From the Department of Medicine, Division of Respiratory Medicine
Karolinska Institutet, Stockholm, Sweden

ASPECTS OF INFLAMMATION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

A CLINICAL STUDY

J. Magnus Löfdahl

Stockholm 2006
Till Britta,
Helmer och Tilda
ABSTRACT

Development of chronic obstructive pulmonary disease (COPD) involves an amplified inflammatory response to tobacco smoke that results in a limitation of airflow. At present, the mechanisms underlying these inflammatory processes are not fully understood and insight into these mechanisms could be obtained through comparison of the processes in patients with COPD and appropriate controls.

Here, in order to characterize inflammatory responses in different body compartments, bronchoscopy was employed to obtain bronchoalveolar lavage (BAL) fluid and mucosal biopsies and, in addition, blood samples were taken from patients with COPD (n=23), smokers without this disease (n=16) and non-smokers (n=15). In addition, all of the participants underwent dynamic spirometry, computerized tomography (CT), body plethysmography and determination of the diffusion capacity of their lungs (DLCO).

The volume of BAL fluid recovered from the patents was lower than from both groups of controls. From patients with an emphysema index of <1% (i.e., for whom less than 1% of the pixels in a CT scan using 10-mm slices demonstrated an attenuation of less than 950 Hounsfield units), a larger volume of BAL fluid was recovered than from those with a corresponding index of >1%. Thus, this recovery correlated negatively to the index of emphysema and positively to other, indirect measures of emphysema (i.e., the DLCO and the FEV1/VC ratio).

Examination by flow cytometry showed that in comparison to smokers without the disease, the alveolar macrophages in BAL fluid from patients with COPD exhibited an altered phenotype, i.e., attenuated expression of the co-stimulatory molecule CD86 and of CD11a, an adhesion protein. Other differences in the expression of surface molecules by alveolar macrophages from smokers and non-smokers suggest that cigarette smoke exerts direct effects on these cells.

Moreover, exploration of the infiltration of the bronchial epithelium by inflammatory cells revealed elevated numbers of lymphocytes in patients with COPD. The numbers of CD4+ cells in these patients was higher than in either control group, while their numbers of CD8+ cells were elevated only in comparison to non-smokers.

In addition, the oxidative burst produced by leukocytes in the peripheral blood of patients and smokers without disease was less pronounced than in non-smokers. Vascular alterations were also present in the patients with COPD, as reflected in an enhanced level of sICAM-1. At the same time, the ability to mobilize the adhesion molecule CD11b was reduced in those patients who had smoked during the 12-hour period immediately preceding bronchoscopy.

These findings emphasize the importance of characterizing patients with COPD in an appropriate manner. Furthermore, the alterations observed in the phenotypes of the inflammatory cells of patients with COPD indicate that the innate immune responses of these individuals may be impaired, e.g., their macrophages may be less capable of activating T-lymphocytes and their circulating phagocytes less responsive to stimulation. Moreover, the present investigation reveals that the amplification of inflammation associated with COPD also involves the airway epithelium, a region of the bronchial mucosa previously explored less extensively than the submucosa, and, finally, confirms that COPD influences not only the airways, but systemic inflammatory responses as well.
LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:


III. Löfdahl JM, Engstrand-Roos E, Pourazar J, Dahlen B, Elmberger G, Blomberg A, Sköld CM. Increased intraepithelial CD4+ and CD8+ T-cells in COPD. *Submitted*

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<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<td>BALT</td>
<td>Bronchial-associated lymphoid tissue</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CT</td>
<td>Computerized tomography</td>
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<td>CXCL</td>
<td>CX chemokine ligand</td>
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<tr>
<td>CXCR</td>
<td>CX chemokine receptor</td>
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<tr>
<td>DLCO</td>
<td>Diffusion capacity of the lung</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ERV</td>
<td>Expiratory reserve volume</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume during a period of one second</td>
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<tr>
<td>VC</td>
<td>Vital Capacity</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formylmethionyl-leucyl-phenylalanine</td>
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<tr>
<td>FRC</td>
<td>Functional residual capacity</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GMA</td>
<td>Glycol methacrylate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>GOLD</td>
<td>Global Initiative for Chronic Obstructive Pulmonary Disease</td>
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<tr>
<td>GRO-α</td>
<td>Growth-related oncogene alpha</td>
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<tr>
<td>GSH</td>
<td>Gluthathione</td>
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<tr>
<td>HRCT</td>
<td>High resolution computerized tomography</td>
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<td>HU</td>
<td>Hounsfield Unit</td>
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<tr>
<td>IC</td>
<td>Inspiratory capacity</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>INF-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTB-4</td>
<td>Leukotriene-B(_4)</td>
</tr>
<tr>
<td>MCP-1α</td>
<td>Monocyte chemotactic protein-1α</td>
</tr>
<tr>
<td>MEF</td>
<td>Maximal expiratory flow</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MPO</td>
<td>Myeloperoxidase</td>
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<td>NE</td>
<td>Neutrophil elastase</td>
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<tr>
<td>NHLBI</td>
<td>Heart, Lung and Blood Institute in the United States</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OG</td>
<td>n-octyl-β-D-glucopyranoside</td>
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<tr>
<td>oxLDLs</td>
<td>Oxidized low-density lipoproteins</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>REE</td>
<td>Resting expenditure of energy</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
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<tr>
<td>RV</td>
<td>Residual volume</td>
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<tr>
<td>sICAM-1</td>
<td>Soluble intercellular adhesion molecule-1</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>TLC</td>
<td>Total lung capacity</td>
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<tr>
<td>TNF-alpha</td>
<td>Tumor necrosis factor-alpha</td>
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<td>WHO</td>
<td>World Health Organization</td>
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1 INTRODUCTION

1.1 HISTORICAL BACKGROUND

In comparison to other common diseases, chronic obstructive pulmonary disease (COPD) was recognized late in the twentieth century, presumably due to the lag time involved in the development of this disease as a consequence of tobacco smoking, which became increasingly popular from the 1920’s onwards. By the 1950’s, an association had been observed between chronic symptoms of bronchitis and mortality associated with malfunction of the respiratory system. Tobacco smoking was known to be associated with this type of bronchitis and the post mortem morphology of airway and parenchyma revealed an association between signs of chronic bronchitis and emphysema [1].

With the goal of classifying this disease, definitions of chronic bronchitis and emphysema that incorporated the concept of obstruction of airflow were proposed [2, 3]. In addition, a definition of chronic bronchitis derived from findings with standardized questionnaires [4, 5] was suggested. Research made in the 1950’s and 1960’s gave rise to two main hypotheses concerning the etiology of COPD, which later came to be known as the British and the Dutch hypotheses.

1.1.1 The British hypothesis concerning the etiology of COPD

It was proposed, primarily by British researchers, that smoking causes hypersecretion of mucus and hampers immunological defenses against respiratory infections. Moreover, it was suggested that this impairment of pulmonary defenses leads eventually to the repeated occurrence of acute and/or chronic infections [6] and subsequently to a decline in pulmonary function [7]. In this context, the importance of early treatment of chronic bronchitis was emphasized in an international [8], as well as in a Swedish context [9-13].

However, in a large-scale prospective population study of working men in West London, the presence of chronic bronchitis was found not to allow reliable prediction of the development of airway obstruction [14]. Furthermore, a proportion of the patients in this investigation who demonstrated obstruction of airways did not fulfill the criteria employed to diagnose chronic bronchitis. These observations, together with the primary finding that smokers exhibit a more rapid decline in pulmonary function, led these investigators to suggest that, in contrast to the British hypothesis, coughing and production of phlegm do not necessarily contribute to the development of airway obstruction. Moreover, in the late 1960’s it had been reported that the primary site of such obstruction was in the small airways [15, 16] and it was emphasized that a continuous, slow narrowing of this “quiet zone” of the airways [17] can occur in the absence of warning symptoms.

1.1.2 The Dutch hypothesis

The Dutch hypothesis proposed that host factors are the primary determinants of an individual’s response to exogenous factors (including tobacco smoke) and endogenous factors [18, 19]. According to this hypothesis, asthma, chronic bronchitis and emphysema are all different expressions of a primary abnormality in the airways, an abnormality which came to be referred to as “chronic non-specific lung disease”. The investigators who postulated this hypothesis suggested that the different symptoms and manifestations of disease developed by individuals with such a primary abnormality are
determined by their exposure to exogenous factors, which provides an appealing explanation as to why only certain smokers develop obstruction of airflow. Certain observations would appear to support this hypothesis, e.g. the fact that hyperresponsiveness of the airways is often associated with a subsequent decline in pulmonary function [20, 21]. However, it has not been possible to relate any histopathological features to “chronic non-specific lung disease”. On the contrary, comparison of biopsies from patients with fixed airway obstruction associated with asthma or with tobacco smoking has revealed significant differences in their inflammatory profiles, thus suggesting that the pathogenesis of these two diseases differ [22].

1.1.3 The definition of chronic obstructive pulmonary disease

In 1964 it was proposed that the diagnosis of COPD be based on exclusion of other pathologies [23]. Increasing use of the term COPD by researchers in the 1980’s and clinicians concerned with respiratory medicine in the 1990’s led to various national definitions [24-26] and, eventually, the proposal of a widely accepted international definition by the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) [27, 28], which has been accepted by the Heart, Lung and Blood Institute in the United States (NHLBI), as well as the World Health Organization (WHO). According to this GOLD definition, the disease is characterized by a slowly progressive, irreversible limitation of airflow and, furthermore, is associated with an abnormal inflammatory response upon inhalation of particles or gases. Subsequently, obstruction was defined quantitatively as a forced expiratory volume during a period of one second (FEV1) that was less than 70% of the forced vital capacity (FVC). In connection with a later extension of this definition by the European Respiratory and American Thoracic Societies [29], it was emphasized that the disease is preventable and treatable, as well as that the most important inducer of the “abnormal inflammation” associated with COPD is tobacco smoke. In addition, this revised definition recognizes COPD as a disease with potential systemic consequences.

1.2 CLINICAL FEATURES

1.2.1 Symptoms

1.2.1.1 The initial asymptomatic phase of COPD

COPD develops over the course of an extended period of years. The lesions associated with this disease occur prior to the onset of symptoms and to obstruction of airflow. Even the bronchioles of young smokers exhibit characteristic pathological features of the COPD [30]. These lesions progress slowly, and those who continue to smoke exhibit a more rapid decline in pulmonary than do those who stop or never smoked [14]. Since the limitation in airflow progress slowly, the patients have time to adapt to the attenuation of their pulmonary function [31], so that a number of individuals who fulfill the criteria for COPD [27-29] are unaware that they have this disease [32, 33]. In fact, certain cases of mild or moderate COPD do not report symptoms [33, 34].

1.2.1.2 Coughing and expectoration

Coughing is a natural response to inhalation of noxious particles and gases, including tobacco smoke. Following ten years of smoking, chronic cough is common and usually represents the first sign of COPD, although it seldom motivates the patient to seek medical advice. Initially, coughing may occur intermittently and may also be unproductive [35]. However, the presence of productive cough for at least three
months, including two consecutive months, is commonly used as the definition of COPD in connection with epidemiological studies [3], so that any phlegm produced but not expectorated, is by definition not considered in association with such studies. During its initial phase, the patients characteristically experience productive coughing predominantly in the morning, but as the disease progresses, such coughing can occur during a longer part of the day. The volume of sputa may be enhanced during periods of infection or in association with changes in the weather. Similarly, the purulence of these sputa may vary, being elevated during periods of exacerbation. As shown by assessment of the cumulative incidence of COPD in groups including young smokers, not all patients with chronic bronchitis have or will develop chronic limitation of airflow [36]. However, investigation of the ten-year cumulative incidence exclusively in middle-aged and elderly smokers revealed that coughing and production of sputum are associated with an elevated risk for the development of COPD [37]. Thus, chronic productive coughing occurs both prior to the onset of disease and during its initial mild stages, increasing in connection with the more severe stages [34]. Moreover, the presence of chronic mucus hypersecretion in patients with manifest COPD is positively correlated to a more rapid decline in pulmonary function [38-41], a requirement for hospitalization [38] and mortality [38]. Chronic coughing and phlegm production is associated with histopathological findings of intraluminal mucus in the bronchi [42-44]. Furthermore, this enhanced production of mucus is associated with hyperplasia of goblet cells [45-47] and hypertrophy of the submucosal glands [42, 44, 46, 48].

1.2.1.3 Dyspnea

Breathlessness is a common first symptom of COPD and often motivates patients to seek medical advice. Initially, this breathlessness occurs in association with exertion, but, as the disease progresses, it characteristically becomes persistent and eventually impairs daily activities. Patients may experience dyspnea in different ways, describing it as an increase in the effort required to breathe, heaviness, hunger for air, gasping for air, etc.

In one investigation of a random sample of adults with a mean age of 49 years, approximately 50% of those found to fulfill the spirometric criteria for COPD reported experiencing dyspnea [49]. In another study where smokers between the ages of 40 and 55 were invited to take a spirometry test, the frequency of COPD among those reporting dyspnea was found to be elevated [50]. Furthermore, dyspnea (as well as other respiratory symptoms) in middle-aged and elderly smokers without airway obstruction has been demonstrated to be an independent risk factor for incident disease [37, 51].

The multiple factors which contribute to the pathogenesis of dyspnea include static and dynamic hyperinflation. As COPD becomes progressively more severe, the properties of supporting tissues and elastin fibers within the lung are altered, resulting in enhanced tissue compliance and a reduction in elastic recoil during expiration. Consequently, this disease is associated with pulmonary hyperinflation [52] and such hyperinflation is positively correlated to patient dyspnea [53, 54]. Additional contributing factors involved in the pathogenesis of dyspnea includes an imbalance between ventilatory demand and capacity and an impairment in gas exchange in the form of both hypoxemia and hypercapnea.
1.2.1.4 Thoracic wheezing

In connection with COPD, thoracic wheezing may occur as a result of either turbulent airflow through bronchioles with a reduced luminal diameter and/or the presence of intraluminal mucus. Such wheezing occurs intermittently in mildly obstructed patients, whereas in more severe stages of the disease the wheezing becomes more persistent [55, 56]. Moreover, in certain patients who do not themselves experience wheezing, this symptom may be detected in connection with medical examination (i.e. objective wheezing). In one study [33], such objective wheezing was the only symptom examined that was able to predict a FEV1/FVC ratio of <70 % in “high-risk” current smokers (40-70 years of age) who had not previously been diagnosed as having COPD. However, the absence of both subjective and objective wheezing does not exclude a diagnosis of COPD.

1.2.2 Pulmonary physiology

1.2.2.1 Dynamic spirometry

The most characteristic abnormality in pulmonary function associated with COPD is a persistent reduction in the airflow rate during forced expiration. Accordingly, this disease is defined on the basis of measurements of this forced expiratory flow rate by dynamic spirometry [24-28].

The rate of airflow during forced expiration is determined by the balance between the total resistance offered by the total cross-sectional area of the airways and the strength of the elastic recoil. In connection with COPD, the resistance to expiratory flow is elevated as a consequence of remodeling of the airways, whereas the elastic recoil of the lungs is reduced to an extent proportional to the alterations in alveolar morphology. Therefore, the volume of air exhaled during forced expiration for one second (FEV1) becomes gradually smaller in individuals susceptible to tobacco smoke as remodeling of the airways proceeds [14, 57-61].

In addition to this reduction in FEV1, the forced vital capacity (FVC) gradually becomes smaller as well, especially during later stages of the disease, due to progressive alteration in the dynamic collapsibility of the small airways and the degree of emphysema (Figure 1). In contrast to restrictive pulmonary disease, the ratio of FEV1 to FVC is low in patients with COPD [24-28]. Furthermore, the reductions in FEV1 and the ratio of FEV1 to FVC are not completely reversible by bronchodilators or inhalation of corticosteroids, as they can often be in asthmatic patients. However, a partial reversal of the decrement in FEV1 and/or FVC associated with COPD can be achieved with these inhalations.

This decrease in airflow is readily apparent from a flow-volume curve. For patients with mild obstruction, the maximal expiratory flow (MEF) may still be normal, whereas the end expiratory airflow is markedly reduced, thereby producing a flow-volume curve with a “scooped out” appearance. The FVC may be normal or nearly normal in early stages of the disease, but with more advanced obstruction, a reduction in FVC becomes evident and a decrease in expiratory flow is observed throughout the entire flow-volume curve.
1.2.2.2 Whole-body plethysmography

One compartment of the total lung capacity that is of interest when assessing the severity of COPD, but cannot be measured by spirometry, is the residual volume (RV), i.e., the volume of air remaining in the lungs following complete expiration. This parameter is measured indirectly, employing dynamic spirometry in combination with whole-body plethysmography [62]. For this purpose, the patient is seated within the body plethysmograph and asked to breathe through a mouthpiece into a pneumothachograph (Figure 2). Following a quiet expiration, a shutter in this mouthpiece closes and the patient is then asked to pant gently against this shutter.

Changes in the volume of air within the plethysmograph, as well as alterations in the pressure on both sides of the shutter are registered by the pneumothachograph.
Application of Boyle’s law to these data, provides the volume of air within the lungs after a normal expiration, i.e., the functional residual capacity (FRC). Subsequently, the maximal volume of air that can be expired starting from the resting end-expiratory level, also known as the expiratory reserve volume (ERV), is determined. Thereafter, the RV can be calculated by subtracting ERV from FRC (Figure 3).

From the RV value, the degree of hyperinflation, which is of interest in evaluating the clinical impact of COPD, can be approximated. In non-obstructed, healthy individuals, the RV constitutes approximately 25-30 % of the total lung capacity (TLC), whereas in patients with COPD, especially those with pronounced emphysema, this value may be as much as 70 % or more. Correlations between RV and TLC values, on one hand, and emphysema assessed by CT, on the other, have been confirmed in additional studies as well [63, 64].

**Figure 3**
The subdivisions of pulmonary volumes recorded by a spirometer. A subdivision of two or more different volumes is referred to as capacity.

1.2.2.3  *The diffusion capacity of the lungs*

Determination of the diffusion of O₂ and CO₂ from alveolar gas to the blood, which is influenced by a number of factors, provides valuable information regarding impairment of the capacity of patients with COPD to oxygenate their blood. The diffusion capacity of the lung for carbon monoxide (DLCO), which is most frequently assessed utilizing the single breath procedure, is intended to yield an estimate of the rate at which both O₂ and CO₂ diffuse from the alveolus to the blood. In this context, the patient is asked to expire sufficiently to attain RV, and thereafter to rapidly inhale a gas mixture containing 0.3 % CO. After holding his/her breath for ten seconds, the subject then rapidly expires and the concentration of CO in this air exhaled is measured.
Comparison of the initial concentration of CO with the concentration in the expirate, allows the amount of CO that has diffused over the alveolar membrane per second to be calculated.

In individuals suffering from COPD, the DLCO is reduced due to a mismatch between ventilation and perfusion, as well as the presence of emphysema, especially in patients with more severe obstruction. The degree of emphysema (as quantitated by CT) in these patients has been found to be correlated to the DLCO [65, 66]. However, patients with a normal DLCO can still have emphysematous lesions detectable by CT [65].

1.2.3 Radiological presentation

1.2.3.1 Chest radiography

A chest radiograph, or chest x-ray, may reveal abnormalities in the form of increased thickness of bronchial walls in patients with chronic bronchitis [67], but such a finding is of limited value as a diagnostic tool. Furthermore, chronic bronchitis in smokers is associated with unspecific, prominent lung markings (“dirty chest”), perhaps caused by the increase in lung density due to accumulation of inflammatory cells and fibrous tissue in the airways [68]. Both of these features may also be present in smokers with normal pulmonary function, so that radiological investigations are rarely helpful in connection with attempts to achieve early diagnosis of COPD. However, patients with mild disease may also demonstrate signs of hyperinflation during periods of exacerbation. Thus, among 204 patients with COPD hospitalized due to exacerbation of their symptoms, 14% exhibited abnormal findings on a chest radiograph [69]. In addition, chest radiography may also be helpful in excluding the presence of complications of COPD (i.e., pneumothorax and pneumonia) or other diseases, such as heart failure.

In the case of patients with COPD who are more severely obstructed and also suffer from emphysema, chest radiography reveals more specific abnormalities present with more specific abnormalities [70], including an enlarged lung volume and/or pulmonary destruction (i.e., reduced vascularity or bullae). However, the reliability of chest radiography in diagnosing emphysema, especially in patients with mild emphysema, remains controversial.

1.2.3.2 Computerized tomography (CT)

Small airways are the primary site of obstruction in patients with COPD. Different procedures for measuring airway thickness employing CT scans have been developed for research purposes [71-73]. Although these techniques are capable of assessing the severity of disease in patients who have already been diagnosed as having COPD, several other pathological states also cause such thickening, so that it cannot be utilized for diagnostic purposes.

With respect to emphysema, high resolution computerized tomography (HRCT) is a highly reliable diagnostic tool. Of the characteristic HRCT features exhibited by emphysematous patients, occurrence of hypodense areas is the most prominent [74]. Other characteristic signs include the presence of bullae, trapping of air and hyperinflation. Moreover, high resolution CT is even capable of detecting early emphysematous lesions. One investigation found that among smokers without a functional reduction in diffusing capacity, 40% nonetheless exhibited signs of emphysematous lesions in connection with CT [75]. In another study, this technique revealed that 40% of the
smokers with normal pulmonary function and no symptoms had emphysematous lesions [76]. Thus, HRCT may reveal early stages of emphysema in smokers before clinical symptoms have developed.

1.3 THE HETEROGENEITY OF COPD

1.3.1 The “pink puffer” and the “blue bloater”

An early attempt to classify patients with COPD on the basis of their clinical features made a distinction between the “pink puffer” and the “blue bloater”. The pink puffer breathes through pursed lips, suffers from involuntary loss of weight and manages to maintain normal blood levels of CO₂. The blue bloater, on the other hand, has a normal body mass index (BMI) and demonstrates signs of ventilatory insufficiency. This distinction has, however, been questioned, since at autopsy most patients have a combination of airway and alveolar disease [77], so that it is not possible to equate the blue bloater with chronic bronchitis and the pink puffer with emphysema.

1.3.2 The emphysematous phenotype

The limitation in airflow associated with emphysema is due to a reduction in elastic recoil [78], as well as to the loss of alveolar attachments to bronchioles [79]. However, unlike COPD, emphysema has not been defined clinically. Instead, diagnosis of this disease is based on the presence of permanent enlargement of the airspaces distal to the terminal bronchioles, together with destruction of alveolar walls without obvious signs of fibrosis [80, 81].

For both clinical and research purposes, assessment by CT has proven useful in evaluating the extent of emphysema, several studies having correlated CT findings with the degree of disease assessed pathologically [82-84]. Patients with emphysema also exhibit a lowered DLCO, as determined readily by the single-breath CO procedure (see above), and this DLCO value has been found to reliably indicate the extent of emphysema as well [65, 83].

The functional consequences of emphysema are enhanced hyperinflation (readily monitored as a decrease in the inspiratory capacity (IC)) and an increase in both the RV value and the RV/TLC ratio [63, 64]. Static and dynamic hyperinflations have both been associated with various symptoms of COPD. The degree of hyperinflation is correlated positively to dypnea [53, 54] and negatively to functional capacity [85, 86]. Consequently, one patient with COPD associated with severe emphysema may experience disabling dyspnea; whereas another non-emphysematous patient with COPD and a similar degree of obstruction (measured as the FEV1) may be asymptomatic.

The severity of the emphysema may also influence the choice of therapy. Assessment of the distribution of emphysema by CT, in combination with determination of the DLCO and FEV1 values, helps identify patients with a better prognosis in connection with volume reduction surgery [87]. Furthermore, evaluation of the severity of emphysema by CT in combination with an exercise test may help identify subpopulations of patients whose exercise capacity, general health status and even survival would benefit from such surgery [88].

1.3.3 Hypoxemia

In patients with COPD and concomitant hypoxemia, the progression and impact of the disease is different than in those without hypoxemia. Hypoxemia, which occurs preferentially in the later stages of the disease [89], is a negative prognostic factor [90,
In addition, impaired cerebral functions are common in hypoxemic patients [92], in part because of the attenuated supply of oxygen to the brain. Higher cognitive functions, such as abstract thinking and complex perceptual-motor integration, appear to be most severely affected. Quality of life has been reported to be negatively affected by hypoxemia, and treatment with oxygen at home can improve this quality [93, 94].

1.3.4 Frequent exacerbations

Exacerbation of COPD is characterized by sustained aggravation of respiratory symptoms and usually leads to a visit to the doctor. Symptoms of such an exacerbation include enhanced breathlessness, coughing, a change in the volume or purulence of the sputum, wheezing and tightness of the chest. The definition most frequently employed for research purposes is based on a requirement for healthcare, including medication with corticosteroids and/or antibiotics [95]. The average intensity of symptoms (as assessed in a diary), is elevated during exacerbations, which has, accordingly, also been defined on this basis [96]. However, certain patients may not seek medical care for their worsened symptoms [96].

Patients with similar degrees of airflow limitation exhibit considerable variation in the frequency with which they suffer from exacerbation of their disease [97-99]. The clinical impact on those who experience frequent exacerbation is more pronounced and includes a more rapid decline in pulmonary function, as well as a worsened general state of health [100, 101]. Furthermore, frequency of exacerbation seems to be related to regular coughing and sputum production [102] and a lower level of activity at home [103].

1.3.5 Systemic involvement

A low body mass index (BMI) appears to be a specific clinical feature of COPD and is an independent predictor of an increased risk of mortality from this disease [91, 104], perhaps in part because of the increase in the work required for breathing. At the same time, the detection of elevated levels of markers of systemic inflammation indicates that this weight loss may also be a direct systemic consequence of the disease. Thus, the level of tumor necrosis factor-alpha (TNF-alpha), a cytokine that cause cachexia in laboratory animals, is elevated in the blood of patients with COPD who demonstrate weight loss [105]. Such systemic inflammation may also be associated peripheral muscle dysfunction [106] prevalent in patients with COPD [107, 108].

1.3.6 Gender differences

Although originally detected most frequently among men, the prevalence of COPD in women and associated mortality has risen dramatically in the industrialized world. One investigator reported that in the United States this prevalence among women increased from 20.1/100,000 in 1980 to 56.7/100,000 in the year 2000 [32] and this same study actually found a higher prevalence of COPD among women than men. In the U.S. lung health study, smoking women with mild COPD were found to have more hyper-reactive bronchi than the corresponding groups of men [109].

Other investigators have also reported on indications that women are more susceptible to COPD [58, 110-114], although contradictory data have appeared as well [115]. For instance, no gender difference has been observed in overall prevalence have been observed in northern Sweden [49].

Moreover, women also report dyspnea more often than do men with the same degree of airway obstruction [49, 116]. In addition, the frequency of exacerbations appears to
differ between men and women. For instance, health statistics for Stockholm, Sweden, reveal a greater need for hospitalized medical care for women with COPD [117], in agreement with observation of more frequent exacerbations in women than men [118].

1.4 THE RELATIONSHIP BETWEEN PATHOLOGY, PATHOPHYSIOLOGY AND INFLAMMATION IN PATIENTS WITH COPD

Airway obstruction in patients with COPD is associated with pathological changes at different levels of the bronchial tree and in the parenchyma (emphysema). One important cause of this obstruction of airflow is the elevation in the resistance of the small airways (<2 mm in diameter) [16, 119, 120]. Examination of autopsy samples and resected lung specimens has revealed an infiltration of inflammatory cells into the bronchioli of smokers [30, 121]. This inflammation, referred to as small airways disease, has subsequently been found to be negatively correlated to the pulmonary function as assessed by the FEV1 [122]. Additional studies of resected lung parenchyma have demonstrated a correlation between inflammation of the small airways and the severity of COPD [123].

Pathological changes are also observed in the pulmonary parenchyma in cases of COPD with emphysema. Such emphysema results in increased lung compliance, which is associated with reduced elastic recoil, and is an important cause of limitations in expiratory airflow [124]. In addition, emphysema is associated with loss of alveolar attachments to the bronchioles [79, 125], which may potentially contribute to the reduction in elastic recoil, but also to a decrease in the luminal diameter of the bronchioli.

Inflammatory changes in the large airways, i.e., chronic bronchitis, are associated primarily with chronic hypersecretion of mucus [46, 126]. However, such hypersecretion does influence the impact of COPD on health, perhaps contributing to the progressive decline in pulmonary function [38, 127].

Considered together, these observations reveal that inflammatory reactions are closely linked to the pathological changes associated with the development of airflow limitation in patients with COPD. Investigation of inflammatory markers in postmortem samples [30], resected lung parenchyma [121], sputum [128] and bronchoalveolar lavage [129] demonstrates clearly that inflammation is present in everyone who smoke, although not all smokers develop COPD [14]. It is therefore of importance to clarify the interactions between the cells participating in the inflammatory responses to tobacco smoke. Elucidation of differences in the inflammatory processes between “susceptible” non-obstructed smokers may lead to a better understanding of the pathogenesis of COPD, and, furthermore, is necessary for the development of more effective treatment strategies.

1.5 THE INFLAMMATORY CELLS THAT PLAY A MAJOR ROLE IN CONNECTION WITH COPD

1.5.1 Monocytes and macrophages

Regardless of whether the individual is a non-smoker, non-obstructed smoker or suffers from COPD alveolar macrophages are the predominant cell type in bronchoalveolar lavage fluid (BALF) [130]. However, the concentration of alveolar macrophages in BALF is dramatically elevated in all smokers [130]. Human alveolar macrophages respond to atmospheric particles by releasing several proinflammatory mediators, including monocyte chemotactic protein-1α (MCP-1α),
 interleukin-1β (IL-1β), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor α (TNF-α) [131, 132]. This release of MCP-1 and IL-8 promotes the further recruitment of monocytes to the lungs and monocytes from patients with COPD exhibit an enhanced capacity for migration when exposed to other products derived from macrophages, e.g., GRO-α [133]. Proinflammatory factors such as IL-8, leukotriene-B_4 (LTB-4) and IL-1β are of importance to the recruitment of neutrophils to sites of injury in the lungs [134, 135]. Moreover, neutrophils exhibit several responses to TNF-α, including activation, production of cytokines and degranulation. In addition, TNF-α plays a role in the adhesion of neutrophils to and their migration through vascular endothelia [136]. Certain of the cytokines produced in the lung exert systemic effects, as well, e.g., by stimulating the liver to produce acute-phase proteins (TNF-α, IL-1β) and/or influencing the bone marrow to release leucocytes (TNF-α, GM-CSF, IL-6).

Macrophages have a potential role to play in the pathogenesis of emphysema and COPD [137], primarily via the production of matrix metalloproteinases (MMP) that can degrade extracellular proteins. Alveolar macrophages have been shown to produce MMP-12 [138, 139], MMP-9 [138, 140], MMP-1 [140] and MMP-2 [141]. The administration of aerosolized alveolar macrophages to experimental animals induces emphysema [137], and in mice deficient in MMP-12 do not appear to be susceptible to smoke-induced emphysema [142]. In addition, in another animal model, inhibitors of MMP attenuated smoke-induced emphysema [143]. Although macrophages do not themselves synthesize neutrophil elastase (NE), these cells can take up this enzyme and release it later on, thus amplifying matrix degradation by MMPs. The macrophage may also act as an antigen-presenting cell (Figure 4). Alveolar macrophages function as phagocytes in connection with the innate immune response to foreign particles and microbes, engulfing and processing extracellular particles and displaying antigen complexes on their surface. CD4+ lymphocytes may then assist in microbe degradation by recognizing these antigens and secreting interferon-γ (INF-γ). This interaction between macrophages and lymphocytes potentiates the immunological response.

**Figure 4**
A schematic illustration of antigen presentation. A CD4+ T-helper lymphocyte (left) recognizes an antigen complex displayed in conjunction with a class II HLA molecule on the surface of a macrophage. In connection with such antigen presentation, co-stimulatory molecules (e.g., CD86) bind to their ligand (CD28) on the surface of this lymphocyte, resulting in an enhanced response.
1.5.2 Granulocytes

One type of granulocyte that has been proposed to play an important role in the pathogenesis of COPD is the polymorphonuclear leukocyte, also known as the neutrophil. These cells participate both in initial inflammatory responses and in the later stages of immunological defenses in essential ways. In patients with COPD, the numbers of neutrophils present in sputum [144] and broncholalveolar lavage fluid [129, 130] are elevated. Within the bronchial wall, however, elevated numbers as well as equal numbers compared to non-obstructed smokers have been reported, possibly implying a short tissue life span for neutrophils passing through to the airway lumen. Recruitment of neutrophils to the airways and pulmonary parenchyma occurs, at least in part, as a response to cigarette smoke, which in itself enhances the number of circulating neutrophils and promotes sequestration of these cells in pulmonary capillaries [145]). Cigarette smoke also exerts a direct effect on the bone marrow, stimulating neutrophil production via a mechanism that appears to involve GMCS-F and GCS-F released by macrophages [146]. Neutrophils can then migrate through the bronchial wall in response to chemotactic factors, such as LTB-4, IL-8 and GRO-α, that are produced, for instance, by alveolar macrophages and epithelial cells.

Upon activation of neutrophils, several products are released from their granules, e.g., myeloperoxidase (MPO), human neutrophil lipocalin (HNL) and lectorferrin. These cells can also secrete proteinases such as neutrophil elastase (NE), cathepsin G, MMP-8 and MMP-9, which are capable of causing tissue damage and have been proposed to be responsible for a large degree of the destruction of alveoli and remodeling of airways characteristic of COPD [147, 148]. Moreover, factors produced by neutrophils can induce hypersecretion of mucus, which may play a significant role in connection with the pathogenesis of chronic bronchitis. Thus, neutrophil elastase promotes secretory cell metaplasia [147], as well as acute secretion by these cells [149]. In addition, mucus secreted in large amounts may contribute to the obstruction of bronchioli [123] and, in addition, enhance inflammation and susceptibility to infection.

At the same time, neutrophils contribute to the elevation of oxidative stress that occurs in direct response to tobacco smoke. This is due to the fact that activation of these cells leads to elevations in the extracellular level of superoxide anion \( \text{O}_2^- \) and their production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS). These products can combine to form peroxynitrate, thereby further aggravating the oxidative burden. Not only can oxidative stress cause tissue damage directly, but it can also amplify the inflammatory response and inhibit the anti-inflammatory effects of steroids (by reducing the activity of histone deacetylase (HDAC)) [150, 151]. The eosinophil, another type of granulocyte, participates in a central manner in the development of asthma, but has not yet been found to play a prominent role in connection with COPD, at least during stable periods of this disease. In connection with exacerbation of COPD, however, more eosinophils infiltrate the mucosa of the airways [152, 153]. Furthermore, a subset of patients suffering from COPD that could be partially reversed by steroid treatment was found to exhibit mucosal inflammation similar in some ways to that associated with asthma, including eosinophilia and thickening of the reticular basement membrane [154].
These observations suggest that eosinophils might play a role in connection with COPD in certain patients and/or during exacerbation. However, individuals with asthma and COPD demonstrate dissimilar inflammatory characteristics when asthmatic patients suffering from chronic limitation of airflow are compared to those with “classic” COPD caused by cigarette smoke. Consequently, these appear to be two distinct diseases, rather than different expressions of a common underlying pathological mechanism(s).

**1.5.3 Lymphocytes**

The mechanisms by which lymphocytes are recruited to the respiratory tract and lungs are not fully understood. Epithelial cells [155] and alveolar macrophages [156] can attract T-lymphocytes by expressing interferon-induced protein-10 (CXCL-10). Epithelial cells in bronchioli from COPD patients exhibit elevated expression of CXCL-10, and this is associated with an increase in CD8+ cells positive for the receptor of CXCL-10, CXCR-3 [157]. Further examples of chemoattractants capable of acting via lymphocyte CXCR3 are CXCL-9 and CXCL-11 (also known as monokine induced by interferon-γ (Mig) and the interferon-inducible T-cell α-chemoattractant (I-TAC), respectively).

The role of lymphocytes in the pathogenesis of COPD is uncertain. An increase in lymphocytes due to chronic immune stimulation has been suggested. In COPD, acute as well as chronic infection of the lower respiratory tract has been reported [158, 159], and such infections may be responsible for the enhanced inflammatory response associated with the disease. This might be supported by the fact that investigations of the small airways in COPD patients [123] have revealed an increase in B-lymphocytes and in bronchial-associated lymphoid tissue (BALT), whereas other studies has revealed an increase in B-lymphocytes in bronchial mucosa [160].

CD8+ T cells have in theory the potential to damage pulmonary tissue, for example by releasing lytic substances such as perforin and granzyme. It has been observed that CD8+ cytotoxic T-lymphocytes from the sputum of COPD patients express higher levels of perforin than from controls [161]. An important function of CD8+ lymphocytes is the kill structural cells infected by viruses. Such killing can be mediated via cytolysis or induction of apoptosis in infected cells [162]. In mice infected with respiratory syncytial virus (RSV), such cell death mediated by cytotoxic T-cell has been associated with pulmonary damage [163]. Thus, CD8+ cells can induce structural cell apoptosis [164] and the number of CD8+ T-cells within the alveolar wall of COPD patients have been found to correlate to the number of alveolar cells exhibiting signs of apoptosis [164].

**1.5.4 Epithelial cells**

Airway epithelial cells are an example of structural cells that can act also as inflammatory cells. Airway epithelial cells can produce mediators with potential to recruit leucocytes such as TNF-α, IL-1β, GMCS-F and IL-8. Furthermore, the epithelium of small airways may be important as a producer of the profibrotic factor TGF-β [165]. One further indication of proinflammatory activity in airway epithelial cells is the increased expression of the inflammatory associated transcription factor NF-κB in patients with COPD [166]. Epithelial cells can also secrete protective factors such as antioxidants and antiproteases. As the direct effects of tobacco smoke include tissue damage of the epithelium, such protective mechanism may be impaired as a consequence.
1.6 CHANGES IN THE INTENSITY OF INFLAMMATION FOLLOWING CESSION OF SMOKING

As mentioned previously, the number of inflammatory cells in the BAL fluid from smokers without COPD is increased. If these individuals quit smoking, the number of macrophages present in their BAL fluid decreases within two months and has returned to levels similar to those observed in non-smokers within six months [167-169]. The numbers of lymphocytes and neutrophils in their BAL fluid declines somewhat more slowly, returning to the levels in non-smokers within 9 and 15 months, respectively [167]. Although the autofluorescence of their alveolar macrophages, a consequence of ingestion of fluorescent particles in cigarette smoke, also decreases, this autofluorescence still remains significantly elevated for at least 15 months after cessation [169].

Moreover, examination of pulmonary tissue resected from patients without COPD in connection with the removal of nodular lesions has revealed that in smokers who quit, the extent of Goblet cell hyperplasia and squamous neoplasm in the small airways are significantly reduced [45, 170]. However, certain pathological alterations in the airways and parenchyma of these individuals, e.g., fibrosis of the bronchioles and damage to alveolar structures, appear to be irreversible. In addition, in contrast to the changes in BAL fluid, the intensity of inflammation in resected bronchi and broncioli from ex-smokers without COPD is not attenuated as compared to current smokers [45, 170]. Finally, after quitting such individuals also exhibit signs of attenuated systemic inflammation in their peripheral blood, i.e., a reduction in the number of circulating leukocytes [171].

At the same time, when an individual with manifest COPD stops smoking, his/her symptoms and decline in pulmonary function are attenuated, but the effects of quitting appear to differ in different pulmonary compartments in this case as well. On the one hand, examination of sputum [172], BAL fluid [173] and blood [174] from former smokers with COPD reveals a reduction in the intensity of inflammation. In contrast, inflammation of the pulmonary tissues of patients with COPD appears to be at least partially persistent, in parallel with what is seen in smokers with normal pulmonary function. Accordingly, bronchial biopsies from patients with COPD who still smoke contain similar numbers of inflammatory cells or proinflammatory markers as those who have quit [175] and, moreover, the levels of cytokines in pulmonary tissues resected from these two groups of patients are similar [176].

However, the number of bronchial T-lymphocytes in patients with COPD is related to the duration of smoking cessation and, in particular, CD8+ cells, although not other inflammatory cells, are reduced after a prolonged period of time [177]. In addition, the numbers of inflammatory cells present in the bronchial mucosa are reduced more potently by inhalation of steroids and/or treatment with B2 agonists in those patients who are ex-smokers compared to those who have never smoked [178].

1.7 THE ACUTE INFLAMMATORY EFFECTS OF SMOKING

A number of studies have characterized the inflammatory response to chronic smoking, and in recent years, there has also been an interest in the acute effects of tobacco smoke on this response in chronic smokers.

BAL fluid from chronic smokers contains elevated levels of inflammatory cells and inhalation of cigarette smoke results in an additional, acute enhancement in the
numbers of neutrophils present [179]. In addition, the numbers of neutrophils circulating in the peripheral blood increase [180] and these cells express higher levels of adhesion molecules [181] following such acute exposure. The acute effects of smoking on the levels of inflammatory mediators in chronic smokers have also been examined. For instance, the elastase activity present in BAL fluid [182] and the blood level of neutrophil elastase (NE) [183] is elevated immediately after smoking, as is the level of leukotrienes B₄, D₄, and E₄ in peripheral blood [184]. Moreover, the levels of several markers of oxidative stress in exhaled air [185] and BAL fluid [179] from chronic smokers, as well as in the peripheral blood of these same individuals [186] increase acutely in response to tobacco smoke.

1.8 LOCAL PULMONARY INFLAMMATION

1.8.1 In large airways

The bronchial mucosa of patients with COPD is infiltrated with T-lymphocytes, predominantly cytotoxic (CD8+) T-cells [166, 187]. The number of these CD8+ cells is elevated in comparison to corresponding tissue of non-obstructed smokers [187], and demonstrates a negative correlation to pulmonary function assessed on the basis of FEV1 [188]. In more severe stages of the disease, however, the numbers of CD8+, as well as of CD3+ cells in the bronchial mucosa appear to be lower than in patients with less obstruction [189].

Other types of lymphocytes are also present in lung tissue in conjunction with COPD. The bronchial mucosa of 114 patients whose COPD was in stable state contained a larger number of reported B-cells (CD20+) than was present in smokers without this disease, and this was significantly more pronounced in more severe stages of COPD [160]. This presence of B-cells has been suggested to reflect an adaptive immune response that occurs in severe stages of the disease, possibly as a consequence of immune stimulation by microbial antigens.

In addition to lymphocytes, macrophages (CD68+) have been found in biopsies of the bronchial mucosa from patients with COPD [187, 190, 191]. The numbers of these cells were higher in tissue from patients with COPD than in smokers without the disease [187, 191] as well as higher in such smokers than in non-smokers [187, 190]. As COPD becomes more severe, the number of CD68+ cells may increase further in comparison to patients who are less severely obstructed [190].

Although the inflammation in bronchial mucosa in patients with COPD involves predominantly lymphocytes and monocytes/macrophages, infiltration by neutrophils also occurs, especially in the case of severe disease, where this infiltration is more pronounced than in patients with mild disease or smokers without COPD [190]. In addition, during exacerbation of the disease, total numbers of different types of inflammatory cells increase significantly [192]. This observation is interesting, since patients who are more severely obstructed experience exacerbation more frequently [97], which may explain the enhanced infiltration of neutrophils associated with severe disease.

1.8.2 In small airways

The small airways (i.e., those with a diameter <2mm) and the surrounding pulmonary parenchyma are key sites of inflammation in response to tobacco smoke. Even young smokers exhibit pathological changes in their small airways [30] and the small airways of smokers with concurrent chronic bronchitis are infiltrated by a larger number of
inflammatory cells than are those of smokers without bronchitis. Comparison of patients with COPD to smokers without the disease has produced contradictory data in this respect. One such study reports an increase in the total number of inflammatory cells and CD8+ cells in connection with COPD [193], whereas others have observed no such difference [194-196]. Similarly, the numbers of monocytes/macrophages in small airways affected by the pathogenic process of COPD were found to be greater than in smokers without the disease in two studies [195, 196], but no such difference was found by other investigators [188, 193, 194].

The relationship between pathological changes in small airways and the severity of COPD has been examined employing resected lung tissue [123]. In this study, 159 patients with different stages of disease were included and the thickness of small airways and the extent of infiltration by different inflammatory cells determined. Multivariate analysis revealed that progression of the disease correlated most strongly with the total thickening of the bronchiole wall. To a lesser extent, the severity of disease was also correlated to multiple parameters of inflammatory responsiveness, including the number of airways containing acute inflammatory cells (neutrophils and macrophages), lymphocytes and lymphoid follicles, as well as the combined volume of CD8+ cells and B-cells that had infiltrated the airway wall.

1.8.3 In pulmonary parenchyma

The inflammatory effects of smoking on lung parenchyma have been examined in patients undergoing lung resection [197]. The parenchyma of patients who smoked and suffered from COPD contained greater numbers of macrophages and lymphocytes than did those of non-obstructed smokers and non-smokers. In patients with mild to moderate disease, infiltration by lymphocytes only, not by macrophages, was elevated [164, 198], with CD8+ cells predominating. In the lung parenchyma of severely obstructed emphysematous patients with COPD the total extent of inflammation appeared to be more pronounced than in non-obstructed smokers with or without mild emphysematous lesions [159].

The parenchymal inflammation in emphysematous patients is accompanied by enhanced epithelial expression of adenoviral proteins [159]. Furthermore, the number of intraalveolar cells that exhibit signs of apoptosis is elevated in emphysematous patients in comparison to smoking and non-smoking control individuals [199]. On the basis of these observations, it has been suggested that emphysema might be induced in part by CD8+ cytolytic lymphocytes and that this might be a consequence of enhanced apoptosis.

1.9 SYSTEMIC INFLAMMATION IN CONJUNCTION WITH COPD

It is obvious to any physician who treats patients with COPD that this disease is associated with systemic complications. It was recognized at an early state that involuntary weight loss and cachexia occur in certain patients with COPD (“pink puffers”), but not in all. Furthermore, the disease can also be accompanied with arterial hypoxemia, a complication which is possible to treat [93, 94]. Consequently, in a slightly revised GOLD definition of COPD, formulated by the European Respiratory Society and the American Thoracic Society task force together [29], the systemic consequences are now recognized as an important feature of this disease.
1.9.1 Evidence of systemic inflammation in conjunction with COPD

COPD is not only associated with inflammatory responses within the lungs, but there are also indications of systemic inflammation. For example, patients with this disease have elevated plasma levels of C-reactive protein (CRP) [174, 200-203] and of fibrinogen [201, 204-206]. Furthermore, the numbers of leukocytes in the blood of patients with COPD are increased [174, 201]. These circulating inflammatory cells exhibit altered phenotypes with respect to cell surface markers [207-209], as well as the functional capacity to produce inflammatory mediators [208, 210]. The systemic level of oxidative stress (i.e., increased exposure oxidants and/or decreased antioxidant capacities) appears to be elevated in smokers. Neutrophils isolated from blood of patients with COPD have been found to produce reactive oxygen species (ROS) under basal conditions, as well as after stimulation in vitro, in contrast to neutrophils from smokers without the disease and non-smokers [211]. The antioxidant capacity (measured as Trolox-equivalents (TEAK)), is lower in the plasma of patients with COPD than in non-smokers, whereas blood levels of products of lipid peroxidation are elevated in the former [186]. However, this same study detected no difference between patients with COPD and smoking control individuals with respect to these parameters and no correlations between pulmonary function and TEAC [212]. Products resulting from ROS-dependent peroxidation of arachidonic acid, another measure of systemic oxidative stress can be detected in the urine. One investigation found higher levels of such products in the urine of patients with COPD than in that of smokers without the disease [213].

A handful of studies indicate that COPD is also associated with elevated plasma levels of various proinflammatory cytokines, such as tumor necrosis factor (TNF)-α [105, 203, 214], as well as of the soluble receptors for this cytokine [214]. Moreover, elevated serum levels of IL-6 [203], as well as indications of an elevation in serum IL-8 have been reported [215, 216]. It is clear that exacerbation of COPD is associated with aggravated inflammation of the airways [217], and the aggravated inflammation during such exacerbation is also evident systemically. One prospective study that monitored 93 patients with COPD for almost one year found a direct relationship between symptomatic exacerbation and rises in systemic IL-6 and fibrinogen [218]. An increase in the systemic level of sIL-1 RII, a receptor for the inflammatory cytokine IL-1, has also been observed during recovery from exacerbationed COPD [174].

1.9.2 Mechanisms underlying this systemic inflammation

The mechanisms behind the systemic inflammation present in patients with COPD are poorly understood. Several independent mechanisms may be involved, and their relative contributions may vary between individuals. Smoking is associated with numerous extrapulmonary pathologies, including cardiovascular disease, and tobacco smoke can contribute significantly to the systemic inflammation associated with COPD. Indeed, tobacco smoke in itself (i.e., in the absence of COPD) induces systemic inflammation. Thus, biomarkers of systemic oxidative stress have been detected in passive [219] as well as active smokers [220], and smokers exhibit leukocytosis and neutrophilia [221]. Furthermore, passive smoking appears to induce endothelial dysfunction [222, 223].
The neutrophilia induced by smoking is associated with early signs indicative of stimulation of the bone marrow [221]. This stimulation may be mediated by proinflammatory factors produced locally in the lungs in response to tobacco smoke. The inflammatory mediators TNF-α, IL-1, IL-6, IL-8 GM-CSF and G-CSF produced by lung tissue in patients with COPD, can reach the bone marrow via the circulation and may stimulate this tissue to release neutrophils. These systemic consequences have, in fact, been associated with the pathogenesis of atherosclerosis in smokers [224]. As discussed above, the proinflammatory mediators may leak into the systemic circulation, or, alternatively, this local proinflammatory environment may activate inflammatory cells transiting through the pulmonary circulation. However, one comparison of the levels of inflammatory markers in sputum and plasma from patients with COPD and smokers without this disease detected no correlation, and the authors concluded that the local pulmonary and systemic inflammations associated with COPD are induced and modulated independently [216]. In another study, healthy subjects were exposed to lipopolysaccharide (LPS) and the inflammatory response locally in the lungs was compared to the systemic response [225]. Correlation between the local inflammatory response and reactivity of the airways and between a rise in body temperature and a systemic inflammatory response were detected. However, a rise in body temperature was correlated with a systemic inflammatory response. However, LPS-induced reactivity of the airways and such an elevation in body temperature were not associated in any of the subjects, and consequently these authors also propose that the mechanisms underlying local pulmonary and systemic inflammation are different.

A recurring complication of COPD is hypoxia in peripheral tissues, which may, in itself or in combination with other factors, contribute to the systemic inflammatory response. As mentioned above, TNF-α produced by i.e., alveolar macrophages, is an important mediator of the early phase of inflammatory processes, and exposure of human mononuclear cells to hypoxia in vitro stimulates their secretion of this cytokine [226-229]. Thus, hypoxia may contribute to the inflammatory response by macrophages, resulting in elevated levels of TNF [226-229] and interleukin 1 (IL-1) [227, 228]. In one investigation the relationship between arterial hypoxia and markers of systemic inflammation in patients with COPD and matched smokers without this disease were examined [230]. The serum levels of TNF-α and of its soluble receptors sTNF-R55 and sTNF-R75 were found to be elevated in the patients. Furthermore, these inflammatory parameters were correlated with the degree of arterial hypoxia. The skeletal musculature has been proposed to be involved in the systemic inflammation associated with COPD. Specifically, an inflammatory response has been observed in connection with exercise. Thus, during exercise, the levels of markers of oxidative stress in the blood of healthy volunteers [231], as well as patients with COPD [232] are increased. Furthermore, analyses of biopsies of diaphragm tissue from patients suffering from moderate-to-severe COPD and from healthy control individuals revealed lower levels of glutathione (GSH) in the patients, suggestive of a reduced antioxidative capacity at rest [233]. Similar differences with respect to the level of reduced GSH in muscle biopsies have been reported [234, 235]. In another study concerning the serum levels of proinflammatory mediators in severely obstructed patients with COPD and healthy controls prior to and after exercise, an increase in the level of TNF-α was observed in the patients, but not in the control group [236]. However, contradictory results in this respect have also been obtained [235]. Together, this handful of studies indicates that skeletal muscle contributes to the systemic
inflammation associated with COPD, although the clinical relevance of exercise-induced inflammation remains to be clarified.

1.9.3 Clinical consequences of systemic inflammation in conjunction with COPD

The systemic inflammation present in patients with COPD may be associated with various changes, including weight loss, atrophy of skeletal muscle, endothelial dysfunction, cardiovascular disease and osteoporosis, among others. Such weight loss appears to be a consequence, at least in part, of the increase in resting expenditure of energy (REE) demonstrated by certain of patients patients with COPD [215]. Although this subgroup does exhibit evidence of systemic inflammation, elevated consumption of oxygen and energy by the respiratory muscles themselves has also been proposed to contribute, to varying degrees, to their enhancement in REE. The systemic inflammation in the patients suffering from involuntary weight loss involves elevated levels of acute phase reactants and of circulating cytokines (primarily IL-8 and TNF-α). Both TNF-α [237, 238] and the local hypoxia associated with this inflammation [237] may promote muscle atrophy, possibly via apoptosis in skeletal muscles, which has been found to be associated with weight loss in patients with COPD [106].

Furthermore, systemic inflammation is known to be involved in the pathogenesis of cardiovascular disease [224], i.e., circulating levels of inflammatory markers, including CRP, show a positive correlation to the risk for future cardiac pathology [239, 240]. Patients with COPD in a stable state exhibit elevated circulating levels of CRP and these levels, together with those of other prothrombotic factors such as fibrinogen [218], rise even further during exacerbation [241]. Together, these findings may account for the elevated risk for pathological vascular events associated with COPD and, in particular, for the enhanced risk of death within a few months after hospitalization for acute exacerbation of this disease [242]. There is also a possible relationship between systemic inflammation in patients with COPD and osteoporosis. The risk of osteoporosis in connection with steroid treatment is well known [243]. Patients with COPD seem, however, to have an increased risk of osteoporosis even in the absence of steroid treatment, and this elevated risk is associated with enhanced circulating levels of TNF-α [244], although no cause-and-effect relationship between these two parameters has been established.

1.10 REMODELING OF THE AIRWAYS AND LUNG PARENCHYMA

Remodeling involves processes leading to structural alterations of airways and parenchyma. These processes may be preceded by inflammation, but in the case of certain diseases, such as idiopathic pulmonary fibrosis, remodeling can occur in the absence of substantial inflammation [245]. The remodeling associated with the development of COPD is initiated by the direct tissue injury caused by tobacco smoke, which is further aggravated by inflammatory and oxidative products released by epithelial cells and macrophages. Although inflammatory processes of this nature are normally followed by more-or-less complete healing, repeated tissue damage may give rise to deficiencies in healing and abnormal reconstruction of airways and parenchyma that may lead to impaired pulmonary function.

To at least some degree, the remodeling processes associated with COPD are irreversible, in large part because of the development of tissue fibrosis, in connection
with which the fibroblast plays a key role. Fibroblasts are activated and attracted to the site of damage by chemoattractants released by the epithelial cells, such as TGF-β and fibronectin. In connection with chronic tissue injury, the fibroblasts proliferate and begin themselves to produce and release components of the extracellular matrix, including collagen and proteoglycans. In addition, certain subgroups of fibroblasts (referred to as myofibroblasts) possess contractile properties [246] and are thus capable of causing contraction of the wounded mucosa.

In the case of small airways, the fibrotic processes described above can lead to a deposition of collagen in the periphery of the bronchiolar wall, so-called peribronchiolar fibrosis, which represents an irreversible aspect of the thickening of the walls of these airways. Such thickening, to which infiltration by inflammatory cells also contributes, is an important example of a remodeling process associated with COPD that is related to deterioration of pulmonary function [123]. Further examples of this type of remodeling in the small airways include the loss of alveolar attachments [79] and metaplasia of goblet cells, both of which are also thought to contribute to the limitation in airflow, although probably to a lesser extent than wall thickening.

The remodeling of pulmonary parenchyma connected with COPD includes destruction of alveolar walls, i.e., emphysema. Development of this condition is promoted by the activities of proteases capable of degrading the extracellular matrix. One protease of significance in this regards is neutrophil elastase (NE), which is synthesized by neutrophil granulocytes and released when these cells are stimulated [247, 248]. Additional proteases such as cathepsins and matrix metalloproteinases (MMPs) also contribute to pulmonary remodeling [142]. Apoptosis mediated by lymphocytes may also contribute to the development of emphysema, as suggested by the correlation between the enhanced numbers of cells undergoing apoptosis and CD8+ T-cells in alveolar walls affected by this condition [164].
Figure 5
Simplified schematic illustration of chemotactic factors capable of attracting the key inflammatory and profibrotic cells to an intrapulmonary site of inflammation. Neutrophils are attracted by interleukin-8 (IL-8), growth-related oncogene-α (GRO-α) and leukotriene B4 (LTB4); monocytes by macrophage-chemotactic protein (MCP-1); CD8+ lymphocytes by interferon-γ-inducible protein (IP-10), monokine induced by interferon-γ (Mig) and the interferon-inducible T-cell α-chemoattractant (I-TAC); and fibroblasts and stimulated by, i.e., IL-8 and tumour growth factor β (TGF-β). The release of proteases, including matrix metalloproteinase (MMP), neutrophil elastase (NE) and cathepsins, contributes both to tissue degradation and hypersecretion of mucus, CD8+ T cells have the capacity to induce apoptosis whereas fibroblasts are involved in the development of peribronchiolar fibrosis present in obstructive bronchiolitis.
1.11 ASSESSMENT OF COPD FOR RESEARCH PURPOSES EMPLOYING COMPUTERIZED TOMOGRAPHY

Computerized tomography (CT) can provide transversal anatomical images of, e.g., the thoracic organs. In such an image the X-ray attenuation of each predefined volume of tissue (voxel) represents a picture element (pixel). These attenuation values, expressed in Hounsfield Units (HU), are defined relative to the values for air (-1000) and water (0) and modern CT scanners can detect attenuation values between -1024 and +3000. The attenuation data, also referred to as tissue density, are organized into a matrix by a computer program to produce the CT images.

1.11.1 Quantitative assessment of larger airways

Automatic and objective procedures for quantifying both the diameter and thickness of the wall of airways are now available. The most commonly used of these methods is the “full–width-at-half maximum” technique. The software allows the investigator to control a marker on the computer screen of the CT. This marker is placed in the lumen of the airway to be examined. The marker registers the attenuation inside this lumen. Thereafter, this marker is moved from its initial position towards the airway wall and this movement is repeated in all directions. The density from the central point to the pulmonary parenchyma outside of the airway wall is recorded. The point at which the attenuation values along the line of movement are half of the maximal value is defined as the inside of the airway wall. Where these values subsequently fall below the half maximum is defined as the outside of this wall. The distance between the inside and outside positions is thus the thickness of the airway wall [71].

In patients with COPD, changes in airway diameter and wall thickness are correlated to the extent of airflow obstruction as measured by FEV1 (% of the predicted value) [71]. In addition, such patients with concurrent chronic bronchitis exhibit more pronounced thickening of the airway wall than do patients without chronic bronchitis [249]. This “half-max” approach tends to overestimate the area of the wall and underestimate the luminal area, due to the limited spatial resolution of CT and the fact that not all airways are situated parallel to the “z-axis” of the scanner. However, procedures for correcting this inaccuracies have been proposed [250, 251] and methods for more accurate measurement of airway wall thickness will hopefully be developed.

1.11.2 Quantitative assessment of smaller airways

Quantitatively, the most important site of airflow obstruction is in airways with a diameter of less than 2 mm [15, 16]. Thus, the correlation between the thickness of the walls of large airways and pulmonary function (see above) is somewhat surprising [71]. The positive correlation between the thickness of the walls of large airways and of bronchioles has been examined in patients undergoing lung surgery to remove nodular lesions [72]. In this study, all of the patients where subjected to a CT scan prior to surgery and wall thickness in the resected lung tissue was characterized histologically. Assessment of wall thickness by CT was found to agree with the wall thickness of both large and small airways as assessed histologically. Certain of the inflammatory changes that contribute to thickening of the airway wall occur in both large and small airways, e.g., the presence of increased numbers of CD8+ T lymphocytes and B-lymphocytes. One investigation concluded that thickening of the walls of bronchi is correlated to measures of airflow obstruction, as well as to inflammatory changes in bronchioli [73],
thus suggesting that, at least, to some extent, the same inflammatory process occur in parallel in both airways of different sizes.

There have been some attempts to assess the thickness of the walls of small airways, but these procedures are characterized by more errors and a larger degree of variability than in the case of large airways. However, measurement of lung density during expiration has been found to be correlated to the collapse of small airways rather than the degree of emphysema [252], thus allowing prediction of the diameter of these smaller airways diameter. During expiration, air is trapped in the most distal airways as the bronchioli close. Patients with COPD exhibit enhanced narrowing of bronchioli, which leads to an increased heterogeneity in lung density during expiration. However, the value of this approach is still uncertain, mainly due to the difficulties involved in holding the breath when the volume of the lungs is low.

### 1.11.3 Emphysema

As discussed above, emphysema is defined as the abnormal and permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls, in the absence of obvious fibrosis [25]. For quantitating the degree of emphysema in a noninvasive manner, different techniques for the assessment of CT scans have been proposed.

#### 1.11.3.1 Subjective quantification

Subjective evaluation of emphysema is based on visual assessment of the CT scan concerning the relative area demonstrating reduced attenuation and vascular disruption. Such visual grading of the extent of emphysema correlates with the extent determined on the basis of macroscopic lung sections [74, 253-255], as well as with microscopic grading [254]. The limitation of this approach is that it does not objectively assess the emphysema. In addition, this grading system indicates the severity, rather than the extent of the emphysematous lesions [256].

#### 1.11.3.2 Objective quantitation of emphysema by measurement of attenuation

The tissue density of the lungs, as revealed by CT, has been employed in attempt to objectively quantitate the degree of emphysema in patients with COPD used in different techniques [82-84, 257-260]. The distribution of lung tissue densities is shifted towards lower values in patients with emphysema compared to those patients without [260], with microscopic evaluation of emphysema being correlated to the lowest 20% of the density histogram [83].

Another common approach is to employ computer software that highlights pixels with values of tissue density below a predefined threshold [82, 84, 257-259]. Subsequently, this software automatically calculates the area of the highlighted region in relationship to the total pulmonary area. This technique has been reported to be able to predict the macroscopically determined degree of emphysema [84].
2 AIMS

The general aim of this thesis was to characterize the inflammatory response in different body compartments in patients with COPD.

The specific aims were:

- To investigate the recovery of bronchoalveolar lavage (BAL) fluid instilled during bronchoscopy in patients with COPD, smokers with normal pulmonary function and non-smokers, and to correlate this recovery to measures of emphysema.

- To analyze the phenotype of alveolar macrophages regarding the expression of cell surface molecules, employing flow cytometry.

- To characterize the inflammatory cells infiltrating the epithelium of bronchial mucosa in patients with COPD. Specifically, to immunohistochemically analyze samples of airway mucosa obtained by bronchoscopy.

- To evaluate the expression of cell surface molecules and functional properties of circulating phagocytes in COPD.
3 CONSIDERATIONS CONCERNING THE SUBJECTS AND METHODOLOGY

Here, only a brief description of the approaches employed in the studies on which this thesis is based is given. More detailed descriptions can be found in the original articles.

3.1 PATIENTS AND CONTROL SUBJECTS (PAPER I-IV)

The studies described here all involved the same groups of patients with COPD and control subjects. Different aspects of inflammation in different body compartments were investigated in the four papers.

3.1.1 Characteristics of the patients and control subjects

Twenty-three patients with COPD, 39-69 years of age with a mean age of 57, were recruited for these studies. All of these patients had a tobacco consumption of more than 10 pack-years and exhibited a post-bronchodilator FEV1/VC ratio of <70, as well as FEV1 value <70% of the predicted. The chest X-rays of all patients demonstrated no signs of pulmonary diseases other than COPD.

Age-matched smokers without COPD (n=16) and non-smokers (15) served as control subjects. These smokers had the same level of cigarette consumption (assessed in terms of pack-years) as the patients.

None of the patients or control subjects had a history of allergy or asthma. All patients and controls were in a stable condition and none of the participants had been administered corticosteroids during the three-month period immediately preceding the studies.

Ten of 23 patients with COPD fulfilled the criteria for a diagnosis of chronic bronchitis, i.e., coughing and production of mucus on most days of each of at least three months during the previous two years.

3.1.2 Tests of pulmonary function

All of the participants performed a dynamic spirometry in a standardized manner. Slow vital capacity and forced vital capacity were determined both before and 10 minutes after inhalation of two 0.5 mg doses of terbutalin (Bricanyl®) and reversibility was also calculated.

The thoracic gas volume at end-tidal expiration was measured in a constant-volume body plethysmograph by panting at a slow frequency against a closed shutter. The diffusion capacity of carbon monoxide (DLCO) was obtained by the single-breath procedure. All these parameters are expressed as percentages of the values predicted according to the relevant reports by the European Coal and Steel Community.

3.1.3 Assessment of emphysema

In the present project, all of the patients with COPD underwent examination by computed tomography (CT). Spiral CT was performed with a slice thickness of 10 mm and the severity of emphysema was expressed as an emphysema index. The CT images were analyzed with a semi-automatic image-processing program that detected and outlined the boundaries of the lung. Subsequently, the computer program calculated the area of the CT image that exhibited tissue attenuation within the predetermined range of HU (i.e., from –1024 to –950 HU) (Figure 7).
Figure 7
Computerized tomography (CT) of two patients with COPD studied here. These patients exhibited the same degree of obstruction, as determined by FEV1. High resolution CT of patient A (with a FEV1 value equal to 49% of the predicted) demonstrated a low degree of emphysema (upper panel A) and tissue density as presented by the histogram from the corresponding level (lower panel A). In contrast, patient B (FEV1 = 48% of predicted) exhibited severe emphysematous lesions (upper panel B) and a dramatically lower tissue density (lower panel B). The vertical bars in the histograms in the lower panels indicate the threshold value (-950 Hounsfield units) for the emphysema index.

3.2 BRONCHOSCOPY (PAPERS I-III)

Bronchoscopy was performed on an outpatient basis, following an overnight fast. For this purpose, morphine-hyoscine was injected intramuscularly prior to the examination and the bronchoscope then inserted nasally after topical anesthesia. The participants did not receive any other medication prior to this bronchoscopy.

3.2.1 Bronchoalveolar lavage (Papers I and II)

Bronchoalveolar lavage (BAL) was performed by wedging the bronchoscope into a subsegment of the middle lobe. A 50-ml aliquot of sterile phosphate-buffered saline (PBS) at 37°C was instilled and gently suctioned out again using a negative pressure of -40 to -50 mmHg, following which this procedure was repeated four more times. The fluid was collected in a silicone treated bottle kept on ice, which was immediately transported to the laboratory.

3.2.2 Biopsies of bronchial mucosa (Paper III)

Biopsy specimens were removed with a pulmonary biopsy forceps with smooth-edged jaws. Four to six biopsies of the endobronchial mucosal were taken from each subject, all from the sub-segmental carinae of the upper left lobe or the apical segment of the
lower left lobe. These biopsies were then immediately immersed in ice cold acetone with the inclusion of the protease inhibitors. The biopsies were subsequently processed into glycol methacrylate (GMA) resins.

3.3 ANALYSES OF BRONCHOALVEOLAR FLUID (PAPER II)

3.3.1 Handling and differential counting of bronchoalveolar fluid

The fluid obtained by bronchoalveolar lavage (BAL) was filtered through a Dacron net to remove large debris and the volume recovered then measured. The cells present in this fluid were pelleted by centrifugation and the supernatants poured off. The pellets were subsequently resuspended in RPMI medium, the cells were counted in a Bürker chamber and total cell viability was determined on the basis of Trypan blue exclusion. For differential counts, cell smears were prepared by cytocentrifugation, after which the cells were stained with May-Grünwald-Giemsa reagents and 500 cells in each smear classified.

3.3.2 Immunostaining and quenching of the auto-fluorescence of alveolar macrophages

The cell suspension obtained as described above was placed in conical tubes (1x10^6 cells/tube) and washed by centrifugation. An appropriate amount of monoclonal antibodies (MoAbs) were then added to each pellet, followed by incubation on ice in the dark. A panel of antibodies recognizing proteins involved in various macrophage functions was employed. A secondary antibody was added, and the immunostained cells were fixed by incubation with paraformaldehyde (PFA). In the case of subjects who smoke, the autofluorescence of their alveolar macrophages interferes with the detection of fluorochrome-labelled antibodies. In order to facilitate flow cytometric analysis of these cells, their autofluorescence was first quenched. This quenching technique includes incubation with n-octyl-β-D-glucopyranoside (OG) followed by treatment with crystal violet (Figure 6).
3.3.3 **Flow cytometric determination of the phenotype of alveolar macrophages**

Before analysis of each cell suspension in the flow cytometer, the instrument was carefully cleaned, and in addition, it was calibrated daily with standardized fluorescent particles. The fluorescence of the macrophage population in each sample, gated on the basis of the light-scattering properties of these cells, was quantitated in arbitrary units and expressed as mean fluorescent intensity (MFI). The MFI of untreated and quenched alveolar macrophages from each participant was also determined. Subsequently, the MFI of each cell suspension labeled with antibody was corrected by subtracting the background fluorescence, which was obtained by labeling with irrelevant antibody of the same isotype.

3.4 **ANALYSES OF LEUCOCYTES AND SERUM (PAPER IV)**

A sample of venous blood was drawn from all participants in the morning after fasting overnight. The erythrocytes were lysed with NH₄Cl and the leukocytes subsequently washed.

3.4.1 **In vitro stimulation**

Leukocytes were stimulated *in vitro* by incubation with TNF-α or, in the cases of controls, with buffer alone. These cells were then incubated in fMLP (N-formylmethionyl-leucyl-phenylalanine) or in buffer alone.
3.4.2 Assay of intracellular production of oxygen radicals by leukocytes

To assay the intracellular production of oxygen free radicals, leukocytes were incubated in 2’, 7’-dichlorofluoresceindiacetate, after which they were stimulated with fMLP or TNF-α or incubated with buffer alone (controls). This activation was terminated by the addition of cold phosphate-buffered saline.

3.4.3 Immunostaining of leucocytes

In order to evaluate the expression of CD11b and CD35 on the surface of leukocytes, these cells were incubated with the appropriate monoclonal antibodies. Parallel incubation of the same preparation of cells with isotype-matched irrelevant antibodies at the same concentrations were used to determine background fluorescence. Thereafter, the cells were analyzed by flow cytometry.

3.4.4 Flow cytometry analysis of plasma leukocytes

For assessment of the intracellular oxidative burst, as well as of the level of expression of surface molecules, stained cell preparations were subjected to flow cytometry. Lymphocytes, monocytes and granulocytes could be distinguished from one another on the basis of their characteristic light-scattering properties. In order to distinguish between the neutrophils and eosinophils present in the granulocyte population, depolarised side-scatter was measured after dichroic plastic sheet in the forward-scatter position. By subsequently shifting the filters for detection, two-colour analysis could be performed.

3.4.5 Serum analyses

Determination of the levels of sICAM, TNF-RII and oxidized low-density lipoproteins (oxLDLs) in serum was achieved by enzyme-linked immunosorbent assays (ELISA).

3.5 STATISTICAL ANALYSES (PAPERS I-IV)

In all four papers the descriptive statistics are presented as medians and interquartile ranges, except for age, where the mean ± SD is given. Statistical comparisons of differences between two groups were performed with the Mann-Whitney test. Differences between three groups were analyzed using the Kruskall Wallis test, followed by post-hoc analysis with the Nemenyi test (Papers I-III). After correction on the basis of the Nemenyi test, a p-value of <0.05 was considered to be statistically significant.

Correlations between assessments of inflammation and clinical features were calculated according to Spearman (Papers I-III). To compare assessments analysis of variance for repeated measures was employed, of different stages of activation, followed by the Tukey honest significant difference test (Paper IV).
4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Results

The recovery of BAL fluid from the patients with COPD was lower than from the other two groups (p<0.001). This recovery from patients with low degree of emphysema (i.e., an emphysema index <1) was higher than from patients with more pronounced emphysema (index >1) (p<0.001). The recovery of BAL fluid was negatively correlated to both the index of emphysema (p<0.001) and positively correlated to the DLCO (p<0.01) and the FEV1/VC ratio (p<0.05). There was no significant correlation between the recovery and the FEV1 value itself. Comparison of the prognostic power of these different variables employing stepwise regression analysis revealed that the emphysema index possessed higher prognostic power than either DLCO or the FEV1/VC ratio.

4.1.2 How does the threshold value for emphysema index influence the assessment of emphysema?

In the present study, calculation of the emphysema index was based on a threshold value of -950 HU and a CT slice thickness of 10 mm. Previous studies have employed differing values for these parameters. By choosing a higher attenuation value as the threshold, the relative area of the lung investigated that present with pixels below the threshold increases, and the emphysema index will increase accordingly. Similarly, analysis of thinner CT slices (e.g., 2 mm instead of 10 mm), the overall attenuation will decrease, thus resulting in a higher emphysema index. One combination of these parameters that is commonly used is a 1mm slice thickness and a threshold of -950 HU [82, 261]. In fact, this combination was found in one study to predict the macroscopic degree of emphysema most accurately [82]. However, that study [82] did not examine only patients with COPD and only mild degrees of emphysematous lesions were found. Studies involving only patients with COPD and more severe emphysema have calculated the emphysema index using a slice thickness of 10mm and a threshold of -950 HU [262-264], as was done here.

4.2 PAPER II

4.2.1 Results

This investigation focused specifically on the phenotypes of alveolar macrophages, cell recovery and differential counts in BAL fluid. Macrophages from patients expressed lower levels of the costimulatory molecule CD86 and the adhesion molecule CD11a than the corresponding cells from smokers without COPD. In addition, the CD16 protein associated with the Fc-receptor was expressed at reduced levels by the macrophages from patients.

We also found that the levels of expression of the adhesion molecules CD11c and CD54 by the macrophages from smokers without COPD were higher and lower, respectively, compared to non-smokers. Furthermore, the total we found an increase in BAL cell number in COPD and smoking controls compared to non-smokers. In addition, the neutrophil percentage in COPD was higher compared to smokers. In this study we specifically investigated alveolar macrophage phenotype, cell recovery and differential counts in BAL fluid. The analysis of macrophage phenotype showed a
lower expression of the costimulatory molecule CD86 and of the adhesion molecule CD11a in COPD compared to “healthy” smokers. Additionally, the Fc-receptor associated CD16 was less expressed in COPD compared to non-smokers. We also found that the expression of the adhesion molecules CD11c was higher and CD54 was lower in “healthy” smokers compared to non-smokers. Furthermore we found an increase in BAL cell number in COPD and smoking controls compared to non-smokers. In addition, the neutrophil percentage in COPD was higher compared to smokers.

Figure 8
Correlation between the level of expression of CD86 by alveolar macrophages (presented as mean fluorescence units = MFI) and pulmonary function assessed on the basis of FEV1 (% of predicted). Each point represents either a patient with COPD or a smoker with normal pulmonary function. R:0.73 and p<0.001, according to the Spearman test.

4.2.2 What is the functional relevance of the altered phenotype of alveolar macrophages associated with COPD?

Inflammatory cells can be altered not only with respect to their expression of cell surface molecules, as documented here, but also with regards to their functional capacities. Alveolar macrophages exhibit a decreased capacity for responding to infectious stimuli [265] and one of the major contributors to morbidity from COPD is exacerbation. Certain of the alterations in the phenotype of alveolar macrophages observed here (i.e., in the expression of CD86 and CD11a) are indicative of a reduction in functional capacity.
4.2.3 The potential acute influence of smoking on the phenotype of alveolar macrophages

All of our participants were asked whether they had smoked or not during the 12-hour period immediately preceding the performance of bronchoscopy. Three of the 9 patients with COPD had smoked and the corresponding figure for smokers without COPD was 8 of 10. Therefore, even though the present investigation was not designed with the express purpose of characterizing the acute effects of smoking on the phenotype of alveolar macrophages and it is, of course, impossible to draw definitive conclusions on the basis of such small subgroups, some preliminary observations of potential interest can be made.

In general, all of the participants, both with and without COPD and those who had and had not smoked prior to bronchoscopy, exhibited similar patterns of expression of surface molecules on their alveolar macrophages, with symmetrical distributions centered around the median for all of these individuals combined. The one possible exception to this conclusion may be the expression of the adhesion molecular CD11a by patients with COPD. One of these three patients who had smoked prior to bronchoscopy demonstrated a level of CD11a that was slightly above the median, whereas the alveolar macrophages from the other two exhibited the highest and second highest levels of expression of this same antigen. Clearly, more extensive studies addressing this particular question are required.

4.3 PAPER III

4.3.1 Results

Here, intraepithelial inflammation in the bronchial mucosa of patients with COPD and control subjects was examined. Biopsies of bronchial mucosa were taken by bronchoscopy and the tissue samples thus obtained were analyzed immunohistochemically. We found elevated numbers of both CD4+ and CD8+ intraepithelial lymphocytes in the bronchial biopsies from patients with COPD.

4.3.2 Do these enhanced numbers of intraepithelial T-cells play a role in the pathogenesis of COPD?

T-lymphocytes produce several mediators and possess several functions that might amplify the inflammatory response associated with COPD and thereby contribute to the pathogenesis. In addition, one should be aware that a cross-sectional study only provides a “snap-shot” of the state of inflammation at the time of the sampling and that mucosal inflammation is likely to change over time. Thus, the increment in inflammatory cells observed in connection with stable COPD is probably even more pronounced during exacerbation. The intraepithelial mucosal inflammation present under stable conditions might be a small fraction of the inflammation present during acute infections.

However, this increment in the numbers of intraepithelial T-cells might also be a reaction to chronic subclinical infection, i.e., an adequate and protective reaction rather than a contribution to the pathogenesis of airway obstruction. Further characterization of the intraepithelial lymphocytes associated with COPD with regards to their phenotype, production of cytokines and state of activation should shed light on this matter.
Figure 9
Representative photomicrographs of the bronchial epithelium of patients with chronic obstructive pulmonary disease, illustrating immunohistochemical staining of tissue biopsies for CD4+ (A) and CD8+ (B) cells.
As expected, the staining is in the form of a ring outlining the cells.
4.3.3 The acute and chronic effects of smoking on intraepithelial lymphocytes

All four patients with COPD who had quit smoking had numbers of intraepithelial CD3+ T-cells that were lower than the median for the entire group of patients with this disease. In addition, in three of these the numbers of CD8+ cells situated intraepithelially were also lower, whereas the distribution of their numbers of CD4+ cells was more symmetrical with respect to the median.

In the case of the five patients with COPD who had smoked prior to bronchoscopy, the distributions of their numbers of intraepithelial CD4+ and CD8+ cells did not differ in any apparent manner from those of the entire group of patients with this disease. However, in all five the numbers of CD3+ cells were higher than the median. These possible effects should be explored further employing larger groups of patients.

4.4 PAPER IV

4.4.1 Results

In this investigation, the oxidative burst and expression of surface molecules by peripheral leukocyte subpopulations in patients with COPD as well as serum levels of proinflammatory factors were examined. We found that the oxidative burst by neutrophils, monocytes and eosinophils from these patients and from smokers without the disease was lower than in the case of non-smokers. Although the levels of expression of CD11a and CD35 by leukocytes did not differ between these three groups, leukocytes from patients with COPD, who had smoked on the morning of the examination, exhibited reduced capacity to mobilize their surface receptors. Finally, an increase in the serum level of the soluble adhesion molecule sICAM-1 was observed in the patients.

4.4.2 Oxidative stress and the pathogenesis of COPD

Indicators of oxidative stress have been found to be associated with the pathogenesis of airflow obstruction, i.e., previous comparative studies have detected higher levels of these markers in the blood of patients with COPD than in smokers without this disease. Furthermore, significant correlations between levels of oxidative stress and pulmonary function have been obtained. However, an increased level of oxidative stress may simply be the result of the enhanced inflammation connected with COPD and thus an innocent bystander. To dates, no longitudinal studies have demonstrated that enhanced oxidative stress can predict the development of COPD.

A number of studies have found that patients with COPD exhibit signs of systemic inflammation. Here, decreased production of intracellular oxygen radicals by the leukocytes of patients with COPD and of non-obstructed smokers was observed in comparison to non-smokers. This potentially reduced capacity for generation of intracellular oxygen radicals by phagocytes might explain, at least in part, the acute and chronic infections associated with COPD.

However, contradictory findings with respect to this oxidative burst indicate that the choice of subjects and methodology is an important factor in this context. In fact, our present observations emphasize the importance of controlling for the acute effects of tobacco smoking, as well as for the potential effects of in vitro cell separation.
5 CONCLUSIONS

- The volume of BAL fluid recovered from patients with COPD was lower than from both groups of controls. From patients with a low degree of emphysema, a larger volume of BAL fluid was recovered than from those with high degree of emphysema. This recovery correlated negatively to the index of emphysema and positively to other, indirect measures of emphysema (i.e., the DLCO and the FEV1/VC ratio).

- Alveolar macrophages exhibit a different phenotype among patients with COPD, associated with less expression of the co-stimulatory molecule CD86 and of CD11a (adhesion) than was found in smokers without disease. In addition, smoking per se may have an influence on the phenotype pattern in alveolar macrophages.

- The epithelium of bronchial mucosa displayed increased numbers of lymphocytes in patients with COPD, and the numbers of CD4+ cells were higher in COPD compared to both control groups, whereas the numbers of CD8+ cells were higher in COPD compared to non-smokers.

- Neutrophils in peripheral blood from patients with COPD and smokers with normal pulmonary function showed decreased oxidative burst after ex vivo stimulation, and vascular involvement was determined as increased sICAM-1 in COPD. In patients with COPD, a reduced ability to mobilize the adhesion molecule CD11b on neutrophils, monocytes and eosinophils was observed in those who had smoked the same morning.
6 FUTURE PERSPECTIVES

6.1 LONGITUDINAL FOLLOW-UP ON CROSS SECTIONAL DATA

The four papers in this thesis are all based on cross sectional data from COPD patients and matched controls. The COPD patients have been carefully characterized with dynamic spirometry, body plethysmography and CT. This has given base-line data on airways obstruction, the degree of hyperinflation, the degree of emphysema et.c. All participants have made a follow-up three years after the inclusion to the study. At this occasion, a dynamic spirometry has been performed and the number of exacerbations during the three year period has been registered. One further check-up will be performed six years after the initial cross-sectional study. During the six year control, dynamic spirometry and body plethysmography will be done. In this manner, longitudinal data regarding disease progression will be obtained. By correlating inflammatory markers from different compartments with longitudinal measurements of the disease, the predictive value of inflammatory markers might be determined.

6.2 COMPARATIVE INVESTIGATIONS DESIGNED TO IDENTIFY PROTEIN MEDIATORS OF RELEVANCE TO CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Proteomics is the study of the entire protein complement of the genome (the proteome) in a biological system, requiring different technical and methodological approaches. Proteomics techniques have the capacity to simultaneously study a subset of all proteins as opposed to a single protein, and consequently the potential of identifying patterns of protein expression. Since such patterns of protein expression may be associated with processes involved in disease development, e.g. in COPD, proteomics studies of BAL fluid, lung tissue or blood may reveal markers of early disease.

In attempt to identify mediators that may participate in the development and/or maintenance of COPD, bronchial biopsies, brush samples and BAL fluid from patients with this disease and control subjects will be examined by proteomics. In particular, alterations in the proteomes of the individual inflammatory cell types involved in COPD, as well as of airway epithelial cells and airway exudates will be characterized. This new cross-sectional study including bronchoscopy in patients with COPD and control subjects is planned to start during the spring of 2007.

6.3 FUNCTIONAL CHARACTERIZATION OF ALVEOLAR MACROPHAGES

A handful of studies report an altered alveolar macrophage phenotype in COPD [266, 267]. Studies investigating macrophage functional deficiency in COPD are scarce. One study [265] reported a decreased cytokine response after stimulation ex-vivo. One finding of the present thesis was the decrement of macrophage expression of CD86 and CD11a in COPD. This may indicate a decreased capacity of antigen presentation in COPD patients compared with non-obstructed smokers.

In this context, investigating the capacity of AM antigen presentation to autologous T-cells would be an interesting complementary study. A set-up for this kind of investigation would include bronchoscopy and BAL in patients with COPD, matched smokers and non-smokers for acquiring alveolar macrophages. In addition, T-lymphocytes would be collected from peripheral blood in each participant. The next
step would be the addition of a bacterial antigen most individuals already have been immunized against, i.e. BCG. An estimation of macrophage antigen presenting capacity could then be determined by analyzing INF-γ by use of ELISPOT. For comparison, the same procedure would be performed with peripheral blood monocyte and autologue T-cells. These investigations would potentially provide data on whether or not the expression of macrophage surface molecules correlates with antigen presenting ability. Another aspect of the macrophage functional capacity regards the ability to induce clonal T-cell expansion. Under normal conditions, macrophages are considered to be poor antigen presenting cells. However, alveolar macrophages acquired from patients with chronic inflammatory diseases have been found to induce a clonal T-cell expansion ex vivo [268]. Similar studies could be done on alveolar macrophages from COPD patients, and this could be compared with the response in smokers and non-smokers.

The last few years, macrophages have been proposed to exhibit polarized functional properties [269, 270]. Mirroring the Th1/Th2 nomenclature, this has been referred to as M1 and M2 cells. Classically activated M1 macrophages have an IL-12^high^, IL-23^high^ and IL-10^low^ phenotype. In contrast to this, M2 macrophages have been proposed to share a IL-12^low^, IL-23^low^ and IL-10^high^ phenotype. By analyzing these features in BALF macrophages, different macrophage polarization might be detected in COPD patients compared to non-obstructed smokers.

In three-dimensional collagen gel system the ability of fibroblasts to contract the gel is an established method for investigating potentially profibrotic mediators in vitro. Conditioned medium from macrophages has been reported to contract collagen gels [271]. Thus, one possibility is that BALF macrophages from COPD patients release more profibrotic mediators compared to controls. This would potentially be possible to quantify by acquiring BALF macrophages form COPD patients and matched control, and subsequently quantify the gel contracting ability in a three-dimensional collagen gel system.

6.4 COMPLEMENTARY ANALYSES OF INTRAEPITHELIAL INFLAMMATION

Paper III of this thesis reports increased numbers of intraepithelial lymphocytes in COPD. Lymphocytes situated intraepithelially seem to constitute a distinct subset of cells. Characteristics of these cells include a longer life time, and an increased expression of the adhesion molecule CD103. The expression of CD103 may be of importance for establishing a firm cell-cell contact. Moreover, the expression of CD103 can be induced by TGF-β, a mediator that is found in increased concentrations in COPD. In order to further clarify the mechanisms behind the presence of lymphocytes intraepithelially, multiple immunostaining in consecutive sections of biopsies might contribute. By staining for CD3, CD4, CD8, CD103 and TGF-β, this would allow a characterization of the expression of CD103 in COPD. Furthermore, this would possibly determine whether epithelial infiltration of airway epithelium in COPD is associated with a release of TGF-β. Additional staining for Th1/Th2 cytokine profile might give further information about these cells.
7 SAMMANFATTNING PÅ SVENSKA

Kroniskt obstruktiv lungsjukdom (KOL) karakteriseras av en irreversibel nedsättning av lungfunktionen och orsakas i en majoritet av fallen av rökning. Rökning i sig orsakar en kronisk inflammation i luftvägar och i lungorna. Flera inflammatoriska celler producerar ämnen med potential att bidra till utvecklingen av de sjukliga förändringar som patologiskt-anatomiskt karakterisera KOL. Det övergripande syftet med de arbeten (I-IV) som ingår i föreliggande avhandling är att beskriva den inflammation som finns på olika nivåer i luftrörsträdet, lunga och i perifert blod hos patienter med KOL. Genom att även inkludera ”friska” rökande försökspersoner och icke-rökande försökspersoner syftar studien till att finna inflammatoriska markörer kopplade specifikt till sjukdomen.

Bronchoalveolar lavage in COPD: fluid recovery correlates with the degree of emphysema.


Different inflammatory cell pattern and macrophage phenotype in chronic obstructive pulmonary disease patients, smokers and non-smokers.

Koncentrationen av inflammatoriska celler i bronkoalveolärt lavage (BAL) är förhöjd hos alla rökare. Den dominerande celltypen i BAL är den alveolära makrofagen. Makrofager har en rad funktioner med potentiell betydelse för patogenesen vid KOL. Målet med denna delstudie var att karaktärisera den inflammatoriska profilen och att fenotypa makrofager i BAL från KOL-patienter och kontroller. Sexton patienter med KOL inkluderades. Åldersmatchade ”friska” rökare (n=10) och icke-rökare (n=9) var kontroller. Patienterna genomgick bronkoskopi med BAL i ett av mellanlobens subsegment. Differentialräkning utfördes på cytopspinpreparat från BAL-vätska. Efter släckning av intracellulär autofluorescence bestämdes fenotypen hos de alveolära makrofagerna med flödescytometri. Vid analysen användes en antikroppspanel som speglade makrofagfunktioner som adhesion (CD54, CD11a-c), aktivering (CD14, CD71), fagocytos (CD16), antigenpresentation (HLA klass II) och co-stimulation (CD86,CD80). Den co-stimulatoriska molekylens CD86 visade lägre uttryck hos patienter med KOL jämfört med ”friska” rökare. Adhesionsmolekylens CD11a var mindre uttryckt på AM vid KOL jämfört med båda kontrollgrupperna.
Adhesionsmolekylen CD 11c skiljde sig inte åt mellan AM vid KOL jämfört med ”friska” rökare, men båda dessa grupper visade högre uttryck av jämfört med ickerökare. CD54 var mindre uttryckt på AM från rökande kontroller jämfört med icke-rökare, medan CD16 (fagocytos) befanns ha ett lägre uttryck vid KOL jämfört med icke-rökare. Analyserna för övriga antigen visade inte signifikanta skillnader mellan KOL och kontroller (CD11b, CD14, CD71, CD80 och HLA klass II). Det nedsatta makrofaguttrycket av den co-stimulatoriska molekylen CD86 och adhesionsmolekylen CD11a vid KOL speglar potentiell en nedsatt co-stimulerande resp. adhererande kapacitet. Resultaten av denna studie pekar på att vidare studier avsände makrofagfenotyp är intressant vid framtagandet av markörer för sjukdomsutveckling vid KOL.

**Increased intraepithelial CD4+ and CD8+ T-cells in COPD**

Kroniskt obstruktiv lungsjukdom (KOL) är förknippad med en ökning av antalet T-lymfocyter i luftvägar och lungvävnad. Luftvägsslemhinnans epitelceller bidrar till det initiala immunologiska svaret då luftvägarna exponeras för inhalerade partiklar och smittämnen. Inflammatoriska celler med förmåga att infiltrera epitelet kan förstärka den inflammatoriska reaktionen. Syftet med denna studie var att karakterisera den epiteliala inflammationen i luftvägsslemhinnan hos patienter med KOL. Bronkoskopi med slemhinnebiopsier utfördes på tjugotvå patienter med KOL (FEV1: 51% av förväntat) (median). Rökare med normal lungfunktion (n=15), matchade avseende ålder och tobakskonsumtion, samt åldersmatchade ickerökare (n=15) inkluderades. Slemhinnebiopsierna bäddades in i glykolmetakrylat (GMA) varefter de färgades för immunhistokemi och snittades. Antalet CD4+ lymfocyter i luftvägsepitel från patienter med KOL befanns vara förhöjt jämfört med rökare och icke-rökare, medan CD8+ T-celler fanns i ökat antal jämfört med icke-rökare. Det förhöjda antalet intraepiteliala lymfocyter är förenligt med ett aktivt medverkande till sjukdomsuppförsen vid KOL. Lymfocytynärvaron kan också tolkas som ett led i infektionsförsvaret vid KOL eftersom sjukdomen är förknippad med en ökad frekvens akuta och kroniska infektioner.

**Reduced intracellular oxygen radical production in whole blood leukocytes from COPD patients and asymptomatic smokers.**

Kroniskt obstruktiv lungsjukdom (KOL) är förknippad med kronisk inflammation i lungorna, men också med systemisk inflammation. Målet med detta delarbete var att undersöka leukocyter i perifert blod avsände fenotyp och funktion. Tjugo patienter med medelsvår KOL rekryterades, tio rökande kontroller och tio icke-rökande kontroller inkluderades. Flödescytometrisk analys av ytmargor och mättning av frisättning av oxidativt aktiva substanser utfördes på helblod efter stimulering med TNF och bakterieantigenet N-formyl-methylene-leukyl-phenylalanin (fMLP). Friättsätningen av oxidativt verksamma substanser var nedsatt hos neutrofiler, monocyter och eosinofiler vid KOL såväl som hos rökande kontroller jämfört med icke-rökare. Som ett mått på kärlengagemang bestämdes även löslig intercelulär adhesionsmolekyl 1 (sICAM-1), och halten var förhöjd vid KOL såväl som hos rökare jämfört med ickerökare. Vidare undersöktes leukocytens uttryck av yttantigen före och efter stimulering med TNF och fMLP. Hos de KOL-patienter som rökt på morgonen för provtagning befanns ökningen av uttrycket av adhesionsmolekylen CD11b efter stimulering vara reducerat jämfört med dem som inte hade rökt.
Sammanfattning

Föreliggande avhandling innehåller delarbeten som alla baserar sig på en tvärsnittsstudie av patienter med KOL, rökare och icke-rökare. Samtliga deltagare är noggrant karakteriserade vad gäller klinik, lungfysiologi och radiologi. Detta har sedan korrelerats till de fynd som erhållits från undersökning med bronkoskopi inkluderande bronsköljning, bronkoalveolärt lavage och slemhinnebiopsier. Särkild tonvikt har legat på förhållandet mellan den kliniska presentationen av sjukdomen och profilen på det inflammatoriska svaret.
8 ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone that has contributed to this book, especially to:

Docent Magnus Sköld, my tutor, for his excellent scientific guidance, for sharing vast knowledge, great sense of humour, patience and, when needed, lack of patience, enabling me to pursue this thesis project during a highly educational period of my life.

Göran Elmberger, my co-supervisor, for shearing his great clinical knowledge in lung pathology, and for support.

The Professors Anders Eklund and Johan Grunewald, for their help in various ways, providing me with an excellent scientific environment and giving me the opportunity to do research under decent conditions.

Gunnar Unge, Veronica Agrenius and Olle Andersson, former and present heads of the department of respiratory medicine, Karolinska University Hospital, for their providence of facilities, and for providing special advice when I was writing the paragraph regarding gender differences in COPD (Olle!).

My co-authors: Lennart Nathell, Jan Wahlström, Ester Engstrand-Roos, Jamshid Pourazar, Barbro Dahlén, Joachim Lundahl and Anders Blomberg for skilful work and cooperativeness.

Benita Dahlberg for her excellent technical knowledge, and for nice company.

Gunnel de Forest, Margitha Dahl, Heléne Blomqvist and Elisabeth Henriksson for outstanding helpfulness.

Magnus Nisell, for providing important support during the days immediately prior to the printing of this book, and for nice company.

Pär Gyllfors and Anders Planck, friends and collaborators in the “farm-hands room” for friendship and numerous fruitful discussions.

Eva-Marie Karlsson and Lousie Rutberg for superb secretarial assistance and support.

To all patients who willingly participated in the studies and made this thesis possible.

To Per Näsman for helping me with statistics, and Joe DiPierre for correcting my English.

To all other members, past and present, at the Department of Respiratory Medicine (Reidar, Gunnar, Marie, Maria, Therese, Marianne, Lars-Olof, Muntasir, Helga, Maria A, Maryam, Michael, Carl-Henric, Valiant, Magnus N, Kalle, Maria D, Hirsch, Eva) and all members, past and present, at the Pulmonary research laboratory (Malin,
Charlotta, Mikael, Farah, Ernesto, Maria, Lukas, Bettina, Åsa, Jenny, Fariba, Kia), for help, inspiring scientific discussions and for creating such a nice atmosphere.

To Christer, Maria, Signe and Gunnar for indispensable support and for always showing interest in my work (Gunnar!)

My parents, Siv and Lennart, my brother Mattias and sister Malin for encouragement during all years.

To my beloved Britta, Helmer and little Tilda for endless love, support, encouragement and for incomprehensible amounts of patience.

To all of you:
-THANKS!-

This work was supported by grants from the Swedish Heart Lung Foundation, King Oscar II Jubilee Fund, the Cancer and Allergy Foundation, the Swedish Foundation for Health Care Sciences and Allergy Research (Vårdal foundation), Karolinska Institutet and Boehringer-Ingelheim/Pfizer
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