Systemic inflammatory markers as predictors of longitudinal outcomes in COPD

Results from the Bergen COPD Cohort Study

Gunnar Reksten Husebø

Thesis for the degree of Philosophiae Doctor (PhD)
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SCIENTIFIC ENVIRONMENT

The Bergen Respiratory Research Group is associated with the Department of Clinical Science, Faculty of Medicine and Dentistry at the University of Bergen and the Department of Thoracic Medicine at Haukeland University Hospital. The main research focus is on obstructive lung diseases, and the group has a broad experience with large epidemiologic studies of this patient category. The group has interdisciplinary capacities, and several researchers have a clinical background, holding positions in both institutions.

Professor Tomas Mikal Lind Eagan was the main supervisor of this PhD-project. Co-supervisors were Professor Per Sigvald Bakke and Assistant Professor Rune Nielsen (formerly Rune Grønseth).

The PhD project was conducted as a part of the Bergen COPD cohort study (BCCS) and the related Bergen COPD exacerbation study (BCES). These studies were initiated, conducted and supervised by Professors Eagan, Bakke and Jon Andrew Hardie, with start of patient inclusion in 2006.

For the whole period, I have shared my time between my PhD project and clinical work as a medical doctor. The first part of my project was funded by the Department of Thoracic Medicine by a 50 % research position. In 2015 I received a 6 year 50 % PhD-grant from the University of Bergen funding the last part of my project.

TERMS AND ABBREVIATIONS

6MWT	6-minute walk test
A1AT	alpha-1 antitrypsin
ACOS	asthma/COPD overlap syndrome
AECOPD	acute exacerbation of COPD
ALK-1	activin receptor-like kinase 1
AM	alveolar macrophage
AMP	antimicrobial peptides
ARDS	acute respiratory distress syndrome
ATS	American Thoracic Society
AUC	area under the curve
BAL	bronchoalveolar lavage
BCCS	Bergen COPD cohort study
BCES	Bergen COPD exacerbation study
BMI	body mass index
BNP	brain natriuretic peptide
cAMP	cyclic adenosine monophosphate
CC16	club cell secretary protein 16
CCL	chemokine ligand
CCS	Charlson comorbidity score
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CXCL	CXC ligand
DLCO	diffusing capacity for carbon monoxide
DNA	deoxyribonucleic acid
ECG	electrocardiogram
EGF	epithelial growth factor
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
ERS	European Respiratory Society
FEV1	forced expiratory volume in 1 second
FFMI	fat free mass index
FMI	fat mass index
FVC	forced vital capacity
GDF-15	growth differentiation factor 15
GEE	generalized estimation equation
GFRAL	GDFN family receptor α-like

GM-CSF	granulocyte-macrophage colony-stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HR	hazard ratio
HRCT	high resolution computed tomography
HU	Hounsfield unit
ICS	inhaled corticosteroids
IFN	interferon
IL	interleukin
ILC	innate lymphoid cells
IP-10	interferon gamma-induced protein 10
IQR	interquartile range
IRR	incidence rate ratio
LAA	low attenuation area
LABA	long-acting beta2 antagonist
LAMA	long-acting muscarine agonist
LLN	lower limit of normal
LPS	lipopolysaccharide
MAIT	mucosal associated t cells
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MKP-1	MAPK phosphatase-1
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MRC	Medical Research Council
NK	natural killer
NLR	neutrophil/lymphocyte ratio
NSE	neutrophil elastase
PAH	pulmonary artery hypertension
PCA	principal component analysis
PCR	polymerase chain reaction
PDE	phosfodiesterase
PI3K	phosphoinositide 3-kinase
PRR	pattern recognition receptor
PSFTPB	Pro-surfactant protein B
RCT	randomized controlled trial
RET	receptor tyrosine kinase
RNA	ribonucleic acid
ROC	receiver operating characteristic
ROS	reactive oxygen species

SAD	small airway disease
SARS	severe acute respiratory syndrome
SD	standard deviation
SGRQ	St. George's Respiratory Questionnaire
SPD	surfactant protein D
sRAGE	soluble receptor for advanced glycation end products
TGF-ß	transforming growth factor
TIMP	tissue inhibitors of metalloproteinases
TLR	toll-like receptor
TNF-α	tumor necrosis factor
VEGF	vascular-endothelial growth factor
WBC	white blood cell

ACKNOWLEDGEMENTS

As with many of my colleagues at the Department of Thoracic Medicine, my PhD project started with a part-time research position which allowed for a combination of clinical and academic work. I would like to thank both former and current director of the Department, Kahtan Al-Azawy and Sverre Lehmann for giving me and others this opportunity to start a PhD course by maintaining these positions in times of constant budget cuts. This framework is essential for providing the department with research competence, which is of great importance for the work environment and not at least the patient treatment.

I would also like to thank the Department of Clinical Science, Faculty of Medicine at the University of Bergen for giving me the opportunity to obtain a PhD degree in terms of funding, offering courses and providing the necessary foundation for my project.

This thesis is based on the extensive work done in designing, organizing and conducting the Bergen COPD Cohort Study and its sub-study Bergen COPD Exacerbation Study. I will thank Per Bakke, Jon Hardie and Tomas Eagan for all your impressive work. These studies have been of great benefit to many people, including hundreds of patients. Thanks to all of you who have contributed to the large data collection of these studies, especially Lene Svendsen and Eli Nordeide for keeping it all together for many years. Not at least thanks to all the patients and controls for your altruistic efforts over several years in order to improve knowledge on COPD.

Professor Tomas Eagan has been my main supervisor for the whole period and deserves a majority of the credit for pushing me through the PhD course. Tomas holds the unique combination of having both an extremely high working capacity and an annoying sense of accuracy, which makes him the perfect manuscript editor. Unlike me he does not find any joy in a random and unrestricted use of hyphens or capital letters, and no statistical flaw is likely to pass unnoticed from his scrutiny. Nonetheless, he has an astounding ability to hide his frustration when work progress is

slow, and his nature is truly benign; working with Tomas is never difficult (but he may disagree to that semi-colon).

I will also thank Tomas for his efforts in organizing the research group. Tomas is the cornerstone for both the scientific and the social activities in the group, both of them equally important for our well-being.

A great thank to co-supervisors Per Bakke and Rune Nielsen. Your knowledge and advice have been of great value to my work. Hopefully we can work together on new projects for the years to come. I will also thank my other co-authors Jon Hardie, Marianne Aanerud, Louise Persson Benneche, Pål Aukrust, Thor Ueland, Lorena Lerner, Jeno Gyuris, Corina D'Alessandro-Gabazza and Esteban Gabazza for your invaluable contributions to the papers.

I will thank Øystein Fløtten and the rest of the management at the Department of Thoracic Medicine for all your support and for your excellent and flexible way of organizing our workdays. In addition to those mentioned above, I will also thank Anders Storesund, Andreas Thelle, Atle Riise, Bernt Aarli, Christina Aamelfot, Fabian Gärtner, Frode Lindemark, Kristel Knudsen, Margrethe Schaufel, Ove Fondenæs, Rajinder Sharma, Solfrid Indrekvam, Tehmina Mustafa, Trygve Jonassen, Åse Rogde and the rest of my colleagues at the Department of Thoracic medicine. You make up an extraordinary assembly of brainpower and warm personalities. Thank you for choosing to spend your talents on pulmonology.

To Solveig, Marta, Christina, Øistein, Bahareh, Christine, Einar Marius, Elise and all other members of the research group. Thanks for all great times spent together at meetings and congresses. Hopefully socializing and travelling will be possible again soon.

Finally and most importantly, to Mette, Mikal, Maria and Eira: You give meaning to it all. Thank you for your love, patience and support for all these years.

At last, thanks to my parents, especially my mother for insisting to raise me as a non-smoker. You were right all the time.

ABSTRACT

Background

Chronic obstructive pulmonary disease (COPD) is major cause of morbidity and mortality worldwide. The prevalence is increasing worldwide, as a result of an increase in cigarette smoking the last decades. The main symptom of COPD is chronic and progressive dyspnea, often accompanied with cough and increased amounts of phlegm. A significant share of the patients suffers episodes with exacerbation of the disease, which may negatively impact quality of life, disease burden and survival.

COPD pathophysiology is complex and consists of different disease mechanisms. Inflammation is a central component of COPD, and increased number of immune cells and cytokines are found both in the airways and in the systemic circulation. The COPD pathophysiology is incompletely understood, and there is comprehensive research on inflammatory biomarkers in order to improve diagnosis, identify patients with increased risk of adverse outcome, and to find targets for medical treatment.

Aims

1-Identify diagnostic biomarkers of stable COPD and acute exacerbation of COPD.
2 -Identify inflammatory biomarkers as predictors for longitudinal outcome using longitudinal data:

a. as predictors for future exacerbations
b. as predictors for change in lung function
c. as predictors for mortality and cause of death
d. as predictors for lung cancer

Methods

The Bergen COPD cohort study (BCCS) included 433 COPD patients and 325 controls between 2006 and 2009. The COPD patients were aged between 40-76, all were former or current smokers. The COPD diagnosis was based on a clinical evaluation combined with an obstructive post-bronchodilator spirometry.

Of the 433 COPD patients, 356 patients living in the vicinity of Haukeland University Hospital were also included in the Bergen COPD Exacerbation Study (BCES).

All patients and controls went through an extensive examination at inclusion including medical history, physical examination, lung function testing, bioelectrical impedance measurements, HRCT, blood sampling, and microbiological testing. The patients and a selection of the controls were followed up during study visits each 6 months for 3 years, repeating lung function tests and blood sampling each 6 months, bioelectrical impedance each 12 months. In addition, patients were followed up to 9 years regarding mortality and cause of death as well as lung cancer development.

Acute exacerbations of COPD (AECOPD) were registered both at each 6-month visit, in addition the patients in the BCES were telephoned each month and asked about symptoms regarding AECOPD. A selection of the patients was also examined at exacerbation where additional blood sampling was performed.

The inflammatory biomarkers were evaluated at baseline and at AECOPD using both non-parametric and multiple regression models. For the analysis of the inflammatory biomarkers as predictors of future exacerbations, decline in lung function, mortality and lung cancer development, bi-level longitudinal regression models and cox-regression models were used.

Results

Systemic inflammatory markers were measured in all 433 patients and 325 controls at inclusion, and in 149 patients at AECOPD. Macrophage migration inhibitory factor (MIF) was identified as potential biomarker both for stable COPD as well as AECOPD in Paper 2.

Within the three years of the BCES, 350 of 403 COPD patients suffered 933 moderate and 370 severe COPD exacerbations. A history of exacerbations, female sex, chronic cough and a lower FEV_1 were identified as predictors for future AECOPD in Paper 1. In Paper 3, high levels of GDF-15 were identified as a predictor for a higher future AECOPD count.

The COPD patients experienced an average yearly FEV_1 decline of 61 ml (1.31 %) in men and 36 ml (0.76 % women) in women. High levels of GDF-15 were identified as a predictor of a faster decline of both FEV_1 and FVC in Paper 3. Other factors associated with a faster FEV_1 decline were male sex and cachexia.

Thirty-six COPD patients died with the first three years of follow up, 159 within 9 years. High levels of GDF-15 were identified as a predictor of a higher mortality in Paper 3. Other factors associated with a higher mortality were a low FEV_1, cachexia, obesity and a high degree of comorbidity.

Twenty-eight patients developed lung cancer within 9 years. COPD was significantly associated with a higher lung cancer risk. Within COPD patients, emphysema and obesity was associated with a higher lung cancer risk. Of 44 inflammatory biomarkers, only IP-10 was associated with a higher lung cancer risk, whereas systemic inflammation evaluated by a PCA-analysis did not show any correlation with lung cancer development.

Conclusion

1 Macrophage migration inhibitory factor (MIF) was identified as potential biomarker for both for stable COPD as well as AECOPD.

2 a. High levels of GDF-15 were identified as a predictor for a higher future AECOPD count in addition to several clinical characteristics.

b. High levels of GDF-15 were identified as a predictor of a faster decline of both FEV_1 and FVC.

c. High levels of GDF-15 were identified as a predictor of all-cause mortality as well as mortality due to respiratory disease.

d. IP-10 was significantly associated with a higher lung cancer risk, whereas systemic inflammation did not show any correlation with lung cancer development.

LIST OF PUBLICATIONS

Paper 1

Husebo GR, Bakke PS, Aanerud M, Hardie JA, Ueland T, Gronseth R, Persson LJ, Aukrust P, and Eagan TM. *Predictors of exacerbations in chronic obstructive pulmonary disease--results from the Bergen COPD cohort study.* PLoS One 9: e109721, 2014.

Paper 2

Husebo GR, Bakke PS, Gronseth R, Hardie JA, Ueland T, Aukrust P, and Eagan TM. *Macrophage migration inhibitory factor, a role in COPD.* American Journal of Physiology Lung Cellular and Molecular Physiology 311: L1-7, 2016.

Paper 3

Husebo GR, Gronseth R, Lerner L, Gyuris J, Hardie JA, Bakke PS, and Eagan TM. *Growth differentiation factor-15 is a predictor of important disease outcomes in patients with COPD.* The European Respiratory Journal 49, 2017.

Paper 4

Husebo GR, Nielsen R, Hardie J, Bakke PS, Lerner L, D'Alessandro-Gabazza C, Gyuris J, Gabazza E, Aukrust P, and Eagan T. *Risk factors for lung cancer in COPD - results from the Bergen COPD cohort study.* Respiratory Medicine 152: 81-88, 2019.

INTRODUCTION

Epidemiology of COPD

Chronic Obstructive Pulmonary Disease, COPD, has been a major cause of morbidity, hospitalization, health care costs, and mortality in Norway for many years. There are no accurate historical data on the prevalence of COPD before the 1980s, among else due to a lack of a general definition of COPD before this time. Tobacco smoking was introduced to the general public in Norway in the last part of the 19th century, at a time when life for the common Norwegian was miserable, life expectancy was about 50 years, and it was more likely to die of tuberculosis before anyone had a chance to achieve the slightly less stigmatizing diagnosis of “smoker's lung”. Within 1950, tuberculosis was in decline, but instead, smoking prevalence was 75 % in males, giving rise to the COPD epidemic for the last part of the century. In 1969, the Norwegian Tuberculosis Association realized defeat and fused with the Norwegian Association of Pulmonary Medicine, retraining and embracing airway obstruction instead of acid-fast bacilli.

The currently estimated prevalence of COPD in Norway varies between 150000 and 300000 patients, where the smallest estimate is more updated and probably the most accurate as of today(1). Many patients are unaware of their diagnosis, as only 50000 COPD patients were in contact with a physician in 2015, and only 60000 patients were prescribed COPD drugs (2), but it is still likely that many COPD patients are misdiagnosed with asthma, possibly due to stigmatization and more ample prescription refunds in asthma than in COPD. A more robust, but indirectly measure of COPD-prevalence is hospital admission; 10819 patients were admitted 17386 times in 2015 (2). There are indications that the both the incidence of hospital admission and COPD prevalence in Norway are stabilizing and possibly declining the last years (1).

Whereas the smoking epidemic is on rapid decline in Western countries, the situation is different worldwide. COPD is an increasing cause of morbidity worldwide, and is expected to be the third leading cause of death worldwide by 2020 (3).

Clinical characteristics of COPD

Symptoms of COPD rarely appear before the age of 40, and are usually preceded by minimum a decade of cigarette smoking or other harmful airway exposure. The main symptom of COPD is chronic dyspnea (4). Initially it is only recognizable at exercise, but as the disease progresses, dyspnea may also be present at minor exertions and at rest. Cough with or without increased amounts of sputum/phlegm may be the first symptom of COPD, and is present in up to 30 % of the patients. Accompanying dyspnea, wheezing or tightness in the chest is another common characteristic. The intensity of symptoms may vary, but they never completely resolve even at best (by definition). On the other side of the scale patients may experience episodes of symptom worsening called acute exacerbations of COPD (AECOPD), which may have a large impact on quality of life and prognosis.

COPD patients frequently have other medical concerns than poor lung function. Muscle loss, fatigue and development of cachexia are common findings in advanced COPD. Anxiety and depression are other conditions closely related to COPD, especially at disease progression. In addition, cardiovascular disease, diabetes and lung cancer are examples of common co-morbidities of COPD. In total, the disease burden in patients with COPD may be formidable, and it is not determined by lung affection alone.

Definition of COPD

The concept of COPD as a standalone disease was introduced in the 1960s as an umbrella term for emphysema, chronic bronchitis, chronic asthma and other less well defined pulmonary disorders (5). Initially, there was much effort in the evaluation of airway obstruction as the common and most important characteristic of these conditions. The work of Fletcher showing the associations between smoking, loss of FEV_1, and time of death had much impact on how we diagnose and prognosticate COPD (6), and spirometry is still a cornerstone in the evaluation of COPD patients. When knowledge and research increased in the last decades of the 20^{th} century, it was obvious that COPD did not only affect the airways, and there was a need to reframe the picture of COPD pathophysiology. There have been several international

collaborations in attempting to standardize COPD diagnostics and treatment. The most influential consortium is the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD). Although officially not creating guidelines, the GOLD has since 2001 regularly released Consensus Reports, acting as strategy documents for international research and national guidelines. The GOLD 2006/2007 report, released during the start of our study, defines COPD (7):

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases.

This definition has later been somewhat simplified, but there is an increasing awareness on COPD comorbidity when evaluating and treating COPD patients.

Spirometry is required or highly recommended for the diagnosis of COPD (4, 8). The GOLD criterion for airflow limitation is a Forced Expiratory Volume in 1 second (FEV_1)/Forced Vital Capacity (FCV) ratio of less than 0.7 after bronchodilatation. GOLD further classifies the severity of airway obstruction into four categories:

GOLD 1	Mild	$FEV_1 \geq 80$ % predicted
GOLD 2	Moderate	50 % $\leq FEV_1 \leq$ 80 % predicted
GOLD 3	Severe	30 % $\leq FEV_1$ 50 % $\leq$ predicted
GOLD 4	Very severe	$FEV_1 < 30$ % predicted

The criterion for airflow obstruction, the fixed FEV_1/FVC ratio of < 0.70 is controversial since a fixed ratio is not the ideal for the different age classes. A fixed ratio was deliberately proposed and acknowledged in order to standardize and simplify diagnostics. However, the fixed ratio may lead to missing diagnostics in younger patients, and misclassification in the elderly, since the FEV_1/FVC ratio gradually declines naturally by age. Some advocates for using the lower limit of normal (LLN) of the ratio, adjusted for age to avoid this circumstance (9, 10).

FEV_1 measurements alone was for many years the only guide for the medical treatment of stable COPD, but the 2011 GOLD Revision added criteria based both on symptoms and COPD exacerbation frequency (11). This was a recognition of that these parameters were of importance regarding the prognosis of the disease, but also of the heterogeneity of COPD.

Phenotypes in COPD

Introducing COPD in the 1960s as a general description of several similar airway disorders was a deliberate simplification. This might have been beneficial in order to coordinate and unite international research, but today there is an increasing interest in the differences between patient groups regarding symptoms, prognosis, medical treatment and the pathophysiology behind these differences. The term phenotype is derived from genetics. Although genetics might be of great importance for the differentiation of COPD phenotypes, it is obvious that the differences between different COPD characteristics cannot be explained fully explained by genes and gene expression. A proposal for a definition of COPD phenotypes was made by Han et al in 2010: *"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)"* (12) .

There are no "official" phenotypes in COPD. Traditionally, the two most mentioned phenotypes correspond to the two main characteristics in COPD, namely chronic bronchitis and emphysema. However, the usefulness of these two phenotypes may be limited since there is a significant overlap between these two conditions, and since the presence of one or another not necessarily warrants any special treatment. Another proposed phenotype is asthma-COPD overlap syndrome (ACOS), a group of patients where the distinction between these two categories is difficult (13). ACOS patients may have a positive bronchodilator test, usually not fully reversible. They may have high levels of blood-eosinophils and are often sensitive to inhaled corticosteroids.

Other more recently proposed phenotypes consider the different adverse outcomes of COPD. It is now recognized that the natural history of COPD as proposed by Fletcher

is more heterogeneous, as some COPD patients have the same lung function decline as healthy controls, whereas others have a rapid decline and thus a severe prognosis. Similarly, while some COPD patients have stable symptoms over time, others suffer from frequent and often a vicious circle of self-replicating exacerbations. These "rapid decliners" and "frequent exacerbators" are often the same patients, since frequent AECOPDs is a risk factor for a faster FEV_1 decline and vice versa (14, 15). Common for both categories is reduced quality of life and a higher mortality (16, 17).

Finally, the last decades there has been a shift from considering COPD a disease limited to the airways, to a clinical syndrome with co-existing disorders sharing risk factors and pathogenesis. Patients with COPD have an increased prevalence of several non-communicable disorders like cardiovascular disease, diabetes and osteoporosis (18). A common denominator of many of these conditions is the finding of increased levels of inflammatory markers in the systemic circulation. This feature is present to different degree in several COPD patients, potentially representing a distinct phenotype (19, 20). This increase in systemic inflammation is associated with several characteristics as well as adverse outcome in COPD (20-22). The significance of systemic inflammation in COPD is incompletely understood, but there is a large research interest on this topic, and this is also of high relevance for this thesis.

General pathology and pathophysiology in COPD

The major risk factor for COPD is cigarette smoking, although several other types of noxious airway exposure are also of relevance (23). Examples include indoor cooking with biomass fuel combined with poor ventilation, occupational exposures or airway pollution form traffic or industry. In general, noxious smoke and gases contain hundreds of different compounds with a potential to trigger an inflammatory reaction involving different components of the airways. The inflammatory response involves cells and cytokines related to the both the innate but also the adaptive immune system, the latter especially in more advanced disease. The results of these inflammatory processes are several different pathological changes not only in the lungs, but also in the pulmonary circulation as well as systemic effects involving several different organs (24).

Innate and adaptive immunity in the lungs

The key to understand the pathophysiology in COPD is to understand how the immune systems of the lungs react to different exposure. The immune system has one main task; to clear the airways from harmful substances including microbes, viruses, organic or inorganic toxins, but also inert particles of any kind. This is a difficult task, given the range of different exposure to effectively remove. If the immune response is insufficient, death from microbial invasion may be the result; if the immune response is excessive, tissue damage or dysfunction may occur. Thus it is important to the host that the immune system strikes a balance between these two opposites. If this process is carried out suboptimally over time, COPD may be the result.

The lungs including the alveoli contain by far the largest exposed surface of the human body, which is constantly exposed for a multitude of foreign elements. The innate immune system in the lungs consists of a combination of mechanical, cellular and humoral elements working both alone and together, but also in concert with the adaptive immune system (25). In a healthy and non-inflammatory lung, macrophages are the dominant inflammatory cells. Neutrophils, T- and B-lymphocytes are present only in small numbers but may be rapidly mobilized via the blood stream when called upon by cytokine signaling. The inflammatory response seen in COPD is similar to that of microbial and fungal infection, often denoted *type 1* and *3 immunity* (26). It is characterized with increased number of macrophages, neutrophils, cytotoxic T- cells and T_H1 and T_H17-cells. This is in contrast to the *type 2 immunity* seen in parasitic infection and asthma, dominated by mast cells, eosinophils and T_H2-cells.

Cytokines

The cytokines are essential mediators for all physiologic and metabolic processes in the human body. A cytokine can be defined as any molecule, usually a peptide or a protein, involved in cell signaling. The nomenclature of cytokines is complex and not always consistent (27). Many cytokines are named after its initial discovered function. As many cytokines have several functions, the same cytokine may have been given different names related to different functions, not always descriptive for its most important role.

Chemokines are cytokines with the ability to induce chemotaxis, like migration of immune cells to the site of an infection. Chemokines are further classified into four subfamilies, the CXC, CC, CX3C and XC-families, all interacting with g-protein transmembrane receptors on their target cells.
Interleukins are cytokines involved in the cell signaling between leukocytes. The interleukins are a heterogeneous group of proteins, more than 38 different families of interleukins are described, many of them crucial for the function of the immune system (28).
Growth factors are cytokines involved in the regulation of proliferation, migration and differentiation of cells and tissue. This is a large and heterogeneous group of cytokines, and where the distinction between cytokines and hormones is not always straightforward.
Interferons are cytokines released as a response to virus infections, many of these can also be classified as interleukins.
In addition, molecules not classified as cytokines, like coagulation factors, complement factors or several enzymes may have cytokine functions.

More than 50 different cytokines are described as likely components of COPD pathophysiology (29). Tumor necrosis factor (TNF-α) and interleukin-8 (IL-8) were among the first cytokines identified in COPD (30), and are examples of up-stream pro-inflammatory cytokines. TNF-α is associated with a diverse range of inflammatory responses, whereas IL-8 is closely related with chemoattraction of neutrophils, hence its second name chemokine (CXC motif) ligand 8 (CXCL8). The IL-1 family and IL-6 are other examples of COPD related cytokines (31, 32). IL-1 is related to macrophage activity in COPD, IL-6 may have both pro- and anti-inflammatory effects and is linked to systemic inflammation and release of C-reactive protein (CRP) from the liver.

Immune response of the lungs

The first barrier in the lungs is the epithelium, which is covered by a protective mucus lining, which again is transported out of the lungs by cilia sweeping upwards. In addition to this mechanical component, the mucus also contains antimicrobial peptides (AMPs) and other compounds with antimicrobial and immunomodulating abilities

(33). Furthermore, epithelial cells have the ability to detect pathogens by different semi-specific pattern recognition receptors (PRR), where the toll-like receptors (TLR) are the most studied (34). An activation of any PRR may again lead to release of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-8, activating a more potent immune response. Epithelial cells may also release growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), important for immune cell production and survival. Other important growth factors secreted from the epithelium are transforming growth factor (TGF-ß), epithelial growth factor (EGF) and vascular-endothelial growth factor (VEGF), related to fibroblast and smooth muscle cell proliferation, mucus secretion and vascular repair.

The alveolar macrophages (AM) are the most abundant cells found in bronchoalveolar lavage (BAL) in a healthy lung (35). They are stationary and versatile cells with a relatively long life span. The AM has phagocytic abilities which are important both to the homeostasis of the healthy lung, but also in the defense against microbes. Also important, the AMs have the ability to bind foreign antigens via MHC molecules, which again is presented to T-cells. Thus, the AMs are specialized antigen presenting cells (APCs); a role shared with the dendritic cells also found in the airways. The AMs may be categorized in a pro-inflammatory M1 and an anti-inflammatory M2 phenotype, although this differentiation is controversial (36). Upon recognition of foreign elements, or by cytokine signaling, the AMs may release pro- but also anti-inflammatory cytokines, activating or modulating the immune response. The AMs are a major source of TNF-α, which illustrates their role as a central initiator of inflammation. Neutrophils and monocytes are attracted from the circulation by IL-8, CXCL1 and monocyte chemoattractant protein-1 (MCP-1). The AMs also release CXCL9, 10 and 12, examples of chemokines attracting both T_C-cells and T_H1-cells. Further, AMs are a source of growth factors, reactive oxygen species and also elastolytic enzymes similar to neutrophils, such as matrix metalloproteinases (MMPs). Altogether the macrophages, due to its versatility, have an essential role in COPD pathophysiology.

Neutrophils are present in large numbers in the pulmonary circulation, lining the capillary bed, searching for pathogens or other pro-inflammatory signaling (37). Unlike the AMs they are normally not abundant in the lung parenchyma in a healthy lung, but their numbers can rapidly multiply in case of pathogen invasion or tissue damage. Like the AMs the neutrophils have phagocytic capabilities but are also known for their secretion of granules into infected tissue. These granules contain anti-microbial molecules such as Myeloperoxidase (MPO), Neutrophil elastase (NSE), MMP8 and 9, but also other proteases or radical oxygen species. Such molecules have a potent anti-microbial effect, but they are also considered responsible for the breakdown of the alveoli, leading to development of emphysema, often denoted as a protease-antiprotease imbalance (38).

Eosinophils are traditionally linked to the pathogenesis of asthma more than COPD, but as much as a third of COPD patients have elevated levels of eosinophils in sputum or blood, and the classification of these patients is still debated (39). Eosinophils have similar weaponry as the neutrophils, but eosinophils differ in ways of differentiation and activation which is addressed later, and they may also have a more complex relation to the adaptive immune system. Characteristic for eosinophilic COPD is the suppressive and clinically beneficial effect of corticosteroids in many patients, unlike in COPD patients with a predominant neutrophilic inflammation.

Other cellular components of the innate immune system are NK-cells, innate lymphoid cells (ILC), mucosal associated T-cells (MAIT), all with roles less clearly defined in COPD.

Although the innate immune system is more prominent in COPD pathophysiology, the role of the adaptive immune system has been highlighted the last decades. Cytotoxic T-cells (CD8+) are found in increased numbers in the lungs of COPD patients, especially is this seen together with increasing degree of emphysema and airway obstruction (40). Helper T-cells (CD4+) are less abundant than T_C-cells but are also increased in COPD. Important T_H1-cell cytokines are interferon gamma (IFN-γ), an activator of macrophages with antiviral properties, and IL-2, important for the differentiation of T-cells. The role of T_H2-cells more evident in asthma than in COPD,

and is addressed later. The more recently discovered T_H17-cells, however, are likely to have an important role in COPD (41). T_H17-cells attract and stimulate both macrophages and neutrophils, but also B-cells via IL-17 and IL-22 signaling. Lastly, B-cell lymphocytes, with their ability of antigen-specific antibody secretion are crucial elements in the defense against infection but may also have a role in auto-immune inflammation in a complex interaction with other cells and components of the immune system (42).

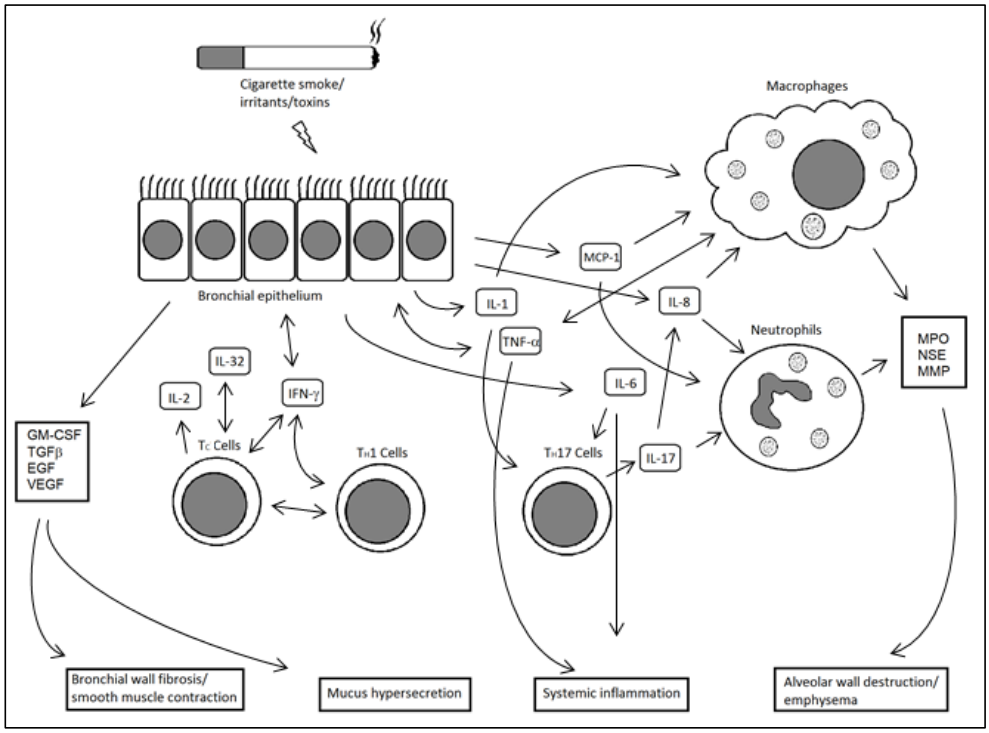

Figure 1. Overview of central components of the immune response in the lungs. Cytokines: IL (interleukin), TNF-α (tumor necrosis factor), MCP-1 (monocyte chemoattraction protein), INF-γ (interferon). Growth factors: granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF-ß), epithelial growth factor (EGF), vascular-endothelial growth factor (VEGF). Proteases/enzymes: myeloperoxidase (MPO), neutrophil elastase (NSE), different matrix metalloproteinases (MMPs).

The innate immune system is the first line of defense and deals with the bulk of invading elements in the lungs, In COPD, several components of both the innate and adaptive immune system may be compromised

Chronic bronchitis

A common definition of chronic bronchitis is based on the presence of cough with phlegm for at least three months a year for two consecutive years (43). However, this definition is not consistently used in COPD research. Alternative definitions exist and may alter the composition of this group (44), whereas the GOLD strategy document does not use the term chronic bronchitis in its definitions or in its therapy recommendations (4). A problem with using a symptom based definition is its lack of specificity, since other diseases such as bronchiectasis or chronic infection provide similar symptoms. The pathophysiology behind chronic bronchitis is complex, as it includes most of the cells and tissue in the lungs in addition to several different mechanisms of immunity. The anatomical characteristics of chronic bronchitis consist of narrowing of the bronchial lumen due to several concurrent disease processes. Some use the term bronchitis to describe the hypersecretion seen in the larger bronchi, while the term *small airway disease* (SAD) or *chronic obliterative bronchiolitis* describes the obstruction of the smallest airways. Clinically it is difficult to differentiate between these two conditions, and whether it is purposeful to separate between them is unclear. Early stage bronchitis is characterized by hypertrophy of mucus secreting Goblet cells and smooth muscle cells which is considered at least partly reversible. In later stage disease, the number of Goblet cells increases, and there is also a reduction of the ciliated pseudostratified epithelium in favor of hyperplasia and/or metaplasia of squamous epithelium (45), both contributing to reduced mucus clearance. In the bronchial wall, the smooth muscle layer is thickened with an impaired ability of relaxation. There is also an increase in fibroblasts, and elastic fibers are replaced by stiffer collagen deposits. The total number of immune cells is increased, neutrophils and macrophages pass rapidly from the blood into the bronchial lumen, while the bronchial wall is dominated by T-lymphocytes (46).

The cytokine pattern is corresponding to the cellular inflammatory response. Sputum samples display elevated levels of the proinflammatory IL-1, IL-6, IL-8 and TNF-α (30), in addition a range of different cytokines are involved to different degree (29). Growth factors may be released by epithelium, smooth muscle and macrophages upon

tissue damage and inflammation, TGFβ, EGF and VEGF are likely involved in bronchial wall repair, but also in pathologic remodeling and cell apoptosis contributing to tissue destruction. It is also worth mentioning the intracellular messenger cyclic adenosine monophosphate (cAMP). This downstream messenger may promote relaxation of airway smooth muscle and thus bronchial dilatation. cAMP is degraded by phosfodiesterases (PDE), in the airways notably PDE4. The role of cAMP and PDE4 in COPD is not well described, but inhibition of PDE4 by roflumilast is established as a therapeutic option in COPD patients (47).

An important characteristic of chronic bronchitis of COPD is the persistent inflammation seen in several patients after smoking cessation (48, 49). This is a large and complex topic involving heterogeneous disease mechanisms. Important factors likely includes memory T- and B-cells, altered transcription of proinflammatory genes, and possibly also changes of the bacterial microbiome (40, 50).

Emphysema

Unlike chronic bronchitis, emphysema is not defined based on symptoms, but on pathoanatomical changes in the distal airways. Emphysema is characterized with destruction of alveolar walls, replacing the alveoli with enlarged airspaces/bullae. A histologic diagnosis of emphysema is usually not available in clinical practice, whereas a plain chest x-ray is insensitive for early stage emphysema. The development of the high-resolution CT-scan (HRCT) has made both the diagnostics and grading of emphysema far more accessible in the clinic.

The likely main mechanism behind this process is the earlier mentioned imbalance between anti-microbial proteases and the counter regulative anti-proteases (38). A main source of protease secretion is neutrophils and macrophages, releasing neutrophil elastase (NSE), different matrix metalloproteinases (MMPs) and a range of different other protease categories (51, 52). An important anti-protease is the α1-antitrypsin (A1AT), an inhibitor of NSE. Patients with genetically caused A1AT-deficiency have a higher risk of emphysema development. Other anti-proteases such as tissue inhibitors of metalloproteinases (TIMPs) are less explored in COPD, but are likely also involved

in the pathophysiology of emphysema (38). A limitation of emphysema research is that emphysematic tissue in later stages is dominated by tissue destruction with a minimal volume and cell count left for sampling.

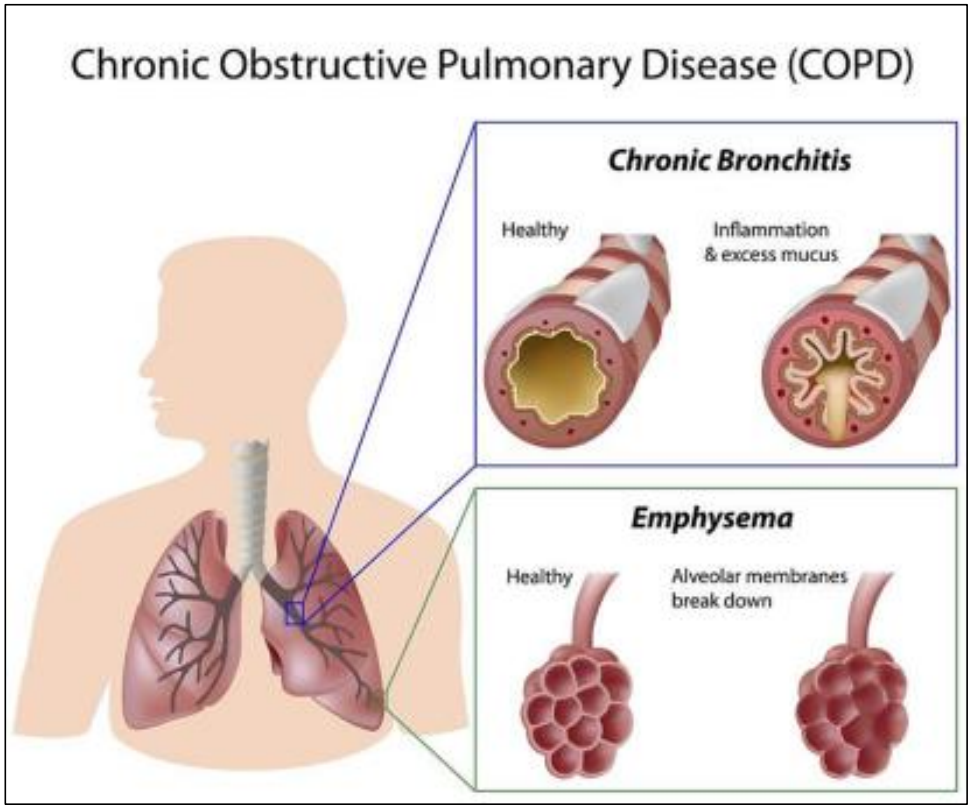

Figure 2. Illustration of chronic bronchitis, with increased amount of inflammatory cells, smooth muscle contraction and fibrosis in the bronchial wall, combined with hypersecretion of mucus, all contributing to airway obstruction. Emphysema illustrated by destruction of alveolar membranes, leading to decreased alveolar surface, loss of capillaries, fibrous remodeling and loss of elastic recoil. (Copyright Shutterstock)

Asthma/COPD overlap

Asthma and COPD have several similarities regarding both symptoms as well as the objective recognition of airway obstruction. Early research on COPD proposed a common etiology and disease mechanism behind these two conditions, the so-called "Dutch hypothesis" (5). This theory has been extensively opposed, and it is today recognized that the epidemiology and the pathophysiology is mostly different for asthma and COPD (53). The immune response in COPD is dominated by

macrophages, neutrophils and T_H1 lymphocytes, whereas in asthma eosinophils, T_H2 lymphocytes and activated mast cells are more common. Similarly, inflammatory signaling in COPD is more dependent on IL-1, IL-8 and TNF-α , while in asthma, the T_H2 cell signature cytokines IL-4, IL-5 and IL-13 are important (29). Further, asthma is considered a large/proximal airway disease, whereas COPD is more prominent in small airways, terminal bronchioles and alveoli. Also, structural changes related to elastin degrading, remodeling and development of fibrosis are typical for late stage COPD and not asthma (13).

Nevertheless, in patients with severe asthma the picture is different. These patients often exhibit an immune cell and cytokine pattern with characteristics from both asthma and COPD. Similarly, COPD patients in the asthma-COPD overlap category may demonstrate eosinophilia and a Th2 cell related cytokine profile, possibly related to genetic factors/altered gene expression in some patients (54, 55). Other characteristics of ACOS are an increased response to corticosteroid treatment and elevated IgE levels compared to standard COPD (54, 56).

Endovascular disease

Although it is not regarded as a clinical phenotype of COPD, the presence of pulmonary vascular disease is of great importance in terms of symptoms and prognosis. The vasculature in the lungs of COPD patients may be affected due to several different disease processes promoting both anatomical and physiological changes. Possible consequences are pulmonary arterial hypertension (PAH) and right ventricular failure, which traditionally have been considered signs of end stage disease.

The effects of cigarette smoking on vasculature in general are well known. Most attention has been given studies of the coronary and systemic circulation, but pulmonary arteries seem to be affected in a similar manner (57). Cigarette smoking is linked to an increased inflammatory response in the pulmonary endothelium, dominated by CD8+ T-cells (58). Pathophysiological changes includes thickening of

the pulmonary endothelial wall and loss of caliber adaptation (59, 60), leading to impaired blood flow adjustment. Another smoking induced mechanism is the earlier mentioned protease-antiprotease imbalance related to emphysema. Lungs with significant emphysema expose a loss of pulmonary capillaries (61), as well as remodeling of the endothelium. Further adding to the disease process, the pulmonary endothelium is particularly prone to vasoconstriction due to hypoxia. In many patients, this may lead to a vicious cycle of increasing hypoxia, additional vasoconstriction and further arterial remodeling and stiffening.

In sum, all these processes leads to an impaired ventilation/perfusion-ratio and development of PAH and consequent right ventricular failure in a share of COPD patients. The significance of PAH development is illustrated by several studies, describing associations between increasing PAH and a lower survival, increasing hypoxia and an increased exacerbation frequency, and where PAH is shown to have a higher predictive value than FEV_1 (62-64). Consequently, PAH development may have a large impact on both symptoms and quality of life in affected COPD patients.

The disease mechanisms behind PAH are complex and incompletely understood, and whether COPD related PAH should be considered a distinct disease in line with primary PAH is debated (65). It is now recognized that PAH may develop also in early disease, and not necessarily in proportion to the degree of airway obstruction (66). In this context, it may be relevant to compare the pathophysiology of COPD with that of general cardiovascular disorders. Conditions such as coronary disease, systemic hypertension and left ventricular failure are all conditions which occur more frequently in COPD patients (67, 68). Smoking is a common risk factor for all these conditions. Similarly, elevated levels of systemic inflammatory markers such as IL-6, TNF-α and CRP are found in patients with cardiovascular disease and PAH (69, 70) as well as in COPD, which is addressed later. Elevated levels of Troponin, BNP and markers of coagulation are other common observations in these conditions (71-73). Thus there are several indications on shared pathophysiological mechanisms between systemic and pulmonary circulation and COPD.

COPD exacerbation and infection

Acute exacerbation of COPD is a common disease characteristic, and is of great importance in terms of prognosis and quality of life (14, 16, 74, 75). AECOPD is usually a clinical diagnosis defined as an episode with worsening of symptoms, where severity assessment may be based on the increasing utilization of health care or on grading of symptoms. The concept "exacerbation" is very difficult to define exactly in pathophysiological terms. To further blur the picture, the differentiation between AECOPD and pneumonia may be unclear due to similarities both in symptoms as well as bacterial findings and inflammatory response (76). There is a multitude of potential triggers of an AECOPD, corresponding to a heterogeneous pathophysiology behind each episode. In a clinical context, an AECOPD can be classified as an infectious or a non-infectious episode. Bacterial and/or viral infection is the cause for a majority of cases, whereas non-infection exacerbation is a less defined group (77, 78).

Bacterial infection has been regarded as the classical cause of symptom worsening of COPD, and isolation of bacterial strains by culture has been described in 50-60 % of AECOPDs (78-80). *Streptococcus pneumoniae*, *Haemophilus influenza*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* are frequently found isolates at AECOPD (79, 81). However, the distinction between stable COPD and AECOPD may be blurred. Pathogenic bacterial strains also may be present in non-exacerbated airways (82), and this has led to uncertainty regarding the causative role of bacteria in AECOPD, and also controversies concerning the necessity of antibiotic treatment (83). Using modern molecular and immunologic techniques, it has been demonstrated that AECOPDs may be associated with acquisition of new bacterial strains or subtypes of the microbes mentioned above (84). However, in many cases of AECOPD the microbiome seems unchanged from the stable state. The concept of respiratory dysbiosis has been proposed as an important factor of the clinically infectious AECOPDs, comprising a dysregulated host immune response leading to an altered microbial growth and sustained inflammation and clinical symptoms (85).

Historically, viral infection was regarded as less important factor in AECOPD. Due to the development of PCR-techniques it is today recognized that viral infection may be a

contributing cause of exacerbation in 40-60 % of cases, where rhinovirus, influenza viruses, respiratory syncytial virus and coronaviruses (other than Covid19/SARS) are most frequently found (86, 87). Viruses may also be found in the airways of stable state COPD (88), but it is more unclear whether this is an element of a persistent virome of the airways, or if they simply represent transient sub-clinical infections. In the case of rhinovirus, experimental studies indicate a temporal relationship between virus-induction and onset of AECOPD (89), and a causal mechanism seems more evident than in the case of bacteria.

The picture becomes even more complicated since many AECOPDs display an increase in both viruses and bacteria. In such cases, viruses are often detected prior to an increase of bacterial load. (90). Further, there are indications that viral infection may hamper the effects of antimicrobial peptides, thus contributing to bacterial growth (91). Dual infection is associated with a higher bacterial load, decline in lung function and longer exacerbation duration (90-92).

The inflammatory response in bacterial AECOPD is similar to that of pneumonia. Sputum and bronchial samplings display an increase in airway neutrophils as well as the neutrophil-related cytokines IL-8 and TNF-α, NSE (93), and also IL-1β, IL-6 and MIP-1 (94). Neutrophils, NSE and IL-8 are also increased at viral AECOPD, whereas eosinophils (92), IP10 and CCL5/RANTES seem more related to viruses (78). IFN is another important factor in the immune response against viral infection. In COPD some studies show that IFN secretion is lower in COPD patients than in non-COPD controls, indicating an impaired antiviral immunity in COPD (91).

Non-infectious exacerbation is a heterogeneous category. In many cases the patient can identify a trigger of the exacerbation, such as specific allergens or air pollutants. Increased urban air pollution is associated with both decline in lung function and a higher AECOPD rate (95, 96). Similarly, COPD patients with characteristics of allergy have an increased risk of exacerbations (97). Allergen exposure as a trigger of exacerbations is a central disease characteristic of asthma, and similar disease mechanisms may be responsible for the high AECOPD rate seen in ACOS-patients (55).

The number of chemical substances with a potential to harm the airways seems indefinite. Both organic and inorganic substances, often acting in synergy with physical factors such as temperature, wind or moisture, may trigger a range of immune responses in the lungs. Air pollutants such as NO_2, SO_2 or O_3 may directly damage lung tissue by the formation of reactive oxygen species (ROS) (98), but in most cases tissue damage is related to inappropriate or excessive immune responses to the different allergens and pollutants. The pathophysiological mechanisms are heterogeneous, and the impact of different types of allergy makes the picture highly complex. Due to this, it is difficult to identify a common inflammatory pathway of non-infectious AECOPD.

The role of eosinophils at AECOPD is increasingly receiving attention. Its role is not as obvious as in asthma, but it has emerged as a clinical useful marker at AECOPD (99). The role of eosinophilic inflammation in non-infectious exacerbations is yet unclear, and it does not appear to be a specific marker for this category as a whole. However, it is recognized that exacerbations characterized with increased sputum eosinophil cell count differs substantially from those related to viruses and bacteria both in symptoms and in response to corticosteroids (78).

In some cases no particular inflammatory pathways seems to be activated, denoted as pauci-inflammatory exacerbations. Parameters of both local and systemic inflammation are to a lesser degree elevated. Some may argue that worsening of symptoms without a subsequent elevation of local or systemic inflammatory parameters should not be defined as an AECOPD (100), and the physician should investigate for an alternative diagnosis. On the other hand, compared with AECOPDs with an obvious trigger, the pauci-inflammatory exacerbations are more frequent in patients with a lower FEV_1, a faster FEV_1 decline, a higher pack-year number smoked and a higher AECOPD rate (78), all central characteristics of COPD.

Due to similarities both in symptoms and risk factors, cardiovascular disease is an important AECOPD differential diagnosis in this context. Elevated serum troponin and ischemic ECG changes are both common observations at AECOPD (101, 102). It is also recognized that AECOPD is a risk factor of myocardial infarction (103), and that

elevated serum troponin at AECOPD is a risk factor for all-cause mortality as well as an indicator of ischemic heart disease requiring revascularization (104, 105). Similarly, venous thromboembolism, most notably pulmonary embolism, is a common complication to AECOPD, especially in non-infectious events (106, 107).

In general, hypercoagulability is a common observation in AECOPD as well as in several inflammatory disorders (108-110). There is a complex interaction between inflammation and coagulation with interlinked pathways of activation (111). It is not unlikely that some cases of non-infectious AECOPD actually are misdiagnosed events of ischemic heart disease or pulmonary embolism. Nevertheless, in other cases the picture is more unclear. Symptoms, biomarkers and diagnostic imaging often seem to indicate concurrent lung and cardiovascular pathology, and it may be impossible and perhaps unwanted to differentiate between the different systems.

Systemic inflammation in COPD

The immune system is constantly aware of any traumatic, toxic or infectious injury, and an insufficient or passive reaction to any threat can be fatal. On the other hand, an active immune system can also be harmful, as inflammation itself is implicated in the pathogenesis in numerous disorders (112). Although the term "systemic inflammation" is often referred to in COPD research, neither words of the term are well defined. The differentiation between low-grade inflammation and an alert immune system can be difficult in the absence of clinical symptom, and is often defined in research as increased levels of one or more inflammatory biomarkers.

The term systemic can be defined as the opposite of localized, thus potentially involving all organ systems in the human body. Any "systemic" analysis or measurement, however, reflects in most cases blood or plasma/serum-sampling from the systemic circulation. An important question is to which degree systemic inflammation in COPD is representative of the inflammatory process in the lungs. A review by Sinden et al supports the concept of inflammation "overspill" from the lungs to the systemic circulation (113), but there is not necessarily a proportionate "overspill" of all immune system components.

Systemic inflammation was early recognized as an inflammatory characteristic in atherosclerosis, a frequent co-morbidity in COPD (114). In addition, diabetes, cachexia, osteoporosis, conditions often seen in COPD patients, have all been linked to increased systemic inflammation, thus it has been rational to also investigate this picture in also in COPD.

Biomarkers

The US National Institutes of Health defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (115). According to this definition any measureable parameter can be a biomarker in COPD, including clinical characteristics and lung function measurements, where FEV_1 is an example of a frequently used biomarker. In medical science, however, research on biomarkers is frequently related to measurements or quantification of cells or molecules in tissue or blood. In 2009, Sin and Vestbo proposed criteria for useful biomarkers in COPD (116). First, the biomarker should have a biological plausible role in the pathophysiology of COPD. Second, the biomarker should be independently and consistently be associated with both COPD as well as hard clinical outcome like hospitalization or mortality. Third, and possibly most difficult to obtain, the biomarker should be modifiable by intervention, and a change in biomarker status should result in change of important clinical outcome. In addition to these criteria, clinical useful biomarkers in COPD should have the ability to aid in the diagnosis of COPD and AECOPD and its phenotypes.

A common way of assessing clinical biomarkers is by the use of ROC (Receiver Operating Characteristic)-curves and AUC-values. Whereas good clinical biomarkers have AUC-values between 0.9 and 1.0, the best COPD biomarkers have AUC-values around 0.7, thus with limited clinical value (117).

Diagnostic biomarkers of COPD

A large number of potential biomarkers have been evaluated in COPD research. Due to its central role in COPD pathogenesis, components of the immune system have been

of particular interest, and much emphasis has been put in the search of the most relevant markers. Many of the traditional markers of inflammation are elevated in patients with airway obstruction due to COPD. C-Reactive Protein (CRP) is a widely used clinical biomarker of inflammation (118). Several studies report elevated CRP at stable COPD, and there is an inverse relationship between increasing CRP and declining FEV_1 (119-121). Further, increased CRP levels are associated with obesity, increasing dyspnea and functional impairment. Although CRP seems to have an independent association with airway obstruction, its role in COPD pathogenesis is unclear. CRP is produced in the liver in response to several inflammatory cytokines and has a role in complement activation (118). CRP is not considered an upstream initiator of inflammation and it is not specific for lung pathology. Thus, despite being a sensitive biomarker in COPD, its lack of specificity implies a limited value in COPD diagnostics. TNF-α is mentioned several times as a central biomarker in COPD, but also for several different other conditions. TNF-α is found to be elevated in plasma and higher levels are also associated with a lower FEV_1 (122). Similarly IL-1, IL-6 and IL-8 may be regarded as general biomarkers of COPD.

The mentioned markers are not specific for lung inflammation, which obviously is a shortcoming in the search of the ideal diagnostic biomarker. Surfactant protein D (SP-D) is a lung specific protein with possible anti-inflammatory function, and where serum levels are significantly elevated in COPD patients (123, 124). Another lung specific protein is club cell secretary protein 16 (CC16, formerly known as Clara cell protein 16), which is secreted from Clara cells in the bronchioles. Serum CC16 is significantly reduced in COPD patients, which may be related to epithelial damage or dysfunction (125). Endocan, or endothelial cell specific molecule-1, is another marker of pulmonary epithelial injury, where elevated serum levels are found in COPD, but also other lung conditions (126). Common for all these markers, although specific, they are not yet clinically useful due to a too low sensitivity.

Diagnostic biomarkers of AECOPD

The identification of an acute exacerbation of COPD is not always straightforward, since acute dyspnea may represent several different diagnoses requiring urgent

treatment. If comparing COPD and coronary atherosclerosis, AECOPD may be the equivalent to myocardial infarction. But where the cardiologist may rely on measurement of serum Troponin as a specific biomarker of myocardial damage with AUC-value above 0.9, no similar biomarker is available as a diagnostic marker of AECOPD. CRP is a widely measured parameter in suspected AECOPD. In a systematic review of biomarkers by Chen in 2016, 26 of 28 AECOPD biomarker studies report elevated levels of CRP at AECOPD (127). CRP is readily available as a frequently used clinical marker of inflammation, but correspondingly, its specificity is lacking. Similarly, leucocyte cell count, and especially neutrophils, is frequently elevated at AECOPD, whereas lymphocytes are not seen to rise at a similar degree. The Neutrophil/Lymphocyte Ratio (NLR) is proposed as a sensitive marker of AECOPD, and may also be considered as a marker of bacterial infection (128, 129). TNF-α is described to be elevated at COPD exacerbation, which is expected due to its role. TNF-α has also been given much attention as a possible target of medical treatment, but with negative results (130).

In 2006, Hurst et al examined 36 different inflammatory biomarkers both at stable COPD and at AECOPD (131). CRP and IL-6 were the two markers associated with the largest increase at AECOPD, but none of the examined inflammatory markers had the statistical ability to perform as an independent AECOPD biomarker. Bafadhel et al performed a cluster analysis of several sputum and serum/blood biomarkers at AECOPD in 145 patients (78). CRP and IL-1 were identified as markers of bacterial exacerbation, CXCL10 (IP10) was related to viral infection, whereas eosinophil count was a third significant marker. Again, no marker had a ROC-value above 0.7 in determining an AECOPD.

Biomarkers as predictors of future AECOPD

Parallel with research on the multifaceted mechanisms of AECOPD, several studies have also attempted to identify risk factors for encountering future AECOPD, thus finding the characteristics for the “frequent exacerbator” phenotype. In this research, the most apparent predictors of future AECOPD have been clinical observations, and one of the best predictors of exacerbations is simply a history of previous

exacerbations (132). Increasing disease severity, measured by the degree of airway obstruction (131, 132), is associated with increase future AECOPD. Similarly, indications of pulmonary hypertension, measured by right heart catheterization (63), but also by a CT measurement of the pulmonary artery/aorta (PA/A) ratio >1, (64) are good predictors of future AECOPD. However, this information is more difficult to apprehend in a clinical setting. Measurement of plasma BNP is a more accessible, but also non-specific marker where high levels indicate a shorter time to AECOPD (133).

Other clinical parameters associated with future AECOPD include gastroesophageal reflux, depression, and/or a low measured quality of life index (134-136). In addition, the presence of the traditional phenotypes chronic bronchitis and emphysema are both related with a higher AECOPD frequency (137-139).

Many inflammatory biomarkers, especially those involved in present AECOPD, have also been evaluated as predictors of future AECOPD. Plasma fibrinogen was described as one of the first predictors of AECOPD, but also levels of WBC, CRP, IL-6, IL-8 and TNF-α are indirectly associated with future AECOPD (15, 20, 21, 140). Among the differential counts of WBC, eosinophils may be associated with a higher AECOPD count, but this an uncertain finding since many studies reporting significant findings do not properly exclude patients with asthma or ACOS (141). Neutrophils and leucocytes are also evaluated independently as predictive biomarkers with uncertain findings, whereas the NLR may be more sensitive (128). Other described predictors of future AECOPD are serum uric acid and the lung specific SPD (142, 143), whereas Fetuin-A, a liver-synthesized inhibitor of systemic inflammation has an inverse relationship with AECOPD frequency (144). In addition to blood sampling, increased levels of sputum inflammatory markers are also shown to be related to the AECOPD frequency (145).

Biomarkers as predictors of decline in FEV_1

Airway obstruction has always been the main characteristic in COPD, and a major research question has been why some patients have a faster decline in lung function than others. The initial theory by Fletcher et al proposing an ever accelerating decline

of FEV_1 as a common attribute of all COPD has been questioned the last decades, and today it seems obvious that the decline of FEV_1 may vary significantly between patients or phenotypes (146, 147). Several biomarkers associated with a low FEV_1 have also been evaluated as predictors for a faster lung function decline in longitudinal studies. A study by Donaldson et al (2005) found an association between elevated sputum levels of IL-6, IL-8, neutrophils and eosinophils and a faster decline in FEV_1 (22). Similarly and in the same study, plasma fibrinogen was associated with a faster FEV_1 decline, thus in a relative small number (n=147). Higashimoto et al (2009) described an association between both serum CRP, matrix metalloproteinase-9 (MMP-9) and plasma fibrinogen and a faster decline in FEV_1 (148), whereas in the larger Eclipse study, fibrinogen was only associated with baseline FEV_1, and not longitudinal change (146). Also in the Eclipse study, IL-6, IL-8, CRP, TNF-α and SPD were evaluated regarding the same outcome with negative results, however baseline levels of club cell secretory protein 16 (CC-16) was associated with a significant change in FEV_1; 4 ml /SD increase (p=0.04), thus low CC-16 were associated with a faster decline, hence its potential protective role (149). Pro-surfactant protein B (PSFTPB) is another lung specific marker. Leung described an association between high levels of PSFTPB and a faster FEV_1 decline (150).

A drawback of several of these proposed biomarkers is the lack of validation in other studies. The OLIN-study evaluated serum levels of the protease MMP-9 and the antiprotease Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) in a large cohort of COPD patients, and found no association with decline in FEV1 over time (151). In the more recent SUMMIT-trial, the biomarkers CRP, SPD, soluble receptor of activated glycogen end-products (s-RAGE), and CC-16 were evaluated in a subpopulation with moderate COPD, no associations between any biomarker and decline in FEV_1 were found (152).

Biomarkers for increased mortality in COPD

COPD is a major cause of death worldwide. Exacerbations of COPD and declining FEV_1 are already mentioned as major factors associated with a higher mortality, and several of the biomarkers related to frequent AECOPDs and a faster drop in FEV_1 have

also been evaluated in this context. CRP and fibrinogen are both examples of biomarkers associated with a higher mortality in COPD (152-154). A study by Agusti et al investigated CRP, WBC, IL-6, IL-8 and TNF-α, and found a higher mortality in patients with persistent high levels of two or more markers. The NLR is another marker associated with higher mortality (155).

The connection between COPD and cardiovascular disease has already been mentioned, and patients with these two conditions have a significantly worse prognosis compared with those with single disease (156). Even though it is specific for cardiac muscle injury, elevated levels of Troponin has emerged as an independent predictor for increased mortality, this is particularly obvious when measuring Troponin at AECOPD (71), but also when evaluating Troponin at stable COPD (157). B-type natriuretic peptides (BNP) are another class of cardiovascular biomarkers. They are frequently elevated in COPD, but their role as predictive biomarker is more uncertain as different studies report diverging results (72).

Closely related to cardiovascular disease is the coagulation system. In addition to the already mentioned fibrinogen, elevated D-dimer at AECOPD is linked to a higher mortality (158). We have also demonstrated a similar association when measuring D-dimer in stable COPD as a part of the BCCS (159).

Biomarkers in clinical use

In summary, clinically useful inflammatory biomarkers in COPD are sparse. Perhaps the most obvious shortcoming is the lack of biomarkers specific for lung damage or COPD pathophysiology. In clinical practice, CRP and leukocyte counts are widely used biomarkers, but then as markers of infection not specific for COPD. The eosinophil count is established as an indicator of expected corticosteroid treatment effect, but is unspecific and relevant in only a minority of COPD patients. There are no inflammatory biomarkers which can significantly aid the clinician when diagnosing COPD or AECOPD.

Inflammatory biomarkers as therapeutic targets in airway inflammation are mostly linked to the *type 2* immune response. Apart from eosinophils, especially IL-5, but also

IL-4 and IL-13 are recognized as treatment targets in asthma (160, 161), but not ACOS or COPD. Attempts have been made to target other cytokines central in COPD inflammatory pathways such as TNF-α, IL-1, IL-8 and IL17, but without success (130, 162-165).

There has been a massive interest on COPD biomarker research the last decades. Hundreds of cytokines and other components of COPD pathophysiology have been identified and described according to the different characteristics and outcome of COPD. None of these are close to fulfill the requirements of the ideal COPD biomarker (116) due to the limitations mentioned. There is a great need for better biomarkers in COPD in order to better understand a multifaceted pathophysiology, to improve diagnostics, and finally to develop effective treatment of this challenging disease.

Summary of selected studies reporting associations between inflammatory biomarkers and COPD related outcomes	Author	Year	Country	Number of participants	Study subjects	Biomarkers studied	Study outcome	Main findings
C-reactive Protein As a Predictor of Prognosis in Chronic Obstructive Pulmonary Disease	Dahl M	2006	Denmark	1302	Airway obstruction with no known COPD diagnosis	CRP	Hospitalizations and mortality	CRP predictor of AECOPD rate and mortality
Persistent Systemic Inflammation is Associated with Poor Clinical Outcomes in COPD: A Novel Phenotype	Agusti	2012	International	1755 +297+202	COPD patients + smokers and healthy controls	WBC, CRP, IL-6, IL-8, Fibrinogen TNFα	Mortality, exacerbations	Persistent systemic inflammation associated with diagnosis of COPD, future AECOPD and mortality
Serum biomarkers and outcomes in patients with moderate COPD: a substudy of the randomised SUMMIT trial	Celli	2019	International	1673	COPD patients	CRP, sRAGE, SPD, Fibrinogen, CC-16	FEV1-decline, mortality, AECOPD	CRP and Fibrinogen associated with mortality, no biomarkers associated with FEV1-decline or AECOPD
Blood Biomarkers as Predictors of Long-Term Mortality in COPD	Mendy	2018	USA	431	COPD patients	CRP, neutrophils, Eosinophils	Mortality	CRP and neutrophils associacted with increased mortality, eosinophils reduced mortality
Predictive value of neutrophil to lymphocyte ratio in patients with acute exacerbation of chronic obstructive pulmonary disease	Teng	2018	China	698	AECOPD, clinical diagnosis	Neutrophil/ lymphocyte-ratio (NLR)	Mortality	NLR associated with increased mortality
Serum uric acid as a predictor of mortality and future exacerbations of COPD	Barziokas K	2014	Greece	314	COPD patients	Serum-uric acid	Mortality, time to first exacerbation	Uric acid associated with mortality and future exacerbation count
High levels of interleukin-6 in the exhaled breath condensate of patients with COPD	Bucchioni	2003	Italy	16 + 12	COPD + controls	Exhaled condensate levels of IL-6, CRP	COPD	IL-6 elevated in exhaled condensate in COPD patients
Plasma fibronogen as a Biomarker for Mortality and Hospitalized Exacerbations in People with COPD	Mannino	2015	International	6376	COPD patients + airway obstrucion	High vs low fibrinogen	3 year mortality, 1 year exacerbations, time to first exacerbation	High fibrinogen preditor for mortality and exacerbations
Blood fibrinogen as a biomarker of chronic obstructive pulmonary disease	Duvoix	2012	UK	n/a	COPD patients	Fibrinogen	Multiple	Fibrinogen associated with COPD, emphysema, faster FEV1-decline, mortality and exacerbation count
Fibrinogen, COPD and Mortality in a Nationally Representative U.S. Cohort	Mannino	2012	USA	8507	COPD patients + airway obstrucion	High vs low fibrinoge	All-cause mortality	Høy fibrinogen prediktor for mortalitet

Table 1. Summary of selected studies reporting associations between inflammatory biomarkers and COPD related outcomes.

Summary of selected studies reporting associations between inflammatory biomarkers and COPD related outcomes	Author	Year	Country	Number of participants	Study subjects	Biomarkers studied	Study outcome	Main findings
Prognostic role of D-dimer for in-hospital and 1-year mortality in exacerbations of COPD	Hu	2016	China	434	AECOPD	D-dimer	Hospital mortality and 1 year mortality	D-dimer predictor for mortality at hospital admitted AECOPD
High-sensitivity troponin I and all-cause mortality in patients with stable COPD: an analysis of the COSYCONET study	Waschki	2020	Germany	2085	COPD patients	Troponin	Mortality	Troponin associated with increased mortality in stable COPD
Troponin T elevation and long-term mortality after chronic obstructive pulmonary disease exacerbation	Brekke	2008	Norway	897	AECOPD	Troponin	All-cause mortality	Troponin measured at AECOPD associated with increased mortality
Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease	Di Francia	1994	France	16+14	30 COPD patients in two weight groups + controls	TNFα	COPD diagnosis, weight loss	TNFα associated with diagnosis of COPD and weight loss in COPD patients
Inflammatory Response and Body Composition in Chronic Obstructive Pulmonary Disease	Eid	2001	UK	80	COPD patients	CRP, NEAPC, IL6, TNFα, STNF-R1/2	Body composition	Increased levels of inflammatory markers associated with weight loss
Obstructive and restrictive lung disease and markers of inflammation: data from the Third National Health and Nutrition Examination	Mannino	2003	USA	15697	2366 COPD patients + controls	CRP, Fibrinogen	COPD diagnosis	CRP and Fibrinogen elevated in COPD patients
Surfactant protein D, a clinical biomarker for chronic obstructive pulmonary disease with excellent discriminant values	Akiki	2016	Lebanon	90 + 124 + 180	COPD + asthma + controls	SPD	COPD	SPD elevated in COPD/ specific for COPD
Protective role for club cell secretory protein-16 (CC16) in the development of COPD.	Laucho-Contrera	2015	USA	6 +6 +7	COPD + smokers + non-smokers	CC16	COPD	Reduced CC16 in COPD and smokers
Use of Plasma Biomarkers at Exacerbation of Chronic Obstructive Pulmonary Disease	Hurst	2006	UK	90	COPD patients	36 inflammatory markers	AECOPD	CRP best biomarker at AECOPD
Changes in forced expiratory volume in 1 second over time in COPD	Vestbo	2011	International	2163	COPD patients	7 inflammatory markers	Decline FEV1	CC16 associated with faster FEV1 decline

Table 1. cont.

Summary of selected studies reporting associations between inflammatory biomarkers and COPD related outcomes	Author	Year	Country	Number of participants	Study subjects	Biomarkers studied	Study outcome	Main findings
Inflammatory biomarkers in sputum predict COPD exacerbations	Tufvesson E	2013	Sweden	43	COPD with 1+ yearly AECOPD	Sputum leukotriene B4, IL-8, CRP	AECOPD	Leukotriene B4 and IL-8 increased in sputum shortly before AECOPD
Susceptibility to Exacerbation in Chronic Obstruktive Pulmonary Disease	Hurst JR + Eclipse	2010	International	2138	COPD, FEV1<80, ratio<70	Clinical parametres, 6 systemic markers	Future exacerbations	History of AECOPD, low FEV1, SGRQ, reflux and WBC predictors for future AECOPD
Increased Systemic Inflammation is a risk factor for COPD exacerbations	Groenewegen	2008	Netherlands		FEV1 30-70%, ratio below under 89% exp	Fibrinogen	AECOPD	Fibrinogen predictor for future AECOPD
Inflammatory Biomarkers and Exacerbations in COPD	Thomsen	2013	Danmark	6574	COPD patients	1,2 or3 elevated markers (CRP, WBC, fibrinogen)	Moderate/severe AECOPD, 2+ Per year	2-3 increased biomarkers associated with future exacerbations
Macrophage Migration Inhibitory Factor (MIF) is a Novel Determinant of Cigarette Smoke-induced Lung Damage	Fallica et al	2014	USA	18 + 32	Mild COPD	MIF	COPD	MIF reduced in COPD patients
Macrophage Migration Inhibitory Factor Deficiency in Chronic Obstructive Pulmonary Disease	Sauler et al	2014	USA	224 (72>65, 32 COPD)	COPD aged 18-45 +>65 + controls	MIF	COPD	Reduced MIF at COPD in patients >65
Growth Differentiation Factor-15 is a novel biomarker predicting acute exacerbation of chronic obstructive pulmonary disease	Mutlu et al	2015	Turkey	87	AECOPD, stable COPD, controls	CRP, GDF-15	COPD, AECOPD admission	GDF-15 elevated in COPD, additional elevation at AECOPD
Growth differentiation factor-15 is associated with muscle mass in chronic obstructive pulmonary disease and promotes muscle wasting in vivo	Patel	2015	UK	94 + 25	COPD patients from two outpatient cohorts + controls	GDF-15	GDF-15 serum + Muscle biopsy	GDF-15 associated with COPD, low muscle diameter and 6MWT
Association of the biomarkers soluble ST2, galectin-3 and growth-differentiation factor-15with heart failure and other non-cardiac diseases	Mueller	2015	Austria	22+15	22 controls + 15 COPD	GDF-15	COPD	GDF-15 increased at COPD
Elevated plasma fibrinogen associated with reduced pulmonary function and increased risk of chronic obstructive pulmonary disease	Dahl	2001	Denmark	8955	General population	Fibrinogen	COPD, AECOPD admission	Increased fibronogen associated with COPD and hospital admissions

Table 1. cont.

Summary of selected studies reporting associations between inflammatory biomarkers and COPD related outcomes	Author	Year	Country	Number of participants	Study subjects	Biomarkers studied	Study outcome	Main findings
Elevated plasma procoagulant and fibrinolytic markers in patients with chronic obstructive pulmonary disease	Ashitani	2002	Japan	40 +20	COPD patients	Coagulation factors	COPS vs controls change pO2 and FEV1	Coagulation factors associated with COPD, faster decline of FEV1
The effects of hypoxia on markers of coagulation and systemic inflammation in patients with COPD	Sabit	2010	UK	20	Mild COPD	TAT, FPA, tPA, TG + hypoxia	COPD, hypoxia	Increase of TAT, FPA og D-dimer after hypoxia
Circulating Tissue Factor Procoagulant Activity is Elevated in Stable Moderate to Severe Chronic Obstructive Pulmonary Disease	Vaidyula	2009	USA	11+45	COPD patients	TF, fVII, fVIII, TAT	COPD	Increased TF and TAT in COPD
Effect of acute exacerbations on circulating endothelial, clotting and fibrinolytic markers in COPD patients	Polosa	2011	Italy	30	COPD patients	IL6, vWF, D-dimer, pro-thrombin	Exacerbation and stable phase	IL6, pro-thrombin increased at exacerbation
D-dimer as a potential biomarker for the progression of COPD	Zhang	2015	China	43	COPD patients	D-dimer	Stable COPD and AECOPD	D-dimer increased in COPD, additional increase at AECOPD
Airways obstruction and the risk for lung cancer	Tockman	1987	USA	N/A	Smokers	Age, smoking, airway obstruction	Lung cancer	Airway obstruction indicator for lung cancer
Ventilatory function and chronic mucus hypersecretion as predictors of death from lung cancer	Lange	1990	Denmark	13640	General population	Age, sex, smoking	Death from lung cancer	Low FEV1 and chronic bronchitis risk factors for death of lung cancer
Chronic obstructive pulmonary disease mortality in six U.S. cities	Speizer	1989	USA	8427	General population	Age, respiratory symptoms, smoking	Death from lung cancer	Low FEV1 risk factor for death of lung cancer
Incidence of non-pulmonary cancer and lung cancer by amount of emphysema and airway wall thickness: a community-based cohort	Aamli	2017	Norway	947	COPD patients	None	Lung cancer	Emphysema risk factor for both lung cancer and non-pulmonary cancer

Table 1. cont.

Summary of selected studies reporting associations between inflammatory biomarkers and COPD related outcomes	Author	Year	Country	Number of participants	Study subjects	Biomarkers studied	Study outcome	Main findings
Association of radiographic emphysema and airflow obstruction with lung cancer	Wilson	2008	USA	3642	Smokers 50-79 years old	Sex, age, smoking, FEV1, emphysema	Lung cancer	Emphysema independent risk factor for lung cancer
Assessing the relationship between lung cancer risk and emphysema detected on low-dose CT of the chest	de Torres	2007	USA	1166	Smokers 40+ old	Emphysema, age, sex, packyears	Lung cancer	Emphysema risk factor for lung cancer. Adenocarsinoma and upper lobe cancer predominant
Inhaled Corticosteroids and Risk of Lung Cancer among Patients with Chronic Obstructive Pulmonary Disease	Parimon	2006	USA	10474	General population/veterans	Age, Smoking, beta2, ICS	Lung cancer	Dose-dependent inverse association between ICS and lung cancer
Inhaled corticosteroids and risk of lung cancer among COPD patients who quit smoking	Kiri	2008	UK	7079	COPD patients from general practice (GPRD)	Age, sex, smoking, comorbidities, medication	Lung cancer	Dose-dependent inverse association between ICS and lung cancer
Oxidative stress and inflammation in the normal airways and blood of patients with lung cancer and COPD	Barreiro	2013	Spain	52 +21	Lung Cancer vs COPD	Inflammatory markers in biopsy and plasma	Lung cancer	Increased levels of oxidative stress in biopsy and plasma in Lung Cancer
Cytokine profile determined by data-mining analysis set into clusters of non-small-cell lung cancer according to prognosis	Barrera	2014	Mexico	110 + 25	Lung Cancer vs healthy controls	Inflammatory markers/cytokines in plasma	Lung cancer	Increased levels of cytokines in plasma in Lung Cancer
Pretreatment Serum Levels of Cytokines and Cytokine Receptors in Patients with Non-Small Cell Lung Cancer, and Correlations with Clinicopathological Features and Prognosis	Kaminska	2006	Poland	103 + 50	Lung Cancer vs healthy controls	Inflammatory markers/cytokines in plasma	Lung cancer	Increased levels of cytokines in plasma in Lung Cancer

Table 1. cont.

OBJECTIVES

1 Identify inflammatory biomarkers for the diagnosis of COPD and COPD exacerbation using cross-sectional data, and evaluate these biomarkers according to important COPD characteristics.

2 Identify inflammatory biomarkers as predictors for longitudinal outcome in COPD using longitudinal data:

a. As predictors for future exacerbations
b. As predictors for change in lung function
c. As predictors for mortality and cause of death
d. As predictors for lung cancer

MATERIALS AND METHODS

Study design and study population

The studies in this thesis are based on data from the Bergen COPD Cohort Study (BCCS) and the Bergen COPD Exacerbation Study (BCES), with additional data from the Norwegian Cancer registry and the Norwegian Cause of Death Registry. The BCCS consisted of 433 COPD patients and 325 healthy controls. Of the 433 COPD patients, 356 patients living in the vicinity of Haukeland University Hospital were also included in the BCES, which was conducted in parallel with the BCCS. Both studies were observation studies without specific intervention. Inclusion started in February 2006 and was closed December 2009.

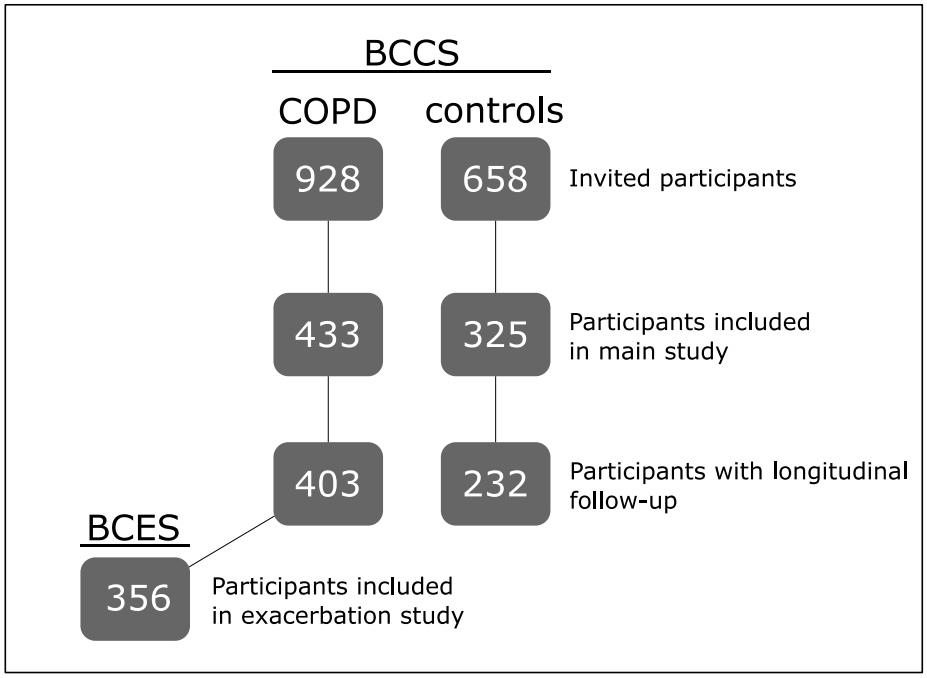

Figure 3. Number of patients and controls invited, included and longitudinally assessed. Out of 433 COPD patients, 8 patients died, 10 withdrew consent and 12 patients used oral corticosteroids, thus 30 patients were. Number of invited controls is an estimate. Never-smoking controls were only examined at baseline.

The Bergen COPD Cohort Study (BCCS)

The BCCS was designed as a case-control cohort study with intentional follow-up each 6 months for 3 years. The COPD patients were recruited from several sources; two prior regional studies on lung patients; the Hordaland County Study (166, 167) and the GenKOLS Study (168), local private practices in pulmonary medicine, and outpatient clinics from regional hospitals. Controls were also recruited from the two studies mentioned above, both current and former smokers as well as never smokers. A total of 928 COPD patients were invited to the study, of these 433 patients were included. 12 patients used oral corticosteroids at the time of inclusion and were excluded from follow-up after the baseline visit. 668 subjects were invited as controls, 325 were included in the study, of these were 46 never smokers not included in the longitudinal follow-up.

Inclusion criteria for COPD patients and controls:

- Women and men, age 40-80 years.
- Approved, written consent to study participation before study inclusion.
- Able to comply with the requirements of the study protocol, and available for study follow-up during the three years of the study.

Inclusion criteria for COPD patients only:

- Baseline post-bronchodilatation $FEV_1/FVC \leq 70$ % and $FEV_1 < 80$ % of predicted.
- Smoker or ex-smoker with a minimum exposure of 10 packyears.

Inclusion criteria for controls only:

- Baseline post-bronchodilatation $FEV_1/FVC > 70$ % and $FEV_1 > 85$ % of predicted.
- Smoker or ex-smoker with a minimum exposure of 10 packyears **or** never smoker.

Exclusion criteria:

- Known respiratory disease (other than COPD for cases), including lung cancer, sarcoidosis, tuberculosis, lung fibrosis, cystic fibrosis or prior lung surgery including lung reduction surgery or lung transplantation.
- Known significant inflammatory disorder, including rheumatoid arthritis or systemic lupus erythematosus.
- Known severe alpha-1-antitrypsin deficiency (Pi- SZ or ZZ alleles).
- Known severe and/or uncontrolled disease including severe psychological conditions that might disrupt study attendance or affect the safety of the study subjects.
- Participation in randomized controlled intervention study involving medications or exposure to radiation.
- Known alcohol or drug abuse.
- Blood transfusion within the last 4 weeks.
- COPD exacerbation (cases only) requiring treatment with oral corticosteroids or antibiotics, or hospitalization, within the last 4 weeks before study baseline. Any course with antibiotics of corticosteroids must be finished at least 2 weeks before study baseline (patients could be re-screened for inclusion later).
- Unable to walk.

The BCCS was based on an extensive data collection at study inclusion, combined with longitudinal measurements of several important COPD characteristics each 6 months for 3 years. The most important aim of the BCCS was to identify and evaluate baseline parameters and COPD characteristics as predictors for different longitudinal outcome like change in lung function, Quality of Life, mortality, respiratory failure, 6MWT change of body composition and more, not all relevant for this thesis, but published by other members of our research group.

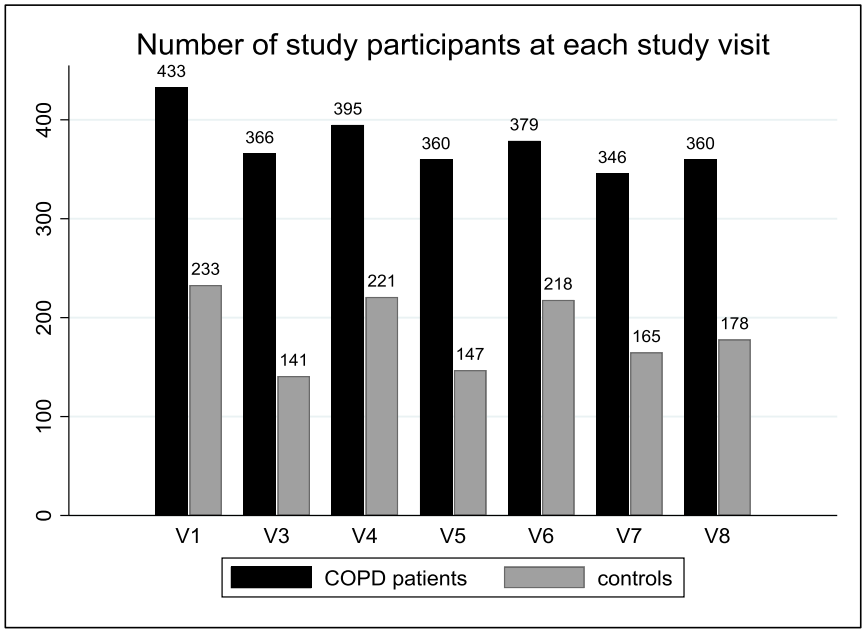

Figure4. Number of COPD patients and controls attending at study visits. Intervals between each study visit were 6 months, except Visit 2 which took place in a subgroup of the patients after 3 months.

The Bergen COPD Exacerbation Study (BCES)

The inclusion and exclusion criteria were the same for both the BCCS and the BCES, with the only difference that the BCES only included 356 COPD patients living in the vicinity in the city of Bergen, serviced by Haukeland University Hospital. All patients were given written and verbal information of the study, and all patients gave a written consent for study participation. The BCES and the BCCS were conducted in parallel with concurrent dates of study initiation. Similarly, the aims of the BCES were to identify and evaluate predictors for COPD exacerbations, but also to investigate exacerbations with bronchial and systemic sampling, assessing both biochemistry and microbiology. Thus, the BCES comprised the unscheduled examinations at COPD exacerbation, whereas the successive registration of exacerbation frequency was a part of the BCCS.

Data collection

Both patients and controls underwent examinations and measurements at inclusion, but also at the study visits. At baseline, all study subjects were examined and interviewed by study physicians. A thorough medical history was obtained, including medication use, clinical symptoms, comorbidities, socioeconomic status, family history, respiratory symptoms including exacerbation and smoking history. In addition, lung function, HRCT, 6-minute walking test, bioelectrical impedance measurement for body composition, blood sampling, sputum sampling and nasopharyngeal aspiration were performed. All patients and a selection of the controls were also intended followed up during study visits each 6 months, for a total of 7 half-yearly visits including baseline during the three years of the study. Patients were examined and interviewed by a study physician regarding symptoms, exacerbations or comorbidities since last visit and change in medication. Lung function was performed at all visits, bioelectrical impedance measurements yearly. Additionally, blood sampling or other microbiological sampling not related to this thesis.

Medications and Comorbidities

All medications and their doses were recorded at each visit, including medication use at exacerbation. Inhalation medications were recorded according to brand name as well as generic name. For the statistical analyses, the class of inhalation drug was used, thus not separating between the different LABAs or ICSs. There was only one LAMA on the market at the time of the study, tiotropium.

All relevant comorbidities were recorded. In addition, the Charlson Comorbidity Score (CCS) was calculated for each study subject (169). The different comorbidities of clinical significance were designated a score of 1, 2, 3 or 6 based on their severity, and then summed up, not including age as a component. All COPD patients had a CCS of at least 1 due to their main disease. For the analyses in our study, CCS was categorized in 0, 1, 2, 3 or 4+.

Symptoms and quality of life

Information on symptoms was registered by different self –complete questionnaires at baseline and at later study visits. All questionnaires were checked for completion after having been filled in at the study center, by our study technicians, and participants given a chance to correct if they had forgotten a question for instance. Degree of breathlessness was assessed with the Medical Research Council (MRC) dyspnea scale (170), scoring dyspnea from 1-5. In addition, the St. George's Respiratory Questionnaire (SGRQ) (171) was used as a measure of respiratory symptoms and quality of life. Clinically relevant symptoms such as coughing and cough with phlegm were recorded as dichotomous variables. The term chronic cough was defined as having cough for three months or more altogether during a year.

COPD Exacerbations

In both the BCCS and the BCES, an acute exacerbation of COPD (AECOPD) was defined as an event with acute worsening of respiratory symptoms with duration of more than 2 days, requiring treatment with antibiotics and/or oral corticosteroids, or hospitalization. This was a health care utilization definition, but in addition, in the BCES, additional symptoms were registered for the evaluation of exacerbations (Appendix C). Similarly, the severity of exacerbations was graded according to health care utilization; where a mild exacerbation did not require any change in treatment beyond short acting bronchodilators, a moderate exacerbation required treatment with antibiotics or oral corticosteroids, and any exacerbation requiring hospital admission was defined as severe. As an alternative, acute exacerbations in the BCES were also graded according to symptoms on a scale of 1-5 (Appendix C).

The exacerbation frequency the year before study inclusion was based on self-report. After study start, all subsequent exacerbations were registered at each study visit; in addition, the patients were contacted by telephone each month where they were asked on symptoms regarding AECOPD. Patients included in the BCES were given a laminated card with contact information to study personnel and information on AECOPD criteria. The BCES telephone was operated 12 hours each day all week. Patients fulfilling AECOPD criteria were either hospitalized, or they were scheduled a visit at the outpatient at the Department of Thoracic Medicine at Haukeland University

Hospital the same or the next working day. If the study physician agreed on the AECOPD criteria, clinical information was collected, and in addition blood sampling and sputum sampling was performed. The patients were all treated according to standard medical care for their AECOPD.

Spirometry

Spirometry was performed on all patients and controls in all regular visits. Spirometry was performed with a Viasys-Jaeger Masterscope CT system (Viasys, Höchberg, Germany) before and after inhalation with 0.4 mg Salbutamol by a metered-dose inhaler (Ventoline, GSK) with the use of an inhalation spacer. All subjects were supervised by trained personnel, and procedures were standardized in accordance with the European Respiratory Society (ERS)/American Thoracic Society (ATS) protocols (172). Spirometry was acceptable if three tests were reproducible and technically adequate, up to 12 attempts was allowed if the subject was able to perform. The test-equipment was calibrated using a 3.0-liter syringe before each subject.

The highest measured values for post-bronchodilatation FEV_1 and FVC were used in the analyses. Predicted values in % were calculated with the use of reference values from a general Norwegian population sample (173).

Body Impedance Measurements

In addition to body weight (kg) and height (m), all subjects went through bioelectrical impedance measurements for calculation of fat mass and fat free mass. Four subjects had a pacemaker, and the measurement was not performed. The Bodystat 1500 (made by Bodystat Ltd, Isle of Man, UK) is a benign version of the electric chair, using low voltage/current to measure impedance of body tissue. The test was performed in supine position, extremities not touching and after resting for at least 50 seconds. Two pairs of electrodes were placed on the right arm and right foot before analysis was performed. Patients were asked to not drink water 4-5 hours before testing, not to smoke or perform physical activity within 12 hours. Bioelectrical impedance measurement was performed at study inclusion, but also at subsequent visits each 12 months for three years.

The Bodystat measurements give information on several parameters regarding body metabolism. For our study, fat mass (kg) and thus lean body mass/fat free mass was estimated. The variables Body Mass Index (BMI), Fat Mass Index (FMI) and Fat Free Mass Index (FFMI) were defined as Body Mass (kg)/ squared body height (m), Fat Mass (kg)/squared body height (m) and Fat Free Mass (kg)/ squared body height (m), respectively.

The Bodystat impedance device was calibrated each week using a Bodystat calibrator, where impedance readings were kept within levels prespecified by the manufacturer. In addition, a reliability study was performed for quality control of the impedance measurer. FMI and FFMI were measured 10 times within one hour in 10 patients and 10 healthy volunteers. The variance for FFMI was 0.47 and 0.54, and for FMI 1.14 and 2.09, in patients and controls respectively (174).

For the statistical analyses, the composite variable Body Composition was created, categorizing patients into three categories: normal, obesity and cachexia. Obesity was defined as having a FMI above 13.5 in women and 9.3 in men, whereas cachexia was defined as having a FFMI below 14.0 in women and 17.0 in men, according to the upper and lower 95 % confidence limit in a normal population (175).

Radiology

HRCT scans were performed on 384 COPD patients soon after inclusion. The CT scans of the thorax were performed using a GE Healthcare multidetector-row CT scanner. Scans were performed with patients in a supine position with full inspiration, without intravenous contrast. Exposure settings were 40 mAs and 120 kVp, images were reconstructed with 1.25 mm continuous slices. CT scans were analyzed with Pulmonary Workstation 2.0 software (VIDA Diagnostics, Iowa City, IA, USA). Emphysema was defined using a threshold technique, measuring the percent of lung parenchyma with an x-ray attenuation below -950 Hounsfield units (176, 177). The degree of emphysema was presented as % of low attenuation areas (% LAA), whereas the cut-off value for a dichotomized variable of emphysema was set at LAA > 10 %.

Laboratory analysis

Peripheral venous blood was drawn for both plasma and serum samples at all visits. Plasma was collected by sampling venous blood into pyrogen-free EDTA collection tubes and centrifuged within 30 minutes at 2150 *g* for 15 minutes at 4°C. The serum samples were coagulated at room temperature for 30-45 minutes, followed by centrifugation at 2500 x g for 15 minutes at 4°C. The plasma and serum samples were aliquoted in 1 mL aliquots and stored in -80°C ultra-freezers until measurements, thus the samples were unthawed up until biomarker measurements.

The biomarkers were analyzed at different occasions after sampling:

NGAL, OPG, CXCL16, TNF-R1, MCP4, NAP2, MBL were measured in 2007 and 2008 by enzyme-linked immunosorbent assays (EIAs) (R&D Systems, Inc, Minneapolis, MN, USA) by our partners in Oslo University Hospital (Aukrust & Ueland et al, Rikshospitalet). The intra- and inter-assay coefficients of variation were <11% for all parameters. Hemoglobin, WBC, Granulocytes, TPC, s-Ferritin, s-Creatinine and CRP were measured in 2007 and 2008 by routine laboratory methods (Modular PP, Roche Diagnostics, Basel, Switzerland). Plasma levels of TNF-α, IL-1, IL-6, were measured in 2009 by our partners at Mie University, Japan (Gabazza et al), using EIA kits (BD Biosciences Pharmingen, San Diego, CA). MIF, ALCAM, CD163, was measured in serum by EIAs provided from (R&D System Inc, Minneapolis, MN) in 2012, again by our partners in Oslo. The intra-assay and inter-assay coefficient of variations were <10% for all parameters.

Plasma levels of IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-SF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, PDGF-BB, MIP-1β and VEGF were measured by Aveo Oncology in 2014 using a magnetic bead multiplex assay (Bio-Plex Pro, Bio-rad laboratories, Inc.). Plasma levels of GDF-15 and Activin A were measured by Aveo Oncology in 2014 using enzyme-linked immunosorbent assays (Quantikine, R&D Systems, Inc). All samples were measured in duplicates, and only accepted if intra-assay variance was less than 10%.

Statistical analysis

The statistical analysis of our study was based on both cross-sectional baseline data at inclusion, and on longitudinal data from the three year follow-up on exacerbations and lung function, and nine years follow-up on cancer diagnosis and mortality. The baseline data was comprehensive for both COPD patients and smoking/non-smoking controls, whereas data from the longitudinal follow-up was relevant only for COPD patients, with the exception of the lung cancer data in paper 4.

Computer software

The statistical analyses were performed in Stata/SE versions 12.1, 13.1 and 14.1 (StataCorp LLC, College Station, Texas, USA). Figures presented in the papers were made in Stata, in GraphPad Prism 7 (GraphPad Software, San Diego, California, USA) and in R version 3.3.2, qgraph package (R Foundation for Statistical Computing, Vienna Austria).

Descriptive analyses

Baseline data from both patients and controls consisted of dichotomous, multi-categorical and continuous variables. For the description of the study populations, categorical variables were presented in numbers and percentages, continuous variables with normally distributed data were presented as mean and standard deviation (SD) values, whereas continuous non-normally distributed data were presented as median and inter quartile range (IQR) values. For the baseline comparison between groups, χ-square test was used for categorical variables, t-test for normally distributed categorical variables, Wilcoxon rank-sum test for continuous variables in two categories, and Kruskal Wallis test for continuous variables in three or more categories. For comparison of paired data, a Wilcoxon equality test on matched data was performed.

Regression models in paper 1:

The main outcome variable was the yearly exacerbation count for each patient, including only moderate and severe exacerbations. The exacerbation count was

analyzed according to a negative binomial distribution with repeated measurements for each year. A random-effects negative binomial model was fitted using the *xtnbreg*-command in Stata. The effect estimates of each variable were reported as Incidence Rate Ratios (IRR) with a corresponding 95 % confidence interval. Several clinical variables as well as inflammatory markers, all with potential associations with the exacerbation rate, were first evaluated bivariately. A p-value of 0.05 was required for statistical significance for all clinical variables. Variables tested were sex, age, body composition (normal/obese/cachectic), smoking habits at study start, exacerbation count 12 months before inclusion (0-1 and 2+), GOLD stage 2-4, hypoxemia ($pO_2 < 8$ kPa), Charlson Comorbidity Score (CCS), chronic cough, use of inhaled steroids and the five inflammatory markers WBC, CRP, NGAL, sTNF-R1 and OPG. All variables were later evaluated in the multiple regression model using a backwards stepwise approach, removing variables with the highest p-value and keeping variables with a p-value below 0.05. All discarded variables were later tested against the model and kept if the p-value was changed to < 0.05. Sex, age, body composition and smoking were considered as important adjustment variables and were kept fixed in the model regardless of p-value. In general, this approach was used for all regression modelling in our project with minor adjustments.

The secondary analysis in paper 1, on the duration of AECOPD, was done using a generalized estimation equation (GEE) model. The outcome was a binary variable of exacerbation duration with a cut-off at >21 days. The model was specified with binomial distribution with a logit link function and exchangeable correlation of the variance, using the *xtgee*-command. Effect estimations were given as odds ratios (OR). Variables tested in the model were the same as in the above model, with the addition of time since study start, exacerbation severity in three categories and season. Similarly, a stepwise approach as above was done finding the best fit for the model.

Regression models in paper 2:

For the comparison of serum MIF levels between COPD and controls, a linear regression model was fitted, using the regress-command in Stata. MIF-levels were used as the outcome variable. Due to a skewed distribution the variable was log-

transformed, and the exponentiated result coefficients were presented as geometric mean ratios. Variables tested were age, sex, body composition, smoking habits, and CCS. A stepwise approach was used for finding the best model. In addition, the inflammatory markers, WBC, CRP, NGAL, sTNF-R1 and OPG were tested in the model in order to evaluate potential confounding effects. Similarly, MIF-levels were evaluated according to COPD characteristics in COPD patients only, using a similar linear regression model with the same variables as above, and in addition including exacerbation frequency, GOLD stage and use of inhaled steroids.

Regression models in paper3:

The distribution of GDF-15 was skewed with several high values, and even after log-transformation the distribution was not normal according to a distributional diagnostic plot (*qnorm*-command). Thus the GDF-15 variable was dichotomized in high versus low levels with cut-off at median, for its use as the outcome variable in the logistic regression models.

Survival analysis was done in COPD patients only using a Cox proportional hazards regression model, with death as the failure indicator and time since inclusion (in years) as the time parameter, using the *stset*- and *stcox*-commands. Effect estimates were presented as Hazard Ratios (HR). Variables tested were sex, age, body composition, smoking habits, GOLD classification, CCS, and exacerbation frequency.

Analysis of exacerbation frequency was done using a random effects negative binomial regression model similar as the model in paper 1, using the same variables mentioned above.

Analysis of change in lung function (both FEV_1 and FVC) and change in free fat mass index (FFMI) was done with a random effects linear regression model, using the *xtmixed*-command (*mixed*-command in Stata 13 and later). The outcome variables were FEV_1 and FVC given in % of predicted, and FFMI-measurements from all available measurements; thus up to 7 repeated measurements per subject during the 3-year follow-up. The identifier variable was each patient, whereas the time variable was specified as years after inclusion. The covariance structure of the random effects was

set as unstructured, thus all variances and covariances were distinctly estimated. All variables were analyzed with a time-interaction, thus the effect estimates were presented both as standard coefficients representing baseline estimates as well as time-interaction coefficients representing effect estimates due to change over time. Variables tested in the model were the same as in the models above, all also with the time-interaction. All three models were also presented as figures showing the *modeled* decline in FEV_1, FVC and FFMI in high vs low levels of GDF-15, based on the estimated coefficients.

Regression models in paper 4:

The main outcome of this study was the diagnosis of lung cancer. Cox proportional hazards regression models were fitted using a diagnosis of lung cancer as the event variable and time from inclusion to cancer diagnosis as time indicator. For the comparison between COPD patients and smoking controls; age, sex, smoking status at inclusion, body composition, and CCS were evaluated as independent variables. For the analysis in COPD patients only, the presence of emphysema, use of inhaled steroids, GOLD classification, use of LAMA or LABA, exacerbation frequency, and chronic bronchitis were also evaluated.

For the analysis of inflammatory markers, 44 different markers were evaluated one at a time added to the model above. Due to multiple testing of biomarkers, a Bonferroni adjusted p-value below 0.05/45=0.0011 was demanded for statistical significance. In addition, a principal component analysis (PCA) was performed, using the *pca*-command in Stata. PCA is a data reduction method that extracts the variance of multiple and often correlated variables into a smaller number of principal components. The eigenvalue of the components corresponds to the variance explained by each component. All variables were standardized (divided by their standard deviation and subtracted the mean) before using an orthogonal transformation. For the statistical analysis in the regression model, we used the 11 largest principal components, which explained 70.8 % of the total variance. The 11 components were analyzed one at a time added to the main model. Furthermore, the 4 largest components were also visualized in scatterplots comparing lung cancer and non-cancer patients, and also a

correlation diagram showing relations between 4 principal components and important COPD characteristics.

SUMMARY OF PAPERS

Paper 1

Predictors of exacerbations in chronic obstructive pulmonary disease

Acute exacerbations of COPD (AECOPD) are important contributors to both morbidity and mortality in COPD, but there are great differences in the frequency of exacerbations between patients. We aimed to unveil risk factors for exacerbations in a COPD cohort with longitudinal follow-up, evaluating both clinical variables as well as systemic inflammatory markers. Similarly, we aimed to find predictors for the duration of the exacerbations.

We used data from the Bergen COPD Cohort Study and the Bergen COPD Exacerbation Study, including 403 patients with longitudinal follow-up each 6 months for three years. Exacerbations were counted consecutive, and graded in mild, moderate and severe exacerbations based on treatment necessary. Baseline variables evaluated as predictors were the variables sex, age, body composition and smoking history, as well as the COPD characteristics exacerbation count before inclusion, GOLD stage, Charlson comorbidity score, hypoxemia, chronic cough, cough with phlegm, use of inhaled steroids, as well as the systemic inflammatory markers Leucocyte count, C-reactive protein (CRP), Neutrophil gelatinase associated lipocalin (NGAL), Soluble TNF-reseptor-1 (sTNF-R1) and Osteoprotegrin (OPG). For the statistical analysis we fitted a negative binomial regression model, allowing for random effects due to repeated measurements, estimating the annual incidence rate ratio (IRR) for the three study years. For the analysis of exacerbation duration, we fitted a generalized estimation equation logistic regression model, adjusting for the same variables, but in addition adjusting for season and AECOPD severity.

Significant predictors for an increased rate (IRR) of exacerbations were female sex [IRR 1.45 (1.14-1.84)], age per 10-year increase [1.23 (1.03-1.47)], >1 AECOPD last year before baseline [1.65 (1.24-2.21)], GOLD III [1.36 (1.07-1.74)], GOLD IV [2.90

(1.98-4.25)], chronic cough [1.64 (1.30-2.06)] and use of inhaled steroids [1.57 (1.21-2.05)]. The inflammatory markers were not associated with the IRR. For predictors of exacerbation duration more than three weeks, significant predictors after adjustment were: hypoxemia [0.60 (0.39-0.92)], years since inclusion [1.19 (1.03-1.37)], AECOPD severity; moderate [OR 1.58 (1.14-2.18)] and severe [2.34 (1.58-3.49)], season; winter [1.51 (1.08-2.12)], spring [1.45 (1.02-2.05)] and sTNF-R1 per SD increase [1.16 (1.00-1.35)].

In summary, several COPD characteristics, especially an increased exacerbation rate before study inclusion, were associated with an increased AECOPD frequency. Increased duration of exacerbations was linked to increased AECOPD severity, winter- or spring season. Hypoxemia was inversely related to exacerbation duration.

Paper 2

Macrophage migration inhibitory factor, a role in COPD?

Macrophage migration inhibitory factor (MIF) is a pluripotent cytokine, and has among else a unique ability to antagonize the anti-inflammatory effects of corticosteroids, making it an interesting study object in COPD research. We aimed to evaluate MIF as a biomarker in COPD.

We used baseline data of 424 patients and 325 healthy controls from the Bergen COPD Cohort Study. Patients were instructed to contact the hospital if they experienced worsening of symptoms, and serum samples were taken from 146 patients at their first moderate or severe exacerbation. Baseline MIF-levels were compared between healthy controls with both non-parametric analyses as well as multivariable linear regression analysis, adjusting for the baseline variables sex, age, body composition, smoking habits and comorbidities. Similarly, we evaluated serum MIF levels both at stable phase as well as AECOPD according to the specific COPD characteristics GOLD stage, exacerbation count before inclusion, chronic bronchitis, presence of emphysema on CT scan and the use of inhaled steroids. Lastly, we compared paired measurements of serum MIF at stable phase vs exacerbation, evaluated with non-parametric analysis.

Median MIF in COPD patients was 20.1 compared to 14.9 in controls ($p<0.01$). MIF was bivariately associated with sex, body composition and CCS ($p<0.05$ for all). In the regression analyses, MIF was significantly higher in COPD patients, coefficient 1.32 ($p<0.01$) and 1.30 ($p<0.01$) unadjusted and adjusted respectively. In addition, in 146 patients at AECOPD, MIF was significantly elevated, with median 23.2 (14.1-42.3) compared to measures at stable disease 19.3 (12.4-31.3), $p<0.01$. We also found a likely association between the use of inhaled corticosteroids and increased MIF levels, adjusted coefficient 1.15, $p=0.054$.

In summary, serum levels of MIF were significantly higher in COPD patients compared with controls. We also identified an additional increase in MIF-levels at AECOPD and a likely association between use of inhaled corticosteroids and increased MIF-levels.

Paper 3

Growth differentiation factor-15 is a predictor of important disease outcomes in patients with COPD

Growth differentiation factor-15 is a cytokine with known association with cachexia, especially in cancer, but also cardiovascular disease and all-cause mortality. We aimed to evaluate GDF-15 as a potential biomarker in COPD using both cross-sectional as well as longitudinal data.

We used data from 413 patients from the Bergen COPD Cohort Study and the Bergen COPD Exacerbation Study. Plasma GDF-15 was evaluated against relevant baseline variables. For this study we also used data from longitudinal follow-up. AECOPD were registered consecutively for three years. Lung function and body impedance measurements were undertaken each 6 months for up to three years. Mortality- and cause of death data for up to 9 years were obtained from the Norwegian Cause of Death Registry. GDF-15 as a predictor of exacerbation IRR, change in lung function and body composition were analyzed with a negative binomial regression model and linear regression models respectively, all addressing random effects, and lastly a cox-regression model evaluating mortality.

Increased levels of GDF-15 were associated with cachexia, current smoking, increasing co-morbidities, exacerbations before inclusion and lastly low FEV1 at baseline. Also, when evaluating longitudinal data, increased levels of GDF-15 were associated with a higher three year exacerbation rate; IRR 1.39 (95% CI 1.1-1.74, p=0.006, a faster decline in both $FEV_{1;}$ 4.29 % vs 3.25 %, and FVC; 2.63 % vs 1.44 % (high vs low levels, p<0.01 for both), and a higher mortality; HR 2.07 (1.4-3.1, p<0.001), especially due to pulmonary and cardiovascular cause of death.

In summary, increased levels of GDF-15 were associated with several COPD characteristics at baseline, and were also a predictor for an increased exacerbation rate, a faster decline of lung function, and a higher mortality.

Paper 4

Risk factors for lung cancer in COPD

Lung cancer share risk factors with COPD and is a common and dreaded co-morbidity among COPD patients. We aimed to evaluate the consecutive incidence of lung cancer after study inclusion, and to identify predictors of lung cancer development.

We used data from 433 COPD patients and 279 controls, all smokers, from the Bergen COPD Cohort Study. The main outcome of the study was a diagnosis of lung cancer during 9 years of follow-up. Lung cancer diagnosis was obtained from the Norwegian Cancer Registry. Baseline predictor variables evaluated was age, sex, lung function, body composition, smoking history, emphysema assessed by CT and clinical symptoms. I addition we evaluated 44 serum/plasma biomarkers both individually as well as combined in a principal component analysis. A cox-regression model was fitted for the evaluation of time to lung cancer diagnosis.

When comparing COPD patients with smoking controls, COPD was an independent risk factor for lung cancer, HR 4.98 (1.45 – 17.1, p=0.01). When analyzing lung cancer patients only, the best predictors were the presence of emphysema, HR 4.35 (1.74 – 10.8, p<0.01) and obesity 3.25 (1.25 – 8.45, p=0.02). Use of inhaled steroids was associated with a reduced risk of lung cancer. Of the 44 biomarkers, only IP-10

was associated with an increased risk of lung cancer, whereas all 44 biomarkers combined the principal component analysis were not linked to increased lung cancer risk.

In summary, COPD was an independent risk factor for lung cancer, and especially in patients with emphysema. Systemic inflammation, measured by serum/plasma inflammatory biomarkers, was not associated with increased lung cancer risk.

DISCUSSION OF METHODS

Study design

The two studies BCCS and BCES consisted of two COPD patient cohorts, where the BCES cohort was simply a subgroup of the larger BCCS cohort. In addition, a cohort of healthy controls was included in order to compare with the two groups. However, the term “healthy control” is a misnomer since the study aimed to include controls with non-COPD morbidities at a similar amount as the general population, thus without "filtering" except for comorbidities conflicting with interpretation of say blood markers or participation, like dementia, cancer, or severe arthritis. The choice of study design is always a compromise between available resources, flexibility in the choice of research questions, and statistical strength. A randomized controlled trial is the gold standard in terms of resolving causal relationships, but is expensive, and the number of exposure variables available for analysis is limited. A longitudinal cohort study allows for the simultaneous investigations of a large number of variables, and allows at the same time for assessment of temporal relationships with higher statistical power than in a cross-sectional study (178).

Some main advantages of our cohort should be mentioned. First, the patients and the controls went through rigorous and multidisciplinary examinations at the visits, providing us with a large number of variables available for the statistical analyses. Second, the large size of the study with 758 study subjects provided solid statistical strength, and allowed for stratification and adjustment for several variables in the regression models. Third, the longitudinal design of the study gave us the possibility to study temporal associations between variables of interest and different outcome, in addition, repeated measurements of central variables both increase data quality and describe changes over time.

The obvious disadvantage with non-intervention research study such as a cohort study is that causal relationships cannot be clearly resolved. Even a strong association over time may occur due to confounding or other directions of causality, even though

efforts are made to account for this. Besides, any observations from even a large cohort study such as the BCCS should be verified in other cohorts, thus a replication cohort would strengthen and increase the value of our findings. These are, however, not readily provided. Therefore, the term "predictor" and its derivatives frequently used both in this thesis and in the papers should be interpreted with these limitations in mind.

We used a mixture of cross-sectional and longitudinal data in our study. Our main focus was on the longitudinal evaluation of COPD related outcome resulting in paper 1, 3 and 4, whereas the more complete data collection at study entry, thus with a more cross-sectional structure, was used for the selection and evaluation of variables necessary for the longitudinal models. Ideally, all non-fixed variables (such as the inflammatory markers) should be measured repeatedly during a study. Single measurements carry the risk of obtaining random extreme values, whereas an average of repeated measurements gives a better indication of the true value, explained by the statistical phenomenon of regression to the mean.

Internal validity

The term internal validity is a general description of to which extent the findings of the study truly represent the reality of the studied population, and to which degree they are affected by systematic errors or biases that may provide inaccurate results. Systematic errors may occur at all stages of a study; when designing inclusion criteria and recruiting study subjects, when collecting data, or when analyzing and presenting data. The nomenclature of systematic errors is not always consistent, and some study procedures may be source of several different errors simultaneously. The terms selection bias, information bias, confounding and reliability are presented in more detail, as well as how the statistical analyses were conducted in order to account for potential biases.

Selection bias

Any cohort of study objects should be as representative as possible in order to generalize or extrapolate any findings on to the intended study population. Our study

population was based on patients recruited from local outpatient clinics, but also from the earlier Bergen GenKOLS (study on genetics in COPD) and the Hordaland County Cohort study (based on a general population)(179, 180). 928 subjects with a presumed COPD diagnosis were invited, whereas 433 were included in the studies. Thus, 495 subjects were not included, giving a potential for a selection bias on the study cohort. We have no accurate numbers on subjects with non-response/lack of willingness to participate in the study, but this was presumably a significant share of the invited, whereas others were excluded by various reasons. A sub-analysis from both the Hordaland County Cohort study and from the OLIN-study indicate that both education and employment may affect the willingness to participate in studies, also the symptom load may be larger in non-responders (181, 182). Similarly, the "healthy volunteer effect" suggests that patients willing to participate in studies are more likely to report a healthier lifestyle, possibly in an attempt to compensate for already acquired disease (183). Further, COPD patients with a mild disease, GOLD class 1 (FEV1>80 %) were excluded by design. Advantages of this is to better exclude for instance asthma patients misdiagnosed with COPD, and also more clearly separate COPD patients from smoking controls. Similarly, but on the other end of the severity scale, patients with a too poor performance status were not included since they were not able to attend to follow-up.

The controls of the study were younger, especially the non-smokers, which was important to adjust for in the statistical analyses. Also, co-morbidities were less frequent in controls. This, however, was difficult and possibly undesirable to adjust for since co-morbidity might be a fundamental characteristic of COPD.

Information bias and statistical analysis

Information bias, also referred to as observational bias or misclassification bias, relates to any error in the measurement or collection of data, or misinterpretation of these. Other terms related to information bias are confounding, reliability and statistical analysis of data, which are discussed separately.

Especially when collecting categorical and/or binary data, all variables should be precisely defined in advance. In our study, questions regarding symptoms such as breathing difficulties and chronic cough were standardized. The interpretation of the questions, however, will always depend on the study subject as well as the person conducting the interview. There are several gender differences when reporting smoking habits, dyspnea and symptoms such as cough and sputum/phlegm production (184, 185). Our study included 46 % female COPD patients. And, sex was always an adjustment variable in the statistical analyses in order to account for potential gender differences. Another example of potential information bias was the definition, counting and categorization of COPD exacerbations. The exacerbation screening was based on standardized questions on symptoms, but in the analyses we used definitions based on health care utilization (appendix). A result of this was that the counts on moderate and severe exacerbations were considered reliable, whereas the number of mild exacerbations, more based on the recollection of symptoms, was regarded as less certain, and thus not included in the regression models. The definition of COPD exacerbations is debated. Symptom based definitions, often based on diary cards, is an alternative to our approach (77), but there is no international consensus on this topic (hence the need of better biomarkers).

All patients had their COPD diagnosis confirmed by spirometry. The definition of COPD based on the FEV_1/FVC-ratio of 70 % is controversial and frequently debated (186). This topic is beyond the scope of this study but is an important issue in a larger debate on how to grade and differentiate COPD patients and its phenotypes. It should be noted, that younger COPD patients may "miss" their COPD diagnosis, whereas some elderly may be misclassified as COPD patients. In our study, all patients also had a clinical diagnosis of COPD, thus misclassification was less likely. Lung function (FEV_1) was analyzed as a continuous variable in the longitudinal analyses but categorized into GOLD stage 2-4 as an adjustment variable, for easier interpretation of the data. An alternative approach could be to evaluate the GOLD A-D assessment from 2011, with more emphasis on symptoms and exacerbations. This classification, however, is less used clinically, and also did not add information when evaluated in exploratory analyses. The additional measurements of lung function FVC and the

FEV_1/FVC-ratio were also evaluated in the statistical analyses as adjustment variables but were consistently of less significance than FEV_1.

The diagnosis of emphysema was based on CT scans and a computerized analysis of the tissue density. Our definition of emphysema gave us a binary variable, not taking into account that emphysema develops gradually, and that its severity varies. Also, the threshold of 10 % lung tissue and the cut-off at -950 HU is debated (187), especially since it does not take into account the geographical distribution of emphysema. A more sophisticated approach might have given more detailed insight into emphysema severity, but emphysema severity per se was not a major topic in our study, and thus we used a simplified approach.

An important assumption of our study was that the measurement of multiple serum/plasma (systemic) inflammatory biomarkers was descriptive of COPD, a lung disease. The role of systemic inflammation in COPD is well described (19), and it seems inconceivable to evaluate local or systemic inflammation independent of each other. In addition, systemic inflammation is associated with both co-morbidity as well as worsened prognosis in COPD patients (20), thus systemic inflammation should be regarded as a fundamental aspect of the disease. A main and yet unresolved question is to which degree peripheral blood sampling and measurement of systemic inflammatory markers truly reflects the pathophysiology of COPD, both regarding the abnormal inflammation of the lungs, but also COPD as a systemic disease. The concept of inflammatory overspill is already mentioned, and there is ample evidence of proteins being able to move from the lungs to the systemic circulation, but also in the other direction (113). Thus increased levels of a biomarker in the lung parenchyma may well go along with a similar increase in the circulation. On the other hand, there is no evidence that a lung/circulation overspill is applicable for all inflammatory biomarkers, or to the same degree. Thus, serum/plasma biomarkers may mirror components of, but very likely not the complete lung inflammation in COPD. Lastly, the issue concerning the time scale of our biomarker analyses needs to be addressed. It is uncertain to which degree systemic inflammation is persistent over months and years. We evaluated baseline measurements of biomarker levels as predictors of events

of several years ahead, but we cannot exclude that biomarker levels have changed significantly during follow-up, possibly interfering with the outcome. A few studies support the concept of persistent inflammation in COPD (20), but research in this field is not fully evolved.

The more technical parts of biomarker analysis are mentioned later, but some important factors affecting biomarkers levels need to be addressed. Gender is already mentioned as a source of bias, and this is also relevant in the matter of biomarker levels. Estrogen levels and other hormonal differences may partly explain differences in degree of inflammation, toxin degradation, but also a higher susceptibility to cigarette smoking than in males (188), and may be an underexplored topic. Further, age and increasing comorbidity are strongly correlated, and they are both important factors influencing biomarker levels, of particular interest is cardiovascular disease (189). Similarly, medication of COPD, especially corticosteroids, but also drugs prescribed for other conditions may have an impact on inflammation and should be accounted for. The last factor mentioned here, and possibly the most difficult to accurately adjust for, is body composition. Height, weight, amount and distribution of fat and muscle, diet and physical activity, are all factors influencing one's body composition. Body Mass Index (BMI) is a frequently used metric of body composition since it is easy to measure, but BMI does not account for the amount of fat or muscle in the body. The consequences of having a high BMI in COPD are debated (190, 191). Our approach was to use a combination of the Fat Mass Index (FMI) and the Fat Free Mass Index (FFMI), where obesity was defined according to a high FMI and cachexia by a low FFMI. This method is more likely to account for the likely beneficial effects of muscle tissue vs fat on the different outcome. In addition, the amount of body fat does not only affect systemic inflammation directly, but may also bind inflammatory biomarkers, thus influencing systemic levels. Nevertheless, the issues concerning body composition, nutrition and physical performance in COPD is complex, and have many unresolved research questions, and the perfect “lifestyle” adjustment variable does probably not exist.

Statistical analysis and confounding

The statistical analyses of the project used a combination of data from different study settings: There was an extensive data collection at inclusion, providing a basis for cross-sectional analyses. In addition, data measurements of important COPD characteristics were repeated both at each 6-month visit as well as ad hoc measurements at AECOPD. Two of the four papers use data from the healthy control groups for comparison to COPD patients. All four papers use longitudinal data from COPD patients to a different extent in the analyses.

Many statistical analyses, especially regression models, are susceptible to confounding. Confounding refers to unknown or hidden variables that influence both the outcome as well as adjustment variables. Confounders should be controlled for when fitting statistical models if possible. Some general principles to variable adjustment and analysis were followed in all four papers. Age, sex and body composition are already mentioned, and these were considered central adjustment variables in all analyses of several reasons; the most important being that these variables frequently are correlated or associated with most central outcome variables, but also with serum/plasma levels of the inflammatory markers. Smoking is another central variable and potential confounder in COPD research. Smoking habits were analyzed both as a categorical never/prior/current smoker, but also in terms of smoking load, as pack-years smoked. Smoking also affects the prevalence of cardiovascular morbidity, thus it is an essential adjustment variable in several statistical models. In the analysis of lung cancer prevalence, non-smoking controls were excluded from the analysis entirely since lung cancer development in non-smokers is rare and may occur due to different pathological processes than in smokers.

Another example of confounding in COPD, which may also classify as an interaction or selection bias issue, is the analysis of any effect of COPD medication on different outcomes. The use of COPD medication may rely on several different factors like phenotype, symptoms and lung function, all with potential effects on the outcome. The use of propensity score matching may reduce this bias and is sometimes used in the analysis of medical treatment in observational data. This implies adding adjustment

scores in the regression models based on any variables predicting medical treatment. However, this is difficult in the setting of COPD, since most factors predicting treatment will also be associated with the outcome; this will be further discussed later.

Any regression model is always a compromise between the best possible fit to the available data set and how the real-life data truly is. A regression model aiming for the best possible fit will usually include a high number of adjustment variables, but will be at risk of overfitting (192). A potential example might be to include a high number of inflammatory biomarkers in the same regression model. Consequences may be identification of statistically significant associations, which are generated randomly and not due to biological factors (often referred to as data mining). Also, a too complex model will reduce the statistical strength in terms of recognizing the true and biological relevant associations between an adjustment variable and the outcome. On the other hand, the consequences of a too simple or parsimonious model might be to exclude essential confounders from the model, providing wrong results. The large number of participants of our study allowed us to liberally adjust for known biological correlations that were not statistically significant in terms of p-values. When analyzing multiple biomarkers however, adjusting for confounding was more complicated due to interactions and the pitfalls of data mining mentioned above. Thus, although using interaction testing and assessing biological plausibility, we cannot exclude that all confounding factors are accounted for regarding to inflammatory biomarkers.

The use of longitudinal data and repeated measurements requires statistical methods that take into account the inherent dependencies between subjects examined repeatedly. These methods are known by several names, like mixed models, multi-level models, random effects models, panel data analysis or simply longitudinal data analysis, and most of them utilize regression analysis in some form (193). Many statisticians look upon these models as special versions of ordinary regression analysis. However, ordinary one-level regression makes the central assumption that any measurement on an individual or on a group is independent of the next individual or group. Multi-level models adjust for many of these factors and should be regarded as the best models describing real life situations. However, a major problem with multi-

level models is a rapid increasing complexity when adding levels and groups, both in terms of model adjustment options, but also in interpretation of the results.

The analysis of predictors of the exacerbation rate was done with a bilevel/ random effects negative binomial regression model. Traditionally, Poisson regression has been used for the analysis of count data, but in later years the slightly more complicated negative binomial regression is preferred since lack of computational power is no longer an issue, and also since it allows for better a statistical fit for the distribution of AECOPD (194). AECOPD tend to cluster in time and thus the individual yearly incidence rates vary. The ideal time unit should be short enough not to include too many AECOPD clusters, but also long enough to avoid too small counts (too many zeros), thus the time unit of one year was a compromise.

For the analysis of duration of exacerbation, we used a generalized estimation equation (GEE) regression model. There is no consensus-based definition on a "long duration" exacerbation, and regression methods based on other distribution models could be considered. A GEE-model may be considered a compromise as it has a god statistical robustness, but with a potential cost of lack of statistical strength. The cut-off at 3 weeks is arbitrary but corresponds roughly to the clinical perspective where a AECOPD patient is expected to recover within reasonable time and should be reassessed if not. Alternative approaches could include symptom diaries or lung function testing, but that would require study resources unavailable to us.

Analysis of decline in lung function, but also change in FMI and FFMI, was done with a random effects linear regression model. An important question on the study of lung function decline is whether it is linear or varies with time. Whereas the (idealized) Fletcher curve indicates a deteriorating FEV_1 decline with time (6), data from the COPDGene-study indicates that the FEV_1 decline is faster in mild/moderate than in more severe COPD (147). Models with exponentiated time variables or linear splines were evaluated and might have provided a slightly better statistical fit. However, we chose a linear decline model as they are more statistically robust and interpretation of results is far easier. When modeling the variance, several subjects had one or more

missing visits during follow-up, and an unstructured correlation structure was chosen for better robustness thus potentially trading off statistical strength (195).

Survival analysis and time to lung cancer diagnosis were done with cox-regression (proportional hazards) analysis. Cox-regression is susceptible to inaccurate data (192). We used data from official and compulsory health care registries with close to 100 % completeness. Co-morbidities were, in addition to age, potential and important confounding factors, thus these were evaluated both individually and as a composite variable (CCS).

In addition to the statistical analysis of the individual inflammatory biomarkers, we intended to also analyze systemic inflammation as whole. At the time of preparation for the 4th paper, we had additional biomarker measurements available for analysis; but far too many for be included simultaneously in standard regression models for several reasons, but most importantly due to widespread interactions between the markers. Methods for analysis of large and complex data sets have evolved tremendously the last decade, providing researchers with plentiful opportunities, but also potential pitfalls as complexity increases. Our approach was a principal component analysis based on the variance of inflammatory markers between the established categories and phenotypes. An alternative approach might have been a cluster analysis with emphasis on identifying new groups/clusters of patients with similar inflammatory patterns, and then compare this to the recognized categories. Nevertheless, the analysis of complex data sets with a large number of variables sets great requirements on both the data quality as well and the interpretation of the data. In general, a principal component analysis should be looked upon as a hypothesis generating rather than testing method (196).

Reliability

Any measure has a high reliability if it generates the same results when repeated under similar conditions. Factors that may affect reliability should be identified in order to improve the quality of the results, but also in terms in interpreting the measurements. The term reliability is most relevant when applying tools for measurements involving

several steps, and where increasing complexity may reduce reliability. Reliability may differ according the instruments used, different methods and between users.

Lung function measured by spirometry was a main outcome in our study. The result of a spirometry is highly dependent of the study subject as well as the technician. All spirometries were performed according to ATS/ERS standardization (172). The different spirometries were identical, were calibrated as descried earlier, and were maintained according to specifications.

Body impedance measurement is less affected by study subject performance, but on the other hand the compliance to restrictions on food, drink, smoking and physical activity could not be accounted for. As for spirometry, tests were done according to standardized protocols and the instruments were calibrated and maintained as recommended. The reliability test mentioned earlier indicated a high reliability on these tests (174).

The laboratory measurements in the inflammatory biomarkers were done at different times, at different laboratories and with different methods, and thus with potentially various reliability. The in-house measurements of Hemoglobin, WBC, Granulocytes, TPC, s-Ferritin, s-Creatinine and CRP used hospital routine methods with high reliability, estimated < 5 %. For the biomarkers measured separately with commercial EIA kits, the samples were measured in duplicates, intra- and inter-assay were analyzed and were below 11 % for all. For the 25 biomarkers measured in the magnetic bead multiplex, the listed intra- and inter-assay variance was between 5-15% and 5-11 %. In our study only one measurement was done for each biomarker, thus reliability was not verified.

In addition to reliability, other validity concerns regarding measurements of the biomarkers should be addressed. Only a few of the biomarkers were measured at laboratories accredited for providing validated reference values. Second, the serum and plasma samples were exposed for super-freezing and thawing, and for some biomarkers transportation on dry ice to overseas laboratories for analysis, thus potentially affecting the levels of the biomarkers. Altogether, the differences in

biomarker levels between study subjects should be interpreted relatively rather than according to absolute values. Second, several of the measurements in the multiplex analysis had measurements below the lower limit of detection, and some had measurements above. This was only a minor problem when using the separate EIAs for the other biomarkers. Third, there are by nature several correlations between many inflammatory biomarkers, the measurements from the multiplex seemed to have a higher degree of correlation between them then the other markers, which was difficult to quantify and adjust for. In sum, these issues may influence the results of the statistical analyses and should be accounted for as mentioned earlier.

External validity

The term external validity refers to transferability of the results and conclusions of the study to a general population, in this case COPD patients overall. Some variants of potential selection bias are already mentioned; by design, COPD patients with mild disease were excluded, thus our results may not be representative for patients with mild or early disease. Similarly, our findings may not reflect the reality of COPD patients with severe disease and short life-expectancy as these were excluded for inclusion, but on the other hand, mortality was still high in our cohort, thus this category was to some degree represented.

Most patients were selected from earlier studies or outpatient clinics and were not randomly recruited from the general population, potentially excluding patients not in contact with the health care system. In Norway, access to health care is universal and inexpensive, thus a large health care access bias is unlikely. Bias due to non-response is already mentioned, where COPD patients with a lower socio-economic status and a larger symptom burden may be under-represented. Nevertheless, it seems unlikely that this patient category, or other mentioned above, should differ dramatically from those included in the cohort in terms of epidemiology and pathophysiology.

DISCUSSION OF MAIN RESULTS

Predictors of AECOPD

The "frequent exacerbator" does not exist as an independent category in COPD classifications or guidelines. Nevertheless, most pulmonary physicians are well acquainted with these patients as they are regular customers in both primary and specialized health care. The clinical observation that there is a frequent exacerbator phenotype has been confirmed by earlier studies (197), and it is recognized that one of the best predictors of exacerbations simply is a history of previous exacerbations (132). It is also recognized that the prognosis of the frequent exacerbator is worse, both when it comes to decline in lung function (14, 74), but also in life-span (16, 75). Inflammatory biomarkers may help identifying these patients before they enter a vicious circle of repeated COPD exacerbations and accelerated disease progression, but they may also help us understanding the special pathophysiology of the frequent exacerbator as well as acting as a guide to the optimal treatment.

The inflammatory biomarkers analyzed in our first study were WBC, CRP, NGAL, sTNFR1 and OPG (198). These markers were chosen due to known associations with either COPD, or important COPD-characteristics, based upon prior cross-sectional studies on the baseline data from the Bergen COPD Cohort Study (199, 200). WBC, CRP and OPG were all bivariately related to the subsequent AECOPD count, but not after multivariable adjustment. Our negative findings were in accordance with some other studies (15, 140), describing bivariable associations between inflammatory markers and future AECOPDs, but not after statistical adjustment for other factors. In some cases, disease severity is a confounding factor for positive findings bivariately, whereas other studies do not report using regression adjustment methods. However, as mentioned in the introduction, there are several studies describing biomarkers with independent associations with future AECOPD (15, 20, 21, 128, 140, 142-144). One problem for many studies is the lack of replication studies, thus the findings have not been confirmed by others. A more recent and large study by Keene et al (2017)

addresses this issue (201). Using prospectively collected plasma/serum samples from two large COPD cohorts, a total of 119 different inflammatory biomarkers were measured by multiplex panels. When analyzing each cohort alone, several biomarkers could independently predict AECOPDs, but when comparing the two cohorts, there was minimal overlap between them. Also, any one inflammatory biomarker had limited predictive value, especially compared to clinical characteristics.

There may be several reasons for the negative findings in our, but also other studies. One explanation is the heterogeneity of AECOPDs. Potential triggers of AECOPDs include different viruses, bacteria, air temperature or pollution, and in many cases a combination of these (202), which may vary significantly between patients. Also, the increased susceptibility to having AECOPD may be more related to fixed, structural changes in the lungs than to an inflammatory response, which is not necessarily reflected by blood biomarkers.

Women had more exacerbations them men with an IRR of 1.45 in our study, but when only looking at hospital admitted AECOPDs, there was no gender difference. The gender difference overall has been observed earlier but is difficult to explain. In the TORCH-study, female COPD patients reported a significant lower quality of life, but at the same time their lung function measurements were better than in male patients, and women trended towards a lower mortality (203). On the other hand, women reported a lower prevalence of chronic cough (and also cough with phlegm), another independent risk factor for AECOPD, a phenomenon also observed by Lindberg et al (204). A post-hoc evaluation or our data also indicate a potential interaction between male gender and chronic cough as risk factors of AECOPD, consequently this potential under-reporting of cough in females may partly explain a higher AECOPD frequency in females.

We found an association between the use of inhaled corticosteroids (ICS) and an increased exacerbation count. This finding was valid both for fluticasone propionate and budesonide, the dominant ICSs on the market at the time, and there was also a dose-response relationship. This finding is obviously in contrast with the findings of the large randomized controlled trials (205, 206) providing the basis for ICS treatment

in COPD. These RCTs may be criticized for their differentiation of AECOPD and pneumonia, as the latter is not properly incorporated in their statistical models. It is generally accepted that use of ICS increases the risk of pneumonia (207), and while having a protective effect against AECOPD, these two conditions may be difficult to distinguish between clinically. However, even though our results were statistically significant after adjustment for other known AECOPD risk factors, our findings could be a result of a selection/treatment bias, since COPD patients receiving ICS treatment are those with the highest risk even before study inclusion. A propensity score matching can in some cases adjust for medication use in the statistical models, but in our case, all relevant variables were already incorporated in the model. The topic of ICS use in COPD is still debated, and thus not fully resolved. There may be a need to better phenotype both COPD patients and AECOPDs to establish the optimal treatment protocols for the use of ICS.

We did not have access to all variables at the time of publication of paper 1 (198), and as additional data became available, these were evaluated against the same regression model used in this study. Emphysema has been proposed as a risk factor of AECOPD (139), but in our study, the presence of emphysema on CT scan was not associated with AECOPD frequency. We also evaluated additional inflammatory markers analyzed by our international collaborators, and we did find a positive association between plasma levels of GDF-15, which is described later in this chapter. Furthermore, we conducted an investigation on coagulation markers in COPD as a part of the same study but not included in this thesis. Here we also identified Thrombin AntiThrombin-complex (TAT) as a predictor of future AECOPD (paper in submission)(208), which may be an illustration of the interactions between the coagulation and the immune system. Finally, we also analyzed blood eosinophil count post hoc, but without significant results in our model. Blood eosinophils have emerged as a predictor of both future AECOPD as well as treatment effect of corticosteroids (141). Our study specifically excluded asthma/ACOS patients where eosinophilia is more common, which may partly explain our negative findings.

In addition to analyzing AECOPD frequency, we evaluated factors with potential associations to AECOPD duration as this might be another measure of disease severity. This topic is less explored before, and there is neither an established cut-off of a long lasting exacerbation, nor a general consensus on when an AECOPD is finished based on symptoms or other measures. AECOPD duration is likely to depend on its cause, and it is likely that infections, especially viral, are related to a longer duration of symptoms (88). Consequently, our observation of a longer duration in winter and spring may be related to the seasonal nature of virus infections. Alternative explanations include inorganic factors such as air temperature or air pollution, where the first has a significant impact on the other at low temperatures. Of the inflammatory biomarkers, only sTNF-R1 had a small but significant correlation with AECOPD duration. The TNF-α system is a central mediator in both bacterial and viral infection. Levels of soluble-TNF-receptor 1 (sTNF-R1) is related to TNF-α but have a longer half-time. The association between sTNF-R1 and AECOPDs is an uncertain finding, but it might be explained by a higher susceptibility for infection in some patients. Lastly, the observation of a longer AECOPD duration in patients with chronic bronchitis may also fit into a picture of infection as a main factor of delayed recovery.

In short, our findings were that clinical parameters are superior to systemic inflammatory markers in predicting future exacerbations; this is also in accordance with most other studies on this topic. The already mentioned study by Keene (201) may indicate that a single, clinically useful inflammatory biomarker of future COPD exacerbations in general does not exist, and perhaps it is more fruitful to look into specific triggers of lung infection (202). A consequence of the lack of a systemic biomarker of AECOPDs is that assessment of clinical characteristics becomes more important. This is also reflected in several recently proposed AECOPD prediction models, which are mostly based on clinical parameters (209).

Macrophage Migration Inhibitory Factor (MIF) as a biomarker in COPD

The name Macrophage Migration Inhibitory Factor (MIF) is not a good descriptor of the function of the molecule, as MIF attracts monocytes and macrophages rather than inhibits them. Today MIF is generally described as a pro-inflammatory cytokine (210).

MIF has been rather extensively evaluated as a biomarker the last decades, and increased MIF-levels are found in different inflammatory disorders, non-communicable diseases, cancer, but also more lung specific conditions such as asthma and acute respiratory distress syndrome (ARDS) (210-215). MIF is found in a range of different immune cells, but as its name implies, macrophages (and monocytes) are central components in MIF-signaling, as they are both a source and a target of MIF. Macrophages contain preformed MIF available for rapid release upon stimulation (216), but MIF is also found in the pulmonary endothelium and in a range of other immune cells than macrophages. MIF is also secreted by the anterior pituitary in concert with ATCH acting more like a hormone than a cytokine, and it has several unique interactions with glucocorticoids which we address later. Systemic MIF levels have been seen to rise after lipopolysaccharide (LPS) or endotoxin-stimulation, and similarly levels of the central mediators TNF-α, IL-1, IL-6 and IL-8 were seen to rise after adding MIF before stimulation (217). Also, inhibiting MIF-signaling by MIF-antibodies or by using MIF-/- mice seems to be protective against LPS or endotoxin-induced inflammation, again indicating a central role of MIF as a pro-inflammatory mediator (218, 219). In a more clinical setting, increased levels of MIF are associated with idiopathic pulmonary fibrosis (IPF), bacterial sepsis and ARDS (219-221). Altogether, there are ample clinical and biochemical justifications to evaluate MIF as a biomarker also in COPD.

In contrast to findings for other conditions, two early studies found an association between decreased MIF levels and COPD. Both Fallica (222) and Sauler (223) found lower plasma levels of MIF in 32 and 32 COPD patients versus 19 and 40 controls, respectively. We found increased levels of MIF in 424 COPD patients compared to 325 controls, with median levels 20.1 vs 14.9 ng/ml, and also an additional increase during exacerbations. A study by Russell et al found increased MIF-levels in sputum and bronchial alveolar lavage (BAL) of COPD patients, but not in serum (224), whereas Milara et al found increased MIF-levels in peripheral neutrophils in COPD patients (225). We believe our study, due to its size and statistical adjustment for available co-factors, gives a better picture of serum MIF levels in COPD, in addition our findings are in accordance with the finding of increased MIF-levels in several

other inflammatory conditions. Nevertheless, our findings should more clearly be replicated in other studies, preferably in larger cohorts with prospective sampling.

Several factors may influence MIF levels and thus partly explain diverging finding. We found that increasing co-morbidity was associated with higher MIF-levels. Further, the studies of Fallica and Sauler found lower MIF-levels at increasing GOLD-stage or a higher degree of emphysema, a similar trend to what we found in our GOLD 3 and 4 patients. Another potentially important factor not adjusted for in the mentioned studies is genetic polymorphism. A second study by Sauler and Zhang describes differences in DLCO in COPD patients dependent on high vs low expression of different alleles of the MIF gene (226). Finally, plasma MIF levels are closely related to plasma levels of cortisol, fluctuating in a circadian rhythm (218). Thus, time of plasma sampling may significantly affect measurements if not standardized, and this may not be accounted for in the performed studies.

Regarding COPD progression the most interesting characteristic of MIF is perhaps its unique ability to be induced by, but also to inhibit the effects of, glucocorticosteroids. Corticosteroid resistance is a hallmark of COPD, and a major problem for its treatment. There are several places of interaction between MIF and corticosteroids. A main MIF signaling cascade goes via the CD74 receptor found in most immune cells via the ERK1/2-MAPK cascade promoting release of pro-inflammatory cytokines and prostaglandins. Glucocorticoids can inhibit immunologic cells by binding to the glucocorticoid receptor, an inductor of MAPK phosphatase-1 (MKP-1), which again inhibits the ERK1/2-MAPK cascade. MIF may on the other hand suppress the effects of MKP-1 directly, as well as hamper glucocorticoid inhibition on pro-inflammatory DNA/RNA transcription (227). In addition is the already mentioned centrally regulated pituitary excretion of MIF. We found an association between the use of inhaled corticosteroid and serum MIF, although small and borderline significant. We could not find any similar effect when looking at MIF levels at AECOPD and the use of oral steroids, but serum sampling was conducted in an early stage of AECOPD.

When evaluating MIF as a diagnostic biomarker of COPD and AECOPD according to the criteria of Sin and Vestbo (116), MIF checks off on biological plausibility, and it is

independently associated with COPD as well as AECOPD which is an important clinical outcome. The problem with MIF as a diagnostic biomarker, however, is the strength of the association between the biomarker and the diagnosis. As mentioned in the introduction, there are several findings of elevated biomarkers, especially at stable COPD, but also at AECOPD. The sensitivity of MIF at COPD and AECOPD is at best mediocre as there is significant overlap between controls and COPD, and between COPD and AECOPD. Further is the lack of specificity, as MIF is not an organ specific marker (like cardiac Troponin for example). Not mentioned in the paper, the ROC-value of MIF as a discriminator between stable COPD and AECOPD in our cohort was 0.69, thus not discriminating enough to be a diagnostic marker in clinical practice.

The last and probably most important characteristic of a useful biomarker according to Sin and Vestbo is related to treatment and subsequent change in biomarker status leading to a different outcome. Roflumilast was developed as a PDE4-inhibitor, and is in approved use worldwide in the treatment of COPD, with a preventive effect on the AECOPD frequency. The study by Milara evaluates the in-vitro effect of roflumilast on corticosteroid resistance in neutrophils from COPD patients. In-vitro treatment with roflumilast is described to reduce MIF-induction after cigarette smoke extract-stimulation (225), thus antagonizing the MIF-level increase induced by corticosteroids, and thereby resulting in a decrease in levels of pro-inflammatory IL-8 and MMP-9. Although PDE4-inhibition with roflumilast induces several other complex mechanisms, reduced MIF-levels may have a role in its anti-inflammatory effect.

Ibudilast (MN-166) is another PDE4 inhibitor, which is primarily designated as a MIF-inhibitor. It is approved for use in Asia for treatment of asthma, but not yet for COPD. As of 2020, ibudilast may be more relevant in the treatment of respiratory failure, and studies on its effect on Covid-19 related ARDS are ongoing (228). Both MIF antibodies as well as small molecule inhibitors of MIF and its CD74 receptor are available (229). MIF is increasingly recognized as a factor in lung diseases (230), thus it is likely that we will see more clinical trials targeting MIF in pulmonary research.

Growth Differentiation Factor 15 (GDF-15) as a biomarker

Although GDF-15 was discovered more than 20 years ago, it is a relatively unknown cytokine in COPD and pulmonary research. GDF-15 has been known by different names, which is indication of scientific interest from several different branches of medical science (231). GDF-15 has been broadly studied as a biomarker in cardiovascular diseases and studies of metabolism and body weight regulation, and is considered a potential prognostic biomarker in these fields (232). GDF-15 differs substantially from other members of the Transforming Growth Factor β (TGF-β) superfamily, but it seems to involve the intracellular Smad-pathways characteristic for the TGF-βs. Much effort has been made in the search for the pathophysiological mechanisms related to GDF-15, however they are yet not fully revealed.

Traditionally, the pathophysiology of any disease is extensively studied before trying to develop any medication, but in some cases the drug development precedes the understanding of the disease mechanisms. The AV-380 antibody has been identified and developed as an inhibitory antibody of GDF-15. Due to the association between GDF-15 and weight loss, AV-380 has been investigated as a potential therapy of cachexia (233); since GDF-15 (designated MIC-1 in that study) was described to induce cachexia in a murine model, and the weight loss was shown to be reversed MIC-1 antibody (AV-380) treatment. Due to a high share of cachexia among COPD patients, and due to the availability of longitudinal assessment of body-composition in our cohort, it was rational to investigate the potential role of GDF-15 as a COPD biomarker.

In our study, we found not unexpectedly a clear relationship between GDF-15 and cachexia at baseline. However, we did not find indications of any predictive value of GDF-15 on the rate of weight loss during the study. More noteworthy was our observations of high GDF-15 levels as a predictor of a higher mortality, a faster decline in FEV_1, and a higher exacerbation count. When analyzing these findings, it is difficult to provide a common mechanistic explanation for the differences in effect. Even though GDF-15 seems to be involved in a number of different conditions, the role of GDF-15 is incompletely understood (234, 235). An important question that

emerges when analyzing our findings, is whether GDF-15 has a significant, up-stream role on our outcome, or if it is a by-product of other, more important disease processes.

With the exception of the recently discovered GDFN family receptor α-like (GFRAL) predominantly found in the central nervous system, the biological targets of GDF-15 elsewhere in the human body remain unrevealed (236). This is in contrast with the widespread distribution of GDF-15 to several different tissues in the body. GDF-15 is expressed in cardiac myocytes and adipose tissue (231). Of more relevance for lung disorders, it can be induced from macrophages, airway epithelium or vascular endothelium after stress-responses, including pro-inflammatory cytokine stimulation (234, 235).

Our observation of GDF-15 as a biomarker of mortality is in accordance with other studies, as it is described as a predictor for both all-cause as well as cardiovascular mortality (237, 238). A smaller study supported this finding in a cohort of COPD patients (239). In our study, the analyses on cause-specific mortality showed the strongest association between high levels of GDF-15 and respiratory cause of death, whereas the trend of a higher risk of cardiovascular death was not statistically significant. However, it is difficult and perhaps unwanted to evaluate the mechanisms leading to a cardiovascular or a pulmonary cause of death separately as these may interact significantly, especially those potentially related to GDF-15. In cardiovascular disease, GDF-15 is induced by coronary ischemia including myocardial infarction. Further, high levels of GDF-15 are associated with heart failure, systemic hypertension, cardiac hypertrophy and pulmonary hypertension, the latter also being a significant risk factor for COPD related death (240). The biomechanics of GDF-15 in heart disease seems to involve intracellular Smad-pathways, which again is related to a possible cardioprotective role in murine and in-vitro models (241), but this is not verified in real-life studies. Another pathway leading to GDF-15 expression is induction by p53, a protein related to cancer but also cellular senescence (242) and thus also mortality. Altogether, GDF-15 seems to have value as a prognostic marker on mortality in general, whereas its causative role in all-cause or COPD-related death remains unclear.

The relation between GDF-15 and both lung function decline and future AECOPD frequency were novel findings in our study. A few other smaller studies have evaluated GDF-15 as a diagnostic biomarker in COPD, reporting elevated levels in COPD patients compared with controls, and an additional increase at AECOPD (243-246). Not reported in our paper, we also found a significant difference in GDF-15 plasma levels between the COPD patients and a selection of the controls (median levels 0.86 vs 0.63, p=0.001, Wilcoxon rank-sum test). We did not, however, have GDF-15 measurements at AECOPD for comparison. A recent and unpublished study by Rydell et al (ATS-congress 2020 abstract) also describe an association between high plasma GDF-15 levels and a faster FEV_1 decline, supporting our findings (247).

Studies by Wu et al describe increased GDF-15 expression from airway epithelium after cigarette smoke exposure, associated with mucus production and cellular senescence (248, 249). A more recent study also describes an exaggerated GDF-15 related inflammatory response after rhinovirus exposure (250). In these studies, both the Smad1 and PI3K-pathways seems to be involved in intracellular signaling, and the Activin receptor-like kinase 1 (ALK1) is a proposed mediator of signaling. These findings are not confirmed by others, and causal relationships are not clarified, but they provide some clues regarding the role of GDF-15 in COPD. Its role as a potential predictor of future AECOPD requires verification in other studies, as lack of consistency between studies is a common problem as discussed earlier in this chapter (paper 1). It may be even more difficult to identify biomarkers related to lung function decline, since studies reporting likely association are sparse (22, 146, 148, 149, 251) and reproducibility is limited. Most of the biomarkers described are not lung-specific, and as with GDF-15 they are also related to cardiovascular disease and other important co-factors related to COPD characteristics.

The association between cachexia, especially related to cancer, and high GDF-15 levels is thoroughly studied, and the recent discovery of GDNF family receptor α–like (GFRAL) and its co-receptor receptor tyrosine kinase (RET) sheds light on the biomechanics proposed to explain its effects. Binding of GDF-15 to the GFRAL/RET complex, probably involving ERK-signaling, has been shown substantially decrease

food intake in murine models, leading to cachexia. Also, GFRAL receptor knock out models, and the above mentioned GDF-15 antibody studies support these findings (233, 236), and thus act as a basis for pharmaceutical trials. The blood-brain barrier might hamper the use of antibodies or other large molecule drugs, but development and utilization of small molecule GFRAL-receptor antagonists may be a better approach in medical treatment of cachexia, especially cancer related/ GDF-15 induced (252). Correspondingly, GFRAL-receptor agonists or the use of natural GDF-15 itself and their potential beneficial effects on obesity is also of great interest, and significant research effort is now put into this topic (252).

When it comes to the other findings in our study, it is less clear whether GDF-15 related pharmacological treatment may have an effect on mortality, lung function decline or exacerbation frequency. As long as no peripheral receptor or any other verified biological target of GDF-15 is discovered, it is difficult to provide a rationale for drug development targeting these outcomes. Still, GDF-15 may have a role in developing and understanding risk-models in COPD, especially in patients with cardiovascular co-morbidity.

Risk factors for lung cancer

The last paper in this thesis concerns both COPD and lung cancer (253). Lung cancer is one of many diseases which are observed more commonly in COPD patients, but it is by far the most serious when it comes to morbidity and reduction in life span. Smoking or noxious airway exposure is regarded as the main risk factors for both conditions. However, regardless of the amount of smoking, it is a common observation that patients developing COPD have a higher, independent risk of also developing lung cancer. Thus, it is rational to search for similarities in the pathophysiology of these diseases, both in order to understand the specific mechanisms linking COPD and cancer, but also due to the need to identify COPD patients with a high lung cancer risk.

In this context, the term predictive biomarker should not be confused with the term tumor marker. The latter is usually referred to as a substance (often a protein) produced or excreted directly from cancer tissue, or indirectly correlated to these, such

as an antigen. It is also worth mentioning the development towards the use of liquid biopsies in the diagnostics of cancer. Liquid biopsies utilize the presence of tumor DNA in the circulation, where small DNA-fragments are detected using PCR or NGS techniques. Their sensitivity in early cancer or pre-malignant lesions is nevertheless unclear, and their role as predictive markers is even more uncertain.

There is extensive evidence of a link between systemic inflammation and cancer development generally (254). In lung cancer this relation has been less clear, but there is now research describing different levels of inflammatory biomarkers between patients with established lung cancer versus healthy controls, smokers and COPD patients (255-260). Biomarkers with predictive value on later lung cancer are harder to discover. A study by Spitz et al describes a lung cancer risk prediction model where markers of DNA repair increase sensitivity of the model (261). Efforts have also been made in looking into alternative biomarkers such as urine samples or exhaled air, by the measurement of volatile organic compounds using gas chromatography and/or mass spectrometry (262), but also here the predictive abilities seem limited.

Of the 44 different biomarkers we measured, only interferon gamma-induced protein 10, (also known as CXCL10, CXC motif chemokine ligand 10 or IP-10) was significantly associated with lung cancer development. IP-10 is secreted by several immune cell types as a response of induction by interferon- γ, INF-γ. IP-10 has several roles, but in the context of COPD it is best known as a marker of viral infection (263, 264). The best described target of IP-10 is the CXCR3 receptor, found abundantly on T-cells and NK-cells, and IP-10 has been linked to tissue damage and emphysema development related to T-cell granzyme release upon CXCR3-activation (265). On the other hand, IP-10 is also associated with angiogenesis and regulation of cell growth and apoptosis and may have a protective role. Elevated levels of serum IP-10 has been observed in patients with lung cancer as well as other cancer types (266). However, the potential role of IP-10 in lung cancer development is unclear, and its value as a predictive biomarker should be confirmed in other studies.

For the other biomarkers in our analysis, there was no significant association with lung cancer among our COPD patients. Similarly, in the principal component analysis

assessing a combined biomarkers analysis, none of the largest components were significant associated with lung cancer. As shown in the correlation diagram from Paper 4, systemic inflammation was to a higher extent related to the clinical phenotypes of chronic bronchitis and frequent exacerbations, whereas the correlations to lung cancer and emphysema were weaker.

An important topic of Paper 4 was the search for different patterns of systemic inflammations related to the different COPD phenotypes. When analyzing the three largest principal components, it is difficult to identify patterns of significance due to high number and large diversity of the biomarkers contributing to the components (loading of the eigenvectors). The fourth largest principal component had a relatively higher correlation with emphysema than the other components. When looking into the variables related to the fourth principal component, neutrophils and NGAL were the most important factors. Neutrophils are related to several different immune responses but are a main source of proteinases. NGAL may prevent inhibition of matrix metalloprotease 9 (MMP9), a protease related to emphysema development. It should be noted that a principal component analysis is not designed to resolve causal relationships.

Our findings indicate a different and more significant impact of systemic inflammation on chronic bronchitis and frequent AECOPD than on emphysema and lung cancer development. However, our collection of biomarkers may not completely embrace the COPD pathophysiology. Several of our markers may be designated general or pluripotential inflammatory markers, whereas the number of markers more specific to emphysema may be limited. Emphysema-specific systemic biomarkers seem to be sparse, either because they do not exist, or because they are not yet discovered.

Other important factors related to both emphysema and lung cancer are inorganic toxins, free radicals, and other toxic substances found in cigarette smoke and polluted air. Such compounds may in addition to trigger an inflammatory response directly damage cells and tissue as well as DNA, contributing to development of both emphysema and malignant lesions. However, the amount and composition of these

compounds are not easily detected, and the impact of these compounds is likely not reflected by the measurement of systemic inflammatory biomarkers.

Our observation of a lower lung cancer rate in COPD patients using inhaled corticosteroids (ICS) is also described in earlier studies (267, 268), and has been subject for debate. An association between chronic inflammation and cancer development has been described for many different cancer types. Thus, it is tempting to hypothesize that the anti-inflammatory effect of corticosteroids may prevent cancer development. In COPD however, one must consider the potential treatment selection bias related to the different prescription of inhaled corticosteroids to the various COPD phenotypes. A patient with chronic bronchitis may be more likely to get ICS treatment, whereas the degree of emphysema is not necessarily reflected in coughing, exacerbations, or a low FEV_1, which are common factors leading to ICS prescription. A systematic review by Raymakers describes potential beneficial effects of ICS on the cancer rate in several population based studies, but not in RCTs (269). Related to the prescription bias, another factor necessary to consider is time-related bias, thus duration of ICS exposure related to study inclusion. A recent study by Suissa et al adjusted for time-related bias, and reported no significant effect of ICS on the lung cancer rate (270).

The lack of symptoms in early phase disease is a major problem when dealing with lung cancer. Development of symptoms is usually synonymous with metastatic disease, which usually renders curative treatment with surgery impossible. Screening with low-dose CT has long been debated and is now increasingly recommended (271, 272). The main obstacle for lung cancer screening is of course the high costs, and a main objective for a study of risk factors for lung cancer is the identification of easily accessible clinical parameters that can be used for the development of screening protocols. The most obvious and most frequently used variables in different protocols are age and smoking, the latter including time of smoking, amount in pack-years and active vs former smoking. Other proposed variables are passive smoking, occupational exposure and other lung disease such as COPD or emphysema (273, 274). Screening protocols for lung cancer should not only aim to include subjects with increased cancer

risk but should also allow for differentiation regarding re-screening. Compared to screening for several other major cancer types, lung cancer screening has a short expiration date which necessitates repeated CT-scans. The initial CT scan may also reveal emphysema, and our findings along with others may support a higher screening frequency in these patients compared to those with structurally normal lungs.

CONCLUSIONS

1 Systemic levels of inflammatory biomarkers were compared between COPD patients and healthy controls using cross-sectional data, and between stable COPD and COPD exacerbation. Macrophage migration inhibitory factor (MIF) was identified as potential biomarker for both for stable COPD as well as AECOPD.

2 Systemic levels of inflammatory biomarkers as well as clinical characteristics were evaluated as predictors for longitudinal outcome in COPD patients.

a) A history of exacerbations, female sex, chronic cough and a lower FEV_1 were identified as predictors for future AECOPD. High levels of GDF-15 were identified as a predictor for a higher future AECOPD count.

b) High levels of GDF-15 were identified as a predictor of a faster decline of both FEV_1 and FVC. Other factors associated with a faster FEV1 decline were male sex and cachexia.

c) High levels of GDF-15 were identified as a predictor of all-cause mortality as well as mortality due to respiratory disease. Other factors associated with a higher mortality were a low FEV_1, cachexia, obesity and a high degree of comorbidity.

d) COPD was significantly associated with a higher lung cancer risk. Within COPD patients, emphysema and obesity was associated with a higher lung cancer risk. Of 44 inflammatory biomarkers, only IP-10 was significantly associated with a higher lung cancer risk, whereas systemic inflammation evaluated by a PCA-analysis did not show any correlation with lung cancer development.

PERSPECTIVES

A perfect biomarker is specific and sensitive, predicts adverse outcome and is a target as well as an effect indicator of pharmacological treatment. In COPD, such a biomarker does probably not exist, but that does not mean that the search of biomarkers if futile. For a pulmonary physician, it is natural to compare the development in COPD research with that of lung cancer, the other major disease category in pulmonology apart from COPD. In lung cancer, there has been a significant change in both diagnostics and treatment the last 15 years. The traditional categorization based on histopathological patterns seen in the microscope, although still referred to by the pathologists, is of increasingly less clinical importance when choosing the optimal treatment. Today, the morphological lung cancer diagnosis is usually supplemented with an increasing number of biomarkers indicating the presence or absence of genetic mutations, translocations or expression of specific proteins, with the common attribute that they are all indicators of likely treatment effect from targeted therapy, specific for each marker. There is not necessary any logical relationship between these markers and the radiological or microscopic appearance, clinical symptoms or any other of the classical characteristics of lung cancer, but there is no doubt that this shift towards personalized medicine has been a major step forward in in lung cancer treatment. We will likely see a similar shift in COPD research for the next decades.

As of today, the only established clinical biomarker is the eosinophil blood count. High blood levels of eosinophils is an indicator of treatment effect of corticosteroids, both inhaled and peroral treatment in both stable disease as well as AECOPD. Eosinophils, however, are not specific for COPD and they are only relevant to a fraction of COPD patients. There are, however, numerous biomarkers associated with different aspects of COPD pathophysiology, which have potential clinical usefulness. As we gain a better knowledge on the COPD inflammatory pathways, it is likely that additional clinical useful biomarkers will emerge. Some as direct therapeutic targets, others as markers of treatment indication, or hopefully, some as markers of both.

Our project identifies novel biomarkers in COPD. The role of both MIF and GDF-15 in COPD should be investigated further. They are not COPD-specific, but both have the potential as targets for therapeutic intervention. On the other hand our project also underpins the concept of COPD as a heterogeneous disease. It is likely that many COPD biomarkers will be relevant only for fractions of the patients, depending on the dominant phenotype, inflammatory pathways and several other factors.

As mentioned in the introduction, COPD is an umbrella diagnosis containing different conditions with similar symptoms and airway obstruction as the common denominator. This has probably been beneficial in order to get attention from media, politicians and the general population to a low-status condition. A common disease definition has also likely contributed in uniting research environments and societies of thoracic and pulmonary medicine around the globe. This simplification has nevertheless had a backside, since it has contributed to an incomplete understanding of COPD. For the last decades it has been obvious that airway obstruction is just one of many symptoms of the disease instead of an all-important attribute. As a result, COPD is again being looked upon as a collection of similar conditions, having fundamental differences (12, 275, 276). As a consequence, future research on COPD should to a greater extent differentiate between disease phenotypes and take into consideration significant comorbidities such as cardiovascular disease when designing studies (156, 277).

There are several knowledge gaps in COPD, and future research should focus on closing these. Differences between smokers in terms of COPD development, differences between COPD patients in AECOPD frequency, lung function decline and life span, and differences in treatment effect are only a few. Differences due to genetics have been an obvious hypothesis, but apart from the genetic mechanisms behind emphysema due to A1AT-defiency, findings have been limited. Nonetheless, improved DNA-sequencing techniques have identified several genes associated with COPD (278). Epigenetic studies on COPD have only scratched the surface (23), but is a research area likely to accelerate the next years.

Genetics and epigenetics related to COPD is an exceedingly comprehensive research topic. However, the largest and most diverse source of genes in the lungs does not

originate in the patients genome, but to the microbiome residing in the airways. The distal airways were until recently considered sterile. New technology, especially techniques utilizing 16s-RNA sequencing have drastically changed this view (279). The role of bacteria, viruses and fungi in stable and exacerbated COPD is understudied, but is under investigation in several research environments including ours (280). There are likely complex interactions within the microbiome, and between the microbiome and the human immune system. Illuminating the functional role of the microbiome may be vital to fully understand the immune responses in COPD, and may also lead to new treatment strategies targeting the microbiome.

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APPENDICES

Appendix A	Questionnaire on symptoms and exposures at study inclusion/first visit
Appendix B	Questionnaire on exacerbation history and changes in medication and/or clinical status at subsequent visits 3-8
Appendix C	Questionnaire for the outpatient clinic/study physician at an event of suspected acute exacerbation of COPD
Appendix D	Questionnaire for monthly telephone interviews recording acute exacerbations of COPD
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Appendix F	Heatmap illustration of correlations between systemic inflammatory markers and COPD outcome and characteristics

(Appendix A-D: English translation, selected questions relevant for the papers of this thesis)

Appendix A

Visit 1 Bergen COPD Cohort study

Questionnaire 2

Questionnaire on symptoms and exposures at study inclusion/visit 1

Subject ID: Date of birth: Date: Visit nr:

A. Airway symptoms

1. Do you usually cough or clear your throat in the morning? (yes/no)
2. Do you usually cough during the day? (yes/no)
3. Do you usually have phlegm when coughing? (yes/no)
4. Do you have cough for three months or more altogether during a year? (yes/no)
5. Are you more breathless than people of your own age when walking uphill? (yes/no)
6. Are you breathless walking at a normal pace on level ground? (yes/no)
7. Are you breathless while at rest? (yes/no)
8. Do you experience breathlessness at rest? (yes/no)
9. Do you experience attacks of breathlessness? (yes/no)
10. Have you ever have had wheezing (a wheezing sound) in your chest? (yes/no)
11. Have you had wheezing (a wheezing sound) in your chest during the last 12 months? (yes/no)
12. Have you been breathless in conjunction with wheezing in your chest? (yes/no)
13. Have you had wheezing when not having symptoms of a cold? (yes(no)
14. Were you hospitalized before you were 2 years old because of lung disease (asthma, bronchitis, bronchiolitis, pneumonia)? (yes/no)

Appendix B

Visit 3-8 Bergen COPD Cohort study

Questionnaire 1

Questionnaire on exacerbation history and changes in medication or clinical status at subsequent visits 3-8

B COPD exacerbations and newly diagnosed diseases

Number of COPD exacerbations since Visit 1___

For exacerbation 1, register:

From date____ to____

Antibiotics: ☐No ☐Yes

Prednisolone/corticosteroids: ☐No ☐Yes

Hospital admission: ☐No ☐Yes

1. Name of medication _____ Dose _____

Start date (dd.mm.yyyy) _____ Stop date _____

2. Name of medication _____ Dose _____

Start date (dd.mm.yyyy) _____ Stop date _____

For exacerbation 2, register:

From date____ to____

Antibiotics: ☐No ☐Yes

Prednisolone/corticosteroids: ☐No ☐Yes

Hospital admission: ☐No ☐Yes

1. Name of medication _____ Dose _____

Start date (dd.mm.yyyy) _____ Stop date _____

2. Name of medication _____ Dose _____

Start date (dd.mm.yyyy) _____ Stop date _____

Have you been diagnosed with heart disease since Visit 1? ☐No ☐Yes
If yes, specify_____
Have you been diagnosed with hypertension since Visit 1? ☐No ☐Yes
Have you been diagnosed with diabetes since Visit 1? ☐No ☐Yes
Have you been diagnosed with cancer since Visit 1? ☐No ☐Yes
If yes, specify_____

C Regular medication

Have you been given influenza vaccine last season? ☐No ☐Yes

Regular use of medication ☐No ☐Yes

Have you started/stopped any medication since Visit 1? ☐No ☐Yes

If stopped any medication, describe name and date of stop.

_____

Name of all regular medication: Started before or after Visit 1?

1. Name_____Dose_____ ☐Before ☐After

If AFTER Visit 1: Start date (dd.mm.yyyy)_____

2. Name_____Dose_____ ☐Before ☐After

If AFTER Visit 1: Start date (dd.mm.yyyy)_____

D Smoking habits

Has the patient changed smoking habits since Visit 1?

☐No, still non-smoker ☐No, still smoker ☐Yes, reduced smoking

☐Yes, started smoking ☐Yes, quitted smoking ☐Yes, increased smoking

Appendix C

Ad hoc visit Bergen COPD Exacerbation Study

Exacerbation questionnaire

Questionnaire for the outpatient clinic/study physician at an event of suspected acute exacerbation of COPD

B. Airway symptoms (physician)

Constitution of the exacerbation:
(exacerbation is defined as the presence of 2 major symptoms or 1 major combined with 1 minor symptom for 2 consecutive days)

Major symptoms:

Do you have increased dyspnea?	☐Yes	☐No
Do you have increased amount of phlegm?	☐Yes	☐No
Have you had a change of color of the phlegm?	☐Yes	☐No

Minor symptoms

Do you have symptoms of a cold (nasal congestion/discharge)?	☐Yes	☐No
Do you have wheezing?	☐Yes	☐No
Have you had body temperature > 38℃	☐Yes	☐No
Have you had cold attacks or shiverings?	☐Yes	☐No

Date of onset of exacerbation (dd.mm.yyyy):_____

Worsening of dyspnea – the last 24 hours

☐Grade 1 = I am just as breathless as I usually am.
☐Grade 2 = I have been more breathless than usually when making errands outside the house (going to the grocery store etc.)
☐Grade 3 = I have been more breathless than usually during daily housework or similar activities.
☐Grade 4 = I have been more breathless than usual while at rest.
☐Grade 5 = I have been so breathless that I had to sit in an upright position during the night.

C. Other symptoms

Have you had chest pain during this exacerbation? ☐Yes ☐No
If yes, did they fluctuate with respiration? ☐Yes ☐No

D. Medication (physician)

Regular medication:
Name:_____ ____Dose:_____
Name:_____ ____Dose:_____
Name:_____ ____Dose:_____

E. Clinical findings

Respiratory rate (counted during 30 sec. minimum):_____
Heart rate:_____ Regular heart rate? ☐Yes ☐No
If irregular, measure ECG. Result ECG, atrial fibrillation? ☐Yes ☐No
Temperature (1 decimal):_____ Method applied (ear, mouth, rectal):_____
Systolic blood pressure:_____ Diastolic blood pressure:_____
Expiratory wheezing sounds? ☐Yes ☐No
Crepitations? ☐Yes ☐No
If crepitations, localization (mark all applicable):

Right	Left
☐Apical	☐Apical
☐Middle field	☐Middle field
☐Lower field	☐Lower field

Appendix D

Monthly telephone contact Bergen COPD Cohort Study

Questionnaire 15

Questionnaire for monthly telephone interviews recording acute exacerbations of COPD

How long time has passed since the last telephone contact?

☐4 weeks ☐8weeks ☐12 weeks ☐16 weeks ☐>16 weeks

Date last visit:_____

Questions

1. Have you during the last 4 weeks felt unwell or had an exacerbation of your COPD?

If yes, during the last weeks, which symptoms of your disease have worsened?

a) Cough during the day. ☐Yes ☐No

b) Cough during the night. ☐Yes ☐No

c) Phlegm. ☐Yes ☐No

d) Breathlessness ☐Yes ☐No

e) Fatigue, ability to do ordinary activities. ☐Yes ☐No

2. If telephone contact was not achieved at the previous attempt, i.e. it is more than 4 weeks since last contact: Have you since the last contact had an exacerbation of your COPD, i.e. more than 4 weeks ago? ☐Yes ☐No

If no, go to question 9, if yes, continue:

3. Have you made contact with a physician during the last 4 weeks due to worsening of your COPD? ☐Yes ☐No

4. Have you dialed the phone number on the laminated card (94807715) due to an exacerbation of your COPD during the last 4 weeks (or since last time we phoned you if more than 4 weeks ago)? ☐Yes ☐No

5. Have you consulted a physician at the outpatient for an acute examination as a result of a telephone contact with us? ☐Yes ☐No

6. Have you during the last 4 weeks had an exacerbation for which you received treatment from anyone other than the exacerbation team? ☐Yes ☐No

If yes, by whom did you receive treatment for your exacerbation?
a) your regular physician
b) another general practitioner
c) a specialist outside the hospital
d) a physician at the hospital

7. Have you during the last 4 weeks been admitted to an emergency ward due to your COPD? ☐Yes ☐No

If yes, which places:_____

8. Have you been hospitalized and stayed overnight at a hospital as a consequence of your COPD? ☐Yes ☐No

If yes, how many times?_____ If yes, how many days and nights?_____

If yes, which hospital?_____

9. Have you had a regular follow-up/consultation with a physician for your COPD during the last 4 weeks? ☐Yes ☐No

If yes, what type of physician?_____

10. Have you during the last 4 weeks taken any new medication for your COPD?
☐Yes ☐No

11. Have you just supplemental oxygen during the last 4 weeks?
☐Yes ☐No

If yes, how many hours have you used oxygen therapy?_____

Have you used oxygen therapy during exercise or physical activity? ☐Yes ☐No
Have you used oxygen therapy while at rest? ☐Yes ☐No
Have you used oxygen therapy during the night? ☐Yes ☐No

Appendix E

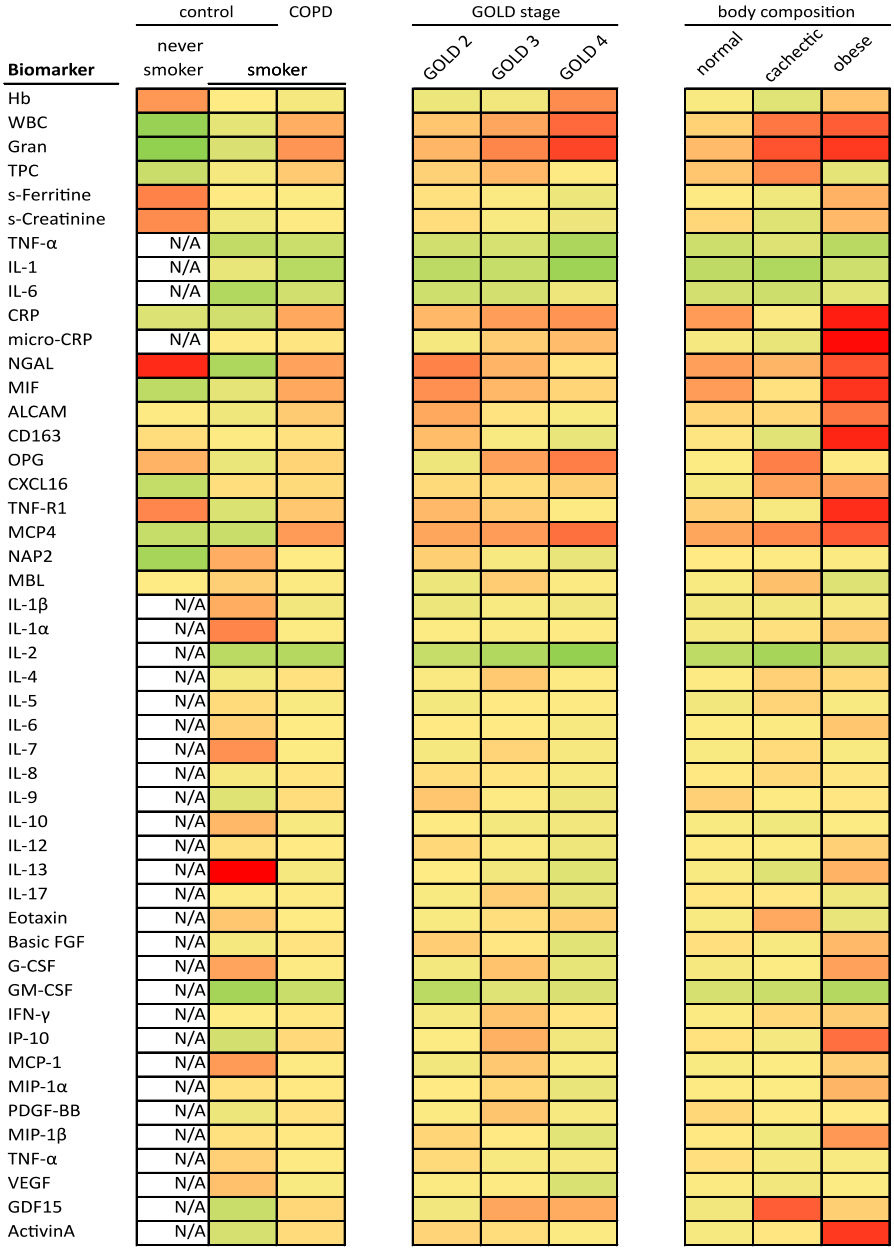
Overview of systemic inflammatory markers measured in COPD patients and controls

Biomarker	Full name	Alt. name	Analyzed
Hb	hemoglobin		Bergen
WBC	leucocyte		2007-2008
Gran	granulocytes, neutrophils		
Eos	eosinophils		
TPC	trombocyte platelet count		
Creatinine	creatinine		
Ferritine	ferritine		
NGAL	neutrophil gelatinase-associated lipocalin	LCN2	Oslo
CRP	C-reactive protein		2007-2008
OPG	osteoprotegrin	TNFRSF11B	
CXCL-16	CXC-motif ligand 16		
TNF-R1	tumor necrosis factor-receptor 1	TNFRSF1A	
MCP-4	monocyte chemotactic protein-4	CCL13	
NAP-2	neutrophil activating protein 2	CXCL7	
MBL	mannose-binding lectin		
micro-CRP	high sensitive C-reactive protein		Japan
TNF-α	tumor necrosis factor α	cachectin	2009
IL-1	interleukin 1		
IL-6	interleukin 6		
MIF	macrophage migration inhibitory factor	GIF	Oslo
Alcam	activated leukocyte cell adhesion molecule	CD166	2012
CD163	cluster of differentiation 163		
GDF-15	growth differentiation factor 15		USA
Activin-A	activin-A		2014
IL-1β	interleukin 1 β		USA [#]
IL-1rα	interleukin 1 receptor α		2014
IL-2	interleukin 2		
IL-4	interleukin 4		
IL-5	interleukin 5		
IL-6	interleukin 6		
IL-7	interleukin 7		
IL-8	interleukin 8	CXCL8	
IL-9	interleukin 9		
IL-10	interleukin 10		
IL-12	interleukin 12		
IL-13	interleukin 13		

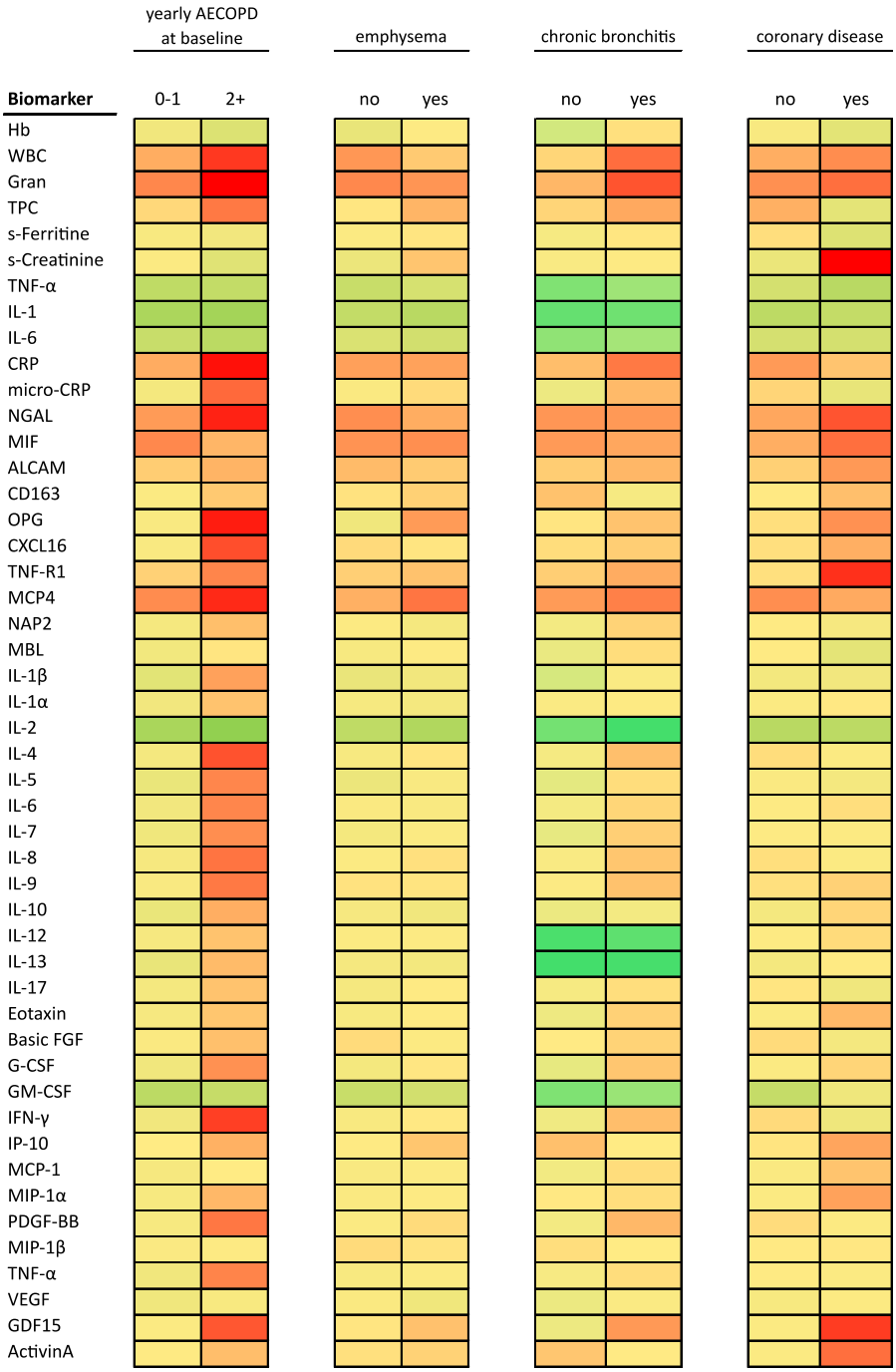
IL-15	interleukin 15	
IL-17	interleukin 17	
Eotaxin	eotaxin	
Basic FGF	basic fibroblast growth factor	
G-CSF	granulocyte colony stimulating factor	
GM-CSF	granulocyte-macrophage colony stimulating factor	
IFN-γ	interferon gamma	
IP-10	interferon gamma-induced protein 10	CXCL10
MCP-1	monocyte chemoattraction protein 1	CCL2
MIP-1α	macrophage inflammatory protein 1α	CCL3
MIP-1β	macrophage inflammatory protein 1β	CCL4
PDGF-BB	platelet derived growth factor-BB	
CCL5	chemokine (C-C motif) ligand 5	RANTES
TNF-α	tumor necrosis factor α	cachectin
VEGF	vascular-endothelial growth factor	

[#] IL1, IL6 and TNF-α were measured in duplicates, last measurements were not used in the analyses. IL15 and CCL5 were excluded from analysis due to technical errors.

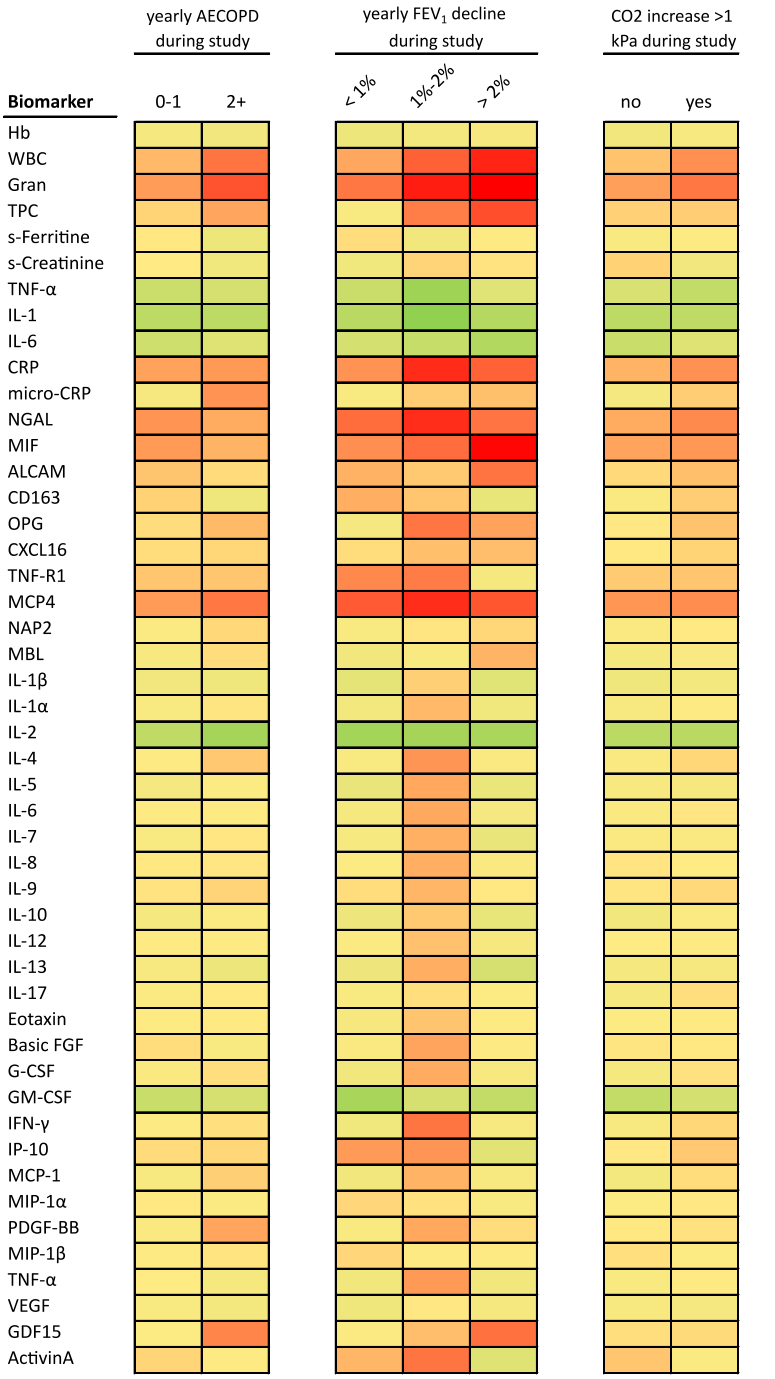
Appendix F Heatmap illustration of correlations between systemic inflammatory markers and COPD outcome and characteristics



Heatmap illustration of correlations between systemic inflammatory markers and COPD outcome and characteristics. All correlations are relative within category and biomarker. Colors depend both on biomarker levels and distrubution skewness.



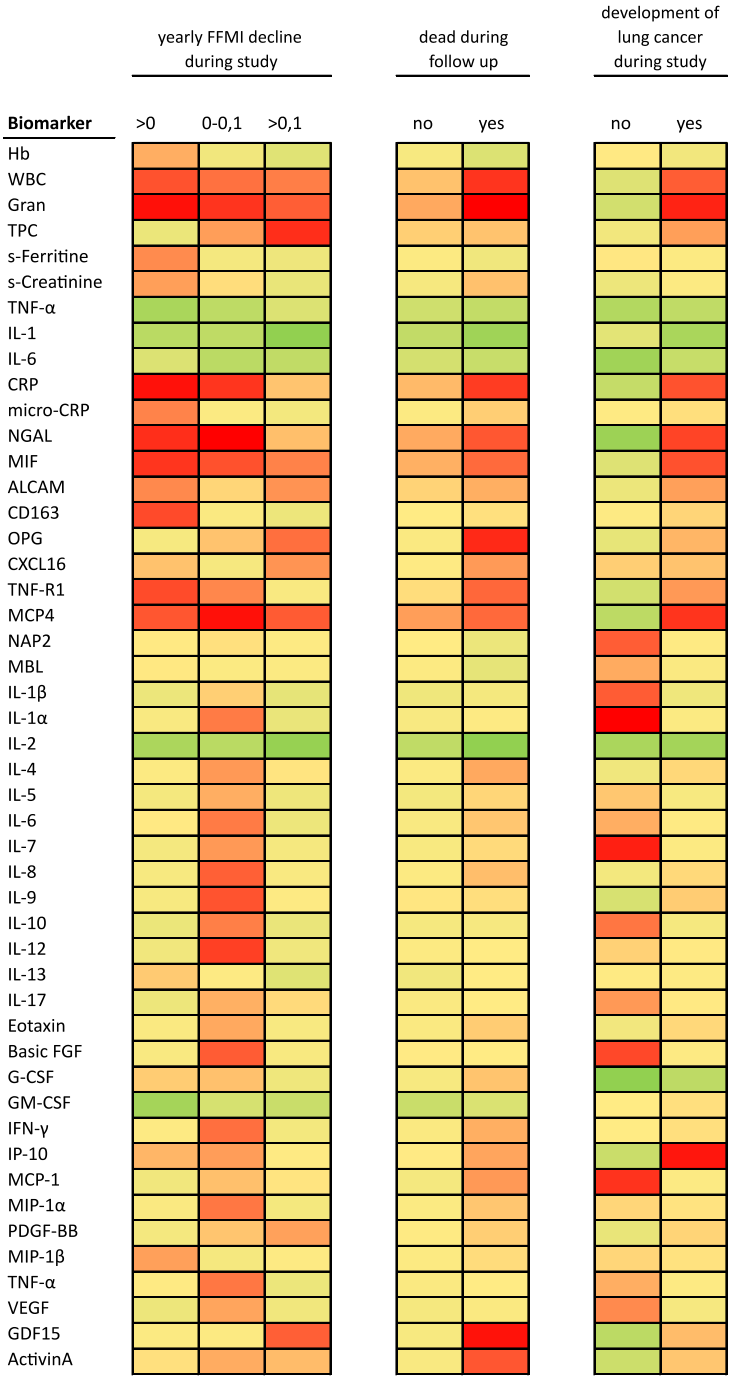
Heatmap illustration of correlations between systemic inflammatory markers and COPD outcome and characteristics. All correlations are relative within category and biomarker. Colors depend both on biomarker levels and distrubution skewness.



Heatmap illustration of correlations between systemic inflammatory markers and COPD outcome and characteristics. All correlations are relative within category and biomarker. Colors depend both on biomarker levels and distrubution skewness.

low levels/ right skewed distribution

high levels/ left skewed



Heatmap illustration of correlations between systemic inflammatory markers and COPD outcome and characteristics. All correlations are relative within category and biomarker. Colors depend both on biomarker levels and distrubution skewness.

PAPERS 1-4

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Predictors of Exacerbations in Chronic Obstructive Pulmonary Disease - Results from the Bergen COPD Cohort Study

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Abstract

Background: COPD exacerbations accelerate disease progression.

Aims: To examine if COPD characteristics and systemic inflammatory markers predict the risk for acute COPD exacerbation (AECOPD) frequency and duration.

Methods: 403 COPD patients, GOLD stage II-IV, aged 44–76 years were included in the Bergen COPD Cohort Study in 2006/07, and followed for 3 years. Examined baseline predictors were sex, age, body composition, smoking, AECOPD the last year, GOLD stage, Charlson comorbidity score (CCS), hypoxemia ($PaO2<8$ kPa), cough, use of inhaled steroids, and the inflammatory markers leucocytes, C-reactive protein (CRP), neutrophil gelatinase associated lipocalin (NGAL), soluble tumor necrosis factor receptor 1 (sTNF-R1), and osteoprotegrin (OPG). Negative binomial models with random effects were fitted to estimate the annual incidence rate ratios (IRR). For analysis of AECOPD duration, a generalized estimation equation logistic regression model was fitted, also adjusting for season, time since inclusion and AECOPD severity.

Results: After multivariate adjustment, significant predictors of AECOPD were: female sex [IRR 1.45 (1.14–1.84)], age per 10 year increase [1.23 (1.03–1.47)], >1 AECOPD last year before baseline [1.65 (1.24–2.21)], GOLD III [1.36 (1.07–1.74)], GOLD IV [2.90 (1.98–4.25)], chronic cough [1.64 (1.30–2.06)] and use of inhaled steroids [1.57 (1.21–2.05)]. For AECOPD duration more than three weeks, significant predictors after adjustment were: hypoxemia [0.60 (0.39–0.92)], years since inclusion [1.19 (1.03–1.37)], AECOPD severity; moderate [OR 1.58 (1.14–2.18)] and severe [2.34 (1.58–3.49)], season; winter [1.51 (1.08–2.12)], spring [1.45 (1.02–2.05)] and sTNF-R1 per SD increase [1.16 (1.00–1.35)].

Conclusion: Several COPD characteristics were independent predictors of both AECOPD frequency and duration.

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Competing Interests: PSB has within the last 5 years received lecture fees from GlaxoSmithKline, Pfizer, Novartis, AstraZeneca, and Boehringer Ingelheim, and received research grants from GlaxoSmithKline and Novartis, MAa has within the last 5 years received lecture fees from GlaxoSmithKline, Boehringer Ingelheim, and AstraZeneca, RG reports grants from The Norwegian Association of Heart and Lung Patients and EXTRA funds from the Norwegian Foundation for Health and Rehabilitation; grants from Boehringer Ingelheim, personal fees from GlaxoSmithKline; and travel grants from AstraZeneca, GlaxoSmithKline, Boehringer Ingelheim and Pfizer, TME has within the last 5 years received lecture fees from GlaxoSmithKline and Boehringer Ingelheim, and received travel grants from InterMune. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common illness worldwide, and its prevalence is increasing. The disease is heterogeneous where some patients are more prone to have exacerbations than other, proposed to be representing a phenotype of its own [1]. COPD exacerbations are associated with accelerated worsening of lung function [2,3], increased disease burden and mortality [4,5], thus making it important to identify and treat these patients.

So far, the best predictors found for future exacerbations are a history of previous exacerbations and decline in forced expiratory volume in one second (FEV1)[6–8], together with direct or indirect indicators of pulmonary hypertension [9,10]. Inflammatory biomarkers may also be of value, alone or several combined [11–15]. Other described predictors are depression [16], gastro-esophageal reflux disease (GERD) [17], and quality of life [18].

However, the existing knowledge of markers that could predict exacerbation of COPD is still limited. The inflammatory markers associated with COPD exacerbations found so far are unspecific,

Table 1. Characteristics of COPD patients according to exacerbation frequency during follow-up.

	Less than 1 exacerbations per year, n = 231	1 or more exacerbations per year, n = 172	p-Value*
Sex, %			0.45
Women	38.1	41.9	
Men	61.9	58.1	
Age, Mean (SD)	62.6 (6.8)	64.3 (6.8)	0.01
Body Composition, %			0.05
Normal	61.0	48.8	
Cachectic	25.1	31.4	
Obese	13.9	19.8	
Smoking, %			0.08
Ex	52.8	61.6	
Current	47.2	38.4	
Exacerbations last year prior to inclusion, %			<0.001
0–1	93.5	70.4	
2+	6.5	29.7	
GOLD 2007 classification, %			<0.001
FEV1 50–80%	58.9	33.7	
FEV1 30–50%	37.2	47.7	
FEV1<30%	3.9	18.6	
Hypoxemia, %			0.07
PaO2>8 kPa	90.9	84.7	
PaO2<8 kPa	9.1	15.3	
Charlson comorbidity Score, %			0.46
1	60.2	54.1	
2	23.8	23.8	
3	10.0	14.0	
4+	06.jan	8.1	
Chronic cough, %			0.002
No	61.5	45.6	
Yes	38.5	54.4	
Cough with phlegm, %			0.02
No	45.9	34.3	
Yes	54.1	65.7	
Use of inhaled steroids, %			<0.001
No	39.4	22.1	
Yes	60.6	77.9	
Inflammatory markers, Median (IQR)			
Leucocyte count (WBC), $x10^9$/l	7.7 (6.3 −9.1)	7.9 (6.6 −9.6)	0.11
C-reactive protein (CRP), ng/ml	3.4 (1.7 −6.8)	4.9 (2.1 −12.6)	0.003
Neutrophil gelatinase lipocalin (NGAL), 10 μg/ml	6.7 (5.1 −9.5)	6.7 (5.2 −9.2)	0.77
Soluble TNF receptor-1 (sTNF-R1), 100 μg/ml	6.8 (5.8 −8.1)	7.1 (5.6 −8.5)	0.43
Osteoprotegrin (OPG), ng/ml	5.5 (3.8 −7.1)	5.9 (4.5 −7.3)	0.10

*χ-square for categorical variables, t-test for means and Kruskal Wallis test for medians
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where abnormal values can be seen in a range of conditions, thus a continued evaluation of novel markers is warranted. Second, although several studies have linked exacerbations with increased inflammation [19,20], it is not fully understood whether this is a cause or a consequence, and for this purpose longitudinal studies are needed. Third, another measure of disease burden apart from exacerbation frequency is their duration, for which some associations have been described [21–24], but patients with delayed exacerbation recovery remains difficult to identify.

This study aimed to find predictors for COPD exacerbations and exacerbation duration, using longitudinal data from a large

cohort study in Western Norway, examining both clinical characteristics and novel systemic inflammatory markers.

Materials and Methods

Study population

433 Patients with COPD were included in the Bergen COPD Cohort Study (BCCS) between February 2006 and February 2008. All subjects in the study received written and oral information prior to inclusion and signed informed consent. The study was approved by the Regional Committee for Medical and Health Research Ethics, region West (REC-West), case number 165.08. The patients were aged between 44–76 years at the time of inclusion. All patients had a clinical diagnosis of COPD, and a ratio of forced expiratory volume in one second (FEV1) to forced vital capacity (FVC) <0.7 at least 15 minutes after bronchodilation, FEV1$<$80% predicted by Norwegian reference values [25], and a smoking history of more than 10 packyears. Exclusion criteria were lung diseases other than COPD, additional active inflammatory disease such as various autoimmune disorders, and having a COPD-exacerbation within 4 weeks prior to inclusion. No patients were using long-term prophylactic macrolides or other antibiotics except one patient who was using a tetracycline for a skin disease. The details of study design, patient selection and data collection have been described previously [26].

Data collection

Briefly, all patients were examined by a study physician. A physical examination, blood gas sampling, and a clinical interview that included exacerbation history, comorbidities and medication history were undertaken. All patients performed spirometry, before and after bronchodilation with 0.4 mg salbutamol, using a Viasys Masterscope. The patients were categorized into Global Initiative for Chronic Obstructive Lung Disease (GOLD 2007) categories II-IV based on post-bronchodilation FEV1.

Body composition was determined with bioelectrical impedance measurements. Cachexia was defined as a fat free mass index (FFMI) less than 17 kg/m^2 in men or less than 14 kg/m^2 in women, which corresponds to the lower 95% confidence limit in a normal population [27]. Obesity was defined as a fat mass index (FMI) of more than 9.3 kg/m^2 in men or more than 13.5 kg/m^2 in women [27].

All patients were examined and interviewed by a study physician at the out-patient clinic every 6 months for 3 consecutive years. At each visit, the study physician performed a detailed clinical interview, where all exacerbations were registered.

The exacerbation count was the main outcome in this study and was prospectively registered by the patients. We defined an exacerbation as a worsening of respiratory symptoms for two consecutive days or more. Exacerbations that did not require any change in treatment were defined as mild, those requiring treatment with antibiotics or systemic steroids by the decision of a physician were considered moderate, and those in need for hospital admission were considered severe [28]. Exacerbation duration was patient reported, based entirely on symptomatic recovery. The cut off for late recovery was set at three weeks; there exists no definition on a long lasting exacerbation, our limit for when to normally expect recovery was based on clinical experience.

Laboratory measurements

Peripheral blood sampling and analyses of total leukocyte (WBC) count, C-reactive protein (CRP), neutrophil gelatinase associated lipocalin (NGAL), soluble tumor necrosis factor receptor-1 (sTNF-R1), and osteoprotegrin (OPG) were performed as previously described [26,29]. WBC and CRP were chosen as inflammatory markers due to their availability as established indicators of inflammation. NGAL [29], sTNF-R1 [26], and OPG [26] have all been shown in cross-sectional analyses from our cohort to be associated with important COPD disease characteristics including FEV1 and exacerbation frequency.

Arterial blood gas analysis was sampled and examined within 5 minutes with a Radiometer ABL520 analyzer [30]. Hypoxemia was defined as a partial oxygen pressure <8.0 kPa.

Missing Values

30 patients only participated in the baseline visit. Of the 30 patients, 9 were excluded due to use of oral steroids, 8 died before any follow-up visits were performed, in 2 patients CT scans revealed lung cancer, and finally 11 patients withdrew their consent to participate. Thus, 403 patients were included in the statistical analyses. Information regarding chronic cough and cough with phlegm was missing in 8 and 2 patients, respectively. Plasma-sampling failed in 12 patients, and for 1, 2, and 7 patients we lacked sufficient plasma to measure sTNF-R1, OPG, and NGAL, respectively. Arterial blood gas analysis failed in 37 cases, most commonly a sampling error where the patient did not want puncture.

Statistical analyses

The exacerbation count distribution was heavily skewed to the right. For the baseline characteristics analysis the exacerbation count was dichotomized into patients with an average exacerbation count of less than 1 per year, and those with 1 or more per year. Mild exacerbations were not included in the exacerbation count analysis, as in concordance with prior studies [6–8], and due to suspected under-reporting [31]. Bivariate associations were examined with t-tests or non-parametric tests for continuous variables, and χ-square tests for categorical variables.

Random effects negative binomial regression models conditional on gamma errors were fitted to estimate the incidence rate ratios (IRR) for each potential predictor variable. Correspondingly, a multivariate model was fitted including all the predictor variables from the bivariate analyses except for cough with phlegm, which showed a strong colinearity with chronic cough. The inflammatory markers also showed strong colinearity with each other, and were therefore tested separately added to the main model. To test for possible interactions by sex, all variables that differed statistically by sex at baseline were tested one at a time with an interaction term in the final multivariate model.

In addition, exacerbations were analyzed according to duration, searching for factors associated with recovery time exceeding three weeks. A generalized estimating equation logistic regression model with exchangeable correlation structure was fitted, testing potential predictor variables both separate and multivariate. Stata 12.1 (StataCorp LP, College Station, TX, USA) was used for the statistical analyses.

Results

350 out of the 403 COPD patients experienced one or more exacerbations during the three years of follow-up. A total of 1696 exacerbations were registered, of which 393 were classified as mild, 933 as moderate, and 370 as severe. Women had more exacerbations than men, the difference consisting of more exacerbations of moderate severity ($p = 0.001$). The median duration for an exacerbation was 14 days (interquartile range 15 days) .

Table 2. Bivariate predictors of the annual incidence rate ratio (IRR) of moderate or severe COPD exacerbations, estimated by a random effects negative binomial model.

Baseline explanatory variables	IRR	(95% CI)	p-Value
Sex			
Men	1		
Women	1.27	(0.99 −1.63)	0.06
Age			
per 10 years increase	1.24	(1.04 −1.48)	0.02
Body Composition			
Normal	1		
Cachectic	1.41	(1.07 −1.85)	0.02
Obese	1.23	(0.88 −1.71)	0.23
Smoking			
Ex	1		
Current	0.83	(0.65 −1.06)	0.13
Exacerbations 12 months before inclusion			
0–1	1		
2+	2.74	(2.08 −3.61)	<0.001
GOLD 2007 classification			
FEV1 50–80%	1		
FEV1 30–50%	1.75	(1.38 −2.23)	<0.001
FEV1<30%	3.59	(2.51 −5.13)	<0.001
Hypoxemia			
PaO2>8 kPa	1		
PaO2<8 kPa	1.61	(1.11 −2.34)	0.01
Charlson comorbidity Score			
1	1		
2	1.07	(0.79 −1.43)	0.67
3	1.21	(0.82 −1.77)	0.33
4+	1.37	(0.86 −2.21)	0.19
Chronic cough			
No	1		
Yes	1.73	(1.36 −2.19)	<0.001
Cough with phlegm			
No	1		
Yes	1.38	(1.08 −1.77)	0.01
Use of inhaled steroids			
No	1		
Yes	2.11	(1.62 −2.74)	<0.001
Inflammatory markers *			
Leucocyte count (WBC)	1.13	(1.00 −1.29)	0.05
C-reactive protein (CRP)	1.13	(1.01 −1.26)	0.04
Neutrophil gelatinase lipocalin (NGAL)	1.01	(0.90 −1.13)	0.89
Soluble TNF receptor-1 (sTNF-R1)	1.05	(0.94 −1.18)	0.36
Osteoprotegrin (OPG)	1.13	(1.01 −1.27)	0.04

*Per 1 SD increase of marker value.

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The baseline characteristics of the study population are presented in Table 1. Patients with a higher exacerbation rate were slightly older, more cachectic or obese, had a higher number of exacerbations before inclusion, had a lower FEV1 in % predicted, and had higher frequencies of chronic cough and cough with phlegm. They were also more frequent users of inhaled corticosteroids (ICS). Of the measured inflammatory markers upon inclusion, only CRP was significantly higher in patients with more frequent exacerbations during follow-up.

Table 3. Multivariate model of the annual incidence rate ratio (IRR) of moderate or severe COPD exacerbations, estimated by a random effects negative binomial model.

Baseline explanatory variables	IRR	95% CI	p-Value
Sex			
Men	1		
Women	1.45	(1.14 −1.84)	0.002
Age			
per 10 years increase	1.23	(1.03 −1.47)	0.02
Body composition			
Normal	1		
Cachectic	1.19	(0.91 −1.56)	0.22
Obese	1.23	(0.90 −1.69)	0.19
Smoking			
Ex	1		
Current	0.93	(0.73–1.19)	0.56
Exacerbations last year			
0–1	1		
2+	1.65	(1.24–2.21)	0.001
GOLD 2007 classification			
FEV1 50–80%	1		
FEV1 30–50%	1.36	(1.07–1.74)	0.01
FEV1<30%	2.90	(1.98–4.25)	<0.001
Hypoxemia			
PaO2>8 kPa	1		
PaO2<8 kPa	1.10	(0.79–1.54)	0.58
Charlson comorbidity Score			
1	1		
2	0.97	(0.74–1.27)	0.81
3	0.98	(0.68–1.42)	0.93
4+	0.98	(0.61–1.57)	0.93
Chronic cough			
No	1		
Yes	1.64	(1.30–2.06)	<0.001
Use of inhaled steroids			
No	1		
Yes	1.57	(1.21–2.05)	0.001
Inflammatory markers added one each, to the above model *			
Leucocyte count (WBC)	1.04	(0.93–1.17)	0.49
C-reactive protein (CRP)	1.03	(0.93–1.14)	0.56
Neutrophil gelatinase lipocalin (NGAL)	0.99	(0.89–1.10)	0.85
Soluble TNF receptor-1 (sTNF-R1)	1.03	(0.92–1.16	0.56
Osteoprotegrin (OPG)	0.92	(0.82–1.03)	0.15

*IRR per 1 SD increase of marker value.

doi:10.1371/journal.pone.0109721.t003

Factors associated with annual exacerbation rate

Table 2 shows bivariate associations between possible predictor variables and annual exacerbation rate. Age, cachexia, number of exacerbations the year before inclusion, GOLD stage, hypoxemia, cough symptoms, and use of ICS were all associated with a higher IRR. Higher levels of CRP and OPG at baseline, but not levels of WBC, NGAL and sTNF-R1, were predictive of a higher exacerbation rate within the follow-up.

The adjusted IRRs are shown in Table 3. Significant predictors of a higher risk for moderate or severe exacerbations were female sex, higher age, a history of frequent exacerbations prior to inclusion, higher GOLD stage, chronic cough and use of ICS. Of all potential interactions between sex and the other variables tested, none were found to be statistically significant.

Thus, mostly the associations seen in the bivariate analyses were confirmed, except for the inflammatory markers, which were not

Table 4. Bivariate predictors of copd-exacerbation duration more than three weeks, estimated by a generalized estimation equation logistic regression model.

Baseline explanatory variables	OR	95% CI	p-Value
Sex			
Men	1		
Women	1.02	(0.79 −1.31)	0.90
Age			
per 10 years increase	0.94	(0.78 −1.13)	0.52
Body Composition			
Normal	1		
Cachectic	1.07	(0.81 −1.43)	0.63
Obese	1.43	(1.02 −2.00)	0.04
Smoking			
Ex	1		
Current	1.24	(0.96 −1.59)	0.10
Exacerbations 12 months before inclusion			
0–1	1		
2+	1.20	(0.91 −1.59)	0.20
GOLD 2007 classification			
FEV1 50–80%	1		
FEV1 30–50%	1.11	(0.84 −1.45)	0.47
FEV1<30%	1.05	(0.72 −1.53)	0.79
Hypoxemia			
PaO2>8 kPa	1		
PaO2<8 kPa	0.83	(0.57 −1.22)	0.34
Charlson comorbidity Score			
1	1		
2	1.51	(1.13 −2.02)	0.005
3	1.26	(0.85 −1.85)	0.25
4+	1.21	(0.77 −1.89)	0.42
Chronic cough			
No	1		
Yes	1.43	(1.11 −1.85)	0.005
Cough with phlegm			
No	1		
Yes	1.18	(0.91 −1.53)	0.22
Use of inhaled steroids			
No	1		
Yes	0.97	(0.72 −1.30)	0.83
Time since inclusion			
Per year increase	1.2	(1.05–1.36)	0.006
Exacerbation severity			
Mild	1		
Moderate (use of antibiotics or steroids)	1.51	(1.12 −2.01)	0.006
Severe (admission to hospital)	2.25	(1.60 −3.17)	<0.001
Season			
Summer	1		
Autumn	1.24	(0.90 −1.71)	0.18
Winter	1.48	(1.09 −2.01)	0.01
Spring	1.36	(0.99 −1.86)	0.06
*Inflammatory markers **			
Leucocyte count (WBC)	1.11	(0.98 −1.25)	0.10

Table 4. Cont.

Baseline explanatory variables	OR	95% CI	p-Value
C-reactive protein (CRP)	0.94	(0.83 −1.07)	0.36
Neutrophil gelatinase lipocalin (NGAL)	1.06	(0.93 −1.21)	0.37
Soluble TNF receptor-1 (sTNF-R1)	1.14	(1.01 −1.28)	0.04
Osteoprotegrin (OPG)	0.97	(0.85 −1.09)	0.59

*IRR per 1 SD increase of marker value.
doi:10.1371/journal.pone.0109721.t004

significant predictors of later exacerbations in the model where a large number of covariables were included.

Factors associated with exacerbation duration more than three weeks

Table 4 and 5 show bi- and multivariate associations between potential predictor variables and exacerbations lasting more than 3 weeks. Variables with significant bivariate associations were obesity, Charlson comorbidity score (CCS) 2, chronic cough, increasing time since inclusion, increasing exacerbation severity, exacerbations during winter and spring (December-February and March-May), and higher levels of baseline sTNF-R1. The same variables remained significant after multivariate adjustment, with the exception of obesity, CCS and chronic cough. The presence of hypoxemia was after adjustment associated with exacerbation duration shorter than three weeks. Age, sex, GOLD-stage, smoking status and use of medication along with the other inflammatory markers did not show a significant association with exacerbation duration.

Discussion

We used prospective follow-up data from a large cohort study from western Norway to identify predictors for COPD exacerbation frequency and duration. The best predictors of future exacerbations in this study were a history of frequent exacerbations prior to inclusion, lower lung function, increasing age, and female sex, confirming the findings of earlier studies [6–8]. Furthermore, we identified some easily accessible clinical variables independently associated with increased exacerbation rates such as chronic cough and the use of inhaled steroids.

Exacerbation duration was significantly associated with exacerbation severity and season, which is in accordance with other studies [21,22,24]. In addition, we identified an association between exacerbation duration and both hypoxemia and sTNF-R1 not demonstrated before.

The main strengths of this study were the prospective design, the large number of patients, and the assessment of a series of different variables. This allowed for the use of complex regression analysis and adjustment for several key variables. Attendance rate at the visits was high, varying between 86 and 97 percent. The longitudinal design allowed for predictive statistical modeling, however, no intervention was done, and the concept prediction should not be confused with causality.

The statistical analysis of exacerbation frequency is complex, due to its distorted distribution and due to clustering both in subject and time [32]. One approach is to compare the frequent vs. the non-frequent exacerbator using logistic regression. However, negative binomial or Poisson regression may be more suited [33]. In our study we treated the exacerbation frequency as a count variable. Both Poisson and negative binomial models were considered, but due to overdispersion of the data the latter model was preferred, though a Poisson model was also fitted producing almost identical results (not shown here).

The exacerbation data was acquired through interview by the study physician, with the aid of the patients' journal present. The majority of patients (n = 350) lived in a proximity to our hospital, which would have led them to attend our hospital in an emergency. Due to the long follow-up, we did not use an exacerbation diary or other grading tools although these methods have been validated [34]. Thus, we believe under-reporting of severe exacerbations were highly unlikely and under-reporting of moderate exacerbations unlikely but probably present to some extent. Regarding severe exacerbations, apart from hospital admission and duration, we had no other clinical information to validate its severity, and due to this we chose to analyze severe and moderate exacerbations together. For the analysis of exacerbation duration, mild exacerbations were included in the model despite the limitations in in the data collection mentioned above, and this should be taken into consideration when interpreting the data.

This study showed a large diversity in both exacerbation frequency and duration. In agreement with earlier studies, a person with exacerbations in the past is more likely to experience exacerbations in the future [6–8]. Earlier studies have shown that women experience more symptoms from their illness, but mortality rates have shown gender equality [35]. A similar picture emerged in our study, where women experienced a higher rate of moderate exacerbations, but not severe exacerbations requiring hospital admission. Whether this represents a genuine increase in exacerbations or an increased tendency among women to seek medical attention remains unclear.

Somewhat surprising, but also seen before [6], was the finding that ex-smokers had no reduction of the exacerbation count compared to active smokers. This could imply that smoking cessation was too late, and that disease progression continued after smoking cessation. Another explanation may be that most of our cohort consisted of a selection of COPD patients having had prior consultations with pulmonary physicians [26], which might affect smoking habits, where perhaps the most symptomatic patients were more likely to have quit prior to entering the study. Different exacerbation rates between smokers and ex-smokers have been seen in COPD patients selected from a more general population [36]. Finally, time since smoking cessation was not a significant variable in our study (data not shown), but our study may not have been powered to examine that properly.

The association between exacerbation frequency and use of ICS may seem paradoxical as large randomized studies have shown a modest, but significant decrease in exacerbation risk with their use [37–40]. Nevertheless, randomized trials often include highly selected study populations, and non-intervention cohort studies as ours add to the existing knowledge. Several studies have shown an association between ICS and pneumonia rate [41–43], and it is

Table 5. Multivariate model of copd-exacerbation duration more than three weeks, estimated by a generalized estimation equation logistic regression model.

Baseline explanatory variables	OR	95% CI	p-Value
Sex			
Men	1		
Women	1.17	(0.88–1.56)	0.29
Age			
per 10 years increase	0.88	(0.70–1.10)	0.27
Body Composition			
Normal	1		
Cachectic	0.92	(0.66–1.29)	0.63
Obese	1.35	(0.93–1.98)	0.11
Smoking			
Ex	1		
Current	1.29	(0.95–1.76)	0.11
GOLD 2007 classification			
FEV1 50–80%	1		
FEV1 30–50%	1.23	(0.90–1.67)	0.19
FEV1<30%	1.18	(0.75–1.87)	0.48
Hypoxemia			
PaO2>8 kPa	1		
PaO2<8 kPa	0.60	(0.39–0.92)	0.02
Charlson comorbidity Score			
1	1		
2	1.14	(0.81–1.59)	0.46
3	1.15	(0.72–1.82)	0.56
4+	1.36	(0.76–2.42)	0.30
Chronic cough			
No	1		
Yes	1.29	(0.97–1.71)	0.08
Time since inclusion			
Per year increase	1.19	(1.03–1.37)	0.02
Exacerbation severity			
Mild	1		
Moderate (use of antibiotics or steroids)	1.58	(1.14–2.18	0.006
Severe (admission to hospital)	2.34	(1.58–3.49)	<0.001
Season			
Summer	1		
Autumn	1.33	(0.94–1.89)	0.11
Winter	1.51	(1.08–2.12)	0.02
Spring	1.45	(1.02–1.35)	0.04
Soluble TNF receptor-1 (sTNF-R1)			
per 1 SD increase of marker value	1.16	(1.00–1.35)	0.05

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possible that use of ICS comes with an increased risk for infectious exacerbations of COPD. We observed an exacerbation IRR of 1.57 in ICS-users after multivariate adjustments, but still we cannot exclude a selection bias since these patients may have received their ICS due to worsening of symptoms not accounted for in our model. Also, in our study, a majority of the patients using ICS (91%) were using them in combination with a long acting beta-2-agonist (LABA), making it difficult to separate the effect of the ICS vs. the LABA. Thus, our observation of increased exacerbation rate in ICS-users cannot be interpreted as a causal effect, but nonetheless, an observation of ICS-use may aid in identifying a patient with increased risk for future exacerbations.

A primary objective in this study was to evaluate the association between systemic inflammation and exacerbation rate measuring inflammatory markers at inclusion. In our study we only measured the inflammatory markers at baseline, and their predictive value

for an event up to three years later is likely to decrease as time goes by. CRP and OPG at baseline were bivariately associated with the exacerbation rate, but not after multivariate adjustment. WBC, fibrinogen and uric acid have independently shown predictive value in other studies [6,11,14], indicating that systemic inflammation may be a prerequisite for exacerbations. CRP has shown predictive value in combination with the WBC and fibrinogen [12], and the specificity and sensitivity may be further improved with the construction of so called inflammomas [15], containing three or more easily accessible markers, or with sputum samples, nonetheless these approaches have still yielded limited clinical value, so the search for additional markers should continue.

It is challenging to predict those patients in risk for a long lasting exacerbation. Dissimilar from the exacerbation rate, factors like prior exacerbations and FEV1 did not seem to affect the duration. Increasing values of sTNF-R1 as a marker of activity in the TNF system was associated with late exacerbation recovery, and may be a marker of chronic inflammation in COPD. On the other hand, sTNF-R1 is associated with important comorbidities difficult to adjust for [26], and this finding must be confirmed in other studies. The association between duration and season can be linked to both lower temperature [44] and seasonality of viral infections [45], and was anticipated; on the other hand, the observation of reduced recovery time in patients with hypoxemia was unexpected. Hypoxemic patients are likely to have more symptoms in their stable state, perhaps making it difficult to distinguish between the exacerbated and the stable state, which might affect their exacerbation reporting. Nonetheless, this observation should be confirmed in other studies.

This study illustrates the vicious circle affecting a large proportion of COPD patients, where one exacerbation predisposes for the next, leading to an ever increasing disease burden. This underscore the need for markers that could further identify patients at risk, as well as the need for proper intervention in these patients. We identify several clinical parameters for recognition of patients at risk for frequent or long lasting exacerbations, making it possible for earlier or more extensive preventive intervention. Finally, there is still a lack of useful inflammatory markers, both to identify patients with high risk of future exacerbations, as well as a diagnostic tool to detect ongoing exacerbations.

Author Contributions

Conceived and designed the experiments: GRH PSB JAH PA TME. Performed the experiments: GRH PSB MA JAH TU RG LJPP TME. Analyzed the data: GRH MA LJPP TME. Contributed reagents/materials/analysis tools: TU PA TME. Contributed to the writing of the manuscript: GRH PSB MA JAH TU RG LJPP PA TME.

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Risk factors for lung cancer in COPD – results from the Bergen COPD cohort study☆

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ABSTRACT

Background: COPD patients have an increased risk of developing lung cancer, but the underlying mechanisms are poorly understood. We aimed to identify risk factors for lung cancer in patients from the Bergen COPD Cohort Study.

Methods: We compared 433 COPD patients with 279 healthy controls, all former or current smokers. All COPD patients had FEV1 < 80% and FEV1/FVC-ratio < 0.7. Baseline predictors were sex, age, spirometry, body composition, smoking history, emphysema assessed by CT, chronic bronchitis, prior exacerbation frequency, Charlson Comorbidity Score, inhalation medication and 44 serum/plasma inflammatory biomarkers. Patients were followed up for 9 years recording incidence of lung cancer. Cox-regression models were fitted for the statistical analyses. The biomarkers were evaluated using principal component analysis.

Results: 28 COPD patients and 3 controls developed lung cancer, COPD patients had a significantly higher risk of developing lung cancer, (HR 5.0; 95% CI 1.5–17.1, $p < 0.01$, adjusted values). Among COPD patients, emphysema (HR 4.4; 1.7–10.8, $p < 0.01$) and obesity (HR 3.3; 1.3–8.5, $p = 0.02$) were associated with a higher cancer rate. Use of inhaled steroids was associated with a lower rate (HR 0.4; 0.2–0.9, $p = 0.03$). Smoking status, pack-years smoked or levels of systemic inflammatory markers, except for interferon gamma-induced protein 10, did not affect the lung cancer rate in patients with COPD.

Conclusion: Patients with COPD have a higher lung cancer rate compared to healthy controls adjusted for smoking. The presence of emphysema and obesity in COPD predicted a higher lung cancer risk in COPD patients. Systemic inflammation was not associated with increased lung cancer risk.

1. Introduction

COPD and lung cancer are two major causes of morbidity and mortality worldwide. COPD is the fourth leading cause of death in the world, whereas lung cancer is the foremost cause of cancer deaths [1]. The incidence of both conditions have been increasing in the last years, and this trend is expected to continue for the next decade [2]. Furthermore, there is a known association between these two common disorders [3]. One obvious explanation for the co-existence of these conditions is their common risk factors, where tobacco-smoking is the most important. However, several studies find that a diagnosis of COPD, regardless of the amount of smoking, is an independent risk factor for development of lung cancer [4,5]. Additionally, lung cancer incidence has been also shown to be associated with the presence of emphysema on CT scan independently of the degree of airway obstruction or smoking history [6,7]. These findings might implicate a pathophysiological link between COPD and lung cancer beyond that of smoking [8].

COPD, however, is a heterogeneous disease where the different phenotypes may overlap, and it is unclear whether COPD patients with predominant airway inflammation have a similar increased lung cancer

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risk. Earlier studies have found an association between systemic inflammation and both frequent exacerbations and a higher mortality in COPD patients [9]. Nevertheless, a potential link between systemic inflammation and lung cancer development has not been sufficiently explored. Research on this topic may be useful both for finding cancer biomarkers for detection of early cancer, as well as gaining a better understanding of mechanisms by which lung tissue in some COPD patients undergo malignant transformation.

Our study aimed to evaluate several different COPD phenotypes and characteristics as risk factors for the development of lung cancer, combining clinical data and systemic inflammatory markers from a large COPD cohort study with longitudinal follow up, merged with data from the Norwegian cancer registry.

2. Materials and methods

2.1. Study population

433 subjects with COPD and 279 healthy controls, all between 40 and 76 years old, were recruited to the Bergen COPD Cohort Study between 2006 and 2009 [10]. Both COPD patients and controls had a smoking history of more than 10 pack-years. All COPD patients had a clinical diagnosis of COPD, a post-bronchodilation test with FEV_1/FVC-ratio < 0.7, and FEV_1 < 80% of predicted value. Exclusion criteria were known cancer within 5 years prior to entry, asthma or lung diseases other than COPD, active inflammatory disorders, and COPD exacerbations 4 weeks prior of inclusion, this latter category could be included later. The Regional Committee for Medical and Health Research Ethics, region west approved the study (REK-Vest, case number 2014/2153). Informed written consent was obtained from all participants.

2.2. Data collection

All subjects were evaluated by a study physician at inclusion, including a clinical interview regarding respiratory symptoms, smoking history, comorbidities and medication use. Comorbidities were pooled to calculate Charlson Comorbidity Score (CCS). All patients performed spirometry, before and after bronchodilation with 0.4 mg salbutamol. COPD patients were categorized according to 2007 Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines. The diagnosis of lung cancer was obtained from the Norwegian Cancer Registry, where registration is mandatory by law, with near 100% coverage among both healthy controls and patients (see also supplementary materials) [11]. Body composition was evaluated with bioelectrical impedance measurements. Cachexia was defined as a fat free mass index (FFMI) less than 17 kg/m^2 or 14 kg/m^2 in men and women, respectively [12], which corresponds to the lower 95% confidence limit in a normal population [13]. Obesity was defined as a fat mass index (FMI) of more than 9.3 kg/m^2 in men or more than 13.5 kg/m^2 in women [13]. Emphysema was assessed by computer tomography (CT) of the lungs, defined as having more than 10% of emphysematous lung tissue, specified as tissue density of less than −950 HU. Chronic bronchitis was defined as having cough with phlegm for more than three months the year before inclusion.

2.3. Laboratory measurements

Peripheral blood sampling was performed as previously described [10]. The analysis of the 44 inflammatory markers was performed with enzyme immunoassays (EIAs) and magnetic bead multiplex assays (see supplementary files for details).

2.4. Statistical methods

The baseline comparison between the study populations (COPD

Table 1
Baseline characteristics of the study population.

Characteristics	Smoking controls (n = 279)	COPD (n = 433)	p-Value[a]
Age, mean (SD)	58.0 (10.0)	63.5 (6.9)	< 0.01
Sex, No (%)			
Female	128 (45.9)	175 (40.4)	0.15
Male	151 (54.1)	258 (59.6)	
Smoking status, No (%)			
Ex	103 (36.9)	243 (56.1)	< 0.01
Current	176 (63.1)	190 (43.9)	
Packyears, mean (SD)	32.1 (21.5)	40.4 (22.7)	< 0.01
Body composition, No (%)			
Normal	248 (88.9)	242 (55.9)	< 0.01
Cachectic	11 (3.9)	123 (28.4)	
Obese	20 (7.2)	68 (15.7)	
Charlson Comorbidity Score, No (%)			
0	197 (70.6)	0	< 0.01
1	61 (21.9)	250 (57.7)	
2	16 (5.7)	102 (23.6)	
3	5 (1.8)	51 (11.8)	
4+	0	30 (6.9)	
Lung cancer, No (%)	3 (1.1)	28 (6.5)	< 0.01

[a] χ^2 or Wilcoxon rank-sum test.

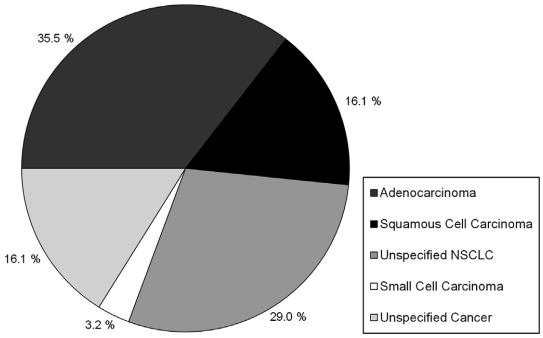

Fig. 1. Distribution of lung cancer histology.

Table 2
Risk factors for the development of lung cancer in COPD patients vs smoking controls, bi-and multivariable cox-regression.

Variables	Bivariable			Multivariable		
	HR	95% CI	p-value	HR	95% CI	p-value
Age per 10 year increase	1.97	(1.24–3.13)	0.004	1.82	(1.04–3.20)	0.04
Sex						
Female	1			1		
Male	1.08	(0.53–2.20)	0.84	0.86	(0.41–1.82)	0.69
Smoking status						
Ex	1			1		
Current	0.57	(0.28–1.18)	0.13	1.18	(0.55–2.57)	0.67
Packyears per 10 units increase	1.12	(0.99–1.25)	0.06	1.03	(0.90–1.18)	0.68
Body composition						
Normal	1			1		
Cachectic	0.92	(0.31–2.72)	0.88	0.48	(0.15–1.48)	0.20
Obese	2.91	(1.31–6.48)	0.009	2.13	0.92 to 4.92)	0.08
Patient category[a]						
Control	1			1		
COPD-patient	6.33	(1.92–20.8)	0.002	4.98	(1.45–17.1)	0.01

[a] All controls and patients were current or former smokers.

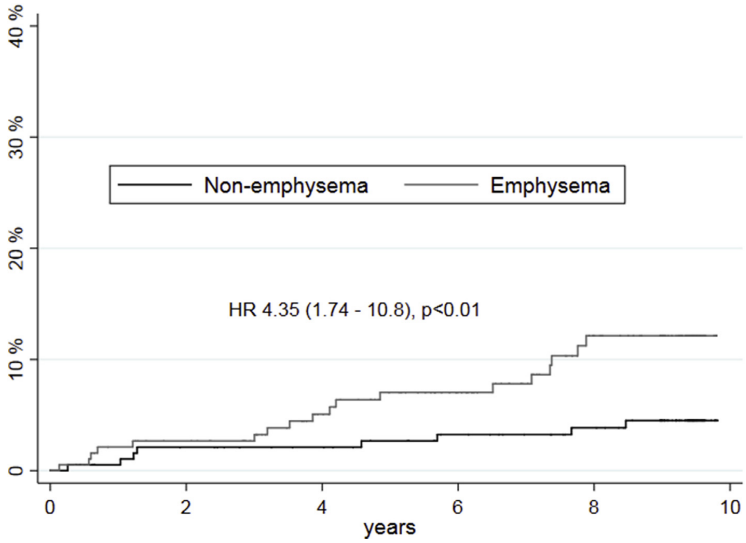

Fig. 2. Incidence of lung cancer in patients with and without emphysema during follow up.

patients vs controls, and cancer vs non-cancer subjects), was done using non-parametric tests (Wilcoxon rank-sum for continuous and χ^2-test for categorical variables).

A cox-regression model was fitted to evaluate risk factors of lung cancer in COPD patients vs smoking controls. Age, sex, smoking status, pack-years smoked, and body composition were adjustment factors. Similarly, for the evaluation of risk factors for lung cancer in COPD patients, a cox-regression model was fitted with adjustment using the same variables as described above. In addition, the COPD characteristics emphysema, chronic bronchitis, 2 or more exacerbations last year before inclusion, and the use of inhalation medication was added to the model one at a time, and kept in the model if the p-value was below 0.05.

The 44 biomarkers were to different degrees correlated, and for the statistical evaluation the markers were added one at a time to the model above. Due to multiple testing of biomarkers, a Bonferroni adjusted p-value below 0.05/44 = 0.0011 was demanded for statistical significance.

The combined analysis of the variety of systemic inflammation between subjects was performed using a principal component analysis (PCA). PCA is a data reduction method that extracts and transforms the variance from multiple inter-correlated biomarkers into a smaller number of independent variables/components [14]. Principal components with eigenvalues above 1 (average) were retained for analysis in the cox-regression model. The first four components, located above the breaking point of the scree-plot, all with eigenvalues above 2, were also visualized using scatterplots and correlation diagrams.

3. Results

The baseline characteristics of the study population are presented in Table 1. COPD patients were older, had different smoking habits and experienced cachexia and obesity more frequently than smokers without COPD. Thirty-one subjects had a diagnosis of lung cancer during follow up of which 28 where in the COPD group. The time between study inclusion and diagnosis of lung cancer varied between 48 days and 8.6 years.

3.1. Lung cancer histology

The different histology patterns are shown in Fig. 1. Non-small cell lung carcinomas were dominant, with only one case of small-cell lung carcinoma.

3.2. Comparison between COPD patients and controls

Table 2 shows unadjusted and adjusted hazard ratios in the combined COPD and control groups. COPD patients had a significantly higher risk of developing lung cancer during follow-up with a HR 5.0 (95% CI 1.5–17.1, p = 0.01) after multivariable adjustment. Smoking status at inclusion or pack-years smoked were not associated with lung cancer.

3.3. Lung cancer risk related to COPD characteristics

Fig. 2 shows the accumulated risk for developing lung cancer in COPD patients with and without CT-defined emphysema.

Table 3 shows the hazard ratios of the association of different COPD characteristics with lung cancer development. Factors associated with a higher lung cancer risk after multivariable adjustments were the presence of emphysema and/or obesity, whereas the use of inhaled corticosteroids (ICS) was associated with a lower risk (Table 3). Use of tiotropium was associated with a higher risk of lung cancer in the unadjusted model, but not after multivariable adjustment. When stratifying on gender, obesity only indicated a higher risk in males (HR 4.76; 1.4–16.0, p = 0.01), but not in females (HR 1.34; 0.2–8.0, p = 0.75). Similarly, the use of ICS indicated a lower risk in patients without emphysema (HR 0.13; 0.02–0.79, p = 0.03), than in patients where emphysema was present (HR 0.47; 0.15–1.50, p = 0.20).

3.4. Biomarkers related to the development of lung cancer

Table 4a shows non-parametric analysis of 44 systemic biomarkers measured at study inclusion. Of the 44 markers, interleukin-6 (IL-6; p = 0.01) and interferon gamma-induced protein 10 (IP-10; p = 0.02) were significantly associated with later development of lung cancer.

Evaluation of biomarkers one at a time in the adjusted cox-

Table 3
Risk factors for the development of lung cancer in COPD patients, bi-and multivariable cox-regression.

Variables	Bivariable			Multivariable		
	HR	95% CI	p-value	HR	95% CI	p-value
Age per 10 year increase	1.76	(0.98–3.16)	0.06	1.77	(0.88–3.55)	0.11
Sex						
Female	1			1		
Male	0.93	(0.44–1.97)	0.86	0.61	(0.27–1.39)	0.24
Smoking status						
Ex	1			1		
Current	0.69	(0.32–1.49)	0.33	1.41	(0.57–3.48)	0.46
Packyears per 10 units increase	1.05	(0.92–1.21)	0.45	0.99	(0.85–1.17)	0.94
Body composition						
Normal	1			1		
Cachectic	0.58	(0.19–1.72)	0.32	0.32	(0.09–1.18)	0.09
Obese	1.99	(0.85 - 4.66)	0.11	3.25	(1.25–8.45)	0.02
Emphysema						
No	1			1		
Yes	2.74	(1.19–6.31)	0.02	4.35	(1.74–10.8)	< 0.01
Use of inhaled steroids						
No	1			1		
Yes	0.74	(0.35–1.58)	0.44	0.40	(0.17–0.93)	0.03
GOLD-status (2007)[a]						
II	1					
III	1.08	(0.50–2.34)	0.84			
IV	0.78	(0.18–3.46)	0.75			
Use of tiotropium[a]						
No	1					
Yes	2.20	(1.05–4.63)	0.04			
Use of long-acting β2-agonists[a]						
No	1					
Yes	0.78	(0.40–1.97)	0.78			
Exacerbations 12 months *before inclusion*[a]						
0-1	1					
2+	0.62	(0.19–2.05)	0.43			
Chronic bronchitis[a]						
No	1					
Yes	1.21	(0.58–2.54)	0.61			
Charlson Comorbidity Score[a]						
1	1					
2	0.83	(0.30–2.27)	0.71			
3	2.29	(0.89–5.91)	0.09			
4+	1.44	(0.33–6.33)				

[a] $p > 0.05$ in multivariable analysis.

Table 4a
Non-parametric analysis of biomarkers in non-cancer vs cancer in COPD-patients.

Biomarker	Mean values		p-value[a]
	non-cancer	lung cancer	
Hemoglobin	14.53	14.19	0.12
Leucocytes	8.13	7.63	0.42
Granulocytes	5.57	5.12	0.46
Eosinophils	2.54	2.57	0.95
Platelet count	293.89	279.48	0.30
Activin-A	0.32	0.33	0.37
ALCAM	73.94	71.87	0.67
Basic FGF	63.91	60.48	0.34
CD-163	315.46	293.59	0.75
CRP	8.50	5.08	0.22
s-Creatinine	67.84	67.36	0.69
CXCL-16	783.28	836.98	0.31
Eotaxin	92.33	87.10	0.17
s-Ferritin	136.29	143.07	0.65
G-CSF	216.23	217.29	0.18
GDF-15	0.98	0.92	0.52
GM-CSF	98.45	56.44	0.39
IFN-γ	330.46	271.55	0.31
IL-1	0.96	0.55	0.59
IL-2	39.41	12.73	0.60
IL-4	15.06	15.86	0.39
IL-5	13.12	12.43	0.97
IL-6	2.96	1.13	0.01
IL-7	30.60	31.92	0.47
IL-8	33.65	35.35	0.39
IL-9	38.32	34.29	0.37
IL-10	80.53	45.93	0.87
IL-12	259.89	77.80	0.82
IL-13	163.04	43.66	0.48
IL-17	93.26	96.41	0.37
IP-10	768.84	1057.31	0.02
MBL	828.55	525.29	0.10
MCP-1	63.57	59.30	0.85
MCP-4	90.59	85.02	0.62
MIF	24.79	21.41	0.50
MIP-1α	8.83	8.17	0.83
MIP-1β	53.88	52.87	0.90
NAP-2	170.89	165.91	0.41
NGAL	75.69	68.55	0.30
OPG	5770.63	6247.31	0.29
PDGF-BB	1128.54	1093.75	0.81
TNF-R1	736.45	752.24	0.78
TNF-α	1.80	1.68	0.59
VEGF	48.20	46.19	0.66

[a] Wilcoxon rank-sum test

regression analysis (Table 4b) showed that only IP-10 is significantly associated with lung cancer (HR 1.80; 1.32–2.45, $p < 0.001$, per 1 SD increase), after multivariate adjustment. Higher levels of IP10 indicated a higher cancer risk in patients with emphysema (HR 2.05; 1.45–2.90, $p < 0.01$), than in non-emphysema patients (HR 0.95; 0.38–2.40, $p = 0.92$).

The combined effect of the 44 biomarkers was evaluated by principal component analysis using components 1–11 with eigenvalues above 1, representing 71% of the cumulative variance of the biomarkers. The values of the different eigenvectors, and the markers included in each principal component, are shown in the supplementary material. The principal components 1–11 were also analysed using the cox-regression model described above, but no significant statistical difference was observed between lung cancer and non-cancer patients (Table 4b). The distribution of component 1–4 of the principal component analysis of the 44 biomarkers in lung cancer vs non-cancer patients is shown in Fig. 3.

Fig. 4 shows the correlations between the different COPD characteristics and biomarkers of systemic inflammation represented by the principal components 1–4. There was a higher degree of correlation between the components and the COPD characteristics of frequent exacerbations and chronic bronchitis, than with lung cancer or emphysema.

4. Discussion

The results of this study showed that both current and ex-smoking COPD patients, irrespective of packyears smoked, had an increased risk of lung cancer. Further, the presence of emphysema and obesity was associated with an increased lung cancer risk, whereas the use of ICS was associated with a reduced risk. Among 44 systemic biomarkers, only IP-10 was significantly associated with the development of lung cancer after multivariable adjustment. The study did not demonstrate any clear association between lung cancer and the COPD characteristics of chronic bronchitis, frequent exacerbations, or markers of systemic inflammation beside IP-10.

A diagnosis of COPD, and especially with the presence of emphysema, was a risk factor for developing lung cancer in our study. Current smoking or a high pack-year count did not increase the risk, suggesting the persistence of lung damage even after quitting smoking, or after a moderate amount of smoking. These findings are in accordance with earlier studies which have demonstrated a relationship between a

Table 4b
Multivariate cox-regression of biomarkers and principal components in non-cancer vs cancer in COPD-patients.

Biomarker	HR [a]	95% CI	p-value
Hemoglobin	0.78	(0.52–1.17)	0.23
Leucocytes	0.80	(0.51–1.25)	0.33
Granulocytes	0.77	(0.49–1.22)	0.27
Eosinophils	0.89	(0.55–1.43)	0.64
Platelet count	0.88	(0.56–1.38)	0.58
Activin-A	0.87	(0.57–1.35)	0.54
ALCAM	0.73	(0.41–1.33)	0.31
Basic FGF	1.04	(0.70–1.54)	0.84
CD-163	0.82	(0.46–1.44)	0.48
CRP	0.66	(0.35–1.24)	0.20
s-Creatinine	0.88	(0.56–1.38)	0.58
CXCL-16	1.24	(0.81–1.89)	0.32
Eotaxin	1.01	(0.65–1.57)	0.96
s-Ferritin	1.09	(0.81–1.46)	0.58
G-CSF	0.98	(0.62–1.55)	0.94
GDF-15	0.99	(0.59–1.65)	0.98
GM-CSF	0.56	(0.13–2.34)	0.43
IFN-γ	0.89	(0.45–1.76)	0.74
IL-1	0.60	(0.29–1.24)	0.17
IL-2	0.72	(0.12–4.24)	0.71
IL-4	1.09	(0.76–1.57)	0.64
IL-5	1.03	(0.70–1.52)	0.89
IL-6	0.25	(0.02–3.61)	0.31
IL-7	1.08	(0.82–1.43)	0.59
IL-8	1.16	(0.80–1.68)	0.44
IL-9	0.93	(0.57–1.51)	0.77
IL-10	0.91	(0.50–1.66)	0.76
IL-12	0.16	(0.00–75.8)	0.56
IL-13	0.46	(0.07–3.06)	0.42
IL-17	1.05	(0.71–1.57)	0.80
IP-10	1.80	(1.32–2.45)	< 0.001
MBL	0.68	(0.38–1.23)	0.20
MCP-1	0.97	(0.64–1.46)	0.87
MCP-4	0.82	(0.82–1.28)	0.37
MIF	0.78	(0.46–1.32)	0.35
MIP-1α	0.99	(0.48–2.02)	0.98
MIP-1β	0.92	(0.60–1.40)	0.68
NAP-2	0.87	(0.61–1.26)	0.47
NGAL	0.70	(0.47–1.11)	0.13
OPG	1.07	(0.72–1.60)	0.74
PDGF-BB	0.96	(0.63–1.48)	0.87
TNF-R1	0.92	(0.61–1.38)	0.69
TNF-α	1.04	(0.68–1.58)	0.84
VEGF	1.00	(0.67–1.51)	0.97
Principal components	**HR**	**95% CI**	**p-value**
PC1	1.00	(0.89–1.14)	0.94
PC2	0.96	(0.78–1.18)	0.69
PC3	0.87	(0.66–1.15)	0.34
PC4	0.74	(0.54–1.01)	0.06
PC5	1.26	(0.90–1.76)	0.17
PC6	0.71	(0.50–1.00)	0.05
PC7	0.89	(0.60–1.31)	0.54
PC8	1.04	(0.69–1.56)	0.85
PC9	1.17	(0.79–1.73)	0.44
PC10	0.83	(0.52–1.32)	0.43
PC11	0.59	(0.38–0.92)	0.02

[a] Per 1 SD increase.

diagnosis of COPD and lung cancer or death of lung cancer irrespective of the amount of smoking [3–5,15]. Subsequent studies have further evaluated this relationship, where the presence of emphysema in COPD patients was associated with both a diagnosis of lung cancer, but also death due to lung cancer as well as non-pulmonary cancer [6,7,16]. The co-existence of emphysema and lung cancer may obviously be ascribed to their common risk factor of noxious airway exposure. However, a common pathophysiology of these two conditions is nevertheless more difficult to explain, with apoptosis and protein degrading as main characteristics of emphysema as opposed to the excessive cell growth in cancer. Possible mechanistic explanations of cancer development include accelerated proliferation of epithelial cells resistant to apoptosis, dysfunction of proteinase-regulation, and increased generation of pro-inflammatory cells, cytokines and reactive oxygen species [8,17].

There is extensive research data linking systemic inflammation and cancer in general [18]. In lung cancer, this relationship is less well described, but several studies have demonstrated elevated inflammatory mediators in patients with established lung cancer [19–22]. An important study question to address was whether indices of systemic inflammation could predict lung cancer in COPD patients. Interrelated phenotypic attributes such as chronic bronchitis and frequent exacerbations were also associated with increased systemic inflammation, but none of them had a significant association with the development of lung cancer. We evaluated inflammatory biomarkers both individually as well as combined with principal component analysis, with mostly negative findings regarding any predictive value of lung cancer. However, the principal component analysis indicated a closer association between inflammatory markers and patients with mainly chronic bronchitis and exacerbations, rather than in patients with emphysema or with high lung cancer risk, suggesting the existence of different pathophysiological/immunological pathways underlying the different COPD phenotypes.

Among 44 serum/plasma biomarkers, only high levels of IP-10 were associated with increased lung cancer risk. IP-10 (CXCL-10) is induced by IFNγ, and is frequently used as a marker of viral infection [23,24]. Its functions include induction of chemotaxis, regulation of cell growth/apoptosis and angiogenesis. Spaks et al. found elevated serum IP-10 in lung cancer patients [25], and high expression is also seen in other cancer types. The role of IP-10 in cancer may depend on its receptor; it may be involved in tumor growth inhibition through angiostatic or immunogenic actions or in direct tumor growth stimulation. The role of IP-10 in either COPD or lung cancer is yet unclear, and thus it should be further investigated.

The association between the use of ICS and a lower risk of lung cancer is in accordance with prior observational studies [26–28], and may indicate a protective effect of ICS. The above mentioned link between inflammation and cancer may be modified by ICS, and the potential protective mechanisms of ICS may include reduced secretion of carcinogenic cytokines or growth-factors in the lungs, as well as inhibition of proto-oncogene expression [29,30]. On the other hand, a similar effect has not been found in randomized controlled studies [31,32], and the observed effect of ICS may be due to a protopathic bias. However randomized controlled trials are neither designed nor have had a sufficient follow up time to evaluate lung cancer risk, thus a potential preventive effect of ICS is still possible although unproven [33,34].

A major finding in the present study was an independent association between obesity and lung cancer development in COPD patients, primarily reflecting an association in males. Obesity is described as a risk factor of several variants of cancer [35], however, regarding lung cancer, several prior studies report opposing findings where some have reported a protective effect of high BMI [36,37]. This is, nevertheless, a complex matter to study, where several confounding factors such as smoking habits, genetics and dietary issues may affect the findings in the different study populations [38,39]. Recently, a large study where 23,732 incident lung cancer cases were identified suggested that central obesity, particularly concurrent with low BMI, could help identify high-risk populations for lung cancer [40], and future studies in COPD patients in relation to cancer development should also include fat distribution as a parameter.

There is an ongoing international debate on lung cancer screening [41]. Early detection of lung cancer using low-dose computer tomography is probably the most important measure in reducing mortality [42]. Most screening protocols consider smoking and aging as the most important risk factors, though some recent studies promotes the inclusion of additional parameters to narrow the screening population [43–45]. Screening appears to be more advantageous in case of

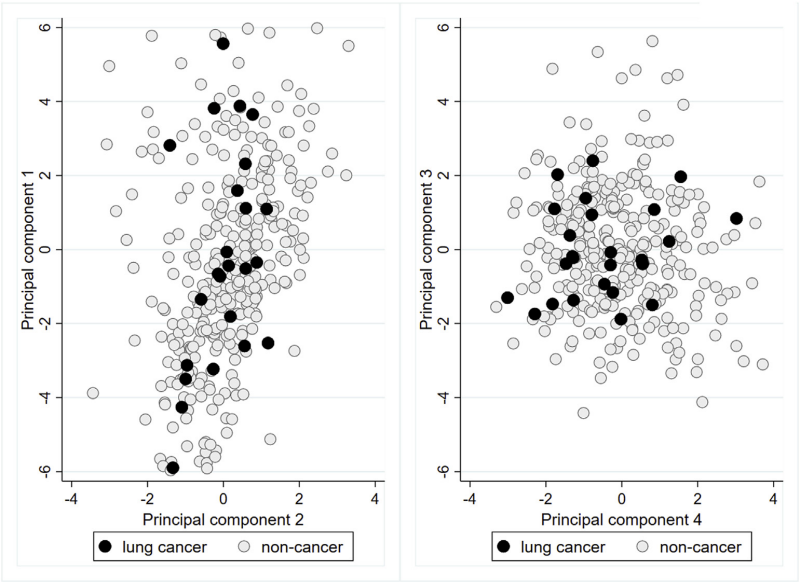

Fig. 3. Systemic inflammation in non-cancer vs lung cancer patients represented by principal components 1–4 of the systemic inflammatory markers, measured at study inclusion. Scatterplots of components 1 vs 2 and 3 vs 4 shows no significant difference between the groups.

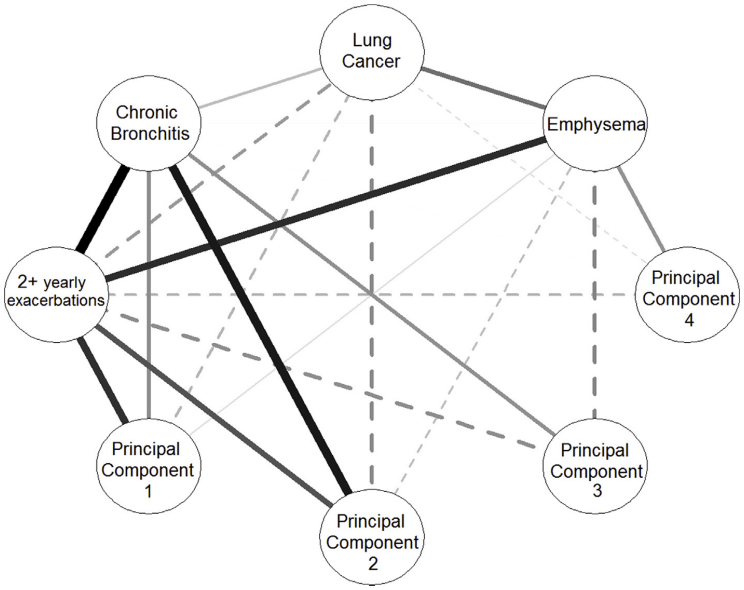

Fig. 4. Correlation diagram showing the relations between COPD characteristics and systemic inflammation at study inclusion represented by principal components 1–4. Thicker line indicates larger degree of correlation, solid line indicates positive correlation, dashed line indicates negative correlation.

emphysema phenotype of COPD, but less in case of COPD with chronic bronchitis or other forms of chronic inflammation. The results of our study support narrowing the screening population based on presence of airflow limitation as well as indications of emphysema.

The strengths of our study were its prospective design with outcomes from a mandatory national registry, and the availability of detailed information on a large number of patients allowing adjustment of multiple variables.

Some limitations should be mentioned. First, the patients were not randomized, and therefore data interpretation regarding causality is difficult. Second, the serum/plasma inflammatory markers were measured only at entry, and thus it is uncertain to what extent this is

representative of lung inflammation or whether the degree of systemic inflammation in the subjects is altered during follow-up. Third, the study population represents an outpatient clinic population, with exclusion of GOLD class 1 patients and, thus the results may not be applicable to a COPD population with early stage disease. Finally, there was no systematic screening of lung cancer. Thus, although the Norwegian Cancer Registry has a high degree of completeness, we cannot exclude cases of non-reported lung cancer among patients or controls.

The present study clearly underscores the necessity of looking at COPD as a heterogeneous disease, where the different phenotypes not only require different diagnostics and treatment, but where they also incorporate different risks of adverse events such as lung cancer development. The idea of using serum/plasma biomarkers in early lung cancer screening may presently seem challenging, but an increasingly easier access to large multiplex bioassays and a better understanding of the pathophysiologic mechanisms behind the COPD phenotypes might change this view in the near future.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Conflicts of interest

No authors report any conflict of interest.

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Appendix A. Supplementary data

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Risk factors for lung cancer in COPD

Supplementary material

Note on missing data

Data on spirometry, body composition, medical history, smoking habits and clinical phenotypes and lung cancer status were complete in all patients. CT scan was performed in 384 patients at baseline, but it was unavailable in 49 patients. A complete biomarker analysis of all 44 inflammatory markers was unavailable in 39 patients.

The main source of data on the diagnosis of lung cancer in 31 patients was the Norwegian Cancer Registry. The Norwegian health care system is predominantly public, and there are no centers for lung cancer diagnostics outside public hospitals and out-patient clinics. All inhabitants can be tracked by the national id-number across different hospitals and public accounts. Submission of data to the cancer registry is mandatory by law and has several validation systems based on both ICD10-registration as well as histological cancer diagnostics. For these reasons we can assume close to 100 % data completeness in both patients and controls.

Note on bioassays

Peripheral venous blood was drawn into pyrogen-free EDTA collection tubes and centrifuged within 30 minutes at 2150 *g* for 15 minutes at 4 ^{o}C. The plasma was aliquoted and stored in -80 ^{o}C ultra-freezers until measurements, thus the samples were never previously thawed. The 44 biomarkers were analyzed at different occasions after sampling:

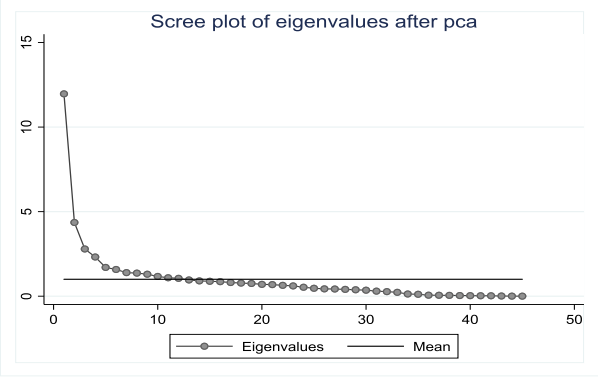
NGAL, MIF, ALCAM, CD163, OPG, CXCL16, TNF-R1, MCP4, NAP2, MBL were measured in 2007 and 2008 by enzyme-linked immunosorbent assays (EIAs) (R&D Systems, Inc, Minneapolis, MN, USA). The intra- and inter-assay coefficients of variation were <11% for all parameters. Hemoglobin, WBC, Granulocytes, TPC, s-Ferritin, s-Creatinine and CRP were measured in 2007 and 2008 by routine laboratory methods (Modular PP, Roche Diagnostics, Basel, Switzerland). Plasma levels of TNF-α, IL-1, IL-6, were measured in 2011 using EIA kits (BD Biosciences Pharmingen, San Diego, CA). The intra-assay and inter-assay coefficient of variations were <10% for all parameters. Plasma levels of IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-SF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, PDGF-BB, MIP-1β and VEGF were measured by Aveo Oncology in 2014 using a magnetic bead multiplex assay (Bio-Plex Pro, Bio-rad laboratories, Inc.). Plasma-levels of GDF15 and Activin A were measured by Aveo Oncology in 2014 using enzyme-linked immunosorbent assays (Quantikine, R&D Systems, Inc). All samples were measured in duplicates, and only accepted if intra-assay variance was less than 10%.

Note on principal component analysis

For the combined evaluation of the biomarkers, a principal component analysis (PCA) was done. PCA is a data reduction method that extracts the variance of multiple and often correlated variables into a smaller number of principal components. The eigenvalue of the components corresponds to the variance explained by each component. All variables were standardized before using an orthogonal transformation. The largest principal components with eigenvalues above mean (=1), are shown in the table below. The total variance explained by the 11 largest components was 70.8 %.

Component	Eigenvalue	Proportion	Cumulative
Component 1	12.1798	0.2768	0.2768
Component 2	4.51165	0.1025	0.3794
Component 3	2.78334	0.0633	0.4426
Component 4	2.26015	0.0514	0.4940
Component 5	1.6773	0.0381	0.5321
Component 6	1.56507	0.0356	0.5677
Component 7	1.37711	0.0313	0.5990
Component 8	1.30888	0.0297	0.6287
Component 9	1.22387	0.0278	0.6565
Component 10	1.16035	0.0264	0.6829
Component 11	1.08142	0.0246	0.7075

A screeplot of the eigenvalues shows a breaking point between components 4 and 5.



For the statistical analysis in the regression model, we used the 11 largest principal components. The correlations between the 4 largest components and lung cancer/other COPD characteristic are shown in figure 3 and 4 in the main text.

The eigenvectors and their loadings according to the original variables of principal component 1-11.

Original variables

PC1	**GCSF**	**IL7**	**IFN-γ**	**BasicFGF**	**MCP1**	**Eotaxin**	**IL10**	**IL2**	**IL12**	**IL13**	**VEGF**	**GMCSF**	**IL4**
	0.2780	0.2769	0.2741	0.2712	0.2712	0.2691	0.2658	0.2636	0.2620	0.2618	0.2496	0.2451	0.2306
PC2	**IL8**	**IL17**	**PDGF**	**MIP1b**	**IL5**	**CD163**	**IL4**						
	0.3330	0.3051	0.2851	0.2389	0.2114	0.2058	0.2038						
PC3	**TNFR1**	**ActA**	**s-Crea**	**TPC**	**GDF15**	**NGAL**	**OPG**	**NAP2**					
	0.4060	0.3310	0.3090	-0.2799	0.2798	0.2368	0.2265	-0.2244					
PC4	**Gran**	**WBC**	**NGAL**	**CRP**									
	0.5175	0.5079	0.2894	0.2350									
PC5	**ALCAM**	**Ferr**	**Hb**	**TPC**	**MIF**	**s-Crea**	**IL1**	**NAP2**					
	-0.3636	0.3517	0.3312	-0.3250	-0.2545	0.2282	0.2266	-0.2112					
PC6	**Hb**	**MIF**	**MCP4**	**OPG**	**Ferr**	**Eos**	**IL6a**						
	0.4582	0.4173	0.3464	-0.2409	0.2342	0.2307	-0.2146						
PC7	**IL1**	**TNFα**	**CXCL16**	**CRP**	**IP10**	**Ferr**	**TPC**	**NAP2**	**IL6a**	**MIP1a**			
	0.4093	0.3843	0.3205	-0.2552	-0.2453	0.2435	0.2331	0.2327	-0.2317	-0.2289			
PC8	**Eos**	**MCP4**	**ALCAM**	**IP10**	**MIF**	**CD163**							
	0.4489	0.4117	-0.3187	0.2695	-0.2655	-0.2332							
PC9	**IL9**	**IL6a**	**NGAL**	**s-Crea**	**OPG**	**TNFα**	**CD163**	**Hb**	**CRP**	**TNFR1**			
	-0.3514	0.3467	-0.3131	-0.2960	0.2714	0.2592	0.2529	0.2445	0.2436	-0.2050			
PC10	**OPG**	**IL6a**	**NAP2**	**MIP1b**	**MIP1a**	**MBL**	**TNFα**						
	-0.4084	0.3659	0.3392	0.2516	-0.2327	0.2110	0.2050						
PC11	**CXCL16**	**Eos**	**s-Crea**	**MBL**	**IP10**	**MCP4**	**MIF**						
	-0.4557	0.4085	0.3795	0.3037	-0.2820	-0.2405	0.2103						

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