papers I, II and IV). For paper IV, BW and BAL samples were diluted 1:2.

**Bronchoscopy**

Bronchoscopy is considered the gold standard of sampling airways (paper IV). Premedication with 1.0 mg of atropine was given subcutaneously 30 minutes before the procedure. Lidocaine was used for topical anaesthesia, and a small dose of oral Midazolam was offered to reduce possible anxiety before the procedure.
The bronchoscope (Olympus BF-1T160, Tokyo) was inserted through the mouth with the subject in the supine position (Photography 1). Lavages were accomplished by injecting 2 x 20 mL of sterile sodium chloride (BW) through the instrument channel, with the bronchoscope tip wedged in a segmental bronchus in the lingual lobe of the left lung or the right middle lobe and then gently suctioned back after each aliquot. BAL was carried out in a similar procedure using 3 x 60 mL of saline. The aspirates recovered from the instillations of BW and BAL were collected into separate siliconised containers and immediately placed in ice water. The recovered fluid was run through a nylon filter and centrifuged at 400 x g for 15 minutes. The BW and BAL supernatants were immediately frozen at −80°C until further analyses. Bronchoscopies were carried out by one medical team at two locations, either Sunderby Central Hospital of Norrbotten, Luleå, Sweden, or University Hospital, Umeå, Sweden; blood samples and blood analyses were carried out by the same personnel regardless of location.

*Photography 1: Bronchoscopy site Umeå (photographer: Ann Helen Karlsson)*
Statistical methods
Dichotomous variables were analysed using Pearson’s $\chi^2$ test (papers I and II). For continuous variables that met the assumption of normal distribution, the independent variable t-test was used; the non-parametric Mann-Whitney U-test was used for those that did not (papers I, II and IV). Mortality risk, expressed as hazard ratio, was estimated using Cox regression models (paper II). Univariate and multivariate associations of proteolytic markers were analysed using regression models in papers I and II.

For paper III, descriptive statistics were used. For statistical comparisons among three groups, the Kruskal-Wallis test was used (paper IV). If the Kruskal-Wallis test indicated significance ($p < 0.05$), the Mann-Whitney U-test was applied for post-hoc comparison between two groups, and when only two groups were compared, the Mann-Whitney-U-test was used (paper IV). A p-value of <0.05 was considered statistically significant for tests in all the papers. The IBM SPSS Statistics for Macintosh (IBM Corp. Armonk, NY, USA), versions 21–23, was used for statistical analyses.

Subjects

Paper I
This cross-sectional study was based on clinical examination of the OLIN COPD study population in 2005. Participation in 2005 was high with 1 806 out of 1 915 subjects still alive engaged in some part of the examinations. A total 164 subjects unable to attend the examinations were interviewed by telephone. Of the subjects, 1 626 participated in spirometries, and 1 621 in the blood sampling. This paper’s study population included all subjects with complete data from spirometry, structured interviews and serum analyses of MMP-9 and TIMP-1 ($n = 1 542$) (Figure 7).
**Paper II**

This longitudinal study was based on clinical examination of the OLIN COPD study population in 2005 and 2010. This paper includes data on basic characteristics and proteolytic markers (serum MMP-9 and TIMP-1) from 2005, spirometry data from 2005 and 2010 and mortality data collected from date of examination in 2005 until 31 December 2010. In 2010, 1379 of the 1542 were still alive, and 1031 participated in clinical examination. In total, 952 subjects participated in the clinical examinations, including spirometry, in both 2005 and 2010 (Figure 7).

**Paper III**

This methodology paper describes the process of recruiting subjects from the longitudinal OLIN COPD study into the pre-defined groups for the KOLIN study. It defines characteristics of study participants and reasons for non-participation. Selection was based on data from the OLIN COPD study at baseline in 2002–04 and at follow-up in 2010 (first recruitment phase) or in 2012/2013 (second recruitment) (Figure 8). Two recruitment phases were organised to reach the aim of 15 subjects in each group; however, this goal was not achieved. For characteristics and number of subjects in groups A–D, see Table 8.

**Paper IV**

In this cross-sectional KOLIN study, 52 individuals recruited from the OLIN COPD study were investigated using bronchoscopy. The study included 12 subjects with COPD and a rapid decline in lung function, 10 with COPD and a non-rapid decline in lung function, 15 current or ex-smokers with normal lung function, and 15 non-smokers with normal lung function see (Figure 8, Table 1). The subjects were stratified into subgroups by merging or dividing the base groups, depending on the hypothesis tested in the three different parts of the study (Table 9).
Ethics

Each paper in this thesis stems from the OLIN COPD study, which is approved by the Regional Ethical Review Board at Umeå University, Sweden (approval number 04-045 M). The KOLIN study was approved as number 2011-147-31M. All studies have been carried out in accordance with the Declaration of Helsinki. Participants gave their informed written consent after being provided with verbal and written information on aims of the studies, participants’ right to withdrawal, and the confidential treatment of their collected data. In the KOLIN study, a clinical medical examination, electrocardiogram, spirometry with reversibility, and routine blood samples were obtained from all subjects; since tobacco smoking involves an increased risk of lung cancer, all smoking or ex-smoking participants underwent a chest X-ray control. Subjects in the KOLIN study also underwent bronchoscopy.

Collection and storage of data and biologic materials were carried out with participants’ written consent. Data have not been made public in any online repository, questionnaires and consent forms are stored locally on paper, and datasets are stored on a select few local computers. The primary medical examination and the bronchoscopy reports were documented in the subject’s medical record, according to clinical practice. All subjects received a study-specific identification code, and the code key was stored behind locked doors within the premises. Data files distributed to researchers were de-identified and included only the study-specific identification code, guaranteeing participants’ confidentiality and anonymity. Participants received no financial compensation except in study IV, in which compensation was offered for taking part in the time-consuming examinations that included a bronchoscopy.

In medical studies, and particularly in epidemiological studies, subjects with previously unknown diseases may be found, and information about a previously undiagnosed disease may be received negatively. The benefit of early diagnosis and possible treatment, however, is expected to exceed the possible harm of being identified as having a disease. Physicians responsible for clinical examinations within the studies have assessed pathological findings and, when appropriate, informed subjects and offered them referrals to appropriate public health care for further consultation.
Results

Paper I

Study population and basic assessments
The study population consisted of 1,542 subjects examined in 2005, 594 subjects with and 948 subjects without COPD. The distribution by GOLD grade among those with COPD was 64% (n = 378) GOLD 1, 32% (n = 190) GOLD 2, and 4% (n = 26) GOLD 3–4. Comparing the non-COPD and COPD groups, significant differences were observed in the distributions of sex, age, body mass index, and smoking habits (Table 3). Median serum MMP-9 values were significantly higher in COPD than non-COPD, whereas differences in serum TIMP-1-concentrations and MMP-9/TIMP-1 ratio between groups were non-significant.

Table 3: Study population characteristics, smoking and proteolytic markers (modified from paper I, copyright: the authors)

<table>
<thead>
<tr>
<th></th>
<th>Non-COPD (n=948)</th>
<th>COPD (n=594)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>447 (47%)</td>
<td>248 (42%)</td>
<td>0.038</td>
</tr>
<tr>
<td>Age (years)</td>
<td>67 (55–71)</td>
<td>69 (57–71)</td>
<td>0.015</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.8 (24.3–29.7)</td>
<td>26.1 (23.5–28.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Packyears</td>
<td>0.8 (0–12)</td>
<td>14 (0–27)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>443 (47%)</td>
<td>149 (25%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>384 (41%)</td>
<td>247 (42%)</td>
<td>0.676</td>
</tr>
<tr>
<td>Current smoker</td>
<td>120 (13%)</td>
<td>197 (33%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-9 (ng/mL)</td>
<td>505 (364–606)</td>
<td>535 (315–653)</td>
<td>0.017</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>316 (229–490)</td>
<td>304 (227–439)</td>
<td>0.252</td>
</tr>
<tr>
<td>MMP-9/TIMP-1 ratio</td>
<td>1.36 (0.85–2.09)</td>
<td>1.50 (0.83–2.32)</td>
<td>0.168</td>
</tr>
</tbody>
</table>

Data presented as n (%) or as median (interquartile range)

Factors associated with proteolytic markers in non-COPD and COPD
Univariate regression in non-COPD revealed that pack-years and current smoking were positively associated with MMP-9. In COPD, similar associations with MMP-9 were displayed by pack-years and current smoking. Productive cough and lower FEV₁% predicted were univariately associated with higher MMP-9 in COPD subjects, but non-significant in non-COPD.
Multivariate analysis revealed that in non-COPD subjects, higher serum MMP-9 was associated with higher pack-years independent of age, sex, productive cough, or FEV$_1$% predicted. Productive cough displayed non-significant associations to MMP-9 for both groups. In COPD, however, lower FEV$_1$% predicted was associated with higher MMP-9 independent of sex, age, pack-years, and productive cough (Table 4).

<table>
<thead>
<tr>
<th></th>
<th>Non-COPD</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female$^a$)</td>
<td>0.038</td>
<td>0.256</td>
</tr>
<tr>
<td>Age$^b$</td>
<td>0.025</td>
<td>0.460</td>
</tr>
<tr>
<td>Productive cough (no prc$^a$)</td>
<td>0.012</td>
<td>0.714</td>
</tr>
<tr>
<td>Pack-years$^b$</td>
<td>0.084</td>
<td>0.013</td>
</tr>
<tr>
<td>FEV$_1$% predicted$^b$</td>
<td>-0.052</td>
<td>0.120</td>
</tr>
</tbody>
</table>

$^a$ Reference value, $^b$ Continuous variable, prc = productive cough

**Paper II**

*Study population and basic assessments*

Lung function decline was determined by spirometry for 952 participants in both 2005 and 2010, 424 (45%) of whom were women. The median (interquartile range) annual decline in FEV$_1$ was -40 mL/year (-64 – -22) in COPD and -46 mL/year (-68 – -16) in non-COPD (p = 0.428). When assessed as change in FEV$_1$% predicted, the annual decline was -0.21 (-1.10 – 0.51) percentage points in non-COPD compared to -0.54 (-1.43 – 0.50) percentage points in COPD (p = 0.020).

*Proteolytic markers and lung function decline*

No significant associations appeared between decline in FEV$_1$ and proteolytic markers in COPD subjects. By contrast, in non-COPD subjects, MMP-9 and MMP-9/TIMP-1 ratio were associated with decline in FEV$_1$ both when assessed as mL/year and as change in per cent predicted (Table 5).
To further investigate this result, a non-response analysis was carried out to compare proteolytic marker values in 2005 between subjects participating and not participating at follow-up in 2010 (those deceased were excluded from the analyses). For both non-COPD and COPD, MMP-9 concentration was higher, MMP-9/TIMP-1 ratios increased, and TIMP-1 concentration was lower in non-participants compared to participants at follow-up.

**Table 5: Linear regression analysis of proteolytic marker levels 2005 in relation to lung function decline.**
(reproduced from paper II, copyright: the authors)

<table>
<thead>
<tr>
<th>Decline in:</th>
<th>non-COPD</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml FEV₁</td>
<td>β</td>
<td>P</td>
</tr>
<tr>
<td>MMP-9</td>
<td>-0.098</td>
<td>0.016</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.100</td>
<td>0.014</td>
</tr>
<tr>
<td>MMP-9/TIMP-1-ratio</td>
<td>-0.128</td>
<td>0.002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>units of FEV₁ % predicted</th>
<th>non-COPD</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>P</td>
<td>β</td>
</tr>
<tr>
<td>MMP-9</td>
<td>-0.117</td>
<td>0.004</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.056</td>
<td>0.169</td>
</tr>
<tr>
<td>MMP-9/TIMP-1 ratio</td>
<td><strong>0.109</strong></td>
<td><strong>0.007</strong></td>
</tr>
</tbody>
</table>

*Proteolytic marker levels in the deceased*

During the observation period, 169 individuals died. Cumulative mortality was lower in non-COPD, (10%, n = 88), than in COPD, (16%, n = 81) (p = 0.008).

Comparing proteolytic marker levels from 2005 between participants and the deceased, MMP-9 level and MMP-9/TIMP-1 ratio were higher among the deceased in both COPD and non-COPD (Table 6). However, comparing proteolytic markers between COPD and non-COPD among the deceased, MMP-9 level (p = 0.001) and MMP-9/TIMP-1 ratio (p = 0.020) were higher in the deceased COPD subjects than the non-COPD (Table 6).
Table 6: Comparing proteolytic marker serum levels 2005 between survivors and deceased and between non-COPD and COPD among the deceased.

<table>
<thead>
<tr>
<th>Proteolytic marker</th>
<th>Non-COPD Participating (n = 860)</th>
<th>Deceased (n = 88)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>501 (346–605)</td>
<td>535 (428–628)</td>
<td>0.026</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>311 (224–521)</td>
<td>327 (250–381)</td>
<td>0.670</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>1.34 (0.81–2.09)</td>
<td>1.71 (1.19–2.16)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>COPD Participating (n = 513)</th>
<th>Deceased (n = 81)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>514 (210–641)</td>
<td>607 (493–681)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>300 (222–479)</td>
<td>311 (260–366)</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>1.38 (0.76–2.29)</td>
<td>1.86 (1.52–2.34)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Deceased non-COPD (n = 88)</th>
<th>COPD (n = 81)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>534.5 (427.5–628)</td>
<td>607 (493–681)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>327 (250–381)</td>
<td>311 (260–366)</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>1.71 (1.19–2.16)</td>
<td>1.86 (1.52–2.34)</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range), MMP and TIMP levels in ng/ml, MMP-9/TIMP-1 displays the ratio between the two markers.

**Mortality associated risk factors**

Risk factors for death in non-COPD subjects included male sex, higher age, ex-smoking, current smoking, heart disease, lower TIMP-1, and higher MMP-9/TIMP-1 ratio. In the COPD group, factors significantly associated with increased mortality were higher age, current smoking, increased MMP-9 levels, and higher MMP-9/TIMP-1 ratio. These results remained significant when adjusted for FEV1 % predicted and productive cough.

**Paper III**

This methodology paper describes the study design and the recruitment process for the sample of a study aiming to understand pathophysiological mechanisms in COPD associated with different phenotypes. The four groups A–D, had preset criteria based on lung function, decline in FEV1, and smoking status (ever smoker >10 pack-years, or non-smoker) (Table 1).
Basic characteristics of groups A–D
Table 8 provides the basic characteristics at the two recruitment phases in 2010 and 2012/13 for all subjects in the study database fulfilling the criteria for lung function, FEV₁ decline, and smoking history.

Non-participation
Groups C and D, but not groups A and B, reached the pre-set aim of 15 participants; group A included 12 participants and group B reached 10 participants after the inclusion of two individuals with a FEV₁ decline of 33 mL/year (thereby exceeding the pre-set criteria ≤30 mL/year). Table 7 provides reasons for non-participation.

Table 7: Reasons for non-participation (modified from paper III, copyright: the authors)

<table>
<thead>
<tr>
<th>Reason</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-participants: Eligible</td>
<td>25:37</td>
<td>19:29</td>
</tr>
<tr>
<td>Deceased</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Exclusion criteria:</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Significant cardiovascular disease</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Other significant diseases</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>History of asthma</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>Declined bronchoscopy</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Social reason</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Not possible to reach by phone</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Paper IV
The study population (described in paper III) included: ever-smokers with COPD and rapid decline (A), ever-smokers with COPD and non-rapid decline (B), ever-smokers with normal lung function (C), and non-smokers with normal lung function (D).

Basic characteristics of group A–D
Table 8 presents basic characteristics of the study population. The study population was stratified in three different ways (parts 1–3), as illustrated in Table 9.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group A (N=12)</th>
<th>Group B (N=10)</th>
<th>Group C (N=15)</th>
<th>Group D (N=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>61 (±6)</td>
<td>67 (±6)</td>
<td>64 (±7)</td>
<td>63 (±8)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>26 (±3.9)</td>
<td>26 (±2.8)</td>
<td>26 (±2.1)</td>
<td>28 (±4.4)</td>
</tr>
<tr>
<td>Female:Male</td>
<td>2:10</td>
<td>4:6</td>
<td>8:7</td>
<td>4:11</td>
</tr>
<tr>
<td>Current smokers: ex-smokers</td>
<td>9:3</td>
<td>3:7</td>
<td>4:11</td>
<td>0:0</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;/VC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.50 (±0.11)</td>
<td>0.54 (±0.08)</td>
<td>0.76 (±0.02)</td>
<td>0.78 (±0.04)</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;% predicted&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.8 (14.1)</td>
<td>65.4 (12.0)</td>
<td>107.3 (13.9)</td>
<td>106.6 (14.6)</td>
</tr>
<tr>
<td>Range</td>
<td>33.0–78.4</td>
<td>45.5–79.2</td>
<td>86.4–135.2</td>
<td>84.3–136.7</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; decline ml/year</td>
<td>−87.3 (±25.2)</td>
<td>−10.1 (±20.3)</td>
<td>−1.0 (±11.2)</td>
<td>−5.3 (±14.0)</td>
</tr>
<tr>
<td>COPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (42%)</td>
<td>8 (80%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Productive cough</td>
<td>3 (25%)</td>
<td>4 (40%)</td>
<td>2 (13%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>mMRC ≥2</td>
<td>2 (17%)</td>
<td>4 (44%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Exacerbation:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mild&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 (8%)</td>
<td>2 (20%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>moderate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>2 (20%)</td>
<td>1 (7%)</td>
<td>0</td>
</tr>
<tr>
<td>Heart disease&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3 (25%)</td>
<td>3 (30%)</td>
<td>3 (20%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (17%)</td>
<td>2 (20%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

Data presented as mean ± standard deviation or n (%)

<sup>a</sup> Based on values best of pre- and post-bronchodilation

<sup>b</sup> Diagnosed by physician

<sup>c</sup> Increased medication or received new medication during the last 12 months

<sup>d</sup> Treated with antibiotics and/or oral steroids during the last 12 months

<sup>e</sup> Heart disease includes any of the following: angina pectoris, percutaneous coronary intervention, coronary artery bypass surgery, myocardial infarction, or chronic heart failure

**Proteolytic markers in airways**

In the COPD group with both ex- and current smokers, MMP-12 concentrations in BW and BAL were higher, compared to both ever-smokers and non-smokers with normal lung function (Figure 9). Comparing COPD current smokers to COPD ex-smokers to clarify the impact of current smoking on MMP-12 revealed no significant difference in BAL-MMP-12. Differences in BAL and BW MMP-9 levels among groups were non-significant.
Table 9: Paper IV subject stratification, basic characteristics and group comparisons (from paper IV, unpublished data)

**Part I**: Comparing proteolytic markers in COPD versus ever-smokers and non-smokers with normal lung function

<table>
<thead>
<tr>
<th></th>
<th>COPD Ever-smokers n = 22</th>
<th>Normal lung function Ever-smokers n = 15</th>
<th>Non-smokers n = 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female:Male</td>
<td>6:1</td>
<td>8:7</td>
<td>4:11</td>
</tr>
<tr>
<td>Age</td>
<td>65±7</td>
<td>67±6</td>
<td>66±8</td>
</tr>
<tr>
<td>Current smokers:Ex-smokers</td>
<td>11:11</td>
<td>3:12</td>
<td>0:0</td>
</tr>
<tr>
<td>Pack-years</td>
<td>36±14</td>
<td>18±9</td>
<td>0</td>
</tr>
<tr>
<td>FEV1% predicted</td>
<td>62±17</td>
<td>108±19</td>
<td>103±17</td>
</tr>
<tr>
<td>FEV1/VC</td>
<td>0.53±0.11</td>
<td>0.73±0.04</td>
<td>0.78±0.04</td>
</tr>
<tr>
<td>Annual decline FEV1, mL</td>
<td>57±42</td>
<td>-a</td>
<td>-a</td>
</tr>
<tr>
<td>Rapid decline:Non-rapid decline</td>
<td>12:10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Per definition an annual decline in FEV1 of <20 mL/year

**Part II**: Separating the effects of proteolytic markers between smoking and COPD

<table>
<thead>
<tr>
<th></th>
<th>COPD current smokers n = 11</th>
<th>COPD ex-smokers n = 11</th>
<th>Normal lung function ex-smokers n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female:Male</td>
<td>2:9</td>
<td>4:7</td>
<td>6:6</td>
</tr>
<tr>
<td>Age</td>
<td>61±5</td>
<td>69±6</td>
<td>67±7</td>
</tr>
<tr>
<td>Pack-years</td>
<td>38±9.3</td>
<td>33±18</td>
<td>18±9</td>
</tr>
<tr>
<td>FEV1% predicted</td>
<td>61±16</td>
<td>62±18</td>
<td>107±17</td>
</tr>
<tr>
<td>FEV1/VC</td>
<td>0.53±0.12</td>
<td>0.73±0.12</td>
<td>0.78±0.04</td>
</tr>
<tr>
<td>Annual decline FEV1, mL</td>
<td>73±44</td>
<td>39±36</td>
<td>-a</td>
</tr>
<tr>
<td>Rapid decline:Non-rapid decline</td>
<td>8:3</td>
<td>4:7</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* per definition annual decline in FEV1 of <20 mL/year

**Part III**: Comparing proteolytic markers in COPD subjects with rapid versus non-rapid decline in lung function.

<table>
<thead>
<tr>
<th></th>
<th>COPD rapid decline n = 12</th>
<th>COPD non-rapid decline n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female:Male</td>
<td>2:10</td>
<td>4:6</td>
</tr>
<tr>
<td>Age</td>
<td>63±7</td>
<td>67±6</td>
</tr>
<tr>
<td>Current smokers:Ex-smokers</td>
<td>8:4</td>
<td>3:7</td>
</tr>
<tr>
<td>Pack-years</td>
<td>37.5±16</td>
<td>33±11</td>
</tr>
<tr>
<td>FEV1% predicted</td>
<td>60±15</td>
<td>63±19</td>
</tr>
<tr>
<td>FEV1/VC</td>
<td>0.52±0.12</td>
<td>0.54±0.11</td>
</tr>
<tr>
<td>Annual decline FEV1, mL</td>
<td>86±29</td>
<td>16±16</td>
</tr>
</tbody>
</table>
Figure 9: (I) Increased concentration of MMP-12 in BAL-fluid and BW in COPD subjects compared to ever-smokers with normal lung function (LF) and non-smokers with normal LF. (II) Exhibited increase in MMP-12 remained elevated in COPD airways even after smoking cessation when compared to ex-smokers with normal LF. (III) Displaying a tendency towards higher concentrations of MMP-12 in COPD with a rapid decline, however without reaching statistical significance.
Figure 10: (A) Displaying a positive association between annual decline in FEV$_1$ and concentration of MMP-12 in BAL-fluid in subjects with COPD. (B) Displaying a negative association between annual decline in FEV$_1$ and serum concentration of TIMP-1 in subjects with COPD.
Proteolytic markers in serum
In terms of proteolytic markers in serum between COPD current smokers and COPD ex-smokers, ex-smokers displayed significantly higher MMP-12 (p = 0.028). By contrast, comparing serum-MMP-12 between COPD ex-smokers and ex-smokers with normal lung function, revealed no significant difference (p = 0.056). COPD subjects with rapid decline in lung function showed the lowest concentration of serum-TIMP-1 median (interquartile range) of 477 ng/mL (295–717); this was lower than the COPD non-rapid decline median (interquartile range) 672 ng/mL (556–764) (p = 0.035).

Proteolytic markers and lung function decline
BAL-MMP-12 in COPD subjects was associated with annual decline in FEV₁ (r = 0.61, p = 0.005) (Figure 10). Lower serum-TIMP-1 was borderline associated with annual decline in FEV₁ (r = −0.42, p = 0.05), with the greatest decline found in subjects with the lowest concentrations of TIMP-1.
Discussion of methodology

In this thesis, the discussion is This section defines concepts in epidemiological research and discusses the study methods.

Concepts of validity and bias
Bias is a systematic error in a study that leads to distortion of the results. Selection bias reflects systematic errors that arise from the manner in which subjects are selected, which may result in a study sample being unrepresentative of what was intended in the study design. Examples of information (or misclassification) bias include reporting bias and recall bias (related to accuracy or completeness for memory of past events), which can occur in the form of random or systematic errors in recorded variables. A confounding factor is a variable that is independently associated with both the exposure and the outcome. Influence of a confounding factor may lead to either over- or underestimation of the true association. Validity refers to the degree to which a study is unaffected by systematic errors. Good validity means that the study correctly measures what was intended. Internal validity relates to how well the study results reflect the subjects’ true condition; it also reflects the confidence that can be placed in associations found within the study. External validity indicates to what extent results from one study can be applied to other settings or populations (generalisability).

Spirometry and spirometric criteria
Spirometers were calibrated with a 3L syringe each morning on working days. Quality control for the spirometric measurements was methodical and managed according to standardised guidelines (187). The internal validity is therefore considered to be high.

Since its launch in 2001, the GOLD document has defined airway obstruction in COPD as a fixed ratio, \( \text{FEV}_1/\text{FVC} <0.70 \). The OLIN COPD study was designed shortly thereafter and consequently, the fixed ratio was used to define airway obstruction. Since KOLIN is based on the OLIN COPD study, it also uses the fixed ratio. Additionally, many guidelines use the fixed ratio (including the Swedish national guidelines) for COPD diagnosis and treatment and thus this ratio is considered clinically relevant.
Using the fixed ratio may overestimate the prevalence of airflow obstruction among the elderly, and include elderly non-smokers without respiratory symptoms (189), which must be considered when interpreting the results. Underdiagnosis of COPD is pronounced (102,103,190), and population-based studies are thus important to describe the clinical picture of COPD in the society.

The studies presented in this thesis used the best value pre- or post-bronchodilation. Furthermore, they used the highest value of FVC or SVC to calculate the ratio, with FEV₁ as numerator. Whether pre- or post-bronchodilator spirometry results are used increases or decreases, respectively, the prevalence rate. Pre-bronchodilator fixed ratio has sometimes been used to define airway obstruction; however, the general consensus is that merely using pre-bronchodilator values exaggerates prevalence, with studies suggesting an overestimation of COPD of 25% to 35% (191,192). Using SVC instead of FVC as the denominator may generate higher prevalence of airway obstruction (193). The 1995 European Respiratory Society guideline (30) and in the 2005 American Thoracic Society/European Respiratory Society report (14) the recommendation is to use the maximum value of either FVC or SVC as an estimate of VC. In subjects with air trapping, however, FVC may be falsely low, resulting in a higher FEV₁/FVC ratio.

Different versions of the GOLD document are referred to in papers I to IV. Spirometric criteria for airway obstruction (post-bronchodilator FEV₁/FVC <0.70) and the spirometric classification of disease severity based on FEV₁ % predicted (GOLD 1–4), however, are similar throughout all versions of the GOLD document.

**LLN**

The LLN criterion is considered a more physiological method to establish airflow limitation. A task force report from European Respiratory Society recommends the use of post-bronchodilator LLN to define airway obstruction in epidemiological studies (38). Instead of using the fixed ratio, LLN definitions are based on the normal distribution and classify the lowest 5% of the healthy population as having an abnormal airflow. Whereas the GOLD classification has been shown to lead to more false positive diagnoses of COPD, LLN could lead to more false negative diagnoses (194). It should be noted that the LLN, like the fixed ratio of FEV₁/FVC, represents an arbitrary definition of COPD.
The European Respiratory Society/American Thoracic Society criterion of $\text{FEV}_1/\text{FVC} < \text{LLN}$ relies on valid reference equations representative of the population under study. Using the LLN for our studies, however, would have violated the study design. If the OLIN COPD study had been designed today, the use of the LLN spirometric criteria would have been considered.

*Adjusting for confounders*

Owing to the large scale of the studies presented in this thesis, an extensive amount of information based on questionnaire and spirometric data existed. To prevent confounding: stratified and adjusted analyses were used. In addition, a number of potential confounders were tested by inclusion in multivariate models without affecting the main associations. Thus, we expect the known main confounders to have been taken into account.

**OLIN COPD study**

Participants in the OLIN COPD study were identified by re-examining population-based cohorts (184). The distribution of disease severity was similar to that described in other population-based studies (195, 196); a majority of GOLD 1 and 2, with only a few cases with GOLD 3–4. This allows us to assume good external validity, which strengthens the generalisability of these results.

*Study design and participation*

Subjects must be recruited in a uniform pre-determined way and participation should ideally be high, since degree of selection bias is related to response rate. In the OLIN COPD study, participation at baseline was high (184), but some individuals were lost to follow-up. In the longitudinal paper II, we noticed a healthy survivor effect, which is considered a sign of selection bias. A healthy survivor effect with subjects lost on follow-up due to mortality and burden of disease, is expected in longitudinal studies.

*Matching*

The reference population without obstructive lung function impairment was pairwise matched 1:1 by age and sex to the COPD group. After inclusion was completed, the pairwise connection was removed. Matching allows for a smaller study population and may also reduce risk of confounding.
The matching was affected for example when spirometry or proteolytic marker data were used as selection criteria. This may have negatively impacted the power of certain analyses and adjustment for age and sex has been used where appropriate.

*Questionnaire and interview*

The questionnaires used in this thesis are well validated (197,198). The structured interviews and lung function tests were managed by the same staff; trained in interview techniques with regular follow-ups for method control; aiming to reduce inter-individual bias. Smoking was self-reported and not confirmed by cotinin tests. All diseases were self-reported and not verified by medical records. The self-reported data on smoking, medication, and comorbidities might have been affected by recall bias for individual subjects but is unlikely to be systematic.

*Study population; paper I and II*

The known under-diagnosis of COPD is related to disease severity and contributes to an underestimation, particularly of GOLD grades 1 and 2, in the population (103). From population-based studies using post-bronchodilator spirometry criteria for COPD, it is possible to include previously undiagnosed COPD subjects. In the 2005-examinations, participation rate was high, and standardised, well validated methods were used. This supports an assumption of good external and internal validity, as well as that observed results should be representative for COPD subjects in the general population.

The Swedish Berglund reference values for FEV$_1$ (16), were used in papers I, III, and IV as they conform well to individuals in northern Sweden without airway symptoms (199). The later-developed local OLIN reference values (21), based on non-smokers without respiratory diseases, were available and consequently used in paper II. Using representative reference values for the population under study strengthens internal validity.

*Blood samples*

Blood samples that were analysed in papers I and II were collected at examinations in 2005 and stored until analysis in 2012. Protein/enzyme levels in serum decrease over time (200). The decline in measurable enzyme should be the same in all vials since the degradation time is identical for a single enzyme and possible storage-effects are expected to affect all samples equally.
Another potential source of error can be excluded by our use of the same ELISA-kit-batches during each study, which strengthens the internal validity. We conclude that storage methods might have influenced the absolute values measured but likely not the results regarding the observed associations.

**Serum concentrations**
The MMP-9/TIMP-1 ratio was calculated based on concentrations of the protein. A number of recent studies have employed this method of addressing the systemic protease-anti-protease balance (201-203). A biologically more relevant manner of estimating the ratio may also include the molar MMP-9/TIMP-1 ratio of active MMP-9, which some studies have used (204). As our studies only determined the total MMP-9 concentration, no data on active MMP-9 are available and, hence, a relevant molar ratio cannot be calculated. However, there are reports confirming that protein concentrations of MMP-9 correlate well with MMP-9 activity (205-207).

**The KOLIN study**
The subjects in the KOLIN study were recruited from the OLIN COPD study, and thus their shared characteristics will not be repeated here.

**Study population; papers III and IV**
The data collected in the longitudinal OLIN COPD study were a prerequisite for identifying COPD phenotypes characterised by rate of decline in FEV$_1$. Various comorbidities impeded the recruitment process, but the process’ detailed description (paper III)—including reasons for non-participation—is a strength when interpreting the KOLIN study results. The recruitment procedure also allowed us to identify COPD phenotypes with respect to smoking and ex-smoking, permitting us to test both the effects of current smoking and of COPD.

The recruitment process was carried out in two steps to increase the study population, since the first recruitment did not identify enough subjects in group A (COPD rapid decline in FEV$_1$) and B (COPD non-rapid decline in FEV$_1$). In KOLIN, an observation time of between six and ten years was used for calculating decline in FEV$_1$. In contrast to papers I and II, the KOLIN study excluded subjects with a restrictive lung function pattern, as they represent a population with an increased prevalence of comorbidities and high BMI that may affect the results (54).
Using a control group with strict criteria for normal lung function created a more homogenous control group, facilitating a more relevant comparison with the COPD groups.

The description of rapid decline in FEV\(_1\) has varied from 45 to 61 mL/year among studies (74,77,78), and defined cut-offs for rapid decline has varied from 40 to 78 ml/years (208-210). Employing 60 mL/year as a cut-off for rapid decline (group A), can be considered appropriate and in line with the existing literature. Furthermore, to clearly differentiate between COPD with rapid decline in FEV\(_1\) and COPD with non-rapid decline the predefined levels of FEV\(_1\) decline in these groups were separated by an empty interval of 30mL.

**Blood samples and bronchoscopies**

All samples of BW, BAL and serum were immediately frozen and stored at -80°C, until analysed. This sample management strengthens validity. Sampling via bronchoscopy provides information about the proteolytic imbalance in different compartments of the lung. BW and BAL are routinely used to sample the respiratory tract and have been well validated (211). BAL represents the alveolar portion (smaller airway diameter) and BW the bronchial portion (larger airway diameter) of the airways.

In COPD, BW and BAL recovery may be low, as a consequence of airway remodelling and emphysema. Diminished BAL and BW volumes in COPD subjects could alter the absolute levels of measured markers. The present studies involved measuring the concentration of proteolytic markers, predicting a smaller effect from low recovery. The decision to add MMP-12 to the collection of proteolytic markers used in existing epidemiological studies was based on earlier findings showing MMP-12 as an important marker for COPD, emphysema, and lung function decline (126,212). MMP-12 could have been used in the epidemiological studies as well, but in the studies’ design phase, MMP-9 attracted significant interest and was chosen as one of a few select markers to study.
Discussion of main results

Proteolytic markers in COPD compared to non-COPD

The initial evaluation of proteolytic markers involved a comparison between the COPD and non-COPD groups, potentially revealing associations with COPD disease mechanisms. Since COPD is considered a systemic inflammatory disease with clinical manifestations and known comorbidities (213), it was of great interest to address proteolytic markers in serum. As a sign of systemic impact in COPD disease, higher serum MMP-9 concentration appeared in the COPD group compared to the non-COPD group (paper I).

Paper I data are in agreement with earlier findings of elevated systemic MMP-9 in COPD (214-216). Elevated systemic MMP-9 in COPD was also found by Piesak and colleagues (207), but also with higher CRP in the COPD group, suggesting an increased inflammatory activity alongside MMP-9. Higashimoto (206), found no statistically significant difference in MMP-9 between COPD subjects and controls in a study of 72 COPD patients, which the author suggests might be since the controls were smokers to a similar degree as the COPD subjects and smoking has exhibited an association with elevated MMP-9 levels.

Study I found systemically measurable MMP-9 with elevated concentrations in COPD. Previous studies (206,207) have described a correlation between MMP-9 concentration and active MMP-9 enzyme, which suggests that quantifying MMP-9 concentration with ELISA well could represent active MMP-9 enzyme.

Compared to these previous studies based on a limited number of cases, the data from paper I offer an advantage in the large number of subjects studied. Furthermore, the previous studies involved hospital patients with generally more severe forms of the disease. The population-based study I included subjects with milder COPD (mainly GOLD spirometric grades 1–2), with the difference still significant in MMP-9 concentration for COPD compared to non-COPD; this implies systemic proteolytic markers are important in the development and progress of COPD.
Proteolytic markers and smoking
To reveal possible confounders and to test the impact of other factors on the level of the proteolytic markers, uni- and multivariate analyses were carried out. This sub-section discusses the results related specifically to smoking.

The exact duration of the smoking effect on systemic markers after cessation is unknown, but Louhelainen and colleagues found increased levels of MMP-9 six months after smoking cessation (217). This is worth taking into account when assessing subjects’ smoking status since recent cessation might affect the levels of the systemic proteolytic markers.

In both the non-COPD and COPD groups of study I, univariate analyses displayed a weak but significant association between pack-years and increased MMP-9. However, in a multivariate model, this association disappeared in the COPD group but remained for non-COPD. This is in line with earlier findings of smoking’s association with MMP-9 in sputum (144) and in blood (218,219). When considering the results of study I—with 42% of the non-COPD group being ex-smokers and 13% current smokers—and Lohelainens results on prolonged elevation of MMP-9, the high prevalence of smokers and ex-smokers could explain the association between smoking and MMP-9 in non-COPD. In the univariate analyses employed in study I, smoking habits were analysed both as smoking status and pack-years. Whereas smoking status more adequately addresses the issue of current smoking, pack-years focuses on the cumulative smoke burden. Of importance, here smoking status was omitted from the multivariate analyses in favour of pack-years, due to the advantage of using a continuous variable that also was present in more subjects than smoking status.

Proteolytic markers in relation to disease severity and clinical symptoms
COPD disease severity is classified by separating spirometry values of FEV₁% predicted into GOLD 1–4. An established positive relationship between a proteolytic marker and spirometric severity grading, in addition to spirometry, could possibly increase the detail level of severity grading and be used to follow disease progression. The cross-sectional findings of study I did not detect any association between systemic proteolytic markers and COPD severity defined by GOLD grade but revealed a weak linear association between both MMP-9 values and MMP-9/TIMP-1 ratio and FEV₁% predicted in COPD.
This finding is unsurprising as FEV$_1$ % predicted constitutes a continuous variable and is suggested to be more sensitive for evaluating markers related to disease severity, compared to GOLD grading, for which each grade includes a wide range of FEV$_1$% predicted.

A population-based study of 888 elderly subjects by Olafsdottir and colleagues revealed that high serum MMP-9 relates to low FEV$_1$% predicted, but the study also demonstrated associations between low FEV$_1$% and higher serum levels of; MMP-9, TIMP-1, and MMP-9/TIMP-1 ratio. This study had a population-based selection of subjects aged 70 but no control group.

Study II compared results between non-COPD and COPD, and thus MMP-9 was associated not just with FEV$_1$% decline generally, but also with FEV$_1$% decline in COPD subjects specifically. Another study revealed borderline significant, negative associations between MMP-9 and FEV$_1$ using serum samples from 50 smoking COPD subjects compared to equal numbers of: COPD non-smokers, smokers without COPD, and non-smoking healthy controls (220). This study had an interesting design with varying subtypes for comparison, however, control subjects had reduced FEV$_1$/FVC ratios and no FEV$_1$% predicted values were presented. The study population and controls were all selected from hospital patients and data on diseases were self-reported. In a cross-sectional twin-study, Engström and colleagues found no association between MMP-9 and FEV$_1$, but a negative association between MMP-9 and RV/TLC (residual volume/total lung capacity) (221). The association between MMP-9 and FEV$_1$ in study II did not exhibit any clear significant result, but the displayed trend was interesting regarding the association of markers with longitudinal spirometry values.

Determining a positive relationship between MMP-9 and clinical symptoms could possibly supplement the evaluation of disease severity and activity. Cough and sputum production are not causally linked to airflow obstruction. Possible airflow obstruction in a patient with pure bronchitis is thought to be secondary to a remodelling of the small airways and increased mucus production. Study II revealed an association between MMP-9 and chronic productive cough in univariate analyses, but as the association did not remain when adjusting for confounders the data will need to be confirmed in further studies.
Vignola demonstrated a positive association between chronic bronchitis and MMP-9 in sputum for 16 patients with chronic bronchitis (174), which is in line with study II findings. It should be noted that productive cough means cough and sputum production for three months in only one year, in contrast to the two-year history required for diagnosing chronic bronchitis.

**Proteolytic markers in relation to lung function decline**

In the second study, decline in lung function was assessed over a period of at least five years. Since lung function values may naturally fluctuate over repeated examinations at shorter intervals (28), longer intervals should minimise the natural variation in measured spirometric values. As progression of COPD is heterogeneous, longer intervals may also better assess lung function decline in a population (222). Furthermore, using FEV₁% predicted as a continuous variable offers better level of detail than GOLD grades when evaluating obstructive severity, since each grade has a wide range in FEV₁% predicted.

Relating increased MMP-9 values to lung function decline measured as change in mL FEV₁ or FEV₁% predicted, revealed a similar pattern in non-COPD and COPD. This association was, however, significant for MMP-9 and MMP-9/TIMP-1 ratio only in non-COPD. In a longitudinal study on 96 COPD patients from a medical department, Higashimoto (223) established a correlation between higher MMP-9 and decline in FEV₁% predicted. Omachi tried unsuccessfully to establish this correlation in a study on 126 patients with alpha 1-antitrypsin deficiency but did find a correlation between increased MMP-9 and both decline in TLCO (transfer factor for carbon monoxide) and decline in lung density. A study on gene polymorphisms in 590 smokers by Joos and colleagues found no association between elevated expression of MMP-9 and rate of decline in lung function but did reveal a correlation between upregulated MMP-1/MMP-12 genes and rate of decline.

The findings of study II displayed an association between proteolytic markers and lung function decline in non-COPD, this association was non-significant in COPD. An association between lung function decline and proteolytic markers in COPD subjects may exist but cannot be affirmed by the present study, due to a loss to follow-up of those with the highest concentrations of proteolytic markers.
Study II findings imply a healthy survivor effect, which might explain the absence of a relationship between proteolytic markers and lung function decline in COPD. Elevated proteolytic markers in participants with lung function decline, however, suggest an enhanced proteolytic effect in relation to declining lung function in general.

In the fourth study, COPD subjects with the highest airway concentration of MMP-12 experienced the greatest decline in FEV$_1$, whereas a negative association existed between TIMP-1 in serum and FEV$_1$ decline. Based on confirmatory data from studies on experimental animals, MMP-12 appears to be a candidate for a direct role in emphysema development. Whereas some support for this assumption also exists in humans (159-161,224), the results from different human studies are conflicting (212).

*Reasons for non-participation in recruitment*

Epidemiological studies on COPD are often cross-sectional, and seldom include an evaluation of possible pathophysiological mechanisms. To better relate proteolytic markers to disease progression, a study was designed comprising two groups of COPD subjects: one with rapid decliners and one with non-rapid decliners. The study design also included two groups with normal lung function, one with non-smokers, and one with ever smokers. Recruitment of subjects with normal lung function reached the set goal, but was unachieved in the COPD groups; the most common reasons for exclusion were medical conditions contraindicating bronchoscopy, and inflammatory conditions or medication expected to affect the outcome (paper III). No available comparisons were found regarding the recruitment from a longitudinal COPD study to a mechanistic study. Few population-based longitudinal studies exist with large enough COPD cohorts to study pathophysiological mechanisms in relation to disease progression (195,225).

Co-morbidities are common among COPD subjects and the examination of non-participants revealed that co-morbidities contributed considerably to problems with recruitment for this study. Even with access to a large population-based COPD cohort, the present study could not meet the intended number of participants for groups A and B.
*Proteolytic markers and COPD phenotypes*

By studying phenotypes, the heterogeneity of COPD could be assessed and possibly result in enhanced diagnostics, better outcome prediction and personalised treatments. Suggested COPD phenotypes are based on clinical parameters such as: emphysema severity measured by computed tomography, spirometry, nutritional status, exercise capacity and exacerbation frequency (61,226). For study IV rapid lung function decline was chosen as the phenotype to evaluate. This was done in part to support the findings on proteolytic markers being significantly elevated in serum, by also evaluating them in the airways of COPD subjects. Furthermore, based on the association between systemic proteolytic markers and rate of FEV1 decline in subjects with COPD (paper I), it was considered relevant to also address airway MMPs in relation to lung function decline.

In smoking COPD subjects, there are at least two obvious factors that may influence the markers of interest; current smoking and active COPD disease. The study design aimed to better assess proteolytic markers by stratification for the influencing factors; smoking, COPD and decline in lung function. By using this design, however, the number of subjects in the different groups decreased considerably and, consequently, so did the power to detect significant associations.

Study IV revealed higher BW and BAL concentrations of MMP-12 in the COPD group, consisting of smokers and ex-smokers, compared to the group with normal lung function comprising ever-smokers and non-smokers. In an outpatient study on sputum from 39 COPD patients, Babusyte (161) and colleagues revealed a higher number of MMP-12 positive macrophages in the COPD subjects compared to the 19 healthy smokers and never-smokers. Demedts (159) and colleagues found higher sputum MMP-12 levels in 28 COPD patients, compared to healthy smokers, former smokers, and never smokers. Contradictory to these findings, Finlay found no differences in gene expression of MMP-12 or any production of MMP-12 protein in cultured macrophages from ten emphysema patients compared to controls (156).

The further results from COPD subjects with rapid decline highlights a trend towards higher concentrations of MMP-12 in BW and BAL, but the data rely on few individuals and thus should be interpreted with caution.
In a conflicting study on emphysema patients, D'Armiento reported differences between emphysema subjects and controls in levels of BAL MMP-9 and MMP-12, but no relation to severity or disease progression. In the findings from the KOLIN study, no difference existed in MMP-9 concentrations between COPD subjects those with normal lung function. This was perhaps a result of insufficient power in the study.

Proteolytic markers and mortality
In both non-COPD and COPD, increased proteolytic markers were found in those deceased over the five-year follow-up (paper II). However, the proteolytic markers among the deceased were higher in COPD than non-COPD, implying that increased proteolytic activity is related to mortality in general, and specifically for individuals with COPD.

In a longitudinal prospective cohort of all available men in one age group (1 082 men), Hansson and colleagues found that higher serum MMP-9 and TIMP-1 levels were associated with greater risk of all-cause mortality (227). By contrast a multicentre study comprising 295 patients with severe sepsis, by Lorente and colleagues found non-survivors had higher serum levels of TIMP-1, and lower serum levels of MMP-9 (203).

The relationship between cause specific mortality and proteolytic markers warrants further investigation, such as a longitudinal study with registered mortality causes linked to each subject. It is also unclear to what extent various parameters and phenotypes define the complex syndrome of COPD, as presented in the population. However, proteolytic imbalance appears to be a significant factor at the population level (228,229).
From epidemiology to clinical aspects

The cross-sectional epidemiology study (paper I) presented distribution and associations of proteolytic marker levels in COPD and non-COPD and the results provided possible hypotheses for further studies. Concepts on risk factors and prognosis were consequently obtained from the longitudinal study (paper II).

The findings from both studies were necessary to establishing the hypotheses addressed in the clinical KOLIN study. Testing the associations between proteolytic markers and COPD in a clinical setting with narrowly defined subgroups (phenotypes) would also further clarify possible underlying disease mechanisms. These findings thus can be used in future longitudinal studies. Even in the present material, with a majority of cases being mild and moderate COPD, the evaluated proteolytic markers display potential as indicators in COPD, regarding both prognosis and mortality.

Participants in KOLIN had more severe disease (all GOLD 2–3) than subjects in the epidemiology studies (94% GOLD 1–2), which could have affected the outcome regarding inter-group associations. In the epidemiological studies, a proportion of the non-COPD subjects had a restrictive spirometric pattern, which could also have affected the results. Such influence is minimised in KOLIN since normal lung function was a prerequisite for inclusion in the control groups. Conforming to the strict inclusion criteria, controls in KOLIN were also more homogenous than in the epidemiology studies.

In the epidemiological studies MMP-9 was revealed to be a serum risk marker for severity and mortality. When assessing proteolytic markers in airways, however, then MMP-12 reveals itself to be an important marker, in line with earlier findings.
Conclusions

In terms of MMPs and their inhibitors, proteolytic markers display associations with symptoms, lung function decline and prognosis and are suggested to impact disease development in COPD.

Specifically, it is concluded that:

- Serum MMP-9 concentrations are higher in COPD compared to non-COPD, implying a systemically measurable sign of lung disease.

- Higher serum MMP-9 and MMP-9/TIMP-1 ratios are associated with lower of FEV$_1$% predicted values, suggesting proteolytic imbalance impacts COPD severity.

- Higher serum MMP-9 and MMP-9/TIMP-1 ratios are longitudinally associated with mortality in COPD subjects, implying a prognostic role for proteolytic imbalance.

- The design of the clinical study with its basis in an epidemiological longitudinal cohort, provides a good foundation to evaluate proteolytic markers and their association with pathophysiological mechanisms in COPD.

- Higher MMP-12 airway concentration is associated with rapid lung function decline in COPD, implying a role for proteolytic markers in COPD progress.
Future directions for research

The following areas present compelling directions for future research efforts:

- Proteolytic markers in relation to emphysema measured by computerized tomography imaging of the thorax;

- Exacerbations in COPD related to proteolytic markers since exacerbations are the most debilitating facet of COPD;

- Proteolytic markers in relation to cardiovascular disease in COPD;

- Cause-specific mortality and possible associations with proteolytic markers; and

- Larger-scale investigation, which could be facilitated by using proteolytic markers in exhaled breath and thus enabling a non-invasive assessment of the airways; this is notable because bronchoscopy examinations require a significant monetary and time investment.
Acknowledgement

Thanks to all the participants in the OLIN COPD study and the KOLIN study; without your contribution, this research would not have been possible.

I had the privilege of working together with many skilled colleagues during the years leading up to this thesis. I am grateful to have been a part of the crew at the Department of Respiratory Medicine at Umeå University and want to thank every colleague I have engaged with during these years.

My warmest appreciation goes to:

Everybody at the clinic and at the lab
Anders Blomberg for your guidance, for teaching me how to use the bronchoscope and for your constant optimism.
Anne Lindberg my co-supervisor who made us all feel welcome when we first visited at her place outside of Luleå in the start of the KOLIN study, your knowledge of epidemiology is inspirational.
Annelie Behndig my co-supervisor for encouraging me when things were tough and for your words of wisdom.
Frida Holmström and Annika Johansson for all the nice moments we have shared, at work and at leisure time. You are great!
Ulrika Nygren, Andreas Fröhlich, Sofia Kemi, Ala Muala, Elisabeth Öberg-Carlsson, Jonas Eriksson-Ström, Ulrika Öhman, Kenneth Nilsson, Eva Norrman, Jon Unosson, Jenny Bosson Damewood and Stefan Barath, who all make (or have made) the pulmonary clinic (and research department) in Umeå such a great place.

We all depended on the expert staff of the lab: Jamshid Pourazar, Ann-Britt Lundström, Greg Rankin, Maria Sehlstedt, you show us what medicine really looks like in practice. Ulf Nilsson, for showing the way with your thesis, and for being a great roommate.
Ragnberth Helleday, for taking the admirable responsibility as head of the department of Medicine and for taking us to a baseball-game when visiting the ATS convention in Denver.
Thomas Sandström, for taking me to meetings where I got to present research and for providing wonderful support as head of the research department. Lena Åström, for always answering my questions and for all the things you do at the research department and the clinic.
Thanks to everybody working at the clinic in Umeå and Sunderby hospital for making the bronchoscopy research work, you are the best!

_Everybody at OLIN_

_Eva Rönmark_ present head of the OLIN studies and _Bo Lundbäck_, former head of the OLIN studies and initiator of the OLIN COPD study. The OLIN studies has really made an impact and now there are 20 theses based on that material. You did something remarkable! _Ann-Christin Jonsson, Sigrid Sundberg_ and _Linnea Hedman_ for data collection, without your thorough work, research would not be possible. _Helena Backman_ for your work on the OLIN reference values, all those statistics are like magic to me. _Viktor Johansson Strandkvist_ for teaching me to handle the Vicatest spirometer and to encourage the participants, it looked so much easier when you did it.

_All the friends and generally nice people I know of_

_Livia Dunér Holthuis_ and _Fredric Travaglia_ for making our arrival to Karlskrona a great one and for all the table-top gaming. _Liv Nordmark_ and _Rikard Norgren_, for taking care of Atlas and Hercules all these times, and for being great friends. _Agnes Holmström_, for being a good friend to me and Ylva and for teaching me to understand the power of the wrinkly face.

All the people at the hospital in _Karlskrona_, you are too many to mention here but you make the hospital a splendid workplace. Everybody at _Nättraby_ primary health care centre, I never thought primary care could be this fun.

_Åsa Gustafsson_ and _Ulrika Bergström_ at FOI for the nice conversations and all the laughs. Still curious about the bronchoscopies you do at your place!

_Apostolos Bossios_ for your great teaching, I guess they miss you in Gothenburg now that you moved to Stockholm. _Hans Stenberg_ for statistical support and for the teaching provided during my early years in med-school. _Björn Gustavii_ and _Carolyn Brimley Norris_, your advice on how to author articles and theses has helped me a lot. _Jill Merriman_, for English proofreading of highest standard. _Julie Knight_ for the great cover image and for the friendly conversation in the process.
Pets and family
Bråndalens Garion Gårdvar ‘Verner’ for taking me out on walks where we met people that you could sniff at and that I could talk to. You made all the work so much easier! Thank you also for modelling for the cover of the thesis. Atlas and Hercules, the bunnies with the world’s most ill-fitting names, you are wonderful, and I love you to bits.

My family: Alice my mother for your great spirit, never seeing anything as impossible and for having me at the ranch where I could get some peace of mind while working. Ulrika my sister for always giving me books to read when I was a kid. Lena my sister for teaching me to read. Anders my brother for always standing up for me. Sigurd my father, for teaching me to walk my own path, wish you were still with us. There is so much to say but all is most easily summarised as: I love you!

Yvonne Claesson for letting me stay at your apartment all this time during the end of my thesis-writing. Rolf Claesson, for proofreading and for helping me when the going gets tough. You both have become family too!

My wife Ylva Claesson Linder for your love and support through the writing of this thesis and for meticulous proofreading and joyful encouragement. I was content with being lonely until I met you and now I do not want to be without you. You make me happy everyday: ‘Cause, honey, your soul could never grow old, it’s evergreen’ as Ed Sheeran put it.

Financial support was gratefully accepted from the Swedish Heart-Lung Foundation, Umeå University, King Gustaf V’s and Queen Victoria’s Freemason Foundation, Visare Norr Fund/Northern County Council’s Regional Federation, Norrbotten and Västerbotten county councils.
References


35. Celli BR, authors of ATS/ERS statement on research questions in COPD. COPD (confusion over proper diagnosis) in the zone of maximum uncertainty. Eur Respir J. European Respiratory Society; 2015 Nov;46(5):1525–6.


42. Fletcher CM. Standardised questionnaire on respiratory symptoms: a statement prepared and approved by the MRC Committee on the Aetiology of Chronic Bronchitis (MRC breathlessness score). The British Medical Journal. (2):1662.


123. Gueders MM, Foidart J-M, Noel A, Cataldo DD. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases. Eur J Pharmacol. 2006 Mar 8;533(1-3):133–44.


