# Studies of Neutrophilic Inflammation in Tobacco Smokers

Kristina Andelid

Department of Internal Medicine and Clinical Nutrition,
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2014

Cover illustration: Neutrophil cells provided by Johan Bylund, University of Gothenburg. Design: Beata Angelbjörk

Studies of Neutrophilic Inflammation in Tobacco Smokers
© Kristina Andelid 2014
kristina.andelid@lungall.gu.se

ISBN 978-91-628- 9216-6

Printed by Kompendiet, Gothenburg, Sweden 2014

“Aime la vérité mais pardonne à l’erreur.”

*Voltaire (1694-1778)*
*Emilie du Chatelet (17/12 1706-1749)*

# Studies of Neutrophilic Inflammation in Tobacco Smokers

Kristina Andelid

Department of Internal Medicine and Clinical Nutrition, Institute of Medicine
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

## ABSTRACT

The general aim of this thesis was to characterize markers of neutrophilic inflammation in smokers with and without obstructive pulmonary disease with chronic bronchitis (OPD-CB), in a stable state and during exacerbations compared to healthy controls. *Methodology*: I) Blood samples were obtained from male smokers without airway symptoms and from never-smokers at year 0 and 6. II) Non-atopic and atopic, occasional smokers plus never-smokers underwent two bronchoscopies, including bronchoalveolar lavage (BAL). III & IV) Smokers with OPD-CB (n=60,) and control groups (n=10 each), underwent blood and sputum sampling every 15:th week and at exacerbations during 15 months. *Results*: I) Blood MPO was higher in smokers than in never smokers at year 6. MPO was negatively correlated with time after cessation of smoking. II) Gelatinases in BAL fluid were unchanged after acute exposure to tobacco smoke. III) The concentrations of IL-17A and GRO-α protein were lower in blood from smokers with severe COPD and in smokers with OPD-CB colonised with opportunistic pathogens. IV) In smokers with OPD-CB, blood MPO and NE proteins were increased during exacerbations; the corresponding mRNA was undetectable. *Conclusions*: Acute exposure to tobacco smoke does not exert a pronounced impact on gelatinases in the airways of occasional smokers. During stable clinical conditions, neutrophils and MPO are increased in smokers without OPD-CB and even more so during exacerbations in smokers with OPD-CB. In smokers with severe OPD-CB, and in those colonised with opportunistic pathogens, specific neutrophil-associated immune signaling is down-regulated at the systemic level. The lack of detectable mRNA for MPO and

NE in the blood of smokers with COPD makes the location of production uncertain for these markers of neutrophil activity.

**Keywords**: smoking, neutrophils, inflammation

# SAMMANFATTNING PÅ SVENSKA

Tobaksrökning medför bland annat inflammation i lungorna som drabbar både luftvägsträdet (bronkerna) och lungblåsorna (alveolerna). Inflammationen i lungorna kan leda till att dessa oåterkalleligen skadas och att kroniskt obstruktiv lungsjukdom (KOL) samt kronisk bronkit utvecklas. Sjukdomen KOL innebär kronisk inflammation i luftvägarna och studier har även visat att såväl rökare utan symptom, som patienter med KOL i samband med försämring, uppvisar tecken till generell inflammation i blodets celler. Patienter med frekventa akuta försämringsepisoder (exacerbationer) förlorar lungfunktion snabbare än andra patienter med KOL.

Kroppens immunförsvar kan delas upp i ett ospecifikt (medfött) och ett specifikt (adaptivt) försvar. Det medfödda, nativa, ospecifika immunförsvaret är kroppens första försvarslinje som aktiveras direkt när främmande och skadliga gaser eller luftburna irritanter samt bakterier eller virus (mikrober), kommer ner i lungorna. Människans medfödda immunförsvar utgörs traditionellt sett av makrofager och neutrofila celler men involverar även en rad andra celltyper. Genom olika mekanismer avger dessa celler substanser som syftar till att oskadliggör mikrober.

Det adaptiva, specifika, försvaret sköts av lymfocyterna som är en typ av vita blodkroppar som lär sig känna igen enskilda mikrober och kan döda dem med hjälp av bland annat antikroppar som bildas av B-celler. Både B- och T-celler är viktiga medarbetare i det specifika immunförsvaret. T-cellerna kan delas upp i hjälparceller ($CD4^+$ celler) som är viktiga för att ”dirigera och leda” immunförsvaret och cytotoxiska celler ($CD8^+$ celler) som kan känna igen en infekterad cell och döda den. Ett fullgott svar från det specifika immunsystemet kan ta flera dagar att bygga upp. Cytokiner är den övergripande beteckningen för signalsubstanser som bildas och avges från alla de celler i kroppen som bidrar till immunförsvaret. Deras funktion är att signalera och överföra information till andra celler. Interleukiner kallas de cytokiner som produceras specifikt av vita blodkroppar.

Den främsta målsättningen med avhandlingsarbetet var att karaktärisera förändringar i blodmarkörer för inflammatoriska celler hos rökare med och utan symtomgivande obstruktiv sjukdom samt hos rökare med akut försämring av KOL samt att bestämma om det finns någon skillnad mellan dessa markörers aktivitet hos rökare med KOL under kliniskt stabila förhållanden jämfört med friska kontrollpersoner. I ett av avhandlingens delarbeten undersöktes även hur akut exponering för tobaksrök påverkar celler lokalt i lungan. Det första delarbetet utgjordes av en långtidsstudie där blodprover tagits från icke-rökare och ”friska” rökare. För det andra delarbetet undersöktes icke-rökare och tillfällighetsrökare med bronkoskop och provtagning från lungorna två gånger med fjorton dagars mellanrum. Två dagar före den andra bronkoskopin fick tillfällighetsrökarna röka totalt 10 cigaretter. För det tredje och fjärde delarbetet undersöktes 60 rökare med KOL och kronisk bronkit med blodprover var 15:e vecka i 15 månader samt vid försämringsperioder. Patienterna fick även lämna upphostningsprover. Dessutom togs blodprover på kontrollpersoner, rökare och icke rökare (10 stycken i varje grupp), vid ett tillfälle.

Resultaten från det första delarbetet visade att koncentrationen av proteinet myeloperoxidas (MPO), som framför allt utsöndras av den neutrofila cellen i samband med aktivering, var högre hos de ”friska” rökarna än hos icke-rökarna efter 6 år. Resultaten från det andra delarbetet visade inga förändringar i koncentrationen av proteinet matrix-metalloproteinas-2(MMP-2) samt-9 (MMP-9) i bronksköljvätska efter akut exponering för tobaksrök. MMP-2 och -9 är enzymer vilka kan bryta ner proteinkomponenter i extracellulärt matrix, den struktur som omger cellerna i kroppen. Resultaten från det tredje delarbetet visade att interleukin (IL)-17 och dess effektormolekyl tillväxt-relaterad onkogen alfa (GRO-α) var lägre hos KOL patienter än hos friska rökare och icke rökare samt hos de KOL patienter som hade opportunistiska sjukdomsalstrande bakterier i sina upphostningar; d.v.s. bakterier som i normala fall hanteras av värdorganismens immunförsvar men som kan vara orsak till sjukdomar när immunförsvaret är nedsatt. Resultaten från det fjärde delarbetet visade att MPO och neutrofilt elastas (NE); ett enzym som produceras av den neutrofila cellen och bryter ner invaderande bakterier och åldrade celler, var ökat hos KOL patienter vid försämringsperioder respektive i stabil fas.

Sammanfattningsvis talar dessa studier för att de neutrofila cellerna och proteiner som utsöndras från dessa är betydelsefulla och ökade i den inflammatoriska och immunologiska processen hos både rökare utan KOL och i än högre grad i samband med försämringsperioder hos rökare med KOL och kronisk bronkit. Hos patienter med svår KOL och hos KOL-patienter med opportunistiska bakterier i sputa ses dock en nedreglering av det neutrofilassocierade immunförsvaret.

# LIST OF PAPERS

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

I. Andelid K, Bake B, Rak S, Lindén A, Rosengren A and Ekberg-Jansson A. Myeloperoxidase as a marker of increasing systemic inflammation in smokers without severe airway symptoms. Respiratory Medicine 2007; 101(5): 888-95

II. Glader P, Eldh B, Bozinovski S**,** Andelid K, Sjöstrand M, Malmhäll C, Anderson GP, Riise GC, Qvarfordt I and Lindén A**.** Impact of acute exposure to tobacco smoke on gelatinases in the bronchoalveolar space. European Respiratory Journal 2008; 32(3): 644-50.

III. Andelid K, Tengvall S, Andersson A, Levänen B, Christiansson K, Jirholt P, Åhrén C, Qvarfordt I, Ekberg-Jansson A and Lindén A. Systemic cytokine signaling via IL-17 in Smokers with Obstructive Pulmonary Disease: A Link to Bacterial Colonisation? Submitted.

IV. Andelid K, Glader P, Yoshihara S, Åhrén C, Jirholt P, Gjertsson I, Ekberg-Jansson A and Lindén A. Systemic Signs of Neutrophil Mobilization during Stable Clinical Conditions and Exacerbations in Smokers with Obstructive Pulmonary Disease: A Link to Hypoxia? Submitted.

# CONTENT

ABBREVIATIONS ........ IV
1 INTRODUCTION ........ 7
1.1 The History of COPD ........ 7
1.2 Risk Factors for COPD Development ........ 11
1.3 Definition of COPD ........ 12
1.4 Exacerbations in COPD ........ 13
1.5 COPD as an Inflammatory Disease ........ 14
  1.5.1 Local Inflammation ........ 14
  1.5.2 Systemic Inflammation ........ 14
1.6 Host Defence ........ 15
  1.6.1 The Innate Immune System ........ 16
  1.6.2 The Adaptive Immune System ........ 20
2 AIM ........ 24
3 STUDY POPULATION ........ 25
4 METHODS ........ 27
4.1 Study Design ........ 27
4.2 Lung Function Tests ........ 28
4.3 Bronchoscopy and BAL. (Paper II) ........ 28
4.4 Blood and Sputum Analyses ........ 29
4.5 Statistical Methods ........ 30
5 RESULTS ........ 32
5.1 Paper I ........ 32
5.2 Paper II ........ 34
5.3 Paper III and IV at Inclusion ........ 36
  5.3.1 Paper III at Inclusion ........ 38
  5.3.2 Paper IV at Inclusion ........ 42
5.4 Paper III and IV at Exacerbations ........ 45
  5.4.1 Paper III at Exacerbations ........ 47

5.4.2 Paper IV at Exacerbations ........ 49
6 DISCUSSION ........ 53
6.1 Paper I ........ 53
6.2 Paper II ........ 55
6.3 Paper III and IV ........ 57
7 SUMMARY AND CONCLUSION ........ 64
8 FUTURE PERSPECTIVES ........ 67
ACKNOWLEDGEMENT ........ 68

# ABBREVIATIONS

| | |
|---|---|
| ACOS | Asthma-COPD overlap syndrome |
| AU | Arbitrary unit |
| AS | Asymptomatic Smokers |
| BAL | Bronchoalveolar lavage |
| BMI | Body Mass Index |
| BW | Bronchial wash |
| CB | Chronic Bronchitis |
| CES | Carboxyl Esterase |
| COPD | Chronic Obstructive Pulmonary Disease |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| $DL_{CO}$ | Carbon monoxide diffusing capacity |
| ELISA | Enzyme-linked immunosorbent assay |
| EXA | Exacerbation |
| $FEV_1$ | Forced Expiratory Volume in one Second |
| FVC | Forced Vital Capacity |
| GOLD | Global Initiative for Obstructive Lung Disease |
| GRO-α | Growth-related oncogene-α |
| HDL | High-density lipoprotein |
| HNL | Human neutrophil lipocalin |
| IFNγ | Interferon γ |
| IL-17A | Interleukin -17A |

| | |
|---|---|
| LPS | Lipopolysaccarides |
| LYS | Lysozyme |
| MMP | Matrix metalloproteinase |
| MPO | Myeloperoxidase |
| mRNA | messenger-Ribonucleic acid |
| NE | Neutrophil Elastase |
| NETs | Neutrophil extracellular traps |
| NS | Never smokers |
| OPD | Obstructive pulmonary disease |
| $PaO_2$ | Partial pressure of oxygen in arterial blood |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| RIA | Radioimmunoassay |
| ROS | Reactive oxygen species |
| TIMP | Tissue inhibitor of matrix metalloproteinase |
| Th cells | T helper cells |
| $T_{regs}$ | T regulatory cells |
| VC | Vital capacity |

# 1 INTRODUCTION

## 1.1 The History of COPD

Chronic Obstructive Pulmonary Disease (COPD) is a growing global healthcare problem, with increasing morbidity and mortality. By 2020, COPD is predicted to rank fifth worldwide in terms of burden of disease and third in terms of mortality (1).

The evolution of knowledge concerning COPD covers 200 years. The stethoscope and spirometer became early tools in diagnosis and assessment of the disease. Later the bronchoscope made direct sampling of tissues and fluid from the lungs possible. Some of the first references to the description of pathological changes in the lungs include Bonet´s description of “voluminous lungs” (Bonet 1679), and Baille´s illustrations of the emphysematous lung, thought to be that of the well-known writer Samuel Johnson (Baille 1789) (2).

In 1816, René Laennec, a French clinician and pathologist at the Hôpital Necker in Paris, invented the stethoscope. Laennec was fascinated by diseases of the chest and he was even trained in the art of chest percussion by Napoleon’s physician Nicolas Corvisart (3). Prior to this invention, the standard medical practice was to place the ear on the patient’s chest to listen to heart- and lung sounds (3). Laennec had just met an obese young woman with suspected heart failure and did not want to place his ear directly on her chest. He made a roll of paper, held it against her chest, and thus created the world’s first stethoscope. Laennec named the device after the Greek words “stethos” (Eng. chest) and “skopos” (Eng. observe). He also described the emphysema component of the disease in 1821 in *Treaties of diseases of the chest* (*De l’Auscultation Médiate ou Traité du Diagnostic des Maladies des Poumons et du Coeur)*. It was in this publication that terms such as rales, rhonchi and crepitance were first described. He performed careful post-mortem examinations of patients he had studied when they were still alive at the hospital, and recognized that emphysema lungs were hyperinflated and did not empty well (2, 3).

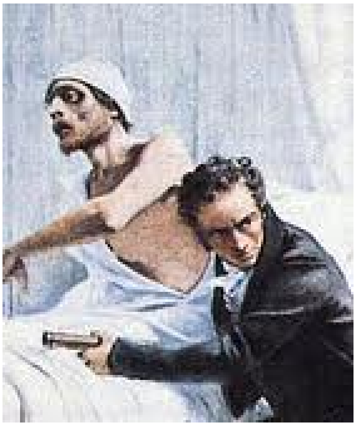


Figure 1. *Laennec a l'Hopital Necker, ausculte un phtisique .Detail from a painting by Theobald Chartran (1849-1907)*

Below we can read an extract from *Treaties of diseases of the chest* (1821) describing emphysema as it appeared during post-mortem examinations:

*"In opening into the chest, it is not unusual to find that the lungs do not collapse, but that they fill up the cavity completely on each side of the heart. When examined, their cells appear full off air, so that a prodigious number of small vesicles are seen upon the surface of the lungs immediately under the pleura. The branches of the trachea are often at the same time a good deal filled with the mucus fluid. This fluid had probably prevented the ready egress of the air, so that it had gradually distended the air cells of the lungs, and had prevented the lungs from collapsing."*

John Hutchinson, who originally studied and practised medicine in London, and later in Australia, invented the spirometer in 1846 (4) (2). This instrument, however, only measured vital capacity. It took another 100 years before the French physiologist Robert Tiffeneau added the concept of timed vital capacity as a measure of airflow and for spirometry to become complete as a diagnostic instrument (2, 5). Tiffeneau is principally known for the "Tiffeneau index", i.e. the ratio of the volume exhaled during the first second of a forced expiratory manoeuvre (forced expiratory volume in 1 second; $FEV_1$) over forced vital capacity ($FEV_1$/FVC x 100) which should be >70 in a normal spirometry (5).

Charles Fletcher, professor of Clinical Epidemiology in London, devoted his life to the study of the natural history of chronic airflow obstruction. In a prospective epidemiological study in men working in West London in 1961, airflow obstruction was estimated by $FEV_1$ measurements taken every six months, over a period of eight years. Fletcher hereby recognised the relationship between smoking and the accelerated rate of decline in $FEV_1$. (6). Even more interestingly, he and his colleagues discovered that smoking cessation would retard the rate of $FEV_1$ decline, to that approaching the reduction rates of non-smokers during ageing (6) (7) (Fig 2). This research provides the scientific basis for smoking cessation education today (8).

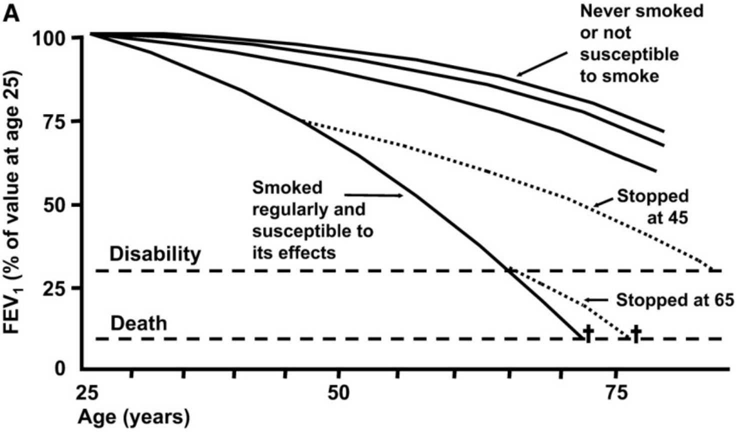


Figure 2. *The Fletcher curve.Adapted from Fletcher, C & Peto, R*

The *bronchoscope* is not regularly used as an instrument in COPD diagnosis. However the possibility of performing bronchial lavage (BL), broncheoalveolar lavage (BAL) and bronchial biopsies from the lower respiratory tract has provided a framework of insights regarding the role of inflammation and inflammatory markers in COPD. Gustav Killian, a German laryngologist, is considered to be the ‘father of bronchoscopy’. In 1896, he passed the bifurcation with the “bronchoscope” in tracheotomised patients. He used a somewhat modified esophagoscope of Rosenheim, and noticed that the bronchi were elastic and flexible. He was “stopped only when the diameter of the tube was surpassing that of the bronchi”. Following the confirmation of his findings in non- tracheotomies corpses, he went on to perform the first direct endoscopy via the larynx in a volunteer. Bronchoscopy was born. In the same year, he was the first to remove a foreign body from a patient via the larynx (9). From then until the 1970s, rigid bronchoscopes were the only option but in 1966 the Japanese researcher Shigeto Ikeda invented the flexible bronchoscope. This instrument contains a fiber optic system that transmits an image from the tip of the instrument to an eyepiece or video camera at the opposite end. The tip of the instrument can be oriented, allowing the practitioner to navigate the instrument into an individual lobe or segment bronchi (10). Rigid bronchoscopy is still often preferred for retrieving foreign objects or in other interventions where a larger lumen is needed.

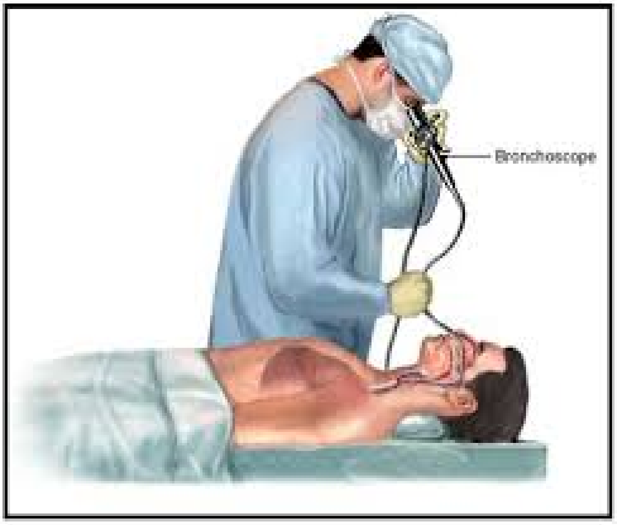


Figure 3. *Flexible bronchoscopy*

In parallel with the technical development new insights were made into the classifications of clinical disorders in pulmonary medicine.The first person to use the term “COPD” is believed to be William Briscoe in a discussion at the 9th Aspen Emphysema Conference in 1965 (2) (11, 12).

From a feministic point of view, this enumeration of men developing COPD diagnostics and research, may seem frustrating; “Why are there no women?”

The answer is that no female physicians were examined prior to the 1880’s (In Sweden 1888) (13).

## 1.2 Risk Factors for COPD Development

The pathophysiology of COPD is complex and the disease involves multiple dimensions related to environmental, genetic and psychological factors. The most commonly known risk factors include:

*Active and passive cigarette smoking:* Today we know that tobacco smoke contains over 5000 chemicals and most of them are formed during the burning of the tobacco (14, 15). Other chemicals in the smoke such as pesticides and microorganisms, surviving the combustion during the smoking may be present in the tobacco itself (15). Tobacco is an agricultural product rich in microorganisms both bacteria and fungi. Studies have demonstrated that the microbiological material in tobacco smoke originates from microorganisms that naturally colonize the tobacco plants in the fields (16). However, it was not until modern molecular biology methods became available, that the large amount of microbes in tobacco was revealed (16).

Several studies have shown that cigarettes harbour gram-positive and gram-negative bacterial types including *Acinetobacter, Bacillus, Burkholderia, Clostridium, Klebsiella, Pseudomonas aeruginosa, Campylobacter, Enterococcus, Proteus and Staphylococcus* (15, 17). In one study *P.aeruginosa* was detected in 100% of all cigarette samples tested (17). Tobacco smoke is also shown to contain endotoxin (lipopolysaccharide, LPS), a family of inflammatory toxins from gram-negative bacteria (18, 19).

It is known that the prevalence of bacterial infections is increased in smokers (20). However, to our knowledge, no one has comprehensively evaluated whether the cigarettes themselves may provide a source of exposure to bacterial organisms (17)

*Biomass smoke exposure:* The use of biomass for cooking and space heating, often in unventilated housing, provides a risk factor for COPD development as the fine particles from solid fuel combustion can be delivered more distally into the lungs (21, 22). Females represent the majority of the exposed population in certain countries throughout the world and this is an important public health problem. Several studies have demonstrated that homes where people had undertaken even simple measures to improve ventilation in the home environment may lower the incidence of COPD (23).

*Genetic factors*: The lung responses to environmental exposure are clearly determined by genetic factors. However, the exact genes responsible for the enhanced risk of developing COPD are not well known. The best described genetic factor in COPD is a deficiency in $\alpha_1$ – antitrypsin. However this particular phenotype only accounts for 1-3% of the patients with COPD (24). The study of numerous other possible genes is continually ongoing (25).

Epigenetics is defined as heritable changes that cannot be explained by changes in DNA sequence (26). COPD has been shown to accumulate in families, and there is evidence that the main risk factor for COPD - cigarette smoking - is associated with epigenetic changes in the bronchial epithelium and that epigenetic pathways regulate airway inflammation (26, 27).

*Psychological factors:* There are complex associations between nicotine dependence, depression and anxiety disorders and smoking cessation. Studies have shown that depression predicts smoking initiation and reduced physical activity (28). Since smoking is a key lifestyle risk factor for COPD and reduced exercise capacity is a marker of poor prognosis in COPD, these associations have obvious mechanistic implications (29).

## 1.3 Definition of COPD

The Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease (GOLD Global Initiative for Chronic Obstructive Disease) document published 2013 (1) defines COPD as a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and lungs to noxious particles or gases. The chronic airflow limitation characteristic of COPD is caused by a mixture of small airways disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), the contributions of which vary from person to person. Exacerbations (see below) and co-morbidities contribute to the overall severity in individual patients (1). The Asthma-COPD overlap syndrome (ACOS) is characterized by persistent airflow limitation with several features usually associated with asthma and several features usually associated with COPD(30)

In conclusion, COPD could be considered as a syndrome of features categorized by several phenotypes which define various COPD subgroups with different presentations and clinical courses (31).

## 1.4 Exacerbations in COPD

The clinical course of COPD is for many patients characterized by *exacerbations (*Lat. *exacerbare meaning: to aggravate);* episodes of symptom worsening, with increased pulmonary and systemic inflammation, and reduced quality of life (32, 33). These exacerbations are important causes of hospital admission and death, and are also associated with increased healthcare costs. In addition, unreported exacerbations constitute a clear source of disease burden in COPD (34). The episodes may be caused by infections due to bacteria, or viruses, or by exposure to pulmonary irritants (35). COPD patients may present different clinical phenotypes and not only treatment, but also procedures for prevention of exacerbations may be different depending on the exhibited phenotype (36). Studies suggest that some patients with COPD are especially susceptible to exacerbations, a phenotype of COPD named "frequent exacerbators" (32, 37). The most important determinant of frequent exacerbations is a previous history of exacerbations (37). Patients with frequent bacterial exacerbations characterized by purulent sputum may have bronchiectasis that is confirmed by computed tomography. They constitute a particular phenotype that has been termed the "infective phenotype" (38) .

Microorganisms, particularly bacteria, are frequently found in the lower airways of COPD patients, both during stable states and during exacerbations. There is emerging evidence that these microorganisms may play an active role in the evolution of the disease even during a stable clinical state including chronic low-grade airway inflammation leading to increased exacerbation frequency and accelerated decline in lung function (38-40). Abnormal presence of bacterial pathogens in the lower airways in stable COPD was recognized in a bronchoscopic sampling study in 1961. This presence of bacteria was called "colonization", which since then has been a commonly accepted term (41). However, some researchers believe that the isolation of pathogenic bacteria in stable COPD should rather be considered a form of chronic infection (38).

## 1.5 COPD as an Inflammatory Disease

### 1.5.1 Local Inflammation

Local inflammation in COPD is due to the inflammatory response of the airways to chronic irritants, the worst being smoke. Under these circumstances three known distinguishable conditions may develop and contribute to the airflow limitation:

*Chronic bronchitis*; characterised by inflammation of the airway walls, hyperplasia of the goblet cells and mucus hypersecrection.

*Small airways disease*; with accumulation of macrophages, T-cells and B-lymfocytes in the airway wall, and neutrophils, both in the lumen and the airway wall (42-44). This condition is characterised by hypertrophia of bronchiolar wall muscles, intraluminal mucus, fibrosis, and the loss of the elastic recoil.

*Emphysema*; characterised by destruction of the alveolar walls and the major condition for the irreversible degradation of the airflow (42, 45). The inflammatory response in COPD thus involves both the innate arm (the non-specific immune system) and the adaptive arm (the acquired immune system) of host defence, see below.

### 1.5.2 Systemic Inflammation

In COPD, systemic inflammation has only been studied during recent decades and is now considered an important link between the pulmonary and the systemic manifestations of this disease. Systemic inflammation has been implied in most of COPD systemic effects including cardiovascular diseases (46), weight loss, skeletal muscle dysfunction (47, 48) and osteoporosis (47).

Cardiovascular disease (CVD) is the second leading cause of death in patients with COPD (49). A number of studies have demonstrated an association between COPD and cardiovascular disease, and the presence of COPD per se may be an independent risk factor for the development of CVD alongside smoking (46, 50-52).

During exacerbations the systemic inflammation is up-regulated and several markers of inflammation in blood have been found to increase. However, there is a need to better understand if the systemic markers of inflammation represent a spill-over from the pulmonary inflammation into the systemic vascular bed, or if it is related to comorbid diseases with specific adverse effects in the lung. Systemic inflammation appears to provide an accelerated decline in lung function (44, 53).

## 1.6 Host Defence

Our defence against viruses, bacteria and different noxious particles in the lung comprises several levels working together. The airway epithelium represents the first line of defence for the lung. The protective arsenal of this epithelium is provided not only in the form of physical barriers, but also receptors and antimicrobial compounds constituting the innate immune system (see below) are also known to be present in the airway epithelium (54).The second level, the innate immune system, is a rapid and unspecific host defence where exposure results in no immunologic memory. The third defence level is the adaptive immune system that is antigen specific and produces memory cells that can prevent re-infection.

The local and systemic inflammation in COPD involves numerous cells from both the innate immune system (neutrophils, macrophages, eosinophils, mast cells and dendritic cells *inter alia*) and from the adaptive immune system (T and B lymphocytes). However, the inflammation also involves the activation of airway and alveolar epithelial cells, endothelial cells and fibroblasts (44). These cells release several inflammatory mediators that contribute to the pathophysiology of COPD including enzymes, antimicrobial peptides, cytokines and chemokines. In the general framework of this thesis, the primary focus is on the neutrophil cell; however certain T-cells and some of the mediators connected to both these cells are studied, and will accordingly be described below.

## 1.6.1 The Innate Immune System

The innate immune system is an older defence system in mammalians including humans. It is essential for detecting the presence of infections and initiating the inflammatory cascade that results in phagocyte recruitment and holding back of pathogens. The innate immune system includes physical barriers such as the skin and the mucosal epithelia of the lungs and the gastrointestinal and reproductive tract. Neutrophils and macrophages are important effector cells in innate immunity since they are able to kill bacterial pathogens in a non- specific manner (55). This system generates an acute-phase-like response, characterized by limited specificity and lack of memory, in contrast to the adaptive immune response. One aspect of the innate immune system is its capacity to generate reactive oxygen species (ROS) and thus, oxidative stress which is strongly linked to inflammation. Epithelial cells, neutrophils and macrophages are cells that can produce ROS when activated. Thus, ROS constitute an important defence against bacterial infections (56).

### Neutrophils

Chronic inflammation involves activation and recruitment of leukocytes, especially neutrophils, which are the first to defend us against invading pathogens. Neutrophils are the most abundant white blood cells and easy to recognize because of their uniquely lobulated nucleus, which has given these cells the alternative name of polymorphonuclear cells (PMNs) (57). Neutrophils are derived from the bone marrow, are transported into the bloodstream and circulate into the tissues. Increased numbers of activated neutrophils are found in sputum and bronchoalveola lavage (BAL) fluid in patients with COPD (58). Bronchial biopsies have further shown an increase of neutrophils in bronchial glands, submucosa and subepitelial tissue in COPD patients in a stable phase (45).The number of neutrophils in the airways seems to be positively related to the severity of the airflow limitation in COPD, probably as a result of the bacterial colonisation common in severe manifestations of the disease (45, 58).

When the neutrophil meets a microbe it can respond in various ways. In particular with; *phagocytosis, degranulation,* and the production of *neutrophil extracellular traps* (NETs) (57, 59).

*Phagocytosis*

Phagocytosis (Greek: *phagein* = ‘to devour’ kytos = ‘cell’, and osis = ‘process’) is central to the function of neutrophils. In this process pathogens are initially engulfed into a plasma membrane-derived vacuole, the phagosome. Following the phagocytosis, microbes are exposed to reactive oxygen species (ROS) and antimicrobial peptides, which effectively kill and digest most microorganisms (see below) (60, 61).

After phagocytosis, when the neutrophils have killed the microorganisms, they undergo a controlled program of cell death known as *apoptosis* (62). The neutrophils may then metamorphose into secondary necrosis, releasing their toxic contents into the neighbouring cells. To avoid this, the intact neutrophil cells are removed by macrophages. This course of events is believed to be crucial for the successful resolution of acute inflammation (63).

### *Degranulation*

Degranulation is defined as the secretion or production of pro-inflammatory substances (mediators) derived from intracellular stored granules or synthesized *de novo* on stimulation by receptors. The mediators are released by exocytosis whereby the granules in the neutrophils fuse with the cytoplasmic membrane to release its content in terms of enzymes, antimicrobial peptides, and other molecules (64). The neutrophil is able to release its contents intracellular into the phagosomes, which contain engulfed small microorganisms (64) (see above), and also to release ROS and cytokines to kill extracellular bacteria and to recruit additional leukocytes to the region of inflammation (45, 64).

Neutrophils contain four types of granules:

1) Azurophil (primary) granules which stores all the cellular myeloperioxidase (MPO) (see below), lysozyme, neutrophil elastase (NE), and defensins,

2) Specific (secondary) granules containing Human neutrophil Lipocalin (HNL), lysozyme and collagenase,

3) Tertiary granules containing adhesion proteins and gelatinase, and

4) Secretory vesicles containing alkalin phosphatase (64).

### *NETs*

Neutrophil extracellular traps (NETs) are web-like extracellular structures of DNA generated by activated neutrophils. NETs contain neutrophil elastase (NE), MPO and other antimicrobial and potentially cytotoxic molecules from the neutrophil cytoplasm that can trap and kill microbes in tissues (59, 65, 66). Isolated human neutrophils release NETs 2–4 h after stimulation with microbes (67), but respond much faster when activated by platelet cells stimulated with lipopolysaccharides(LPS), a process thought to be relevant during sepsis (66, 68). A recent study has also provided morphological evidence that NETs are important constituents of sputum from patients with exacerbated COPD (59).

## Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is a heme-containing peroxidase expressed in neutrophils, and to a lesser extent in monocytes (69). MPO is mainly stored in azurophil granules in the neutrophils and it utilizes hydrogen peroxide ($H_2O_2$) and the chloride anjon ($Cl^-$) to generate hypochloric acid (HOCl), a potent antimicrobial system. After the phagocytic uptake of pathogens in the neutrophil, MPO is released into the phagosome to kill the microbe. Thus neutrophils use MPO as a major defender against bacteria (69-71). This enzyme is also liberated from the neutrophil during activation, and extracellular MPO has been detected in several inflammatory diseases (72, 73).

The major function of MPO is thus the defence of the organism against infections by generating antimicrobial oxidants, free radicals and other ROS (69, 74). However, this activity can also lead to oxidative damage of the endothelium and vessel wall (75) , a process that could contribute to the pathogenesis of atherosclerosis (70, 76, 77). Studies have also found that MPO-derived oxidants harm the endothelial-protective effect of high-density lipoprotein (HDL), leading to endothelial dysfunction. Consequently, endothelial dysfunction has been considered to be associated with the development of atherosclerosis (74, 78).

## Neutrophil Elastase (NE)

Human neutrophil elastase (NE) is a neutrophil-specific serine protease stored in azurophil granules in the mature neutrophil. Neutrophils can be stimulated to release NE upon exposure to various cytokines and chemoattractants, including tumor necrosis factor (TNF)-α, IL-8, and bacterial lipopolysaccharide (LPS) among others (79). Along with other neutrophil azurophil molecules (MPO), NE assists with phagocytosis of pathogens by the activated neutrophils (79), but the effect of NE also implies degradation of extracellular matrix and proteins and damage of the lung parenchyma and airway walls (80, 81). Neutrophil elastase most likely plays a role in the migration of neutrophils towards a site of inflammation and degradation of protein from invading organisms (81). It also degrades elastin, a protein in connective tissue, and a structural lung component that prevents small airways from collapsing. The degradation of elastin is a factor leading to the development of pulmonary emphysema (81, 82). Epithelial tissues are protected from excessive proteolysis by NE and other proteases by proteinase inhibitors. These are produced locally at sites of tissue injury and by the liver, that generates saturating quantities of a-1 antitrypsin, a serine proteinase inhibitor, in the circulation (83). Circulating NE is rapidly bound and neutralized by saturating levels of alpha1-antitrypsin, making direct measurement of elastase activity in the serum challenging (83). In alpha-1 antitrypsin deficiency this protease-anti-protease balance is disrupted, resulting in an increased risk of destructive lung disease (84). The decreased mucociliary clearance in COPD patients also leads to a longer retention of apoptotic neutrophils with consequent necrosis, hence releasing their toxic agents, such as NE, into the affected airways (79). In addition NE also induces mucus gland hyperplasia, secretion of mucus and reduced ciliary beat frequency; changes that may contribute to the ability of bacteria to invade and colonize the COPD airway(85).

### Matrix metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs)

Matrix metalloproteinases (MMPs) are a family of 26 endopeptidases that are involved in the breakdown and remodelling of the extracellular matrix(86, 87). They differ from each other in expression of profile and choice of substrate but all share certain characteristics: They degrade proteins of the extracellular matrix, contain zinc in the active site, require calcium for their stability, and only function at a neutral pH (87). All MMPs can also be secreted in an inactive pro-form that is activated in the extracellular space (87).

The MMPs can functionally be divided into several groups, including gelatinases, collagenases and others; depending on which molecule they degrade (88, 89). MMP-2 and MMP-9 are included in the gelatinase group, and apart from gelatine they also degrade collagen, elastine and fibronectine and other extracellular matrix proteins (87). MMP-2 is produced by several cell types including endothelial cells and macrophages. In adults, MMP-9 is expressed in neutrophils and eosinophils but inflammatory stimulation can lead to expression in many cell types including endothelial cells, macrophages and fibroblasts (87, 90). MMP-2 and MMP-9 have specific inhibitors called Tissue Inhibitors of MMP (TIMP) -2, and TIMP-1(88).

Increased levels of MMPs, especially MMP-9, have been shown in Bronchoalveolar lavage (BAL) fluid of patients with COPD, compared with normal controls (91). High levels of both MMP-9 and its inhibitor TIMP-1 have been found in sputum from chronic bronchitis patients (92). Moreover MMP-9 levels are found to be significantly increased during COPD exacerbations with unchanging TIMP-1 levels and this correlates with influx of both neutrophils and lymphocytes (86).

## 1.6.2 The Adaptive Immune System

In vertebrates, evolution has led to the development of an exclusive adaptive arm of the immune system that makes it possible to recognise and eliminate a specific pathogen (93).The most important effector cells are B cells and T cells. The B cells produce antibodies to antigen stimulation. The T cells have a unique antigen receptor that recognises intruder structures. In contrast to innate immunity both B and T cells generate memory cells following an infection.

### Lymphocytes in the adaptive immune system

Until date, most documentation of pathogenic involvement in tobacco smokers is that of T cells. Indeed T-cells are known to accumulate in the lungs of patients with COPD and also constitute one of the key systemic signs in these patients (94). The ability of certain T cells to produce cytokines that recruit monocytes and neutrophils indicates a close link between innate and adaptive immunity(95). T cells can be divided into two subgroups: CD4+cells (Helper T-cells)their role in the adaptive immune system being to activate other immune cells such as macrophages, neutrophils and B cells (95), and CD8+ cells (Cytotoxic T-cells) which are capable of killing virally infected cells (95). Increased levels of CD8+ and CD4+ T-cells have been found in the lungs and blood of patients with COPD (94, 96).

Upon activation by T cell receptor and cytokine –mediated signalling naïve CD4 T cells may differentiate and mature into several sub-types of T helper cells; Th1, Th2, T regulatory ($T_{reg)}$ ) and, more recently, Th17 cells that all can be distinguished by their unique cytokine production profiles and their functions (97). Th cells thus play critical roles in orchestrating the adaptive immune system.

The *Th1 cells* mediate immune responses against intracellular pathogens(98) Two of their principal cytokine products are interferon γ (IFNγ), and IL-2. IFNγ is important in activating macrophages(99) and IL-2 production is important for CD4 T-cell memory.

The *Th2 cells* mediate host defense against extracellular parasites including helminths and are important in the induction of asthma and other allergic diseases(98). Th2 cells produce IL-4, IL-5, IL-9, IL-10 IL-13 and IL-25. The positive feedback cytokine for Th2 cell differentiation is IL-4 (97) (100).

The $T_{regs}$ constitute a subset of CD4 T-cells that play a major role in controlling autoimmune responses(101). The role of $\mathrm{T_{regs}}$ is to provide protection to the body from an over- activated immune response. $\mathrm{T_{regs}}$ are important both for the production and induction of anti-inflammatory cytokines in chronic inflammation, such as COPD (96). However, studies have found increased levels of $\mathrm{T_{regs}}$ in lung tissue of COPD patients, suggesting possible dysfunction of the regulatory cell resulting in an increased tissue damage in response to inflammation (102). A defect in $\mathrm{T_{regs}}$ function may trigger the development and progression of inflammatory diseases including COPD. Investigations of these cells and their function, both locally and systemically in COPD are still in their early stages (101, 103).

The *Th17 cells* mediate immune responses against extracellular bacteria and fungi. In contrast to Th1 and Th2 differentiation the Th17 differentiation is linked to alternative developmental programs, in the beginning shared with $\mathrm{T_{regs}}$. Differentiation of Th -17 cells consists of three stages: *transforming;* growth factor β (TGF-β)induces differentiation in the presence of IL-6; an *amplification* stage mediated by IL-21 and a *stabilisation* stage due to IL-23.Th -17 cells are defined as CD4+ T-lymphocytes that predominantly secrete the cytokine IL-17A but also IL-21 (a positive feedback amplifier) and IL-22(97). An increase in T-17 cells in peripheral blood have been observed in COPD patients compared to smokers without COPD and healthy subjects and this was associated with an increase in $\mathrm{T_{reg}}$ cells in COPD patients and smokers without COPD (94).

## Cytokines

Cytokines are named from the Greek words *cyto*- (eng. cell) and *kino*- (eng. movement). Thus, in English; “To set cells in motion” (104). The cytokines are intercellular signalling peptides released by cells; affecting the behavior of other cells. Several cytokines plays a role in organising the airway inflammation in COPD through the recruitment, activation and survival of inflammatory cells (105).

### IL-17 A

Interleukin (IL)-17A is a pro-inflammatory cytokine originally believed to be produced exclusively by a unique subset of Th cells – hence named Th17 cells(106). However, it is now recognized that IL-17A can also be produced by cytotoxic T cells, lymphoid tissue cells, mucosal associated invariant T cells, neutrophils, activated monocytes and mast cells (107-109). More important, IL-17A can target a broad variety of structural cells such as epithelial cells, fibroblasts, and smooth muscle cells in mammals. The IL-17 family now consists of six cytokine members: IL-17A-IL-17F (96). IL-17A induces the release of neutrophil-mobilising cytokines. The accumulation of neutrophils is associated with an increase in proteolytic enzymes, including metalloproteinase-9 (MMP-9) and neutrophil elastase (NE) resulting in increased antibacterial activity (106, 110, 111). However, IL-17A can also exert anti-inflammatory effects in stimulating neutrophil apoptosis and macrophage phagocytosis of aged neutrophils in mice (112). Studies have shown increased expression of IL-17A, in the bronchial mucosa of stable COPD patients (107, 113, 114). Only some occasional studies have been done on systemic immune signalling via extracellular IL-17A in COPD, showing diverse results, indicating both increased and decreased extracellular concentrations in blood, thereby preventing more definitive conclusions on its role at the systemic level in this context (115, 116).

### Chemokines

Chemokines are small chemotactic cytokines; their name is derived from their ability to induce directed chemotaxis in nearby cells. The chemokines play an important role in the recruitment of inflammatory cells to the lung from the circulation in COPD (117). The chemokines exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors, that are selectively found on the surfaces of their target cells (105).

### GRO-α (CXCL1)

Among the variety of neutrophil-mobilising cytokines released in bronchial epithelial cells and other structural cells of humans in response to stimulation with IL-17A, growth related oncogene-α (GRO-α, also known as CXCL1), stands out as an effector molecule of interest (113, 118). This chemokine is produced by epithelial- and endothelial cells, fibroblasts as well as monocytes

(119), and it exerts its effects via specific receptors (CXCR) on neutrophils and monocytes (119). In addition, GRO-α is a powerful activator of neutrophils by inducing exocytosis (120). Studies have found increased levels of GRO-α in induced sputum of patients with COPD and this was correlated with the increased proportions of neutrophils (119). It has also been found that interleukin-17 induces the release of several neutrophil-recruiting cytokines including GRO-α, from human bronchial epithelial cells.

# 2 AIM

The general aim of the thesis was to characterise neutrophilic inflammation in response to tobacco smoking in humans

*The specific aims of the individual studies were:*

I. To characterise signs of systemic inflammation in smokers without obstructive pulmonary disease over a long period of time.

II. To determine whether acute exposure to tobacco smoke *per se* causes an impact on gelatinases and their inhibitors in the peripheral airways of human subjects with normal lung function.

III. To characterise systemic cytokine signaling via IL-17A and GRO-α during stable clinical conditions and exacerbations in smokers with obstructive pulmonary disease and chronic bronchitis.

IV. To determine the interrelationship for systemic signs of neutrophil mobilisation during stable clinical conditions and exacerbations in smokers with obstructive pulmonary disease and chronic bronchitis.

# 3 STUDY POPULATION

## Paper I

Subjects were recruited from the WHO population study “Men born 1933 in Gothenburg”, a randomized half of all men born in 1933 and resident in Gothenburg in 1983 (n=1016). From the original cohort, 92 men, 58 smokers and 34 never-smokers were recruited for further investigations concerning respiratory symptoms and inflammatory markers in 1994 (Year 0), see Study design below.

In 2000, the 92 subjects from year 0 were asked to participate in a follow-up study. Sixty-eight subjects came to the follow-up investigation at year 6; 29 current smokers, 28 never-smokers and 11 “quitters”, who had given up smoking since Year 0 according to self-reported data in the local questionnaire. The follow-up examinations took place in 2000 and 2001, when the subjects were 67- 68 years old. The median follow-up time was somewhat more than 6 years (median: 75 months; range: 60-83 months).

Twenty-four recruited subjects did not complete the follow-up examination at year 6. Seven of these were never-smokers and 17 were smokers. Seven subjects had died since year 0; all were smokers. Causes of death were cancer (n=4), myocardial infarction (n=1), bronchopneumonia (n=1) and suicide (n=1).

All subjects were evaluated for inclusion during a telephone interview by a physician at Year 0 and Year 6. Subjects were excluded if they had any airway disease for which they had sought medical attention, congestive heart failure, unstable angina pectoris or any other severe disease. In addition, thorax deformation or treatment with corticosteroids, N-acetylcystein, or acetylic acid (ASA) less than 4 weeks prior to blood analyses, also resulted in exclusion. Patients presenting airway symptoms at Year 6 (but not at Year 0) remained, and were included in our study. In the case of infection both at Year 0 and Year 6, the examination was postponed for 4 weeks.

## Paper II

Three groups of study subjects with normal lung function were recruited for this study: non-atopic-occasional smokers, atopic-occasional smokers and never-smokers. Spirometry was performed to measure and confirm normal lung function in each individual. All atopic-occasional-smokers had a history of subjective symptoms from the upper and/or lower airways. The history of atopy was objectively confirmed through PhadiatopTM testing (Phadia AB, Uppsala, Sweden) of specific immunoglobulin (Ig)E and by assessing total IgE levels in blood. All three groups had been free from smoking and respiratory infections for ≥4 weeks prior to participating in the study.

In total, 29 occasional-smokers were recruited. Seven of these were later excluded from further analysis as they did not meet the study criteria for cotinine levels in urine. Of the remaining 22 occasional-smokers, 13 were non-atopic and nine were atopic. In total, 18 never-smokers were recruited, of which three were subsequently excluded due to infections during the study. The patient characteristics of the 13 non-atopic-occasional smokers, nine-atopic-occasional smokers and 15 never-smokers are shown in Table 1.

### Paper III-IV

Sixty (60) smokers with obstructive pulmonary disease and chronic bronchitis (OPD-CB) were recruited from the outpatient clinic at the Department of Respiratory Medicine at Sahlgrenska University Hospital, Gothenburg, Sweden, and by advertising in the local press. All smokers with OPD-CB were in a stable phase at inclusion and stated to not having undergone any respiratory tract infection at least 4 weeks prior to inclusion. They all fulfilled the GOLD criteria for COPD and were classified accordingly (stage I–IV). The diagnosis of chronic bronchitis was based upon a history of phlegm for at least 3 consecutive months during 2 consecutive years. The OPD-CB subjects were all current smokers with a smoking history of at least 10 pack-years.

Exclusion criteria were: asthma, atopy, lung diseases other than OPD-CB, α1-antitrypsin deficiency, clinically significant heart failure and regular use of oral glucocorticoids. Patients with cancer, documented immunodeficiency, known mental disorder or obvious abuse of alcohol or drugs were also excluded.

Four smokers with OPD-CB did not complete the study. This was due to suicide (1), compliance problems (1) use of oral steroids (1) and diagnosis of neurological disease during the study time (1). As control groups, we included 10 asymptomatic current smokers (AS) with a tobacco load of at least 10 pack-years and 10 healthy never-smokers (NS); all recruited via advertisement and all having a normal lung function.

# 4 METHODS

## 4.1 Study Design

### Paper I

The study was longitudinal. Subjects were examined at two time points, 6 years apart. In both Year 0 (1994), and Year 6 (2000), all subjects underwent lung function tests, blood tests and a symptom questionnaire; a modified version of the European Community Respiratory Health Survey(121).

### Paper II

The study was cross-sectional. All subjects underwent two bronchoscopies including bronchoalveolar lavage (BAL); the first at day 1 (termed BAL1) and a second at day 14 (termed BAL2). On days 12 and 13, all occasional-smokers smoked, in total, 10 filter cigarettes of a commercial brand (tar 10 mg, nicotine 0.8 mg), purchased commercially (not given as a gift). To be considered as occasional-smoker, the subjects had to habitually smoke cigarettes on at least one occasion per month and a maximum of four occasions per month. The dose (number) of cigarettes was chosen based upon the clinical observation that none of the recruited occasional-smokers habitually smoked >20 cigarettes over a 48-h period. It was reasoned that it would be unethical to exceed the number of cigarettes the recruited subjects would habitually smoke on average; therefore, a dose of 10 cigarettes over a 48-h period was chosen and considered as ethically impregnable. The smoking status for each subject was controlled by measuring the urine cotinine level at the time of each of the two bronchoscopies. To be included, all subjects had to display cotinine levels <100 ng·mL−1 prior to BAL1. For the continued inclusion of occasional smokers at the time of BAL2 (i.e. as a confirmation of the intervention smoke exposure), these subjects had to display cotinine levels at least five-fold of those obtained at the first bronchoscopy. Subjects were excluded if they suffered from any infection between the two bronchoscopies.

### Paper III-IV

The study was prospective.  All subjects, including controls, where examined at an inclusion visit (Visit 1), and underwent lung function tests (see below), pulmonary x-ray, physical examination, blood tests and urine cotinine test to check for tobacco use. The smokers with OPD-CB also donated a spontaneous sputum sample for bacteria culture. During the subsequent 15 months, these patients underwent blood tests every 15th week (during Visit 2-5). If the patient had an exacerbation (EXA), an extra visit was arranged. On this occasion, the patient received treatment assessed to be adequate depending on his/ her condition, after blood sampling. We used the definition of COPD exacerbations based on criteria described by Wedzicha and Donaldson(122) originally modified from those described by Anthonisen et al (123).

## 4.2 Lung Function Tests

*Ventilatory lung capacity.* Forced expired volume in one second (FEV1) and forced vital capacity (FVC) were obtained utilizing a calibrated spirometer (Jaeger Masterscope, VIASYS Healthcare GmbH, Hoechberg, Germany and Sensormedics Vmax 22, VIASYS Healthcare, Yorba Linda, CA, USA). Spirometry was performed without prior bronchodilation to avoid selection of non-reversible COPD patients and the European Respiratory Society reference values for spirometry were utilized for evaluation(124).

*Gas diffusion capacity.* Diffusion capacity test (DLCO) was assessed by the single breath method with the standard equipment (SensorMedics® 2200, SensorMedics Co, Bilthoven, the Netherlands)and the reference values according to Salorinne *et al.* were utilized(125).

## 4.3 Bronchoscopy and BAL. (Paper II)

### Sampling and handling

Bronchoscopies were performed according to standard procedure using a flexible bronchoscope. A bronchial wash of 20 mL phosphate buffered saline (PBS) preceded the BAL to avoid contamination from the proximal airways during the procedure. BAL was subsequently performed in the right middle lobe utilising 3× 50 mL of PBS. BAL fluid was collected in a polypropylene tube and kept on ice until it reached the laboratory. The total cell count and trypan blue exclusion were on the carried out followed by centrifugation of the BAL samples to separate the cells from the BAL fluid. The cells were then resuspended in buffer and after preparation in a cytospin subsequently stained with May-Grünwald-Giemsa. Differential counting of 600 cells per sample was carried out according to standard morphological criteria.

### Gelatinases and gelatinase inhibitors in BAL fluid

Zymography was used to identify MMP-2 and MMP-9 bands and to screen for total MMP-2 and MMP-9 activity in the BAL fluid of the first five subjects in each study group. The concentrations of MMP-2 (pro plus active forms), MMP-9 (pro plus active forms), TIMP-1 and TIMP-2 were determined in all of the BAL fluid samples using commercial ELISA kits from R&D Systems (Abingdon, UK) and carried out according to the manufacture's recommendations. The net gelatinase activity was measured in all BAL fluid samples using a fluorescence-conjugated gelatine substrate (D-12054 DQ gelatin from pig skin; Invitrogen, Mount Waverley, Australia).

## 4.4 Blood and Sputum Analyses

### Myeloperoxidase (MPO) (Paper I+IV)

The concentration of MPO protein in serum (Paper I) was determined using a double antibody radioimmunoassay (RIA) (Pharmacia & Upjohn, Diagnostic AB, Uppsala; Sweden) briefly described in paper I (p.890).

The concentration of MPO protein in plasma (Paper IV) was measured in diluted samples 1:10 using ELISA (HK324; Hycult Biotechnology bv, Uden, the Netherlands). The results were subsequently corrected for dilution. The analysis was conducted at the Department of Internal Medicine & Clinical Nutrition Sahlgrenska Academy, University of Gothenburg, Sweden.

### Human neutrophil lipocalin (HNL) (Paper I)

HNL was determined in serum using a double – antibody radioimmunoassay (RIA), (Pharmacia & Upjohn, Diagnostic AB, Uppsala; Sweden) previously described briefly in paper I (p.890). The analysis was conducted at the Department of Medical Science, Clinical Chemistry, University of Uppsala.

### Lysozyme (Paper I)

Lysozyme was titrated in serum by incubating standard solution or samples with Sephadex- bound anti –lysozyme for 2 h before the addition of $I^{125}$ labelled lysozyme, a method previously described briefly in paper I (p.890). The analysis was conducted at the Department of Medical Science, Clinical Chemistry, University of Uppsala.

### IL-17A and GRO-α protein (Paper III)

The concentrations of IL-17A protein in plasma and GRO-α protein in serum were determined by using commercial ELISA kits (3520-1H-20 MabTech®, Nacka, Sweden and DY275 R&D Systems®, Minneapolis, MN, USA, respectively). The analyses were conducted at the Department of Medicine and the Department of Rheumatology respectively, Sahlgrenska Academy, University of Gothenburg, Sweden.

### Neutrophil Elastase (NE) (Paper IV)

The concentration of (NE) protein was assessed using the latex bead concentration method. The analysis was conducted at the Department of Pediatrics, Dokkyo Medical University, Japan (126).

### mRNA (Paper III-IV)

Blood samples were collected in PAXgene Blood RNA Tubes (QIAGEN, Hilden, Germany). Total RNA was isolated using PAXgene Blood RNA Kit (QIAGEN) according to the protocol, previously described briefly in paper III-IV. The analyses were conducted at the Department of Rheumatology and Inflammation Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Sweden.

### Sputum analyses (Paper III-IV)

Samples of spontaneous sputum were conducted and sent to the accredited laboratory of Bacteriology at Sahlgrenska University Hospital Gothenburg, Sweden, for semiquantative analysis regarding growth of bacteria and also a morphological evaluation to ascertain whether the sputum samples were representative for the lower respiratory, using light microscopy.

## 4.5 Statistical Methods

Non-parametric statistical methods were applied. Differences were considered statistically significant for P-values <0.05. The comparisons of multiple groups were conducted utilising Kruskal-Wallis test followed by Mann-Whitney U-test. For comparison between two groups, the Mann-Whiteny U test and Wilcoxson´s signed rank test were applied. The correlation analyses were performed using Spearman´s rank correlation test.

*Table 1. *smokers with phlegm, wheezing / smokers without symptoms (according to questionnaire at Year0) OPD-CB;smokers with Obstructive Pulmonary Disease with Chronic Bronchitis. Quitters; smokers given up smoking since Year 0.*

.

# 5 RESULTS

## 5.1 Paper I

### Higher concentrations of MPO and other inflammatory markers in the blood in smokers

Six years after the baseline samples were taken, the systemic markers myeloperoxidase (MPO), lysozyme and human neutrophil lipocalin (HNL) all showed higher concentrations in the blood of the smokers group compared with the group of never-smokers (Fig 1a-c). In addition MPO showed a significant increase unique for subjects who continued to smoke throughout the observation time (Table 2).

*Table 2. Change for inflammatory markers in blood during a 6 year period.*

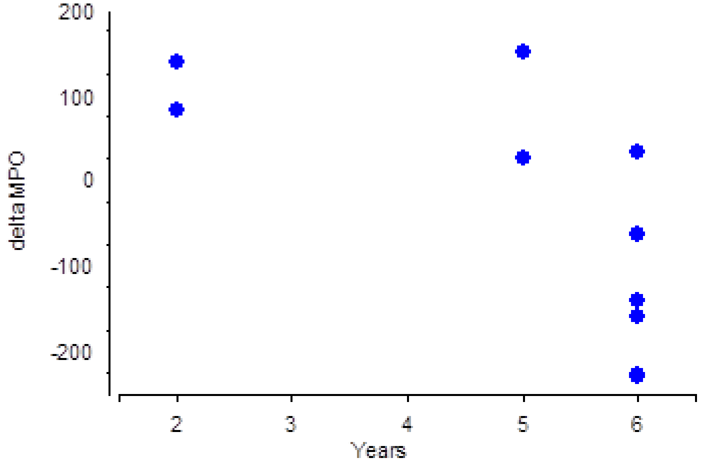
|  | *Smokers* (n=29) | *Quitters* (n=11) | *Never-smokers* (n=28) |
|---|---|---|---|
| MPO(µg/L) | 131(33) | 70(39)$^{ns}$ | 44(23)$^{*}$ |
| LYS (µg/L) | 225(68) | 28(102)$^{ns}$ | 206(36)$^{ns}$ |
| HNL (µg/L) | 31(5) | 7(9)$^{ns}$ | 34(4)$^{ns}$ |

Data presented as mean (SD). ‘‘Quitters’’ refer to smoking subjects who quit smoking during the observation period.

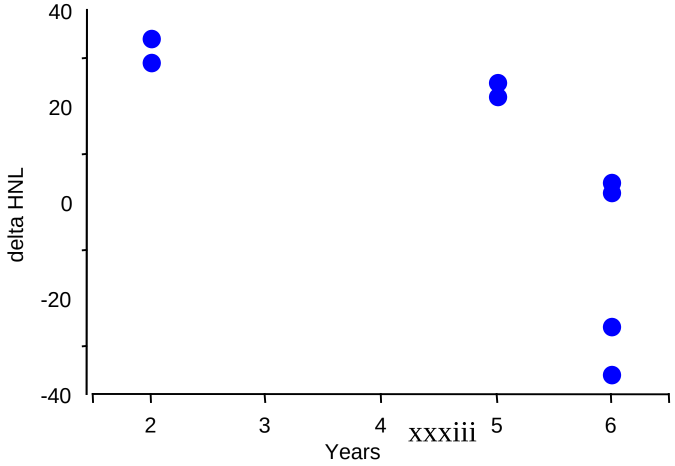
### Negative correlation between MPO, HNL and lysozyme, and the duration of smoking cessation

A strong negative correlation was found between the duration of smoking cessation in quitters, and the change (delta value) in MPO, HNL and lysozyme (Fig 4).

A)



B)



C)

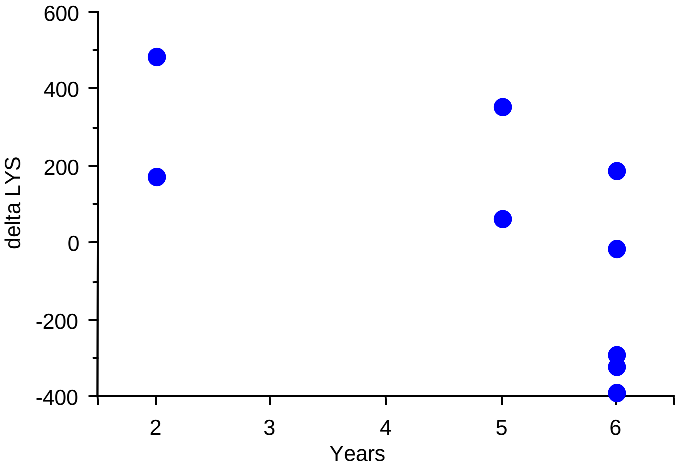


Figure 4. *Change in (delta) MPO, (A) HNL, (B) and lysozyme (C) in blood from quitters versus duration of smoking cessation during a 6-year observation period. n = 9 . (A)P=0.03, Rs=0.77; (B)P=0.01, Rs=0.93; and (C) P=0.05, Rs=0.70.*

## 5.2 Paper II

### No impact on the number of neutrophils or macrophages in BAL

Neither bronchoalveolar lavage (BAL) recovery, total cell count and cell viability, nor percentage of neutrophils, macrophages and lymphocytes differed markedly between BAL1 and BAL2 in any of the study groups or between the study groups. The percentage of eosinophils was higher in BAL2 than BAL1 among never-smokers (p=0.016) and atopic smokers (p=0.039) (Table 3 in supplementary data Paper II)

## No impact on local gelatinases in BAL

### Identity of gelatinases

Zymography was used to identify the dominant gelatinases in BAL fluid. Three main bands were identified at ~70, 90 and 150 kDa in size (fig 1, Paper II). No differences were found in the appearance for the density of the bands for matrix metalloproteinase (MMP)-2 and MMP-9 between never-smokers, occasional-smokers or atopic occasional-smokers. No differences were found between BAL1 and BAL2 in any of the groups. (Table 4 in supplementary data. Paper II).

### Quantity of gelatinases and gelatinase inhibitors

MMP-2, MMP-9, and their inhibitors TIMP-1 and TIMP-2 were quantified in all samples using ELISA. The measurements confirmed the zymography results with no pronounced difference seen between BAL1 and BAL2 or between study groups (Fig 5).

Figure 5. *Quantitative analysis of gelatinases and gelatinase inhibitors in human bronchoalveolar lavage (BAL) fluid. ELISA was utilised to measure concentrations of total a) matrix metalloproteinase (MMP)-2, b) MMP-9, c) tissue inhibitors of MMP (TIMP)-1 and d) TIMP-2 in BAL samples before (BAL1) and after (BAL2) smoking in 13 nonatopic-occasional smokers and nine atopic occasional smokers, and in corresponding BAL samples from a control group of 15 never-smokers not exposed to tobacco smoke between bronchoscopies.*

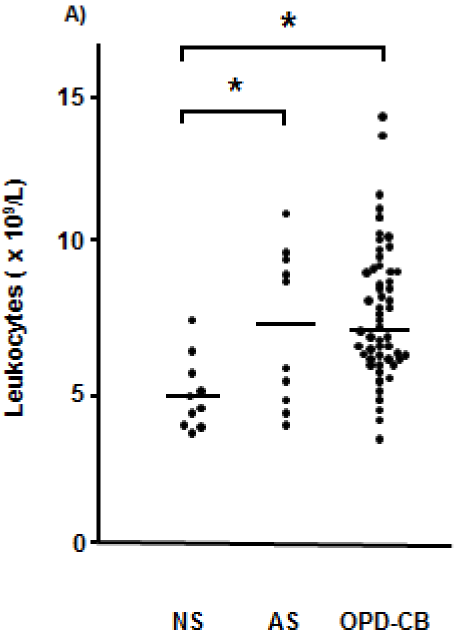
## 5.3 Paper III and IV at Inclusion

### Study population

Lung function values were lower in smokers with OPD-CB than in the control groups (Table 1). The tobacco load (i.e. the number of pack-years) tended to be somewhat higher in smokers with OPD-CB than in the control group of asymptomatic smokers but this difference did not prove statistically significant (Table 1).

### Concentrations of leukocytes, neutrophils and CRP

The leukocyte and neutrophil concentrations were higher in the combined group of OPD-CB and asymptomatic smokers, compared with never-smokers (Fig 5A). CRP concentrations were substantially higher in smokers with OPD-CB than in the control groups (Fig 5B)



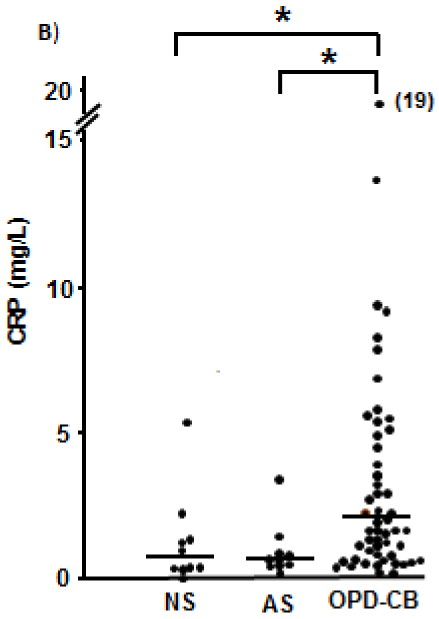


Figure 6. *Concentrations of leukocytes(A) and CRP (B) at inclusion in never-smokers (NS), asymtomatic-smokers (AS) and patients with obstructive pulmonary disease with chronic bronchitis(OPD-CB). Data are presented both as individual (dots) and median values (bold lines).(*$^{*}p<0.05$*, n=10 (NS) n=10 (AS) n=60 (OPD-CB)*

### Severe disease smokers with OPD-CB

In severe disease smokers with OPD-CB (GOLD III-IV), the concentrations of leukocytes; neutrophils and lymphocytes were lower compared with mild disease (GOLD I-II) even though the concentrations of neutrophils were increased in the pooled group of tobacco smokers. (Table 2, Paper III).

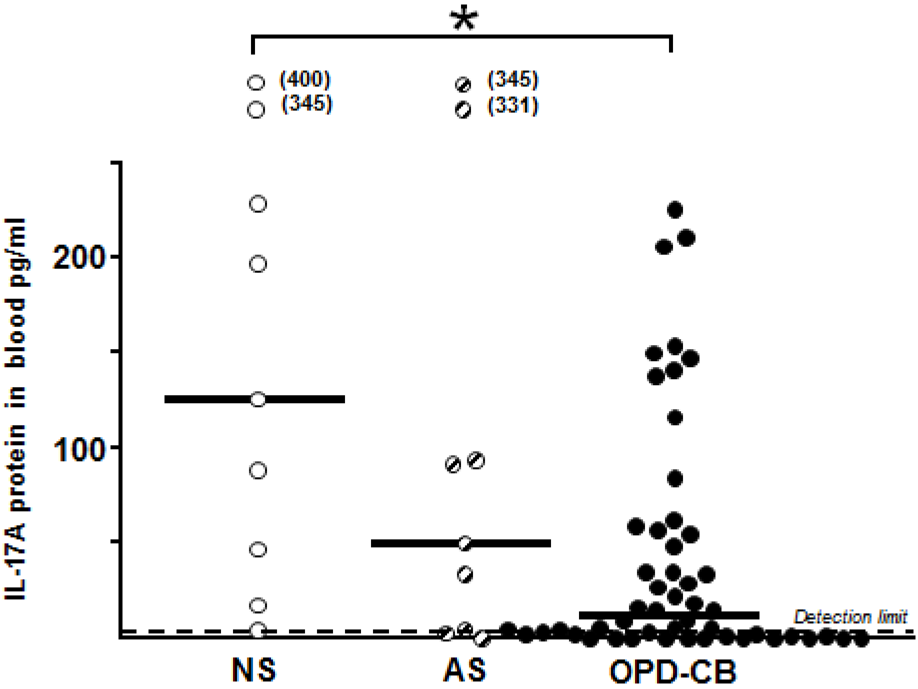
#### Lung-function, tobacco load and inflammatory markers in blood

No correlation was found between concentrations of IL-17A, GRO-α, MPO, NE or CRP and inflammatory cells in blood on the one hand, and ventilatory capacity, gas diffusion capacity or tobacco load on the other hand, at the time of inclusion. However, there was a weak, positive correlation between GRO-α and FEV1 within the whole group (OPD-CB, AS and NS) ($r=0.31$, $p<0.05$, $n= 74$).

## 5.3.1 Paper III at Inclusion

#### Lower concentrations of IL-17A and GRO-α in smokers with OPD-CB

At the time of inclusion in the study, the smokers with OPD-CB displayed markedly lower concentrations of IL-17A and GRO-α protein in blood, compared with never-smokers. The concentrations of IL-17A protein in the group of asymptomatic smokers tended to be lower compared with the never-smokers, whereas the concentrations of GRO-α protein were clearly lower in smokers with OPD-CB compared with the asymptomatic smokers (Fig 7).


*
(400)
(345)
(345)
(331)
IL-17A protein in blood pg/ml
200
100
Detection limit
NS
AS
OPD-CB

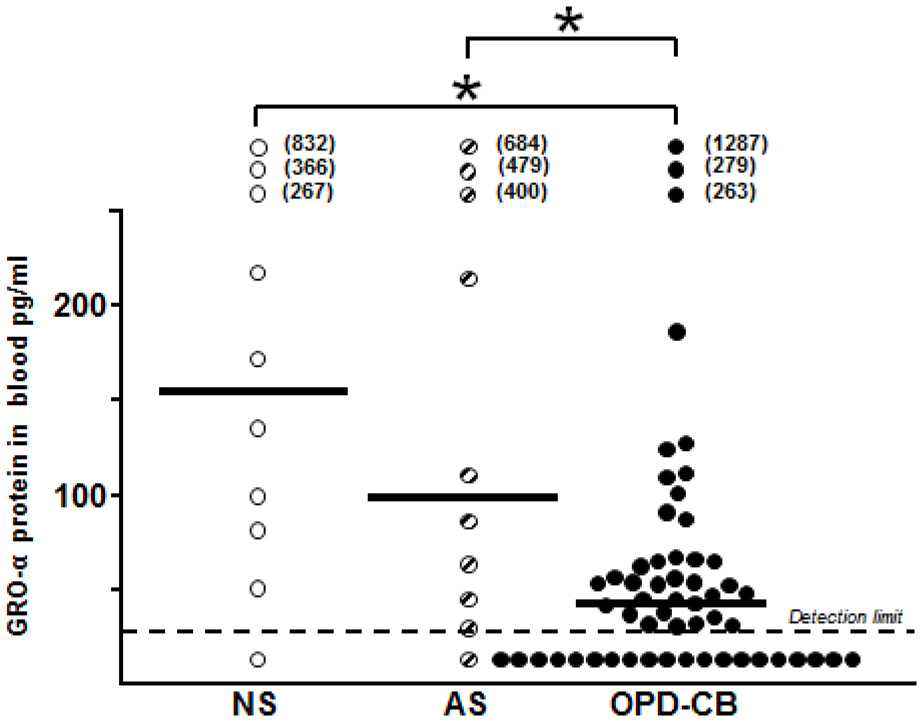


Figure 7. *Concentrations of interleukin (IL)-17 and growth-related oncogene(GRO)-α protein in blood from smokers with obstructive pulmonary disease including chronic bronchitis (OPD-CB) during stable conditions at the time of inclusion, compared with never-smokers (NS) and asymptomatic smokers (AS). Data are presented as individual (dots) and median values (bold lines). *)* p*<0.05*

There was a positive correlation between IL-17A and GRO-α protein in the pooled group of smokers (OPD-CB and AS) (Fig 8). In smokers with OPD-CB, we also detected a correlation between neutrophils and GRO-α protein among subjects with severe disease (GOLD Stage III+IV). (Fig3, Paper III)

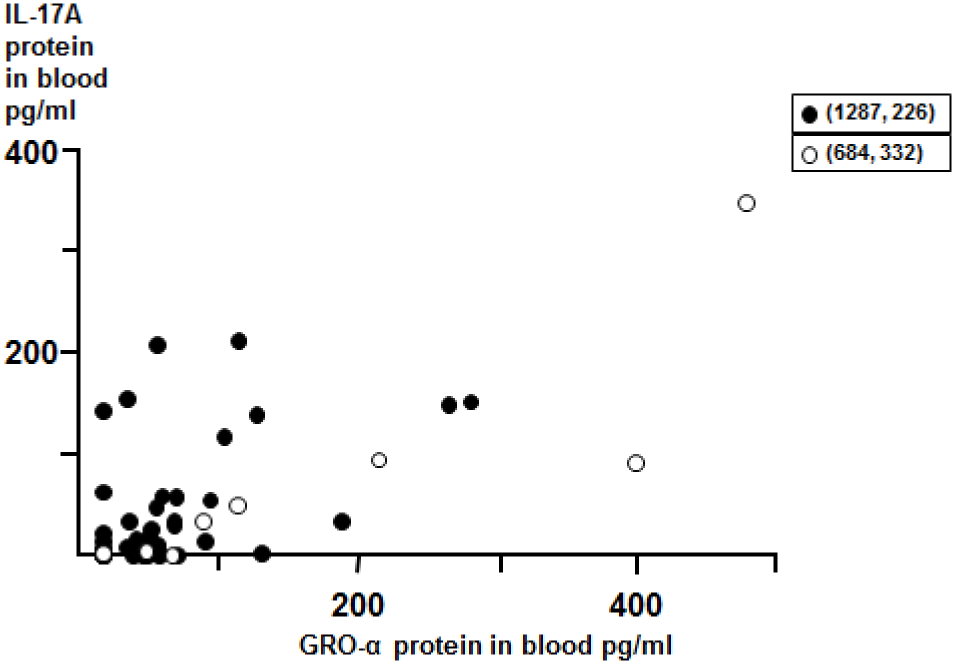


Figure 8. *Fig Correlation (r=0.57, p<0.05, Spearman rank correlation) between the concentrations of IL-17A and GRO–α protein in blood from smokers (ie. smokers with obstructive pulmonary disease including chronic bronchitis (OPD-CB, filled circles) and asymptomatic smokers (AS, open circles) during stable conditions at the time of inclusion).*

### Lower concentrations of IL-17A and GRO-α protein in smokers with OPD-CB with opportunistic pathogens in sputum

Markedly lower concentrations of IL-17A protein was found in the few smokers with OPD-CB displaying growth of opportunistic pathogens *(Escherichia coli, Pseudomonas aeruginosa, Stenotrophomonas maltophilia Enterobacter agglomerans)* (2.0 [0.8-4.1] pg/ml; n=3) compared with the pooled group of OPD-CB displaying growth of common respiratory pathogens *(Haemophilus influenzae, Streptoccus pneumonia, Moraxella catharralis)* or oropharyngeal flora in sputum (35 [0.8-225.7] pg/ml; n=27) (Kruskal-Wallis test: p=0.020).

Smokers with OPD-CB displaying growth of oropharyngeal flora (56 [0.8-225.7] pg/ml; n=15). Smokers with OPD-CB with common respiratory pathogens (32.6 [0.8-211.5] pg/ml; n=12).

The concentrations of GRO-α associated with growth of opportunistic pathogens (15.5 [15.5-15.5] pg/ml; n=3), were clearly lower (Kruskal-Wallis test: p=0.036) compared to those associated with the pooled group displaying growth of common respiratory pathogens or oropharyngeal flora (56.0 [15.5-279] pg/ml; n=21).

## 5.3.2 Paper IV at Inclusion

### Increased concentrations of NE but not MPO

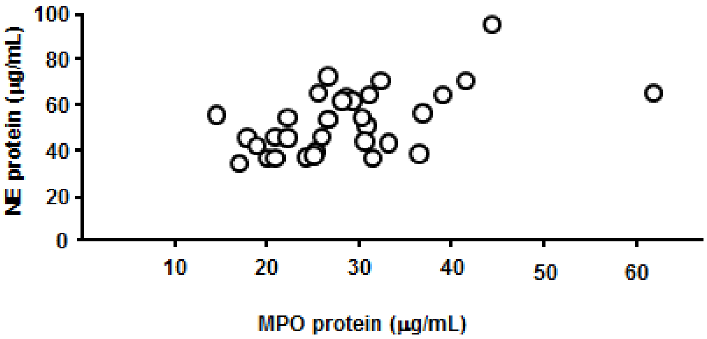
There was no clear difference in MPO protein concentrations between smokers with OPD-CB, asymptomatic smokers and never-smokers at the time of inclusion. However, the concentration of neutrophil elastase (NE) protein was higher in the OPD-CB group and asymptomatic smokers, compared with never-smokers (Figs.1b and 1c, Paper IV). Notably, there was a moderate correlation between the concentrations of MPO and NE protein versus neutrophils (Figs 2a and 2b, Paper IV).

### Positive correlations between MPO and NE

There were strong positive correlations between MPO and NE protein in blood, as well as mRNA normalised to beta actin, among smokers with OPD-

CB at time of inclusion (Fig 9). We also detected moderate correlations between mRNA, normalized to beta actin, for MPO and carboxyl esterase (CES) among smokers with OPD-CB. There was no corresponding correlation between mRNA for CES and NE among these patients (data not shown).

a)



b)

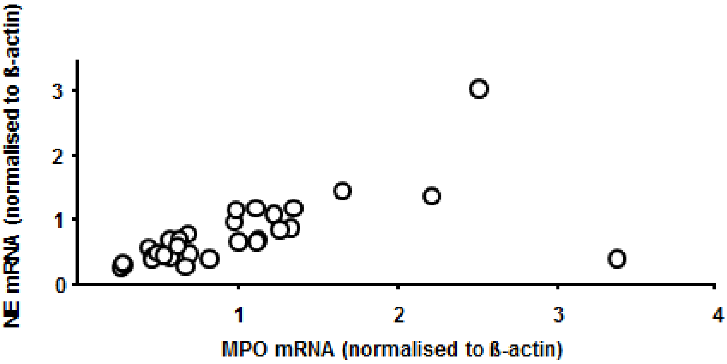


*Fig 9.Correlations between signs of systemic inflammation in smokers with obstructive pulmonary disease and chronic bronchitis (OPD-CB) during stable clinical conditions at the time of inclusion:* ***a)*** *blood concentrations of myeloperoxidase (MPO) and neutrophil elastase (NE) protein at the time of inclusion (Spearman rank correlation: r= 0.72, p< 0.001)* ***b)*** *the levels of mRNA for MPO and NE in blood leukocytes at the time of inclusion (Spearman rank correlation: r= 0.85, p<0.001)*

### Pathogens in sputum and inflammatory markers

There was no reproducible difference in concentrations of MPO, NE, CRP protein or neutrophils for smokers with OPD-CB with, on the one hand common respiratory pathogens or oropharyngeal flora, and on the other those with opportunistic pathogens in sputum.

## 5.4 Paper III and IV at Exacerbations

### Frequency

Among the smokers with OPD-CB, 63% suffered from at least one exacerbation during the 15 month study period. Some of the smokers had a second and even a third exacerbation but these are not included in the data below.

### Increased concentrations of inflammatory cells

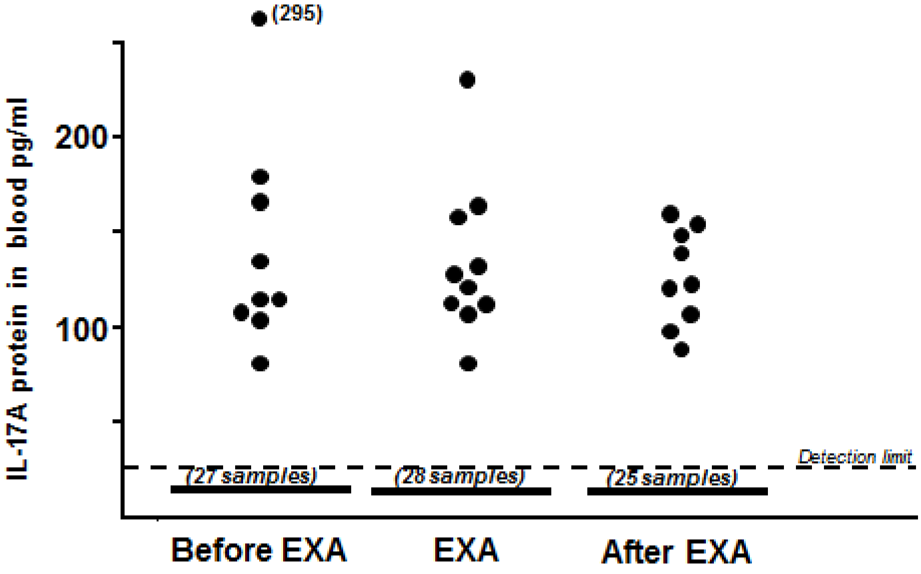
The concentration of leukocytes, neutrophils and CRP, but not lymphocytes, monocytes or eosinophils increased in the group of smokers with OPD-CB during exacerbations (Table 1. Online supplement Paper III and Table 2 Paper IV)

### 5.4.1 Paper III at Exacerbations

#### Low concentrations of IL-17A

In smokers with OPD-CB, there was no detectable increase in the concentrations of IL-17A protein in blood during the first exacerbation (Fig 10)

A)



In contrast, concentrations of GRO-α protein were markedly increased during the first exacerbation, compared with corresponding concentrations at the most recent regular visit prior to the first exacerbation (a maximum of 15 weeks earlier). At the time of the first regular visit after the exacerbation, the concentration of GRO-α protein decreased towards baseline values (Fig 10).

B)

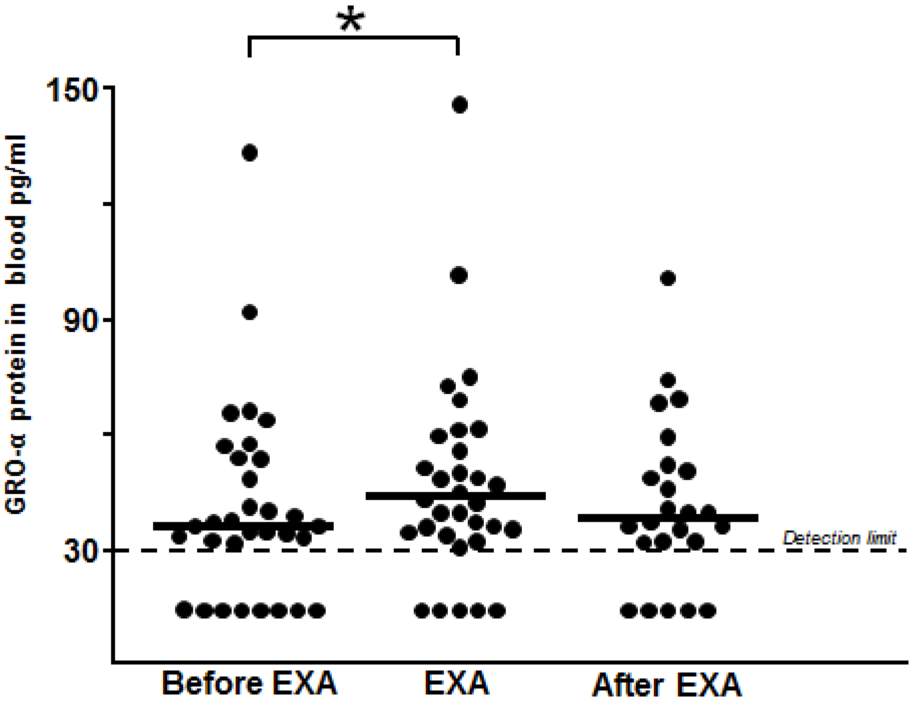


Figure 10. *Comparisons of the concentrations of IL-17A (A) and GRO-α protein (B) in blood from smokers with obstructive pulmonary disease including chronic bronchitis (OPD-CB) before (Before), during (Exacerbation), and after (After) the first exacerbation. Data are presented as individual (filled circles) and median values (bold lines). *) p<0.05; NS p>0.05(Kruskal-Wallis test followed by Mann-Whitney U-test).*

## 5.4.2 Paper IV at Exacerbations

### Increasing concentrations of MPO and NE

MPO concentrations in smokers with OPD-CB were markedly elevated during exacerbations. Likewise, NE protein tended to increase during exacerbations but not in a statistically significant manner (Table 1. Online supplement Paper III).Absolute MPO concentrations in smokers with OPD-CB were clearly higher during exacerbations (EXA) than at the time of the most recent regular visit prior to the exacerbation (a maximum of 15 weeks earlier). MPO protein was normalised at the time of the first subsequent regular visit.

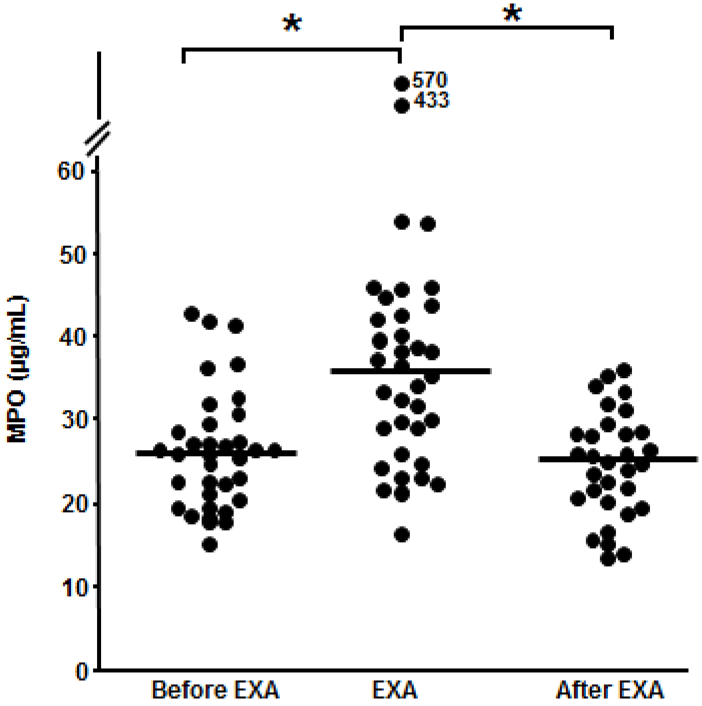
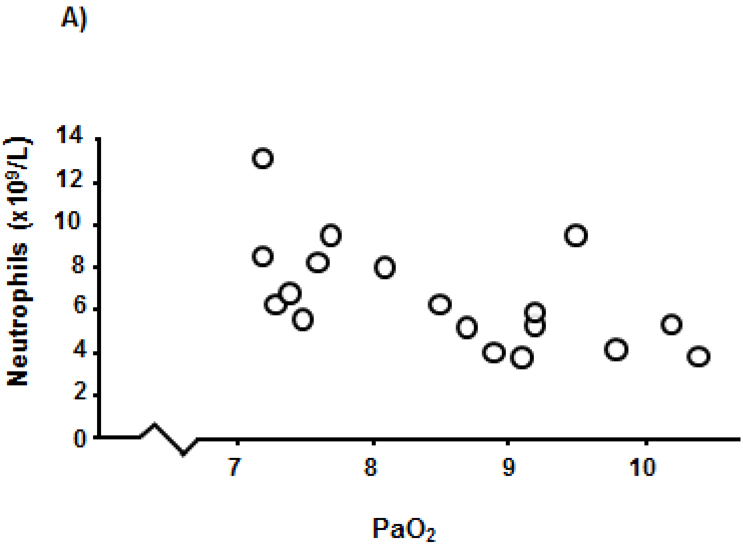


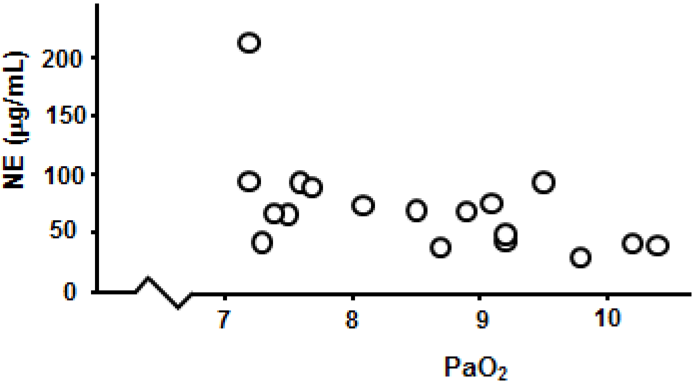
Figure 11. *Fig Blood concentrations of myeloperoxidase (MPO) protein in patients with obstructive pulmonary disease and chronic bronchitis (OPD-CB) before (BeforeEXA), during (EXA), and after (AfterEXA) exacerbations. Data presented as individual (circles) and median (bold lines) values (*Mann Whitney U-test: p< 0.05, n=38).*

## A link between hypoxia and systemic inflammation

Arterial blood gas was analysed at exacerbations in 18 smokers with OPD-CB with oxygen saturation below 94%. There was a strong negative correlation between PaO2 and the concentrations of neutrophils, NE and MPO protein, respectively (Fig12).



B)



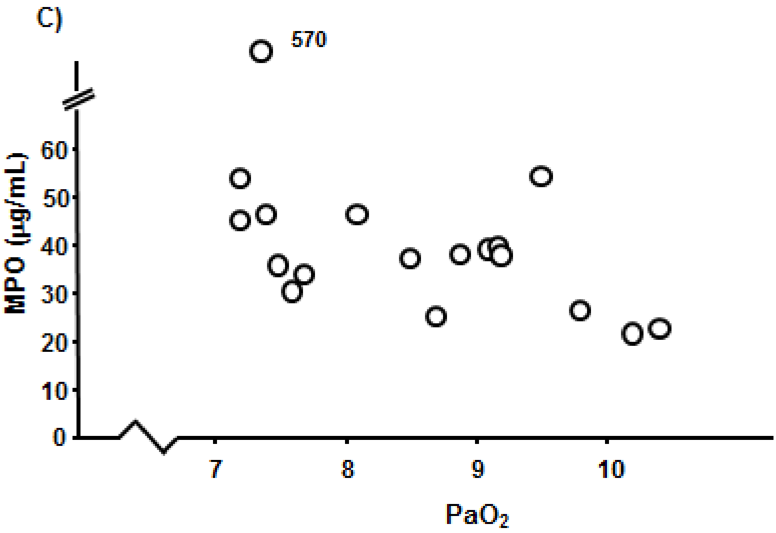


Figure 12. *Correlations between oxygen saturation and signs of systemic inflammation in eighteen smokers with obstructive pulmonary disease and chronic bronchitis (OPD-CB) who developed hypoxia during exacerbations: A***)** *Partial oxygen pressure (PaO2) and blood concentrations of neutrophils (Spearman rank correlation: r= 0.63, p= 0.005;n=18);* ***B)*** *PaO2 and blood concentrations of neutrophil elastase (NE) protein (Spearman rank correlation: r= 0.56, p=0.02 n=18); and* ***C)*** *PaO2 and blood concentrations of myeloperoxidase (MPO) protein (Spearman rank correlation: r=0.5, p=0.03 n=18).*

# 6 DISCUSSION

## 6.1 Paper I

### Subjects

The study group in Paper I consists of subjects originally derived from the population study “Men born 1933 in Gothenburg”(127).In 1994 the men in the study group where approximately 61 years old; in our study (Year 6), they had reached 67 years of age. Thus, this is a group of elderly men, consisting of smokers and never-smokers, recruited from the same population, of the same age and without severe airway symptoms at inclusion 1994, despite a rather heavy smoking among the smokers. Consequently, the smokers selected for this study may represent subjects less predisposed to react to tobacco smoke, as indicated by a preceding study on gender differences among smokers(128). Therefore, the applicability to females and younger men may be questioned. Fourteen percent of the smokers had died in smoking-related diseases during the six year period; none of the never-smokers had died.

### Lung function

In this study we found no significant difference in VC, $FEV_1$ or $D_LCO$ at Year 6 compared with Year 0. However, when looking separately at the group of smokers at Year 6, seventeen of them had a $FEV_1$/FVC <70, and in 13 of those FEV1/FVC was <65. Two of the ex-smokers (“quitters”) also showed a FEV1/FVC <65. Thus, in reality, 62% of the smokers had developed COPD during this 6 year period. There may be several reasons why this is not reflected in our statistics of lung-function measurement. One reason being our limited population of “clinically healthy” male subjects was highly selected. Interestingly, their vital capacity values (VC and FVC) were generally but not significantly higher, and $FEV_1$ lower, at Year 6 compared with Year 0 which lowers the ratio FEV1/FVC. Subsequently at Year 6 the smokers could be grouped in GOLD stage I (n=4) or the upper range of GOLD stage II (n=13). Yet it must be noted,that what is now defined as GOLD stage I, at the time of study initiation (Year 0), was regarded as “preclinical COPD”. However, most importantly, at Year 0, High Resolution Computed Tomography (HRCT) already demonstrated emphysematous changes in 25 (44%) of the smokers, but only one (3%) of the never-smokers(129). Additionally at Year 6, the severity and extent of emphysematous lesions had increased significantly among current smokers(130). This shows that some of the smoking subjects had preclinical COPD at Year 0 and further developed the disease with continued smoking.

### Systemic signs of inflammation during 6 years' time in smokers without airway symptoms

Supporting previously published data from Year 0 our study showed that concentrations of all three markers of primarily neutrophilic inflammation; MPO, lysozyme and HNL, were significantly higher in smokers than in never-smokers.

Smoking leads to elevated white cells count, particularly neutrophils (131, 132). MPO is expressed in neutrophils and to a lesser extent in monocytes (70, 71). Our finding of increasing concentrations of MPO exclusively in the subjects that continued to smoke provides an additional argument in favor of neutrophils being involved in systemic inflammation caused by tobacco smoking. It has been demonstrated in several studies that COPD is associated with increased risk of cardiovascular disease (CVD) and that cardiovascular deaths are a significant cause of mortality in COPD patients(133, 134). Moreover; MPO appears to participate in a range of events involved in the initiation, propagation and subsequent complications of atherosclerotic plaques(75). In our current study, over 60% of the smokers that 6 years earlier claimed to be healthy now had lung function values corresponding to COPD stage I and II. In addition at Year 6, 45% of the smokers also declared having some sort of medication for cardiovascular diseases. Thus, the increase of MPO concentrations in smokers in this study additionally supports an active role of MPO in systemic inflammation caused by tobacco smoke.

In Paper I we were not able to demonstrate a significant correlation between measured inflammatory markers in blood and decline in lung function, as we might have expected given the results of the study at Year 0(135) . This may be due to several factors such as the limited population or that their lung function in most cases was normal or close to normal. Also, the extent of comorbidities in the group of smokers may have had an influence on the results. In support of this hypothesis, a study on systemic biomarkers of neutrophilic inflammation in COPD patients has shown that MPO concentrations in serum correlate with $FEV_1$ /FVC and DLCO respectively in patients without metabolic syndrome but not in patients with metabolic syndrome. Also, in this study, MPO concentrations were increased with COPD severity (136).

As cigarette smoking is known to cause systemic immune alterations both in the number and function of immune cells, it is tempting to believe that smoking cessation could reverse this situation. In Paper I we found a negative correlation between duration of smoking cessation and change in blood MPO, HNL and lysozyme concentrations. This finding is compatible with smoking cessation limiting systemic inflammation; although our group of "quitters" was rather small (n=11). Additional support for this argument was provided by a recently published study of 1819 subjects where the effects of cigarette smoking on 10 "systemic immune and inflammatory markers" (IL-15, IL-16, IL-1Ra, IL-1β, SCF, sIL-6R, sVEGFR3, CRP, CCL17 and CCL11) were characterized. Substantial differences were found in immune markers between current and never-smokers but for many of these, levels in former smokers approached those of never-smokers over time (137)

## 6.2 Paper II

### Study subjects

The three groups of subjects recruited for this study had substantially different characteristics compared with the study groups in Paper I and Paper III-IV. These subjects were young, occasional-smokers or never-smokers, and all with normal lung function. Thus, they were chosen to provide good physical and ethical circumstances for a study of acute exposure to tobacco smoke in lungs hitherto free from chronic inflammation. We went to great lengths to ascertain that the included subjects were occasional-smokers. However, the amount of cigarettes smoked per day, estimated retrospectively by the subjects themselves could have been over- or underestimated according to their motivation to participate in the study. Yet there was a strict control of cotinine levels in urine prior to both BAL1 and BAL2 to assure the smoking status, and a clear increase in the nicotine metabolite cotinine in urine was documented for all included subjects before BAL 2.

## Bronchoscopies

Bronchoscopy as a method is a unique way of reaching the human peripheral airways for sampling, in contrast to the technique of induced sputum that is likely to reflect only proximal airways(138)

The bronchoscopies were performed according to standard procedure using a flexible bronchoscope. The bronchial wash (BW) and BAL represent different compartments of the respiratory tract. The small volume of BW (20 ml) is considered to represent the bronchi, and precedes the BAL to avoid contamination from proximal airways.

## Local signs of inflammation after short-term exposure to tobacco smoke

Studies, both prior to and after this study on tobacco smokers, have indicated an increase in matrix metalloproteinases (MMP), MMP-2 and MMP-9, mainly originating from macrophages and neutrophils respectively, in the lungs of COPD patients (86, 87, 91, 139). One recent study reported a striking correlation between MMP-9 expression and increasing COPD severity in the presence of increasing glucocorticosteroids(139). This latter study found the same up-regulation of NE. It is notable that NE can degrade TIMP-1, the inhibitor of MMP-9, thus further promoting the action of MMP-9 and changing the balance between proteases and anti-proteases in the airways. Additionally COPD patients have shown higher protein levels in the airways during exacerbations than during the stable phase of the disease (86).

The results in Paper II indicated however that acute exposure to tobacco smoke does not cause any pronounced or lasting impact on the local gelatinases or gelatinases inhibitors in the peripheral airways of humans. Neither did the study show any impact on the local number of neutrophils or macrophages, regardless of atopy status. However the percentage of eosinophils was higher in BAL2 compared with BAL1 possibly indicating that the procedure itself can exert an impact on the accumulation of eosinophils.

The main conclusion of Paper II is that acute exposure to a moderate dose of tobacco smoke is not sufficient to cause a lasting impact on gelatinases or their inhibitors in the human airways. These results question studies of acute tobacco exposure in a variety of animal models (140, 141); and our findings correspond well with findings in an earlier study on induced sputum with no substantial changes in MPP-9 levels or neutrophils 24 h after exposure to tobacco smoke(142) .

## 6.3 Paper III and IV

### Study subjects

Even though COPD is a common disease, the recruitment of 60 patients with COPD matching the inclusion criteria for the studies in Paper III and IV proved to be a time consuming process. The majority of patients was recruited from the outpatient clinic at the Department of Respiratory Medicine at Sahlgrenska University Hospital, Gothenburg and had a known COPD diagnosis. Other patients were diagnosed with COPD by their primary care center, and the COPD diagnosis (GOLD stage I-IV) was confirmed by spirometry at inclusion in the study. The fact that all spirometries prior to inclusion in the study were performed without bronchodilation, in accordance with the study design, has been a subject for discussion in our group. During the last decade post-bronchodilator airflow obstruction has been regarded as fundamental to the definition of COPD (1). The absence of this procedure in our study could be seen as a weakness in definition of the OPD-CB study patients. Yet one must bear in mind that an improvement of FEV1 and/or FVC greater than 12%, or 200 ml that is considered consistent with an asthma diagnosis may also be seen in COPD patients(143) and that a subgroup of long standing asthma does not demonstrate reversible airflow obstruction(144).

The differentiation of asthma and COPD is particularly difficult in older people. Studies have shown, in patients over the age of 55 years, that an overlap of asthma and COPD occurs in up to 50% of individuals (145, 146). The traditional approach to distinguish between adult-onset asthma and COPD according to rapid reversibility of airflow obstruction can thus be questioned because of the “overlap” between these two clinical entities, an interesting topic that is currently debated in the literature (147, 148).

Of note the inclusion criteria for our smokers with OPD-CB also included smoking history (> 10 pack years), a detailed medical history involving the age of symptom onset, the time over which the symptoms increased, variability of symptoms, and the exclusion of patients with atopic status. Hence we consider our study group to form as pure a group of COPD patients as possible. All patients were in a stable clinical condition at inclusion.

Pulsoxymetry measurements, but no regular control of blood gas were performed at inclusion in the study. Blood gas analysis was performed only at exacerbations in certain patients with oxygen concentrations <94%.However, with hindsight, this could have been an interesting criterion to follow both at inclusion and at the regular visits as our data in Paper IV have shown some interesting correlations between hypoxia and inflammatory markers.

Together with the smokers with OPD-CB, ten asymptomatic smokers and 10 never smokers were subsequently included as controls. No bronchodilation was performed but all subjects were asked about allergic symptoms. A history of atopy was further evaluated by testing for systemic IgE antibodies using Phadiatop$^{TM}$ testing (Phadia AB, Uppsala Sweden). A positive test result excluded the subject.

## Exacerbation frequency

After inclusion all smokers with OPD-CB in Paper III and IV underwent blood tests every 15th week for 15 months. The patients were instructed to contact the study-nurse if they had experienced symptoms indicating an exacerbation. The definition of COPD exacerbation was based on criteria described by Wedzicha and Donaldson(122, 149), modified from those described by Anthonisen et al(123) requiring two new symptoms to be present for two days, one of which must be one of the major symptoms: increased dyspnea, sputum volume, or sputum purulence. Minor exacerbation symptoms include cough, wheeze, sore throat, nasal discharge, and fever.

Thirty-eight (63%) smokers with OPD-CB had at least one exacerbation during the 15 month study period. Although all patients were vigorously encouraged to contact the study-nurse at exacerbations, some patients waited until their next regular visit. This tendency to underreport exacerbations has been shown in other exacerbation studies (122, 150) and could be explained by the fact that “COPD patients” in general are accustomed to frequent symptom changes and do not regard these as an impairment in the disease. Furthermore, some patients sought medical treatment during weekends when no study nurse was available, and this too, is likely to have influenced our record of patient’s exacerbations in the study.

## Systemic signs of innate activity during stable clinical conditions and exacerbations

The chronic increase of innate activity at the systemic level in smokers and COPD patients, as revealed in peripheral blood, includes increased proportion, number and changed functionality of neutrophils(42, 132, 137, 151). In accordance with this, leukocyte and neutrophil concentrations were substantially higher in the asymptomatic smokers and the OPD-CB group in our study compared to never-smokers. However, concentrations of these cells were lower in smokers with OPD-CB with severe disease (GOLD III-IV) compared with mild disease (GOLD I-II) which could suggest impaired innate activity at the systemic level in severe COPD. Few studies have addressed the ability of neutrophils in COPD patients to ingest and kill bacteria. Yet one recent publication has suggested that neutrophils from patients with COPD may have functional defects (85). This study showed that peripheral blood neutrophils from COPD patients' exhibit increased locomotor speed but reduced chemotactic accuracy. This was however irrespective of disease severity (85).

Both MPO and NE originate from azurophilic neutrophil granules (69, 79). In accordance with this we found strong correlations between ME and NE protein both during stable conditions and exacerbations and moderate correlations between ME and NE on the one hand and neutrophils on the other.

In our study material, smokers with OPD-CB and asymptomatic smokers showed a moderate trend towards higher MPO concentrations in blood compared with the never-smoking group during stable clinical conditions. This is compatible with results from Paper I, showing increasing concentrations of blood MPO over time in asymptomatic smokers (152) . However, other studies have found elevated concentrations of MPO in blood of COPD patients and also significant correlations with lung function (136, 153). The reason why the smokers with OPD-CB in our study did not show higher concentrations in MPO during stable clinical conditions at the time of inclusion, nor any correlation with lung function values, may relate to lack of statistical power or the selection of study subjects.

However, the significant increase of NE in smokers with OPD-CB and asymptomatic smokers in Paper III and IV further supports the idea of a higher degree of neutrophilic inflammation in COPD, irrespectively of the location of these neutrophils and is also confirmed by a recent study on patients with COPD and lung cancer(154).

Several previously published studies have shown that systemic signs of inflammation are up-regulated during COPD exacerbations. C-reactive protein, produced mainly by hepatocytes under the control of IL-6, is involved in COPD pathogenesis through the complement system(155). One earlier study has shown 36 biomarkers confirming the presence of a COPD exacerbation; among them CRP seems to be the most sensitive (37) . CRP also remained at a high level at exacerbation remission(33). As expected, and in support of this, the smokers with OPD-CB in Paper III and IV, showed a clear increase in CRP at exacerbations. Notably, CRP also presented the most pronounced relative change among the inflammatory markers studied in Paper III and IV. Further, the smokers with OPD-CB exhibited a clear increase in the concentrations of neutrophils and MPO protein as well as a trend towards increase in NE during exacerbations. However there was no augmentation in mRNA for MPO or NE during exacerbations; hence we found no evidence for *de novo* synthesis. In accordance with the “spillover” theory (156) this increase in MPO and NE protein could derive from inflammation in the lungs. The concept of “spillover” has however been questioned as there is hitherto no direct evidence of a correlation between lung and plasma cytokines(156, 157). Regarding muscle mass loss in COPD patients a recent study suggests that muscle atrophy is not related to airway inflammation but to deconditioning, exacerbations and hypoxia (48).

## Systemic signs of inflammation and hypoxia during exacerbations

In Paper IV we detected a negative correlation between the concentrations of neutrophils, MPO and NE respectively, versus $PaO_2$ in blood during exacerbations in 18 smokers with OPD–CB and hypoxemia. Earlier studies have shown that hypoxia can induce inflammation, assessed by increased levels of proinflammatory cytokines in persons with mountain sickness, and augmented serum levels of IL-6 and CRP in healthy volunteers spending several nights at an elevation over 3000 m(158, 159). It has also been demonstrated that hypoxia dramatically increases the release of neutrophil elastase in peripheral blood neutrophils in healthy human volunteers. Theoretically this could be a potential for increased tissue injury (160). Another study investigated the effect of an experimental hypoxic challenge in patients with COPD (no patient had a decrease in oxygen saturation <88%) and found an increase in coagulation markers and IL-6(161). This study suggests that patients with COPD may be at increased risk of venous thromboembolism following air travel and acute exacerbations. To the best of our knowledge ours is the first study to demonstrate a link between hypoxemia in blood and inflammatory markers in exacerbating COPD patients.

## Systemic signs of adaptive activity during stable clinical conditions and exacerbations

Inflammation in COPD also shows an increase in the total number of T-cells in the lung parenchyma and both central and peripheral airways (162) . The pro-inflammatory cytokine IL-17A, secreted from Th-17 cells and cytotoxic T-cells ( also from neutrophils and macrophages), is supposed to be involved in COPD development(107). Neutrophil inflammation is central in COPD as described above. Since IL-17A increases neutrophil accumulation and the associated activity in animal airway models it is possible that IL-17A plays a role in this and other diseases characterized by excessive neutrophil mobilization in the airways(82). In line with this, previous studies have demonstrated increased IL-17A expression in bronchial submucosa in COPD patients compared with control subjects (107, 113, 114, 163). In one previous study, IL-17A protein concentrations were increased in sputum from COPD patients (116) but below the detection limit in another study, however with increased concentrations compared with asthma subjects (114).

Some groups have suggested that the alveolar tissue destruction observed in emphysema represents an autoimmune disorder involving T cells in the disease pathogenesis (102). Also, recent animal studies present strong evidence that chronic cigarette smoke exposure is sufficient to initiate an autoimmune response (164).

In Paper III, we found markedly lower concentrations of extracellular IL-17 in blood in stable-state smokers with OPD-CB compared with never smokers, and no change in IL-17 concentrations during exacerbations. This finding is supported by observations in a recent study of elderly COPD patients and control subjects (115). However, conflicting evidence in a new study indicates an increase in systemic cytokine signalling via extracellular IL-17 in COPD patients, correlating with disease severity (116). Another study found increased concentrations of Th-17 cells in peripheral blood in COPD patients compared with non-smokers. However IL-17 protein concentrations were not assessed here (94).

In Paper III we also characterised systemic cytokine signalling via GRO-α, the neutrophil effector molecule for IL-17A, where concentrations in smokers with OPD-CB in a stable state were markedly lower compared with never smokers but increased during exacerbations. Furthermore, we observed a positive correlation between IL-17A and GRO-α in the pooled group of all smokers (OPD-CB and asymptomatic smokers), compatible with the idea that IL-17A signals via GRO-α.

Thus our study provides evidence for down-regulated cytokine signalling via IL-17A and GRO-α in smokers with OPD-CB. Support for these results is provided by a recent study in which low proportions of IL-17A expressing T cells in blood were associated with a reduced gas diffusion capacity in patients with severe COPD(165).

Regarding the seemingly contradictive results of IL-17A and GRO-α concentration during exacerbations in our group of smokers with OPD-CB in Paper III, one could have anticipated that the concentrations of GRO-α should have followed those of IL-17A. However, we cannot rule out an increase of IL-17A from very low to higher but still undetectable concentrations, and also the fact that GRO-α can be mediated by mechanisms other than those mediated by IL-17(118, 119).

## Systemic signs of inflammation and local bacterial colonization during stable clinical conditions and exacerbations

Sputum samples collected from smokers with OPD-CB during stable clinical conditions at the time of inclusion and at exacerbations, displayed growth of the most common pathogenic bacteria associated with COPD i.e. *Haemophilus influenza, Streptococcus pneumonia, Moraxella catarralis and Haemophilus parainfluenzae* (35). However, at inclusion some sputum samples showed growth of opportunistic pathogens including *Pseudomonas aeriguinosa, Escherichia coli* and *Stenotropomonas maltophilia.* Infection with *Pseudomonas aeruginosa* is showed to be associated with increased mortality in COPD patients (166).

In Paper IV we found no association between growth of bacterial pathogens in sputum samples and increase of blood MPO, NE or CRP concentrations neither at inclusion, nor during exacerbations. In Paper III we found an interesting association between smokers with OPD-CB colonised by opportunistic pathogens and low concentrations of IL-17A and GRO-α in blood. As IL-17A is known to play an important role in the antibacterial host defence of human lungs (106, 167, 168) we interpret this finding as a functional sign of immune suppression in these smokers with OPD-CB although reservations can be made with reference to the small sample size.

.

# 7 SUMMARY AND CONCLUSION

## Paper I

The results from this study of elderly male smokers without severe airway symptoms, forward systemic signs of increasing inflammation during a 6-year period and indicates the use of MPO as a reproducible marker of this phenomenon. Indirect evidence for an increase in systemic MPO caused by tobacco smoking is the negative correlation between on one hand duration of smoking cessation, and the change in blood MPO on the other, in a smaller group of smokers that had quit smoking during the period.

## Paper II

In this study of occasional tobacco smokers, the results reveal no pronounced and lasting signs of alteration among a range of local signs of inflammation after limited, short-term exposure to tobacco smoke among occasional smokers. Specifically, there were no signs of clear or sustained alterations in proteolytic homeostasis.

## Paper III

This study of systemic signs of inflammation in smokers with OPD-CB, forward results indicating a clear down-regulation of cytokine signalling via interleukin (IL)-17 and its effector molecule growth-related oncogene (GRO)-α during stable clinical conditions. Even though indicative only due to a small sample size, the results suggest that this impairment in systemic cytokine signalling is linked to the local colonisation by opportunistic pathogens in the airways.

## Paper IV

In this study, the results demonstrate clear signs of systemic inflammation involving circulating blood neutrophils in smokers with OPD-CB during stable clinical conditions and, even more so, during exacerbations. The systemic signs of inflammation involve increased concentrations of neutrophil activity markers as well, including NE during stable clinical conditions and MPO during exacerbations. However, the results failed to prove a matching increase in *de novo* synthesis of MPO or NE in circulating blood leukocytes. An association of hypoxemia and neutrophil mobilisation during exacerbations was also detected.

# General conclusions

The results in this thesis confirm that neutrophils and neutrophil activity markers (NE and MPO) are involved and enhanced in both asymptomatic smokers and in smokers with OPD-CB during stable clinical conditions and/or exacerbations. During exacerbations, some of these systemic signs of inflammations are further augmented, however without any matching *de novo* synthesis in circulating blood leukocytes. Hypothetically, there are several explanations for this principal finding. First, the release of the neutrophil activity markers instead could be effectuated at the post-transcriptional level in circulating blood neutrophils, trough exocytosis or via neutrophil extracellular traps (NETs). Another possibility is that there is an exocytosis of the neutrophil activity markers from cells located in the extravascular compartments of the lungs; in line with the “spillover” theory.

The results of this thesis also include results include systemic signs of impaired cytokine signalling via IL-17A and GRO-α in smokers with OPD-CB during stable conditions but increased concentrations of GRO-α during exacerbations, despite the fact that GRO-α may serve as an effector molecule for IL-17A. That we failed to reproducibly detect IL-17A concentrations during exacerbations could be that even if the concentrations were increased during exacerbations, they still were too low to be detectable. Another reason could be that the release of GRO-α is mediated via mechanisms not only depending upon IL-17A. Our demonstration of impaired immune signalling via IL-17A in smokers with OPD-CB does not lend any support to the use of systematically administered anti-IL-17 antibodies in COPD.

The growth of common airway pathogens in sputum was associated with increased neutrophil concentrations during exacerbations and not with any substantial augmentation of MPO, NE, CRP, IL-17A or GRO-α. Moreover, there were significantly lower concentrations of IL-17A and GRO-α among smokers with OPD-CB colonized with opportunistic pathogens in the airways. Because cytokine signalling via IL-17A, and in part via GRO-α, is believed to be important for antibacterial host defence, it seems feasible that COPD patients colonized with opportunistic pathogens could suffer from an impairment in this type of signalling at the systemic level, as demonstrated here. “Supportive trends” in this direction were observed among our results as well, including the fact that smokers with OPD-CB with severe disease had lower concentrations of blood leukocytes, neutrophils and lymphocytes, as well as a trend towards lower concentrations of IL-17A. However, given that neither of these “supportive trends” proved statistical significance; new studies on these phenomena are warranted.

The increased concentrations of CRP in smokers with OPD-CB (compared to controls) during stable clinical conditions and exacerbations do confirm the results of earlier studies on COPD.  For mechanistic reasons, this systemic sign of inflammation may actually be a better validated sign of systemic involvement in  the neutrophil activity markers studied in the current thesis in the sense that the cellular courses are likely to be mainly extra pulmonary

Finally; acute exposure to a moderate dose of tobacco smoke did not cause any pronounced impact on local signs of inflammation, including a variety of aspects of the proteolytic homeostasis in naïve peripheral airways. This finding are compatible with the fact that it requires repeated exposure over time of tobacco smoking to establish COPD but it does not in any way warrant that occasional exposure to tobacco smoke is harmless. Fortunately, the results in this thesis also indicate that smoking cessation may result in the reversal of increasing systemic signs of inflammation.

# 8 FUTURE PERSPECTIVES

This thesis is based on three different study cohorts illustrating systemic and local signs for inflammation in smokers with and without obstructive pulmonary disease; with the focus set on various aspects of neutrophilic inflammation. The studies have forwarded interesting results but new questions have emerged during the course of these studies, as presented below.

Our demonstrations in (Paper III) forward the question how local and systemic cytokine signalling via IL-17A and other cytokines relates to one another? This is an interesting mechanistic question that deserves further study in studies taking samples from both the systemic and the local level, ideally at the same time.

The evidence of a link between hypoxemia and neutrophil mobilization during exacerbations among smokers with OPD-CB (demonstrated in Paper IV) motivates further mechanistic evaluation in patients with chronic hypoxia. It may prove important to evaluate whether the life extending effect of long-term oxygen therapy in COPD relates to an effect on neutrophilic inflammation in these patients?

The “indicative findings” of an association between impaired systemic cytokine signalling via IL-17A and colonisation by opportunistic pathogens in the airways in smokers with OPD-CB are in need of validation in larger study materials, even though our findings actually proved significance with the applied statistical methods. Bacterial colonisation in the airways is a “true” sign of immunosuppression from a functional point of view and our observation raises the question whether impaired cytokine signalling via IL-17A, or other critical cytokines, does facilitate bacterial colonisation in the airways? If this is the case, then there may be a potential for new therapy in this large group of patients.

# ACKNOWLEDGEMENT

## Till alla som på något sätt har bidragit till detta arbete vill jag framföra ett varmt och djupt TACK!

### Ett speciellt tack till:

**Anders Lindén** min huvudhandledare för mer än tio års arbete tillsammans i olika forskningsprojekt. Trots att du sedan några år bor och arbetar i Stockholm har du alltid satt av tid till samtal och diskussioner om forskningsfrågor. Du har tålmodigt kämpat in i det sista med att försöka strukturera upp mitt skrivande, lära mig vara konsekvent och använda nyckelbegrepp. Alltid lika vänlig! Tack Anders!

**Ann Ekberg Jansson** min bihandledare och chef! Två roller som du på ett utmärkt sätt har förenat. Trots en stor arbetsbelastning har du funnits till hands och har varit generös med råd, omtänksamhet och praktisk hjälp i besvärliga forsknings-stunder. Stort tack!

**Angereds Närsjukhus (ANS)** som generöst har bidragit till mina möjligheter att genomföra denna avhandling.

Alla i Lungteamet på ANS; Kollegerna **Lars Olof Larsson, Niklas Stenersen (**Osthyveln Niklas!), **Claes-Göran Löfdahl**, **Sofia Zachrisson** och alla underbara kompetenta lungsjuksköterskor; **Mary Farrell, Lotta Sjöstrand, Mirela Osmanovic, Anna Gunnarsson** (numera i London)**, Helena Viena, Lotta Hellberg** och **Helén Engberg** -som håller reda på oss! Ett speciellt tack till BMA **Sanna Nilisse** för gott samarbete i vårt gemensamma intresse för sömnapnépatienter. Det är alltid roligt att komma till lungmottagningen för att arbeta tillsammans med er alla! Tack också till stöttande kolleger i övriga team på ANS, ett speciellt tack till **Natalia Mossberg** som skapade kontakt med skickliga språkgranskaren **Shirley Fridlund**.

**Mona Palmqvist** verksamhetschef på lungmedicin SU/Sahlgrenska och sektionschef **Cattis Dellborg** för att jag fortsatt har fått låna lediga skrivbord på Elevhemmet och ha forskningspärmar i kassaskåpet. Tack **Lena Olsson** för ditt inspirerande civilkurage; och att jag har fått sitta vid ditt skrivbord när du inte varit där. Tack **Anita Nordenson**, KOL-kollega, danskompis och

medlem (nu 4 medlemmar!) i den famösa latinska filmklubben. Och tack också till övriga kolleger på Lungkliniken på Sahlgrenska för trevliga samtal om både forskning och livet under dygnets alla timmar!

**Anders Andersson** som på olika sätt har stöttat mig genom hela forskningsresan. Ibland med glada tillrop, ibland med skrämseltaktik. Många kvällar har vi tillbringat på Elevhemmet; fikat, pratat och skrivit på våra alster (inte alltid i den ordningen). Du är dessutom medarbetare i en av mina artiklar och är världsbäst på att göra figurer! Tack för din vänskap!

**Sven Larsson** för introduktion till KOL-forskningens värld via Krokodilstudien! Framför allt ett stort tack för att för att du uppmuntrade mig till att ta fram violan igen efter många års uppehåll. Det du gjorde då har bidragit till underbara musikupplevelser och möten med fantastiska människor och musiker; dels i Partille Kammarorkester men även i andra orkestrar. Din insats har varit ovärderlig och själsligt berikande! Och tack för te, hembakat bröd och mesost varje måndagskväll före repetitionerna tillsammans med **Eva** och dig!

**Ingemar Qvarfordt** för stabila, sakliga och kunniga råd om hur bakterier skall hanteras och beskrivas.

**Barbro Balder,** eminent forskningssköterska i KOL-EXA studien och i bronkoskopistudien! Du var en klippa under alla dessa år! Tack vare din vänlighet, klokhet, kompetens och organistionsförmåga kom patienterna tillbaka på alla återbesök som var så viktiga speciellt i KOL-EXA studien. Tack också för att du tog hand om, och matade in alla kliniska data som ligger till grund för beräkningarna.

**Mariann Robertson** välorganiserad forskningssköterska i KOL-33 studien! Tack för många trevliga stunder även i samband med arbete i andra studier!

**Pernilla Glader, Pernilla Jirholt, Sara Tengvall, Karin Christiansson och Bettina Levänen** som alla på ett skickligt sätt har utför de olika laboratorieanalyserna. Utan er hade det inte blivit något att skriva om!

**Per Hellström;** neuropsykolog och kompis från forskarutbildningskursen som vänligt hjälpte flera av oss genom att göra statistiken (delvis) begriplig. Tack för trevliga stunder över en kopp te med samtal om allt mellan himmel och jord samt ytterst värdefulla råd om hur man hanterar en dator, om forskning i allmänhet, och om hur man uttrycker sig på engelska i forskningssammanhang.

**Johan Bylund** Tack!! för att ha bidragit med en vacker bild på neutrofila celler som har använts på omslaget och konstnärligt bearbetats av:

**Beata Angelbjörk** fin kompis och violinist! Tack för design av omslaget till avhandlingen! Är väldigt glad för den bilden. Och tack för många trevliga och roliga(!) stunder tillsammans både med och utan musik!

**James Gale** för excellent ”fussy” granskning av Introduktionen.

Tack till alla stöttande kompisar under årens lopp speciellt: **Jadwiga Kurowska, Hildur Kalman, Åsa Kahl, och Barbro och Göran Mellander.**

Alla medlemmar i Partille Kammarorkester med **Malin Bergman** som konsertmästare och speciellt tack till violastämman med **Gunnar Rynefors, Gunilla och Bror Jansson.** Violapower!

Stort, stort tack till mina älskade föräldrar **Inga** och **Karl Erik Bergsten** som alltid har stöttat mig, och under gymnasieåren såg till att jag gick kvar i skolan. Tack Mamma för att du alltid har kloka synpunkter vid våra samtal om livet. Tack också till mina fina syskon med respektive: **Cecilia** och **Erland, Hans, Per** och **Carin**, och alla underbara syskonbarn. Tack till **Peter Andelid**!

Till min älskade son **Calle** and his lovely wife **Shuen** in Hong Kong:

I miss you every day

Med kärlek till **State Lycke** min livskamrat

Tack för att du finns!

# REFERENCES

1. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. American journal of respiratory and critical care medicine. 2013;187(4):347-65. Epub 2012/08/11.
2. Petty TL. The history of COPD. International journal of chronic obstructive pulmonary disease. 2006;1(1):3-14. Epub 2007/12/01.
3. Scherer JR. Before cardiac MRI: Rene Laennec (1781-1826) and the invention of the stethoscope. Cardiology journal. 2007;14(5):518-9. Epub 2008/07/25.
4. Spriggs EA. John Hutchinson, the inventor of the spirometer--hisnorthcountrybackground, life in London, and scientific achievements. Medical history. 1977;21(4):357-64. Epub 1977/10/01.
5. Malo JL. A tribute to Tiffeneau's original contribution in the current issue of the journal. Allergy. 2006;61(10):1200-1. Epub 2006/09/01.
6. Fletcher C, Peto R. The natural history of chronic airflow obstruction. British medical journal. 1977;1(6077):1645-8. Epub 1977/06/25.
7. Peto R, Speizer FE, Cochrane AL, Moore F, Fletcher CM, Tinker CM, et al. The relevance in adults of air-flow obstruction, but not of mucus hypersecretion, to mortality from chronic lung disease. Results from 20 years of prospective observation. The American review of respiratory disease. 1983;128(3):491-500. Epub 1983/09/01.
8. Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, et al. Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. JAMA : the journal of the American Medical Association. 1994;272(19):1497-505. Epub 1994/11/16.
9. Prowse SJ, Makura Z. Gustav Killian: beyond his dehiscence. The Journal of laryngology and otology. 2012;126(11):1164-8. Epub 2012/09/06.
10. Ikeda S, Tsuboi E, Ono R, Ishikawa S. Flexible bronchofiberscope. Japanese journal of clinical oncology. 2010;40(9):e55-64. Epub 2010/08/26.
11. Nash ES, Briscoe WA, Cournand A. THE RELATIONSHIP BETWEEN CLINICAL AND PHYSIOLOGICAL FINDINGS IN CHRONIC OBSTRUCTIVE DISEASE OF THE LUNGS. Medicina thoracalis. 1965;22:305-27. Epub 1965/01/01.
12. Briscoe WA, Nash ES. THE SLOW SPACE IN CHRONIC OBSTRUCTIVE PULMONARY DISEASES. Annals of the New York Academy of Sciences. 1965;121:706-22. Epub 1965/03/24.

13. McGovern CM. Doctors or ladies: women physicians in psychiatric institutions, 1872-1900. Bulletin of the history of medicine. 1981;55(1):88-107. Epub 1981/01/01.

14. Pauly JL, Paszkiewicz G. Cigarette smoke, bacteria, mold, microbial toxins, and chronic lung inflammation. Journal of oncology. 2011;2011:819129. Epub 2011/07/21.

15. Larsson L, Pehrson C, Dechen T, Crane-Godreau M. Microbiological components in mainstream and sidestream cigarette smoke. Tobacco induced diseases. 2012;10(1):13. Epub 2012/08/18.

16. Larsson L, Szponar B, Ridha B, Pehrson C, Dutkiewicz J, Krysinska-Traczyk E, et al. Identification of bacterial and fungal components in tobacco and tobacco smoke. Tobacco induced diseases. 2008;4:4. Epub 2008/09/30.

17. Sapkota AR, Berger S, Vogel TM. Human pathogens abundant in the bacterial metagenome of cigarettes. Environmental health perspectives. 2010;118(3):351-6. Epub 2010/01/13.

18. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. Chest. 1999;115(3):829-35. Epub 1999/03/20.

19. Sebastian A, Pehrson C, Larsson L. Elevated concentrations of endotoxin in indoor air due to cigarette smoking. Journal of environmental monitoring : JEM. 2006;8(5):519-22. Epub 2006/05/12.

20. Bagaitkar J, Demuth DR, Scott DA. Tobacco use increases susceptibility to bacterial infection. Tobacco induced diseases. 2008;4:12. Epub 2008/12/20.

21. Varkey AB. Chronic obstructive pulmonary disease in women: exploring gender differences. Current opinion in pulmonary medicine. 2004;10(2):98-103. Epub 2004/03/17.

22. Camp PG, Ramirez-Venegas A, Sansores RH, Alva LF, McDougall JE, Sin DD, et al. COPD phenotypes in biomass smoke- versus tobacco smoke-exposed Mexican women. The European respiratory journal. 2014;43(3):725-34. Epub 2013/10/12.

23. Diette GB, Accinelli RA, Balmes JR, Buist AS, Checkley W, Garbe P, et al. OBSTRUCTIVE LUNG DISEASE AND EXPOSURE TO BURNING BIOMASS FUEL IN THE INDOOR ENVIRONMENT. Global heart. 2012;7(3):265-70. Epub 2012/11/10.

24. Stoller JK, Aboussouan LS. Alpha1-antitrypsin deficiency. Lancet. 2005;365(9478):2225-36. Epub 2005/06/28.

25. Bosse Y. Updates on the COPD gene list. International journal of chronic obstructive pulmonary disease. 2012;7:607-31. Epub 2012/10/12.

26. Adcock IM, Ford P, Ito K, Barnes PJ. Epigenetics and airways disease. Respiratory research. 2006;7:21. Epub 2006/02/08.

27. Schamberger AC, Mise N, Meiners S, Eickelberg O. Epigenetic mechanisms in COPD: implications for pathogenesis and drug

discovery. Expert opinion on drug discovery. 2014;9(6):609-28. Epub 2014/05/23.

28. Patton GC, Carlin JB, Coffey C, Wolfe R, Hibbert M, Bowes G. Depression, anxiety, and smoking initiation: a prospective study over 3 years. American journal of public health. 1998;88(10):1518-22. Epub 1998/10/17.

29. Atlantis E, Fahey P, Cochrane B, Smith S. Bidirectional associations between clinically relevant depression or anxiety and COPD: a systematic review and meta-analysis. Chest. 2013;144(3):766-77. Epub 2013/02/23.

30. Hardin M, Cho M, McDonald ML, Beaty T, Ramsdell J, Bhatt S, et al. The clinical and genetic features of COPD-asthma overlap syndrome. The European respiratory journal. 2014;44(2):341-50. Epub 2014/05/31.

31. Barker BL, Brightling CE. Phenotyping the heterogeneity of chronic obstructive pulmonary disease. Clinical science (London, England : 1979). 2013;124(6):371-87. Epub 2012/11/30.

32. Wedzicha JA, Brill SE, Allinson JP, Donaldson GC. Mechanisms and impact of the frequent exacerbator phenotype in chronic obstructive pulmonary disease. BMC medicine. 2013;11:181. Epub 2013/08/16.

33. Perera PN, Armstrong EP, Sherrill DL, Skrepnek GH. Acute exacerbations of COPD in the United States: inpatient burden and predictors of costs and mortality. Copd. 2012;9(2):131-41. Epub 2012/03/14.

34. Seemungal TA, Wedzicha JA. Exacerbation frequency and FEV1 decline of COPD: is it geographic? The European respiratory journal. 2014;43(5):1220-2. Epub 2014/05/03.

35. Hassett DJ, Borchers MT, Panos RJ. Chronic obstructive pulmonary disease (COPD): evaluation from clinical, immunological and bacterial pathogenesis perspectives. Journal of microbiology (Seoul, Korea). 2014;52(3):211-26. Epub 2014/03/04.

36. Miravitlles M, Soler-Cataluna JJ, Calle M, Soriano JB. Treatment of COPD by clinical phenotypes: putting old evidence into clinical practice. The European respiratory journal. 2013;41(6):1252-6. Epub 2012/10/13.

37. Hurst JR, Vestbo J, Anzueto A, Locantore N, Mullerova H, Tal-Singer R, et al. Susceptibility to exacerbation in chronic obstructive pulmonary disease. The New England journal of medicine. 2010;363(12):1128-38. Epub 2010/09/17.

38. Matkovic Z, Miravitlles M. Chronic bronchial infection in COPD. Is there an infective phenotype? Respiratory medicine. 2013;107(1):10-22. Epub 2012/12/12.

39. Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. Thorax. 2002;57(10):847-52. Epub 2002/09/27.

40. Makris D, Moschandreas J, Damianaki A, Ntaoukakis E, Siafakas NM, Milic Emili J, et al. Exacerbations and lung function decline in COPD: new insights in current and ex-smokers. Respiratory medicine. 2007;101(6):1305-12. Epub 2006/11/23.
41. Laurenzi GA, Potter RT, Kass EH. Bacteriologic flora of the lower respiratory tract. The New England journal of medicine. 1961;265:1273-8. Epub 1961/12/28.
42. Wouters EF. Local and systemic inflammation in chronic obstructive pulmonary disease. Proceedings of the American Thoracic Society. 2005;2(1):26-33. Epub 2005/08/23.
43. Mikko M, Forsslund H, Cui L, Grunewald J, Wheelock AM, Wahlstrom J, et al. Increased intraepithelial (CD103+) CD8+ T cells in the airways of smokers with and without chronic obstructive pulmonary disease. Immunobiology. 2013;218(2):225-31. Epub 2012/06/02.
44. Barnes PJ. Cellular and molecular mechanisms of chronic obstructive pulmonary disease. Clinics in chest medicine. 2014;35(1):71-86. Epub 2014/02/11.
45. Barbu C, Iordache M, Man MG. Inflammation in COPD: pathogenesis, local and systemic effects. Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie. 2011;52(1):21-7. Epub 2011/03/23.
46. Black-Shinn JL, Kinney GL, Wise AL, Regan EA, Make B, Krantz MJ, et al. Cardiovascular Disease is Associated with COPD Severity and Reduced Functional Status and Quality of Life. Copd. 2014. Epub 2014/05/17.
47. Cielen N, Maes K, Gayan-Ramirez G. Musculoskeletal disorders in chronic obstructive pulmonary disease. BioMed research international. 2014;2014:965764. Epub 2014/05/02.
48. Barker BL, McKenna S, Mistry V, Pancholi M, Patel H, Haldar K, et al. Systemic and pulmonary inflammation is independent of skeletal muscle changes in patients with chronic obstructive pulmonary disease. International journal of chronic obstructive pulmonary disease. 2014;9:975-81. Epub 2014/09/24.
49. Rabe KF. Treating COPD--the TORCH trial, P values, and the Dodo. The New England journal of medicine. 2007;356(8):851-4. Epub 2007/02/23.
50. Maters GA, de Voogd JN, Sanderman R, Wempe JB. Predictors of All-Cause Mortality in Patients with Stable COPD: Medical Co-morbid Conditions or High Depressive Symptoms. Copd. 2014. Epub 2014/05/17.
51. Sin DD, Man SF. Chronic obstructive pulmonary disease as a risk factor for cardiovascular morbidity and mortality. Proceedings of the American Thoracic Society. 2005;2(1):8-11. Epub 2005/08/23.
52. Mannino DM, Doherty DE, Sonia Buist A. Global Initiative on Obstructive Lung Disease (GOLD) classification of lung disease and

mortality: findings from the Atherosclerosis Risk in Communities (ARIC) study. Respiratory medicine. 2006;100(1):115-22. Epub 2005/05/17.

53. Hurst JR, Donaldson GC, Perera WR, Wilkinson TM, Bilello JA, Hagan GW, et al. Use of plasma biomarkers at exacerbation of chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2006;174(8):867-74. Epub 2006/06/27.

54. Parker D, Prince A. Innate immunity in the respiratory epithelium. American journal of respiratory cell and molecular biology. 2011;45(2):189-201. Epub 2011/02/19.

55. Hillaire ML, Haagsman HP, Osterhaus AD, Rimmelzwaan GF, van Eijk M. Pulmonary surfactant protein D in first-line innate defence against influenza A virus infections. Journal of innate immunity. 2013;5(3):197-208. Epub 2013/02/09.

56. Mak JC. Pathogenesis of COPD. Part II. Oxidative-antioxidative imbalance. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2008;12(4):368-74. Epub 2008/03/29.

57. Bardoel BW, Kenny EF, Sollberger G, Zychlinsky A. The Balancing Act of Neutrophils. Cell host & microbe. 2014;15(5):526-36. Epub 2014/05/17.

58. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. American journal of respiratory and critical care medicine. 1996;153(2):530-4. Epub 1996/02/01.

59. Obermayer A, Stoiber W, Krautgartner WD, Klappacher M, Kofler B, Steinbacher P, et al. New aspects on the structure of neutrophil extracellular traps from chronic obstructive pulmonary disease and in vitro generation. PloS one. 2014;9(5):e97784. Epub 2014/05/17.

60. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. Microbes and infection / Institut Pasteur. 2003;5(14):1299-306. Epub 2003/11/14.

61. Lu T, Porter AR, Kennedy AD, Kobayashi SD, Deleo FR. Phagocytosis and Killing of Staphylococcus aureus by Human Neutrophils. Journal of innate immunity. 2014. Epub 2014/04/10.

62. Kobayashi SD, Voyich JM, Burlak C, DeLeo FR. Neutrophils in the innate immune response. Archivum immunologiae et therapiae experimentalis. 2005;53(6):505-17. Epub 2006/01/13.

63. Wang J, Huang W, Wang C, Liu R. Dynamic process of phagocytosis and forms of macrophage cell death induced by ingestion of apoptotic neutrophils. Science China Life sciences. 2014. Epub 2014/09/14.

64. Lacy P. Mechanisms of degranulation in neutrophils. Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology. 2006;2(3):98-108. Epub 2006/09/15.

65. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science (New York, NY). 2004;303(5663):1532-5. Epub 2004/03/06.
66. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nature medicine. 2007;13(4):463-9. Epub 2007/03/27.
67. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. The Journal of cell biology. 2007;176(2):231-41. Epub 2007/01/11.
68. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. The Journal of cell biology. 2010;191(3):677-91. Epub 2010/10/27.
69. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. Journal of leukocyte biology. 2013;93(2):185-98. Epub 2012/10/16.
70. Nussbaum C, Klinke A, Adam M, Baldus S, Sperandio M. Myeloperoxidase: a leukocyte-derived protagonist of inflammation and cardiovascular disease. Antioxidants & redox signaling. 2013;18(6):692-713. Epub 2012/07/25.
71. Klebanoff SJ. Myeloperoxidase: friend and foe. Journal of leukocyte biology. 2005;77(5):598-625. Epub 2005/02/04.
72. van der Veen BS, de Winther MP, Heeringa P. Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. Antioxidants & redox signaling. 2009;11(11):2899-937. Epub 2009/07/23.
73. Wang W, Jian Z, Guo J, Ning X. Increased Levels of Serum Myeloperoxidase in Patients with Active Rheumatoid Arthritis. Life sciences. 2014. Epub 2014/09/28.
74. Kataoka Y, Shao M, Wolski K, Uno K, Puri R, Murat Tuzcu E, et al. Myeloperoxidase levels predict accelerated progression of coronary atherosclerosis in diabetic patients: insights from intravascular ultrasound. Atherosclerosis. 2014;232(2):377-83. Epub 2014/01/29.
75. Nicholls SJ, Hazen SL. Myeloperoxidase and cardiovascular disease. Arteriosclerosis, thrombosis, and vascular biology. 2005;25(6):1102-11. Epub 2005/03/26.
76. Klinke A, Nussbaum C, Kubala L, Friedrichs K, Rudolph TK, Rudolph V, et al. Myeloperoxidase attracts neutrophils by physical forces. Blood. 2011;117(4):1350-8. Epub 2010/10/29.
77. Hickey MJ. MPO and neutrophils: a magnetic attraction. Blood. 2011;117(4):1103-4. Epub 2011/01/29.

78. Nicholls SJ, Zheng L, Hazen SL. Formation of dysfunctional high-density lipoprotein by myeloperoxidase. Trends in cardiovascular medicine. 2005;15(6):212-9. Epub 2005/09/27.

79. Roghanian A, Sallenave JM. Neutrophil elastase (NE) and NE inhibitors: canonical and noncanonical functions in lung chronic inflammatory diseases (cystic fibrosis and chronic obstructive pulmonary disease). Journal of aerosol medicine and pulmonary drug delivery. 2008;21(1):125-44. Epub 2008/06/04.

80. Stockley RA. Neutrophils and the pathogenesis of COPD. Chest. 2002;121(5 Suppl):151S-5S. Epub 2002/05/16.

81. Sandhaus RA, Turino G. Neutrophil elastase-mediated lung disease. Copd. 2013;10 Suppl 1:60-3. Epub 2013/04/03.

82. Linden A, Laan M, Anderson GP. Neutrophils, interleukin-17A and lung disease. The European respiratory journal. 2005;25(1):159-72. Epub 2005/01/11.

83. Henriksen PA. The potential of neutrophil elastase inhibitors as anti-inflammatory therapies. Current opinion in hematology. 2014;21(1):23-8. Epub 2013/11/19.

84. Turino GM. The origins of a concept: the protease-antiprotease imbalance hypothesis. Chest. 2002;122(3):1058-60. Epub 2002/09/13.

85. Hoenderdos K, Condliffe A. The neutrophil in chronic obstructive pulmonary disease. American journal of respiratory cell and molecular biology. 2013;48(5):531-9. Epub 2013/01/19.

86. Mercer PF, Shute JK, Bhowmik A, Donaldson GC, Wedzicha JA, Warner JA. MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. Respiratory research. 2005;6:151. Epub 2005/12/24.

87. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. Experimental lung research. 2005;31(6):599-621. Epub 2005/07/16.

88. Gueders MM, Foidart JM, Noel A, Cataldo DD. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases. European journal of pharmacology. 2006;533(1-3):133-44. Epub 2006/02/21.

89. Glader P, Eldh B, Bozinovski S, Andelid K, Sjostrand M, Malmhall C, et al. Impact of acute exposure to tobacco smoke on gelatinases in the bronchoalveolar space. The European respiratory journal. 2008;32(3):644-50. Epub 2008/04/18.

90. Devarajan P, Johnston JJ, Ginsberg SS, Van Wart HE, Berliner N. Structure and expression of neutrophil gelatinase cDNA. Identity with type IV collagenase from HT1080 cells. The Journal of biological chemistry. 1992;267(35):25228-32. Epub 1992/12/15.

91. Finlay GA, Russell KJ, McMahon KJ, D'Arcy E M, Masterson JB, FitzGerald MX, et al. Elevated levels of matrix metalloproteinases in

bronchoalveolar lavage fluid of emphysematous patients. Thorax. 1997;52(6):502-6. Epub 1997/06/01.

92. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, et al. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. American journal of respiratory and critical care medicine. 1998;158(6):1945-50. Epub 1998/12/16.

93. Nonaka M, Satake H. Urochordate immunity. Advances in experimental medicine and biology. 2010;708:302-10. Epub 2010/01/01.

94. Vargas-Rojas MI, Ramirez-Venegas A, Limon-Camacho L, Ochoa L, Hernandez-Zenteno R, Sansores RH. Increase of Th17 cells in peripheral blood of patients with chronic obstructive pulmonary disease. Respiratory medicine. 2011;105(11):1648-54. Epub 2011/07/19.

95. Ivanov S, Linden A. Interleukin-17 as a drug target in human disease. Trends in pharmacological sciences. 2009;30(2):95-103. Epub 2009/01/24.

96. Lane N, Robins RA, Corne J, Fairclough L. Regulation in chronic obstructive pulmonary disease: the role of regulatory T-cells and Th17 cells. Clinical science (London, England : 1979). 2010;119(2):75-86. Epub 2010/04/21.

97. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008;112(5):1557-69. Epub 2008/08/30.

98. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology. 1989;7:145-73. Epub 1989/01/01.

99. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. Science (New York, NY). 1988;240(4851):516-8. Epub 1988/04/22.

100. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. Journal of immunology (Baltimore, Md : 1950). 1990;145(11):3796-806. Epub 1990/12/01.

101. Dancer R, Sansom DM. Regulatory T cells and COPD. Thorax. 2013;68(12):1176-8. Epub 2013/10/24.

102. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, et al. Antielastin autoimmunity in tobacco smoking-induced emphysema. Nature medicine. 2007;13(5):567-9. Epub 2007/04/24.

103. Profita M, Albano GD, Riccobono L, Di Sano C, Montalbano AM, Gagliardo R, et al. Increased levels of Th17 cells are associated with non-neuronal acetylcholine in COPD patients. Immunobiology. 2014;219(5):392-401. Epub 2014/02/18.

104. Cannon JG. Inflammatory Cytokines in Nonpathological States. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society. 2000;15:298-303. Epub 2001/06/08.

105. Barnes PJ. The cytokine network in chronic obstructive pulmonary disease. American journal of respiratory cell and molecular biology. 2009;41(6):631-8. Epub 2009/09/01.
106. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004;21(4):467-76. Epub 2004/10/16.
107. Eustace A, Smyth LJ, Mitchell L, Williamson K, Plumb J, Singh D. Identification of cells expressing IL-17A and IL-17F in the lungs of patients with COPD. Chest. 2011;139(5):1089-100. Epub 2010/09/25.
108. Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, Leipe J, et al. Mast cells express IL-17A in rheumatoid arthritis synovium. Journal of immunology (Baltimore, Md : 1950). 2010;184(7):3336-40. Epub 2010/03/05.
109. Pappu R, Ramirez-Carrozzi V, Ota N, Ouyang W, Hu Y. The IL-17 family cytokines in immunity and disease. Journal of clinical immunology. 2010;30(2):185-95. Epub 2010/02/24.
110. Zuniga LA, Jain R, Haines C, Cua DJ. Th17 cell development: from the cradle to the grave. Immunological reviews. 2013;252(1):78-88. Epub 2013/02/15.
111. Hoshino H, Laan M, Sjostrand M, Lotvall J, Skoogh BE, Linden A. Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. The Journal of allergy and clinical immunology. 2000;105(1 Pt 1):143-9. Epub 2000/01/12.
112. Silverpil E, Glader P, Hansson M, Linden A. Impact of interleukin-17 on macrophage phagocytosis of apoptotic neutrophils and particles. Inflammation. 2011;34(1):1-9. Epub 2010/03/27.
113. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Vicari C, Capelli A, et al. T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. Clinical and experimental immunology. 2009;157(2):316-24. Epub 2009/07/17.
114. Doe C, Bafadhel M, Siddiqui S, Desai D, Mistry V, Rugman P, et al. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. Chest. 2010;138(5):1140-7. Epub 2010/06/12.
115. Zhang X, Zheng H, Zhang H, Ma W, Wang F, Liu C, et al. Increased interleukin (IL)-8 and decreased IL-17 production in chronic obstructive pulmonary disease (COPD) provoked by cigarette smoke. Cytokine. 2011;56(3):717-25. Epub 2011/10/15.
116. Zhang L, Cheng Z, Liu W, Wu K. Expression of interleukin (IL)-10, IL-17A and IL-22 in serum and sputum of stable chronic obstructive pulmonary disease patients. Copd. 2013;10(4):459-65. Epub 2013/03/30.
117. Donnelly LE, Barnes PJ. Chemokine receptors as therapeutic targets in chronic obstructive pulmonary disease. Trends in pharmacological sciences. 2006;27(10):546-53. Epub 2006/08/17.
118. Prause O, Laan M, Lotvall J, Linden A. Pharmacological modulation of interleukin-17-induced GCP-2-, GRO-alpha- and interleukin-8

release in human bronchial epithelial cells. European journal of pharmacology. 2003;462(1-3):193-8. Epub 2003/02/20.

119. Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. Thorax. 2002;57(7):590-5. Epub 2002/07/04.

120. Geiser T, Dewald B, Ehrengruber MU, Clark-Lewis I, Baggiolini M. The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. The Journal of biological chemistry. 1993;268(21):15419-24. Epub 1993/07/25.

121. Burney PG, Luczynska C, Chinn S, Jarvis D. The European Community Respiratory Health Survey. The European respiratory journal. 1994;7(5):954-60. Epub 1994/05/01.

122. Wedzicha JA, Donaldson GC. Exacerbations of chronic obstructive pulmonary disease. Respiratory care. 2003;48(12):1204-13; discussion 13-5. Epub 2003/12/04.

123. Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. Annals of internal medicine. 1987;106(2):196-204. Epub 1987/02/01.

124. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. The European respiratory journal. 1993;6 Suppl 16:5-40. Epub 1993/03/01.

125. Salorinne Y. Single-breath pulmonary diffusing capacity. Reference values and application in connective tissue diseases and in various lung diseases. Scandinavian journal of respiratory diseases Supplementum. 1976;96:1-84. Epub 1976/01/01.

126. Yoshihara S, Yamada Y, Abe T, Linden A, Arisaka O. Association of epithelial damage and signs of neutrophil mobilization in the airways during acute exacerbations of paediatric asthma. Clinical and experimental immunology. 2006;144(2):212-6. Epub 2006/04/26.

127. Rosengren A, Orth-Gomer K, Wedel H, Wilhelmsen L. Stressful life events, social support, and mortality in men born in 1933. BMJ (Clinical research ed). 1993;307(6912):1102-5. Epub 1993/10/30.

128. Larsson S, Nordenson A, Glader P, Yoshihara S, Linden A, Slinde F. A gender difference in circulating neutrophils in malnourished patients with COPD. International journal of chronic obstructive pulmonary disease. 2011;6:83-8. Epub 2011/03/17.

129. Tylen U, Boijsen M, Ekberg-Jansson A, Bake B, Lofdahl CG. Emphysematous lesions and lung function in healthy smokers 60 years of age. Respiratory medicine. 2000;94(1):38-43. Epub 2000/03/14.

130. Vikgren J, Boijsen M, Andelid K, Ekberg-Jansson A, Larsson S, Bake B, et al. High-resolution computed tomography in healthy smokers and never-smokers: a 6-year follow-up study of men born in 1933. Acta

radiologica (Stockholm, Sweden : 1987). 2004;45(1):44-52. Epub 2004/05/29.

131. Andreoli C, Bassi A, Gregg EO, Nunziata A, Puntoni R, Corsini E. Effects of cigarette smoking on circulating leukocytes and plasma cytokines in monozygotic twins. Clinical chemistry and laboratory medicine : CCLM / FESCC. 2014. Epub 2014/07/17.

132. Arnson Y, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. Journal of autoimmunity. 2010;34(3):J258-65. Epub 2010/01/01.

133. Finkelstein J, Cha E, Scharf SM. Chronic obstructive pulmonary disease as an independent risk factor for cardiovascular morbidity. International journal of chronic obstructive pulmonary disease. 2009;4:337-49. Epub 2009/10/06.

134. Onishi K, Yoshimoto D, Hagan GW, Jones PW. Prevalence of airflow limitation in outpatients with cardiovascular diseases in Japan. International journal of chronic obstructive pulmonary disease. 2014;9:563-8. Epub 2014/06/13.

135. Ekberg-Jansson A, Andersson B, Bake B, Boijsen M, Enanden I, Rosengren A, et al. Neutrophil-associated activation markers in healthy smokers relates to a fall in DL(CO) and to emphysematous changes on high resolution CT. Respiratory medicine. 2001;95(5):363-73. Epub 2001/06/08.

136. Cockayne DA, Cheng DT, Waschki B, Sridhar S, Ravindran P, Hilton H, et al. Systemic biomarkers of neutrophilic inflammation, tissue injury and repair in COPD patients with differing levels of disease severity. PloS one. 2012;7(6):e38629. Epub 2012/06/16.

137. Shiels MS, Katki HA, Freedman ND, Purdue MP, Wentzensen N, Trabert B, et al. Cigarette smoking and variations in systemic immune and inflammation markers. Journal of the National Cancer Institute. 2014;106(11). Epub 2014/10/03.

138. Rutgers SR, Timens W, Kaufmann HF, van der Mark TW, Koeter GH, Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. The European respiratory journal. 2000;15(1):109-15. Epub 2000/03/04.

139. Vlahos R, Wark PA, Anderson GP, Bozinovski S. Glucocorticosteroids differentially regulate MMP-9 and neutrophil elastase in COPD. PloS one. 2012;7(3):e33277. Epub 2012/03/14.

140. Leclerc O, Lagente V, Planquois JM, Berthelier C, Artola M, Eichholtz T, et al. Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. The European respiratory journal. 2006;27(6):1102-9. Epub 2006/03/03.

141. Churg A, Zay K, Shay S, Xie C, Shapiro SD, Hendricks R, et al. Acute cigarette smoke-induced connective tissue breakdown requires both neutrophils and macrophage metalloelastase in mice. American journal of respiratory cell and molecular biology. 2002;27(3):368-74. Epub 2002/09/03.

142. van der Vaart H, Postma DS, Timens W, Hylkema MN, Willemse BW, Boezen HM, et al. Acute effects of cigarette smoking on inflammation in healthy intermittent smokers. Respiratory research. 2005;6:22. Epub 2005/03/03.
143. Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S, et al. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. The New England journal of medicine. 2008;359(15):1543-54. Epub 2008/10/07.
144. Vonk JM, Jongepier H, Panhuysen CI, Schouten JP, Bleecker ER, Postma DS. Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up. Thorax. 2003;58(4):322-7. Epub 2003/04/02.
145. Gibson PG, McDonald VM, Marks GB. Asthma in older adults. Lancet. 2010;376(9743):803-13. Epub 2010/09/08.
146. Weatherall M, Travers J, Shirtcliffe PM, Marsh SE, Williams MV, Nowitz MR, et al. Distinct clinical phenotypes of airways disease defined by cluster analysis. The European respiratory journal. 2009;34(4):812-8. Epub 2009/04/10.
147. Abramson MJ, Perret JL, Dharmage SC, McDonald VM, McDonald CF. Distinguishing adult-onset asthma from COPD: a review and a new approach. International journal of chronic obstructive pulmonary disease. 2014;9:945-62. Epub 2014/09/24.
148. Perret JL, Walters EH, Abramson MJ, McDonald CF, Dharmage SC. The independent and combined effects of lifetime smoke exposures and asthma as they relate to COPD. Expert review of respiratory medicine. 2014;8(4):503-14. Epub 2014/05/17.
149. Alahmari AD, Patel AR, Kowlessar BS, Mackay AJ, Singh R, Wedzicha JA, et al. Daily activity during stability and exacerbation of chronic obstructive pulmonary disease. BMC pulmonary medicine. 2014;14:98. Epub 2014/06/03.
150. Wedzicha JA, Singh R, Mackay AJ. Acute COPD exacerbations. Clinics in chest medicine. 2014;35(1):157-63. Epub 2014/02/11.
151. Baines KJ, Simpson JL, Gibson PG. Innate immune responses are increased in chronic obstructive pulmonary disease. PloS one. 2011;6(3):e18426. Epub 2011/04/13.
152. Andelid K, Bake B, Rak S, Linden A, Rosengren A, Ekberg-Jansson A. Myeloperoxidase as a marker of increasing systemic inflammation in smokers without severe airway symptoms. Respiratory medicine. 2007;101(5):888-95. Epub 2006/11/14.
153. Aaron SD, Angel JB, Lunau M, Wright K, Fex C, Le Saux N, et al. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2001;163(2):349-55. Epub 2001/02/17.

154. Vaguliene N, Zemaitis M, Lavinskiene S, Miliauskas S, Sakalauskas R. Local and systemic neutrophilic inflammation in patients with lung cancer and chronic obstructive pulmonary disease. BMC immunology. 2013;14:36. Epub 2013/08/08.

155. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends in immunology. 2004;25(1):4-7. Epub 2003/12/31.

156. Sinden NJ, Stockley RA. Systemic inflammation and comorbidity in COPD: a result of 'overspill' of inflammatory mediators from the lungs? Review of the evidence. Thorax. 2010;65(10):930-6. Epub 2010/07/16.

157. Wouters EF, Reynaert NL, Dentener MA, Vernooy JH. Systemic and local inflammation in asthma and chronic obstructive pulmonary disease: is there a connection? Proceedings of the American Thoracic Society. 2009;6(8):638-47. Epub 2009/12/17.

158. Hartmann G, Tschop M, Fischer R, Bidlingmaier C, Riepl R, Tschop K, et al. High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. Cytokine. 2000;12(3):246-52. Epub 2000/03/08.

159. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. The New England journal of medicine. 2011;364(7):656-65. Epub 2011/02/18.

160. McGovern NN, Cowburn AS, Porter L, Walmsley SR, Summers C, Thompson AA, et al. Hypoxia selectively inhibits respiratory burst activity and killing of Staphylococcus aureus in human neutrophils. Journal of immunology (Baltimore, Md : 1950). 2011;186(1):453-63. Epub 2010/12/08.

161. Sabit R, Thomas P, Shale DJ, Collins P, Linnane SJ. The effects of hypoxia on markers of coagulation and systemic inflammation in patients with COPD. Chest. 2010;138(1):47-51. Epub 2010/02/16.

162. Hodge SJ, Hodge GL, Holmes M, Reynolds PN. Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease. Cytometry Part B, Clinical cytometry. 2004;61(1):27-34. Epub 2004/09/08.

163. Chang Y, Nadigel J, Boulais N, Bourbeau J, Maltais F, Eidelman DH, et al. CD8 positive T cells express IL-17 in patients with chronic obstructive pulmonary disease. Respiratory research. 2011;12:43. Epub 2011/04/12.

164. Motz GT, Eppert BL, Wortham BW, Amos-Kroohs RM, Flury JL, Wesselkamper SC, et al. Chronic cigarette smoke exposure primes NK cell activation in a mouse model of chronic obstructive pulmonary disease. Journal of immunology (Baltimore, Md : 1950). 2010;184(8):4460-9. Epub 2010/03/17.

165. Paats MS, Bergen IM, Hoogsteden HC, van der Eerden MM, Hendriks RW. Systemic CD4+ and CD8+ T-cell cytokine profiles correlate

with GOLD stage in stable COPD. The European respiratory journal. 2012;40(2):330-7. Epub 2011/12/21.

166. Almagro P, Salvado M, Garcia-Vidal C, Rodriguez-Carballeira M, Cuchi E, Torres J, et al. Pseudomonas aeruginosa and mortality after hospital admission for chronic obstructive pulmonary disease. Respiration; international review of thoracic diseases. 2012;84(1):36-43. Epub 2011/10/15.

167. Paats MS, Bergen IM, Hanselaar WE, van Zoelen EC, Verbrugh HA, Hoogsteden HC, et al. T helper 17 cells are involved in the local and systemic inflammatory response in community-acquired pneumonia. Thorax. 2013;68(5):468-74. Epub 2013/01/15.

168. Glader P, Smith ME, Malmhall C, Balder B, Sjostrand M, Qvarfordt I, et al. Interleukin-17-producing T-helper cells and related cytokines in human airways exposed to endotoxin. The European respiratory journal. 2010;36(5):1155-64. Epub 2010/02/27.