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Noninvasive biomarkers for early smoking related lung disease

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ACADEMIC DISSERTATION

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To my family
2.1. Pulmonary function tests .............................................................44
2.2. Sputum induction and processing .............................................44
2.3. Plasma specimens .................................................................45
2.4. NO measurements (Study I) ..................................................45

3. Studies conducted on the sputum cells, supernatants and plasma samples ....46
3.1. Enzyme-Linked Immunosorbent Assay (ELISA)/Enzyme Immunoassay (EIA) (Studies I, II, III, IV, V) ..............................................................46
3.2. Immunocytochemistry for sputum cells (Studies I, III) ..................46
3.3. Western blot analysis (Study III) .............................................47
3.4. Gelatinase assay (Study III) ...................................................48
3.5. Measurement of serine proteinase activity (Study IV) ..................48
3.6. MMP-8 immunofluorometric assay (Study IV) .........................49

4. Statistical analysis ......................................................................49

RESULTS ..........................................................................................51
1. Characteristics of subjects ........................................................51
2. Cell profile ................................................................................54

3. Oxidative/nitrosative stress in smokers ........................................56
3.1. Markers of increased oxidative stress in smokers (Study I) .........56
3.1.1. The expression of oxidative stress markers in induced sputum of non-smokers and smokers ...............................................................56
3.1.2. ECP, lactoferrin and FENO in smokers ....................................57
3.1.3. Correlations between oxidative stress markers, inflammatory cell profile and lung function values .................................................58
3.2. 8-Isoprostane as a marker of oxidative stress (Study II) ...............58

4. Matrix metalloproteinases as non-invasive markers for chronic obstructive pulmonary disease .........................................................59
4.1. Matrix metalloproteinases in smokers and early stages of COPD (GOLD Stage 0) (Study III) .................................................................59
4.1.1. Increased sputum and plasma levels of matrix metalloproteinases in non-symptomatic and symptomatic chronic smokers (Stage 0 COPD) ..............60
4.1.2. Immunocytochemistry of MMPs and TIMP-1 in induced sputum cytospins...61
4.1.3. MMP-9 activity by zymography and Western blotting ................61
4.1.4. Correlations of MMPs and TIMP-1 .......................................62
4.2. Neutrophil proteinases in COPD exacerbation (Study IV) ……………………63
4.2.1. Neutrophil elastase, MMP-8 and -9 in sputum samples…………………63
4.2.2. Neutrophil proteinases correlated with sputum cells and lung function
parameters…………………………………………………………………………………65
5. Age and smoking affect plasma biomarkers (Study V)…………………65
5.1. Surfactant protein A……………………………………………………………65
5.2. Surfactant protein D …………………………………………………………66
5.3. Matrix metalloproteinase -9 …………………………………………………66
5.4. TIMP-1 and MMP-9/TIMP-1………………………………………………66
5.5. SP-A may be a promising marker for COPD……………………………..67

DISCUSSION…………………………………………………………………………68
1. The potential markers of COPD and their significance in the early diagnosis of
this disease (Studies I, II, and III)……………………………………………………69
1.1. The expression of oxidative stress markers in symptomatic and non-symptomatic
smokers………………………………………………………………………………..69
1.2. Matrix metalloproteinases as specific markers of early COPD……………73
2. Changes in inflammatory profile and levels of proteases during COPD
exacerbation and its recovery period (Study IV)……………………………………76
3. Age related alterations and value of plasma surfactant proteins (SP) and
MMP-9 in healthy non-smokers and smokers (Study V)……………………………77

CONCLUSIONS………………………………………………………………………80

ACKNOWLEDGEMENTS…………………………………………………………82

REFERENCES……………………………………………………………………85
LIST OF ORIGINAL PUBLICATIONS


ABBREVIATIONS

ATS  American Thoracic Society
B    regression coefficient
BAL  bronchoalveolar lavage
BM   basement membrane
BMI  body mass index
CI   confidence interval
COPD chronic obstructive pulmonary disease
DLCO diffusion capacity
DTE  dithioethreitol
ECM  extracellular matrix
ECP  eosinophilic cationic protein
EIA  enzyme immunoassay
ELISA enzyme-linked immunosorbent assay
ERS  European Respiratory Society
EXA  exacerbation
FENO fractional exhaled nitric oxide
FEV1 forced expiratory volume in one second
FVC  forced vital capacity
GOLD the Global initiative for chronic Obstructive Lung Disease
HC   healthy control
HS   healthy smoker
iNOS inducible nitric oxide synthase
IIP  idiopathic interstitial pneumonia
4-HNE 4-hydroxy-2-nonenal
MGG  May-Grynwald-Giemsa
MMP  matrix metalloproteinase
MPO  myeloperoxidase
MT-MMP membrane-type matrix metalloproteinase
NE   neutrophil elastase
NO   nitric oxide
ONS  middle aged/elderly (old) non-smokers
OS   middle aged/elderly (old) smokers
PBS  phosphate buffered saline
RNS  reactive nitrogen species
ROC  receiver operating characteristic
ROS  reactive oxygen species
SD   standard deviation
SE   standard error
SEM  standard error of the mean
SP   surfactant protein
TIMP tissue inhibitor of matrix metalloproteinase
VC   vital capacity
YNS  young non-smokers
YS   young smokers
ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a slowly progressive disease characterized by airway inflammation and largely irreversible airflow limitation. Since the inflammatory process starts many years prior to the onset of clinical symptoms and still continues after smoking cessation, there is an urgent need to find simple non-invasive biomarkers that can be used in the early diagnosis of COPD and which could help in predicting the disease progression.

The first aim of the present study was to evaluate the involvement of different oxidative/nitrosative stress markers, matrix metalloproteinases (MMPs) and their tissue inhibitor-1 (TIMP-1) in smokers and in COPD. Another goal was to investigate the role of neutrophil proteases (neutrophil elastase (NE), MMP-8, MMP-9) during COPD exacerbation and its recovery period. Finally the value of some promising new COPD biomarkers identified from unbiased proteomics such as surfactant protein A (SP-A) were compared between young and elderly smokers and subjects with COPD as well as to SP-D, MMP-9 and TIMP-1 in these same individuals.

Induced sputum and/or plasma samples were collected from healthy non-smokers, "healthy" non-symptomatic smokers, smokers with chronic symptoms who displayed normal lung function values i.e. risk for developing COPD in future (GOLD Stage 0), subjects with stable Stage I-III COPD, during and after COPD exacerbation, and from young (age <25 years) and middle-aged/older non-smokers and daily smokers. Cell profiles were evaluated from sputum cytospins. The levels of MMP-8, -9, -12, TIMP-1, 8-isoprostane, surfactant protein-A (SP-A), SP-D, eosinophilic cationic protein (ECP) and lactoferrin were measured by EIA/ELISA or immunofluorometric assay (IFMA), and neutrophil elastase (NE) by spectrophotometrical analysis. The expressions of different oxidative/nitrosative stress markers, MMPs and TIMP-1 in induced sputum were studied also by immunocytochemistry. Molecular forms and gelatinolytic activity of MMP-9 were identified by Western blot analysis and zymography. Exhaled NO (FENO) was measured with a chemiluminescence analyser by using a PC and software devised for this purpose. Spirometry was performed in all studied subjects.
Elevated numbers of inducible nitric oxide synthase (iNOS), nitrotyrosine, and 4-hydroxy-2-nonenal (4-HNE) positive cells and increased levels of 8-isoprostane, lactoferrin and MMP-9 were found already in sputum of non-symptomatic smokers compared to non-smokers, but they did not differentiate smokers from the individuals with Stage 0 COPD. FENO was decreased in smokers and correlated negatively with the total number of neutrophils and positive cells for iNOS, nitrotyrosine and myeloperoxidase (MPO). Sputum levels of MMP-8 and plasma MMP-12 appeared to differentiate Stage 0 COPD subjects from healthy smokers. The levels of 8-isoprostane, MMP-8 and -9 were also measured in stable Stage I-III COPD and they correlated with the severity of the disease. MMP-8, -9 and -12, and TIMP-1 could be detected by immunocytochemistry in macrophages and neutrophils, especially in smokers. Subsequently the levels of neutrophil proteinases (NE, MMP-8 and MMP-9) were studied during COPD exacerbation. The levels of NE and MMP-8 were clearly increased in patients with COPD exacerbation as compared to stable COPD and controls, and declined during the one-month recovery period, evidence for a role for these enzymes in COPD exacerbations.

In the last study, the effects of subject’s age and smoking habits were evaluated on the circulating levels of SP-A, SP-D, MMP-9 and TIMP-1. Long-term smoking increased the plasma levels of all of these proteins. The SP-A level increased whereas that of TIMP-1 decreased with age, while SP-D and MMP-9 concentrations remained unchanged. SP-A most clearly correlated with age, pack years and FEV$_1$/FVC, and based on the receiver operating characteristic (ROC) curve analysis, SP-A was the best marker for discriminating subjects with COPD from controls.

In conclusion, these findings support the hypothesis that especially neutrophil derived oxidants may activate MMPs and induce an active remodeling process already in the lungs of smokers with normal lung function values. The marked increase of sputum levels of NE, MMP-8 and MMP-9 in smokers, stable COPD and/or during its exacerbations suggest that these enzymes play a role in the development and progression of COPD. Based on the comparison of various biomarkers, SP-A can be proposed to serve as sensitive biomarker in COPD development.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of disability and death, being responsible for a significant increase of economic and social burden worldwide. The incidence of the disease is increasing and it has been estimated that by 2020, COPD will be the third most common cause of death in the world (Lopez and Murray 1998). The total burden of COPD is underestimated, since there are no significant clinical symptoms experienced in the early stages of the disease.

COPD is a progressive disease characterized by chronic inflammation of the peripheral airways, chronic bronchitis and destruction of the lung parenchyma (emphysema) and systemic extrapulmonary manifestations (Pauwels et al. 2001, MacNee 2005, Rabe et al. 2007). Even the early stages of the disease with normal lung function values display inflammatory changes and structural abnormalities in the airways and lung parenchyma (Hogg 2004). The changes in lung function tests occur when damage of lung tissue, which is mainly irreversible, is already extensive. At present, there is no valid screening method for early COPD.

One major risk factor for COPD is cigarette smoking (Fletcher and Peto 1977, Rabe et al. 2007), which causes increased oxidative/nitrosative stress. Oxidative stress can activate proteases and this leads to an imbalance of proteases and antiproteases. Oxidative stress and a protease/antiprotease imbalance in turn have been postulated to be one of the major contributors to airway inflammation, the destruction of lung parenchyma and the development of small airway fibrosis in COPD (Rahman and MacNee1996a, Rahman and MacNee1996b, Langen et al. 2003, Hogg et al. 2004).

Markers of oxidative/nitrosative stress have been detected in the sputum and lung specimens from patients with moderate to severe COPD (Ichinose et al. 2000, Silkoff et al. 2001, Rahman et al. 2002, Maestrelli et al. 2003), but it is still unclear whether these markers can differentiate healthy smokers from non-smokers or smokers with symptoms but normal lung function values i.e. an individual at an increased risk for developing COPD (previous term Stage 0) from non-symptomatic smokers.
One major contributor to the increased oxidant burden in COPD is nitric oxide (NO) since cigarette smoke contains the highest levels of NO to which humans are directly exposed (Rahman et al. 1996). Inducible nitric oxide synthase (iNOS) produces the highest levels of NO in human cells and tissues, and it may be involved in the airway inflammation (Barnes 1995). Activation of iNOS and myeloperoxidase (MPO) can lead to the formation of nitrotyrosine (Davis et al. 2001), which has been found in the induced sputum of patients with severe COPD (Ichinose et al. 2000). 4-Hydroxy-2-nonenal (4-HNE), which is a marker of lipid peroxidation, has been detected previously in a lung biopsy from COPD patients (Rahman et al. 2002). Serum ECP is a marker used for measuring asthma severity and changes in disease activity (Tomassini et al. 1996, Amin et al. 2000) and lactoferrin is marker of neutrophil activation (Singh et al. 2002, Rogan et al. 2006). However, the role of these oxidative/nitrosative stress markers in the development and early diagnosis of COPD has remained unclear.

One of the most widely investigated non-invasive markers of nitrosative stress and airway inflammation is fractional exhaled NO (FENO), which is a sensitive and specific marker for eosinophilic inflammation in asthma (Smith et al. 2005), but its significance in smokers and its association with other markers of oxidative/nitrosative stress in the lung are poorly understood.

8-Epi-prostaglandinF$_{2\alpha}$ (8-isoprostane) has been proposed to be a reliable marker for assessing oxidative stress in vivo (Delanty et al. 1996, Montuschi et al. 2004). Recent studies have shown elevated 8-isoprostane in the exhaled breath condensate of COPD patients (Montuschi et al. 2000, Biernacki et al. 2003, Kostikas et al. 2003, Carpagnano et al. 2004), but it has not been evaluated in the sputum of smokers and individuals with mild COPD.

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play a critical role not only in tissue repair and remodelling but also in the pathogenesis of COPD. MMPs are proteolytic enzymes that are collectively capable of cleaving all components of the extracellular matrix (ECM) and basement membranes, and process bioactive mediators such as growth factors, cytokines, chemokines, and cell-surface receptors (Parks and Shapiro 2001).
The levels of MMP-8, MMP-9 and MMP-12 have found to be increased in COPD (Beeh et al. 2003a, Culpitt et al. 2005, Demedts et al. 2006, Elkington and Friedland 2006). Neutrophil elastase (NE) is a serine protease that destroys the alveolar wall by digesting and degrading elastin and collagen proteins in the ECM (Janoff et al. 1977, Shapiro 2002). NE, MMP-8, -9 and/or -12 have not been earlier compared in non-smokers, healthy smokers and GOLD Stage 0 or during the exacerbation of COPD and its recovery period.

Individuals generally start smoking at an early age (13-15 yrs) leading to the belief that significant changes due to smoking may have occurred already in young people (<25 years of age). However, it is unclear whether an inflammatory response is present in young healthy people after a relatively short-term cigarette smoking and does it differ from the airway inflammation that has been found in middle-aged cigarette smokers. Very few studies have focused on the expression of markers of tissue destruction in young smokers.

Surfactant protein (SP)-A is the major pulmonary surfactant-associated protein which has a role in innate host defense and the regulation of inflammatory processes in the lung. The proteomic studies conducted in our laboratory on lung tissues have identified SP-A as a potential marker for COPD (Ohlmeier et al. 2008). Some recent studies have also revealed increased SP-A levels not only in the serum of smokers and patients with COPD but also in individuals with pulmonary fibrosis (Mason et al. 1998, Whitsett 2005, Ohlmeier et al. 2008).

Surfactant protein (SP)-D is a large hydrophilic protein that makes an important contribution to surfactant homeostasis and pulmonary immunity (Kishore et al. 2006). Currently, little is known about the role of oxidative/nitrosative stress markers, MMPs, TIMPs, SP-A and SP-D during the onset of COPD.

This study aimed to investigate potential markers of early COPD measuring the oxidative burden, protease/antiprotease imbalance, levels of SP-A and SP-D from the induced sputum and/or plasma obtained from non-smokers, smokers and subjects with mild COPD.
REVIEW OF THE LITERATURE

1. Definitions and classification

Chronic obstructive pulmonary disease (COPD) is defined as a preventable and treatable disease characterized by poorly reversible airflow limitation that is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases, primarily to cigarette smoke (Celli and MacNee 2004, ATS/ERS 2005). COPD is characterized by chronic inflammation of the peripheral airways, chronic bronchitis, destruction of the lung parenchyma (emphysema) and systemic extrapulmonary disease (Pauwels et al. 2001, MacNee 2005, Rabe et al. 2007). The patient can suffer from one, some or to all of these conditions.

Peripheral airway inflammation or small-airway disease involves various morphological abnormalities, airway narrowing with goblet cell metaplasia, smooth muscle hypertrophy, excess mucus, oedema and inflammatory cellular infiltration. Airway remodelling with subepithelial and peribronchial fibrosis has been postulated as being the critical factor in small-airway narrowing and fixed airway obstruction in the small airways of patients with COPD (Wright 1995, Nadel 2000, Hogg et al. 2004).

Chronic bronchitis is characterized by cough and sputum production that results from the ability of cigarette smoke to induce mucous gland enlargement and goblet cell hyperplasia in the central airways. This inflammation is associated with increased mucus production, decreased mucociliary clearance and increased permeability of epithelial barrier of airways. An individual is considered to have chronic bronchitis if cough and sputum are present on most days for a minimum of 3 months for at least 2 consecutive years when other pulmonary or cardiac causes for the chronic productive cough have been excluded (Celli and MacNee 2004, Fletcher and Peto 1977, Hogg 2004).

Emphysema is defined as permanent destructive enlargement of peripheral airspaces of the lung without any obvious fibrosis, including respiratory bronchioles, alveolar ducts and alveoli, accompanied by destruction of these structures’ walls. The centrilobular emphysema is most closely associated with cigarette smoking (Kim et al. 1991).
Deterioration of COPD is accelerated after acute exacerbations that vary in frequency, but ultimately culminate in severe COPD. Acute exacerbations increase the morbidity and mortality and represent a major healthcare burden with enormous financial consequences (Soler-Cataluna et al. 2005). There is no standardized definition of an acute exacerbation, but the most common symptoms include increased breathlessness, cough, sputum production and purulence, major etiologic factors including viral and bacterial infections (Anthonisen et al. 1987, Sapey and Stockley 2006).

In recent years, COPD has been postulated as a systemic illness and/or as being associated with other smoking-related systemic diseases with manifestations from organ systems other than lungs and airways. The major systemic consequences or co-morbidities are: cardiovascular disease, effects on nutritional status, skeletal muscle dysfunction, exercise intolerance, osteoporosis, anxiety and depression (Aguilaniu et al. 1992, Keele-Card et al. 1993, Agustí et al. 2003, Jones et al. 2003, MacCallum 2005, Wouters 2005). The mechanisms by which these conditions develop are unclear, probably many factors are involved.

COPD can be classified according to the phenotype (Calverley and Walker 2003) and disease severity (Global Initiative for COPD 2010); by GOLD criteria in COPD FEV₁/FVC is below 0.7. The classification according to the disease severity should be done on post-bronchodilator lung function (Table 1). In addition, some of the unequivocal COPD patients have FEV₁/FVC >0.7 due to decline of FVC, and thus the term “undefined” COPD has been suggested (Wan et al. 2011). COPD contains several sub-phenotypes (for example airway or emphysema predominant), which is an area of intensive investigation.

Some of our studies have been done during the short period when the international COPD classification, the GOLD criteria (Pauwels et al. 2001), included individuals with an FEV₁/FVC > 0.7 and respiratory symptoms of chronic cough and sputum as a COPD stage (GOLD Stage 0) (Pauwels et al. 2001). The usefulness of Stage 0 in predicting COPD development is unclear (Vestbo and Lange 2002), but several studies have indicated that Stage 0 has importance, at least in predicting long-term mortality (Ekberg-Aronsson et al. 2005, Mannino 2006, Stavem et al. 2006). Potential markers not only need to be evaluated in terms of disease development but they may also be useful in smoking cessation protocols.
**Table 1. Classification of COPD according to the disease severity.**

This table is modified from international consensus statement by American Thoracic Society (ATS) and European Respiratory Society (ERS) Global Initiative for COPD (Rabe et al. 2007, GOLD 2010).

<table>
<thead>
<tr>
<th></th>
<th>FEV&lt;sub&gt;1&lt;/sub&gt;/FVC</th>
<th>FEV&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLD Stage I (mild)</td>
<td>&lt;0.7</td>
<td>&gt;80% predicted</td>
<td>Chronic cough and sputum  Occasional or remittent dyspnoea  Mild airflow limitation</td>
</tr>
<tr>
<td>GOLD Stage II (moderate)</td>
<td>&lt;0.7</td>
<td>&lt;80%, but &gt;50% predicted</td>
<td>Chronic cough and sputum  Shortness of breath on exertion  Chronic respiratory symptoms or an exacerbation forces the patient to seek medical help</td>
</tr>
<tr>
<td>GOLD Stage III (severe)</td>
<td>&lt;0.7</td>
<td>&lt;50%, but &gt;30% predicted</td>
<td>Chronic cough and sputum production  Greater shortness of breath on exertion  Worsening airflow limitation  Reduced exercise capacity  Fatigue  Repeated exacerbations  Reduced quality of life</td>
</tr>
<tr>
<td>GOLD Stage IV (very severe)</td>
<td>&lt;0.7</td>
<td>&lt;30% predicted or &lt;50% predicted plus chronic respiratory failure*</td>
<td>Severe airflow limitation  Chronic respiratory failure  Cor pulmonale possible  Frequent, possibly life threatening exacerbations  Significantly impaired quality of life</td>
</tr>
</tbody>
</table>

* Respiratory failure: arterial partial pressure of oxygen (Pa<sub>O2</sub>) < 8.0 kPa (60 mmHg) with or without arterial partial pressure of CO<sub>2</sub> (Pa<sub>CO2</sub>) > 6.7 kPa (50 mmHg) while breathing air at sea level.

FEV<sub>1</sub> - forced expiratory volume in 1 second, FVC - forced vital capacity.

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**2. Epidemiology and aetiology of COPD**

COPD is a leading cause of morbidity and mortality throughout the world, being responsible for significant disability and an increasing economic and social burden. COPD is the fourth leading cause of death worldwide, the incidence of the disease
is increasing and it has been estimated that by the year 2020 COPD will be the third largest cause of death and the fifth most common cause of global disability (Lopez and Murray 1998, Calverley and Walker 2003, Celli and MacNee 2004, Larsson 2007). COPD mortality in females has increased significantly in the last 20 years. There are striking differences between the prevalence of COPD in various countries and between sexes even when using identical detection methods (Halbert et al. 2003, Menezes et al. 2005, Halbert et al. 2006, Buist et al. 2007). By GOLD criteria (GOLD stage II or higher), the prevalence of COPD in a large population-based study was 10.1% among the subjects aged ≥ 40 yrs (Buist et al. 2007). In Finland, the prevalence of COPD among the adult population was 4.3% in males and 3.1% in females in 2000-2001 (Vasankari et al. 2010, Kinnula et al. 2011), and based on these studies the prevalence of COPD is no longer increasing in Finland though there is an increasing trend in many countries, especially in those with high smoking frequencies and pollution.

On the other hand, even in European countries including Finland the total burden of COPD is underestimated, because the disease is usually not diagnosed until lung function parameters have become significantly reduced and a major part of the lung has been damaged. Since COPD is associated with a long smoking history, it has mid-life onset. However, recent studies have shown that significant numbers of COPD and chronic bronchitis can be detected even among young smokers who have a 10-year smoking history (Hamari et al. 2010, De Marco et al. 2011).

Tobacco smoke is the most important risk factor for COPD worldwide, as 90% of patients with COPD are smokers (GOLD 2010). Earlier studies have indicated that only 10-20% of heavy smokers develop an irreversible airway limitation suggesting that other environmental or genetic factors contribute to COPD (Fletcher and Peto 1977, Snider 1989), but recent studies have reported that up to 50% smokers might develop the disease (Lundback et al. 2003) and this number may increase even more when different environmental factors i.e. pollen, animal dander, other inhaled irritants than cigarette smoke, cold air are involved (Kotaniemi et al. 2002).
Table 2. The risk factors of COPD besides smoking.

<table>
<thead>
<tr>
<th>Host factors</th>
<th>Exposures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic factors (alpha-1 antitrypsin deficiency)</td>
<td>Socio-economic status</td>
</tr>
<tr>
<td>Sex</td>
<td>Occupational exposures</td>
</tr>
<tr>
<td>Airway hyperreactivity</td>
<td>Environmental pollution</td>
</tr>
<tr>
<td>IgE and asthma</td>
<td>Perinatal events, lower birth weight and childhood respiratory infections</td>
</tr>
<tr>
<td></td>
<td>Recurrent bronchopulmonary infections</td>
</tr>
<tr>
<td></td>
<td>Diet</td>
</tr>
</tbody>
</table>


3. Pathogenesis of COPD

Lung inflammation, an increased oxidant burden, and a protease-antiprotease imbalance in the lungs are considered to play an important role in the pathogenesis and progression of COPD (Snider 1989, Rahman and MacNee 1996a, Rahman et al. 1996, Rahman et al. 1997).

3.1. Inflammation in the lung in COPD

The bronchial epithelium is the first line of defence against inhaled noxious agents, such as cigarette smoke, air pollutants, allergens and microorganisms in the lungs (Puchelle 2000, Gower et al. 2011). The epithelial surface barrier is normally quite
impermeable to the material that lands on it, but these agents can induce inflammatory reactions and change the mucosal permeability. Damaged epithelial cells release inflammatory mediators that recruit and activate inflammatory cells and extracellular matrix (ECM) to produce and release oxidants and proteolytic enzymes including matrix metalloproteinases (MMPs), which degrade basement membrane (BM), enhancing inflammatory cell migration to the site of injury, and mediate epithelial injury (Mendis et al. 1990, Puchelle 2000). Cigarette smoke also directly activates macrophages and polymorphonuclear neutrophils to produce more reactive oxygen species (ROS) and different mediators which in turn lead to the recruitment of other inflammatory cells into the airways (Hoidal and Niewoehner 1982). The major site of action in COPD is the alveolar space, lung parenchyma and small airways.

3.2. Inflammatory cells in COPD

COPD involves several types of inflammatory cells, but the relationship between these cells and the sequence of their appearance and persistence are to a great extent unknown. Studies of bronchial or lung biopsies and induced sputum have shown evidence of inflammation in all cigarette smokers, especially in COPD patients central airways compared to smokers and non-smokers (Saetta et al. 2002, Di Stefano et al. 2004). Neutrophils are mainly located in the lumen of the airways and macrophages being found in the lung tissue (Saetta et al. 2001). In COPD and especially during its exacerbations, there are increased numbers of neutrophils, macrophages, but also lymphocytes and eosinophils in the airways (Rahman and MacNee 2000a, Saetta et al. 2001, Di Stefano et al. 2004).

3.2.1. Neutrophils

Neutrophils are the main inflammatory cells at the sites of acute inflammation. In the lung they are usually recruited from the circulation to the airways. There are several important factors involved in cell migration i.e. the expression of adhesion molecules (DiStefano et al. 1994) and different chemoattractants: IL-8, LTB₄, TNF-
α (Mikami et al. 1998, Aaron et al. 2001, Williams and Jose 2001). Furthermore, nicotine itself may be a chemoattractant for neutrophils (Totti et al. 1984) pointing to links between smoking and neutrophil recruitment to the lung. The degree of neutrophil recruitment and differences in neutrophil function (chemotactic responsibility to a chemoattractant and ability to digest connective tissue) has a major pathogenic importance in the development of COPD (Burnett et al. 1987, Stockley et al. 1994, Mikami et al. 1998). Neutrophils are capable of inducing tissue damage through the release of serine proteases and oxidants. Neutrophils secrete many proteases, including neutrophil elastase (NE), cathepsin G, proteinase-3 and matrix metalloproteinases (MMP-8, MMP-9), which are potent mucus stimulants and participate in processes that may contribute to alveolar destruction (DiStefano et al. 1994, Barnes 2004).

Neutrophils are found to be increased in the airways of smokers (Willemse et al. 2005) and subjects with COPD (Lacoste et al. 1993, Keatings et al. 1996, Confalonieri et al. 1998) and reduced in lung parenchyma of COPD patients (Finkelstein et al. 1995) evidence of their rapid transit from parenchyma into the airway lumen.

3.2.2. Macrophages

Macrophages are the predominant defence cells in the normal lung and also during chronic inflammation such as COPD. One early response to inhaled irritants is the recruitment of macrophages to the lung, where they attempt to phagocytoze and, if possible, destroy the unwanted particles. The phagocytosis of cigarette smoke-derived particles is an important defense mechanism in the neutralization of the toxins while it has been shown that alveolar macrophages isolated from COPD patients and healthy smokers display significantly decreased phagocytic ability compared to non-smokers (Hodge et al. 2007). Macrophages also play an important role in the inflammatory process by releasing proteolytic enzymes, chemotactic factors, proinflammatory cytokines, growth factors (interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor- α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) etc), and reactive oxygen and nitrogen species leading to the recruitment of several cell types from the circulation, including monocytes,
neutrophils and T-lymphocytes, into the inflammatory site. Alveolar macrophages secrete matrix metalloproteinases (MMP-2, MMP-8, MMP-9, MMP-12), cathepsins and neutrophil elastase taken up from phagocytozed neutrophils (Kelley 1990, Punturieri et al. 2000, Russell et al. 2002a, Russell et al. 2002b, Barnes 2004, Murugan and Peck 2009) which degrade the extracellular matrix. Macrophages are elevated in the lungs of smokers and patients with COPD, and there is an association between increased numbers of macrophages in the airways and the degree of small airways disease in patients with COPD as well in individuals with mild to moderate emphysema (Saetta et al. 1993, DiStefano et al. 1998, Ohnishi et al. 1998, Tetley 2002).

3.2.3. Lymphocytes

Lymphocytes are leucocytes that play a major role in cell-mediated immunity. The two major classes of lymphocytes are B-cells, which mature independent of the thymus, and T-cells, which are processed in the thymus. Increased numbers of T-lymphocytes, especially CD8+, but also CD4+, are found in lung parenchyma, peripheral and central airways of patients with COPD (Finkelstein et al. 1995, Majo et al. 2001, Di Stefano et al. 2004, Chang et al. 2011). CD8+ T-cells or cytotoxic T cells are capable of killing damaged or dysfunctional cells, including infected and tumor cells and they can recognize and bind to major histocompatibility complex (MHC) class I molecule making possible antigen-specific activation of CD8+ cell (Milstein et al. 2011). The majority of CD4+ cells are T helper cells that are involved in activating and directing other immune cells by recognizing MHC class II molecules (Harrington et al. 2005). Different chemokines have been described to be responsible for the recruitment of T-cells and blood monocytes increasing the number of macrophages and CD8+ T-cells in the subepithelial areas in the lung of COPD patients. CD8+ T-lymphocytes together with neutrophils infiltrate into the bronchial epithelial surface in COPD (O’Shaughnessy et al. 1997, Saetta et al. 1999). The mechanisms by which T-cells accumulate in the airways of COPD patients and their role in the pathophysiology of COPD are not yet understood, but there are clear correlations between the number of T-cells, the severity of airway obstruction and the extent of alveolar destruction (Di Stefano et al. 2002). There is
an association between alveolar cell apoptosis and CD8+ cells in emphysema (Majo et al. 2001) and it is possible that CD4+ cells have immunological memory and thus they play a role in sustaining the inflammatory reaction in the absence of cigarette smoke (Majo et al. 2001, Retamales et al. 2001).

3.2.4. Eosinophils

Eosinophils are the inflammatory cells that most often occur in patients with asthma, whereas neutrophils are usually detected in individuals with COPD. However, increased numbers of eosinophils and eosinophil cationic protein (ECP) have been occasionally found in the induced sputum and bronchoalveolar lavage fluid (BALF) taken from subjects with stable COPD (Lacoste et al. 1993), but particularly during COPD exacerbations (Di Stefano et al. 1994, Saetta et al. 2001). It has been proposed that in contrast to asthma, the tissue eosinophils that have been found in COPD do not degranulate (Lacoste et al. 1993). There are studies indicating that in COPD patients with eosinophilic airway inflammation, inhaled corticosteroids can relieve the symptoms and may even influence the clinical course of the disease (Brightling et al. 2000, Ford et al. 2010, Lehtimaki et al. 2010).

3.3. Biomarkers/mediators in COPD

3.3.1. Oxidative and nitrosative stress markers in COPD

Cigarette smoke contains high concentration of free radicals (one puff contains more than $10^{15}$ radicals) and other oxidants, which evoke increased oxidative/nitrosative stress in airways and lungs (Church and Pryor 1985, Pryor and Stone 1993, MacNee 2000). Oxidative stress in turn has been considered to be one of the major contributors of airway inflammation, the destruction of lung parenchyma and development of small airway fibrosis in COPD (Rahman et al. 1996, Langen et al. 2003).
In COPD, oxidant stress occurs in small airways, lung parenchyma, and alveolar regions, and is associated with the activation of cytokines and growth factors and activated inflammatory cells that produce large amounts of reactive oxygen and nitrogen species (ROS and RNS). ROS are chemically reactive molecules either inhaled (cigarette smoke) or produced endogenously as natural byproducts in a number of reactions, especially in airway inflammation. ROS themselves constitute both 1) free radicals i.e. they have a free electrode in their outer orbital (examples being superoxide and hydroxyl radicals), and 2) certain other reactive compounds (such as hydrogen peroxide $H_2O_2$), which do not fulfill the definition of the term “radical” (Halliwell 1994). Major producers of superoxide radicals include NADPH oxidases and xanthine oxidase i.e. their breakdown can be spontaneous and/or enzymatic (superoxide dismutases) (Figure 1).

Environmental stress, including cigarette smoke and pollutants, can increase ROS/RNS levels dramatically causing damage to cell structures, especially lipids, proteins and DNA (Hensley et al. 2000). Most of the ROS and RNS produced in the lung tissue come from neutrophils, alveolar macrophages and eosinophils, but also bronchial and alveolar epithelial cells and endothelial cells are capable of producing ROS (Kinnula et al. 1995). In COPD, the presence of ROS leads to airway hyperresponsiveness, mucus secretion and airway smooth muscle contraction, and also to activation of proteases and transcription of many inflammatory genes (Rahman and MacNee 1998, Paredi et al. 2002, Wood et al. 2003). In addition to the activation of several oxidant producing systems and enzymes in COPD, there is a simultaneous decline/inactivation of many antioxidant enzymes in the COPD lung (Harju et al. 2002, Kinnula 2005), which further increases the oxidant burden. Ultimately, the increased oxidant burden causes an oxidant/antioxidant imbalance, which is thought to play an important role in the pathogenesis of COPD (Repine et al. 1997, Van der Vliet et al. 1999). Many oxidants are unstable and have short half-lives, and are therefore difficult to investigate in vivo (Jones et al. 2000).
Figure 1. Sources of ROS/RNS generated endogenously by pulmonary cells and key metabolic pathways for these species. Some of the H$_2$O$_2$ is also decomposed by thiol-containing proteins such as peroxiredoxins, thioredoxins and glutaredoxins. O$_2$: oxygen; O$_2^•−$: superoxide anion radical; H$_2$O$_2$: hydrogen peroxide; •OH: hydroxyl radical; NO: nitric oxide; ONOO$: peroxynitrite; SODs: superoxide dismutases (MnSOD, CuZnSOD, ECSOD); H$_2$O: water; iNOS: inducible nitric oxide synthase; MPO: myeloperoxidase; (Modified from the reviews of Yoshizumi et al. 2001, Davis et al. 2001, Kinnula and Crapo 2003).

Nitric oxide (NO) is part of the normal metabolism and is necessary for the homeostasis of the lung and other organs. NO is also the major contributor to the increased oxidant burden in COPD since cigarette smoke contains the highest levels of NO (up to 300 ppm) to which humans are directly exposed (Pryor and Stone 1993, Rahman et al. 1996). The formation of NO is regulated by three different isoenzymes of NO synthase (NOS), endothelial and neuronal NOSs produce low NO levels: these enzymes play an important role in cell signaling and homeostasis, while activation of inducible nitric oxide synthase (iNOS) leads to high/toxic NO concentrations (Van der Vliet et al. 1999, Ricciardolo et al. 2004). iNOS is also significantly induced by many of the mediators present in airway inflammation (Barnes 1995). Excessive production of NO through iNOS can lead to NO reaction with superoxide anion (O$_2^−$), leading to the formation of the highly inflammatory molecule peroxynitrite.
(ONOO−) and depletion of bioactive NO (Pryor and Squadrito 1995, Hanafy et al. 2001). Peroxynitrite is extremely reactive and can directly combine with various biological targets and components of the cell and with other molecules to form additional types of RNS as well as other types of chemically reactive free radicals (Pryor and Squadrito 1995, Kinnula 2005, Sugiura and Ichinose 2011). In COPD, RNS can cause lung inflammation, oxidative/nitrative stress, activation of matrix metalloproteinases, and inactivation of antiproteases (Van der Vliet et al. 1999, MacNee 2001, Rahman et al. 2006). The degree of protein nitration in lung tissue has been suggested to be related to iNOS expression and it is associated with decreased FEV₁/FVC (Maestrelli et al. 2003). However, the role of iNOS in the pathogenesis and progression of COPD is controversial.

**Fractional exhaled NO (FENO)** has become an extensively investigated non-invasive marker of nitrosative stress and airway inflammation. It is a sensitive and specific marker for eosinophilic inflammation in asthma (Smith et al. 2005), but its significance in smokers and its association with other markers of oxidative/nitrosative stress in the lung needs further investigation. FENO has been measured at single expiratory airflow rate of 50ml/s (ATS/ERS 2005), but more information can be obtained when measuring exhaled NO at multiple flow rates and calculating the NO concentration from small peripheral airways and alveoli (CA,NO) and NO from central airways (J' av, NO) (Lehtimaki et al. 2010). FENO has been found to be decreased in chronic smokers, but to be variable in COPD patients (Kharitonov et al. 1995, Corradi et al. 1999, Rutgers et al. 1999, Balint et al. 2001, Montuschi et al. 2001). In COPD, the levels of exhaled NO from large airways (J' av, NO) have been found to be unchanged (Roy et al. 2007) or decreased (Brindicci et al. 2005) and from small airways (CA,NO) unchanged (Roy et al. 2007) or increased (Brindicci et al. 2005).

One additional marker of oxidative/nitrosative stress is nitrotyrosine that is a stable product of tyrosine nitration mediated by RNS such as peroxynitrite and nitrogen dioxide (Ischiropoulos et al. 1992, Pacher et al. 2007). Alternative pathways for the formation of nitrotyrosine include the myeloperoxidase (MPO) system (Eiserich et al. 1998, van Dalen et al. 2000, Davis et al. 2001) or through the direct reaction of NO with tyrosyl radicals (Gunther et al. 1997). Nitrotyrosine-positive cells have been found in the induced sputum of COPD patients with severe
disease (Ichinose et al. 2000), but it has not been evaluated in mild COPD or in smokers at risk for COPD development. MPO is expressed in neutrophils azurophilic granules, secreted during their activation and therefore has been proposed as a marker of neutrophil activation. MPO possesses potent proinflammatory properties and may contribute directly to tissue injury (Klebanoff 1980). MPO has found to be associated with COPD, its exacerbation and decreased diffusion capacity (Ekberg-Jansson et al. 2001, Barczyk et al. 2004). The number of MPO-positive cells has been found to be increased in bronchial submucosa in patients with severe COPD as compared to mild/moderate COPD, smokers and non-smokers (Ricciardolo et al. 2005), but its role in COPD development remains unclear.

4-hydroxy-2-nonenal

One consequence of oxidative stress is membrane lipid peroxidation in the lungs. One of the specific and stable end products of lipid peroxidation is 4-hydroxy-2-nonenal (4-HNE) and this has earlier been detected in a lung biopsy taken from COPD patients (Rahman et al. 2002). Recent studies have indicated that 4-HNE can act as a second messenger which may play a role in the regulation of expression of the protective enzyme gamma-glutamylcysteine synthetase as well as a variety of other genes like transforming growth factor-β1 (TGF-β1), cyclooxygenase 2 and monocyte chemotactic protein-1, which have been found to be present in the lung tissue of COPD patients (Rahman and MacNee 2000b, Liu et al. 2001, Rahman et al. 2002), but its role in the pathogenesis and early diagnosis of COPD is still unclear.

Eosinophil cationic protein

Eosinophil cationic protein (ECP) is a member of the ribonuclease superfamily and is mainly produced by eosinophils, but also some mono-myelocytic cell-lines (Monteseirin et al. 2007). In addition, neutrophils have the ability to take up ECP from the surrounding environment, store it in their azurophil granules, and release it when activated (Bystrom et al. 2001, Bystrom et al. 2002). It has been found that the serum levels of ECP are correlated with the number of activated eosinophils in the bronchial mucosa of asthmatics (Hoshino and Nakamura 1997). Serum ECP
levels have also found to be useful as an objective measurement of asthma severity and for monitoring changes in disease activity throughout the year (Tomassini et al. 1996, Amin et al. 2000).

**Lactoferrin**
The human lung, but also saliva and other human secretions contain a wide range of antimicrobial compound including the lactoperoxidase system, which produces lactoferrin. Lactoferrin is released from specific granules of neutrophils at the areas of inflammation. Lactoferrin has both antimicrobial and anti-inflammatory properties and contributes to host defence both systemically and at mucosal surfaces (Elass et al. 2002, Singh et al. 2002, Rogan et al. 2006). However, its role in COPD pathogenesis is unclear.

**8-Epi-prostaglandinF_{2α} (8-isoprostane)**
F2-isoprostanes exist in ester linkages of phospholipids in vivo, and are formed in situ by free-radical-catalysed lipid peroxidation of arachidonic acid in cell membrane phospholipids, this being independent of the action of cyclooxygenase (Morrow et al. 1990). They can be released into the circulation, secretions and urine where their levels have been found to reflect the oxidant burden reliably (Morrow et al. 1992, Reilly et al. 1996, Montuschi et al. 2004). 8-isoprostane has been considered as an ideal marker for investigating the pathophysiology of oxidant/antioxidant imbalance because of its biochemical stability (Morrow et al. 1995). However, also isoprostanes have potent biological actions and therefore they may contribute significantly to the progression of oxidant-mediated lung diseases, such as COPD. Several studies have proposed a role for 8-isoprostane in oxidative stress, reflecting the degree of lipid peroxidation (Morrow et al. 1990) and pulmonary oxygen toxicity (Janssen 2001) and it is believed to constitute a component in a common pathway leading to airway obstruction (Paredi et al. 2002). The levels of 8-isoprostane have been shown to be elevated in the exhaled breath condensate of COPD patients despite their age, sex, history of smoking in pack years and lung function impairment, and the concentrations increase during COPD exacerbations and decline after treatment with antibiotics (Montuschi et al. 2000, Biernacki et al. 2003, Kostikas et al. 2003, Carpagnano et al. 2004). The levels of isoprostane can be evaluated in serum/plasma and induced sputum, but there are a

Before the present study, 8-isoprostane had not been evaluated in sputum obtained from individuals with mild COPD or in smokers at risk for developing COPD i.e. those with chronic symptoms (chronic cough, sputum production, previous Stage 0 COPD).

3.3.2. Matrix metalloproteinases in COPD

MMPs are believed to play a critical role in physiological tissue repair and remodelling, including growth, development and wound healing but also in the pathological tissue-destructive processes, including the development of cancer, arthritis, atherosclerosis and pathogenesis of COPD (Saetta et al. 2001, Shapiro 2002, Barnes 2004, Gueders et al. 2006, Nagase et al. 2006).

In vertebrates MMPs comprise a family of 28 matrix degrading enzymes that contain a zinc atom in their active site and are able to cleave all components of the extracellular matrix (ECM) and basement membranes (BM) including collagen, laminin, and elastin. The ECM is a complex network of different molecules sustaining collagens, laminin, fibronectin, entactin/noidogen and proteoglycans, and serving a mechanical role by supporting and maintaining tissue structure and modulating different cell functions including development, migration and proliferation (Mott and Werb 2004). Basement membrane is a thin layer of ECM separating underlying connective tissue from bronchial epithelial cells. The main components of the airways BM are collagen types IV, V and VII, proteoglycans, fibronectin and different isoforms of laminin (Roche et al. 1989, Wetzels et al. 1991, Paulsson 1992).

In addition to their ability to degrade the components of the extracellular matrix, some MMPs also cleave cytokines and antiproteolytic molecules (Banda et al. 1980, Sires et al. 1994, Churg et al. 2003, Parks 2003). They also process bioactive
mediators such as growth factors, cytokines, chemokines, and cell-surface receptors (Shapiro 1998, Parks and Shapiro 2001, Ohbayashi 2002, Kelly and Jarjour 2003). MMPs are activated by many different factors including cigarette smoke and oxidative stress (Rajagopalan et al. 1996, Shapiro 2002, Nelson and Melendez 2004, Kinnula 2005, Rahman and Adcock 2006). In normal healthy tissues, the MMP activity is controlled by cytokines, growth factors, hormones and endogenous tissue inhibitors of metalloproteinases (TIMPs) (Parks et al. 2004, Spinale 2007). MMP family members share common structural and functional elements: 1) 40-50% identity at the amino acid level and domain structures, 2) MMP catalytic activity is dependent on the presence of a zinc ion at the active site, 3) inhibition by specific tissue inhibitors of matrix metalloproteinases (TIMPs) 1-4, 4) MMPs are secreted as inactive proenzymes and are activated at the cell membrane surface or extracellular space by proteolytic enzymes or oxidants by cleavage of the N-terminal domain (Shapiro 1998, Overall and Lopez-Otin 2002, Strenlicht and Werb 2001, Visse and Nagase 2003, Parks et al. 2004).

Depending on substrate specificity, amino acid similarity and identifiable sequence modules, the MMP family can be classified into subclasses (Table 3).

Table 3. Matrix metalloproteinases and their main substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molecular weight latent/active (kDa)</th>
<th>Main substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1 (collagenase-1)</td>
<td>54/41</td>
<td>Collagen types I, II, III, VII, VIII, X, aggrecan, gelatin, pro-MMP-2, pro-MMP-9</td>
</tr>
<tr>
<td>MMP-8 (collagenase-2)</td>
<td>85/64</td>
<td>Collagen types I, II, III, VII, VIII, X, aggrecan, gelatin</td>
</tr>
<tr>
<td>MMP-13 (collagenase-3)</td>
<td>65/55</td>
<td>Collagen types I, II, III, aggrecan, gelatin, Collagen type I</td>
</tr>
<tr>
<td>MMP-18 (collagenase-4)</td>
<td>53/42</td>
<td></td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2 (gelatinase A)</td>
<td>72/66</td>
<td>Gelatin, collagens I, II, III, IV, V, VII, X, XI, XIV, elastin,</td>
</tr>
<tr>
<td>MMP-9 (gelatinase B)</td>
<td>92/85</td>
<td>fibronectin, aggregan, gelatin, collagens IV, V, VII, X, XIV, pro-MMP-9, pro-MMP-13, elastin, aggregan, laminin, fibronectin, proteoglycans</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Stromelysins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3 (stromelysin-1)</td>
<td>57/45, 28</td>
<td>Collagens II, III, IV, IX, X, XI, fibronectin, proteoglycans, elastin, pro-MMP-1, pro-MMP-7, pro-MMP-8, pro-MMP-9, pro-MMP-13 collagens III, IV, V, gelatin, fibronectin, proteoglycans, matrix glycoproteins</td>
</tr>
<tr>
<td>MMP-10 (stromelysin-2)</td>
<td>56/47, 24</td>
<td></td>
</tr>
<tr>
<td>MMP-11 (stromelysin-3)</td>
<td>58/28</td>
<td>Fibronectin, laminin, gelatin, aggregan, elastin, collagens IV, V, IX, X</td>
</tr>
<tr>
<td><strong>Matrilysins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7 (matrilysin-1)</td>
<td>28/19</td>
<td>Collagens II, III, IV, V, IX, X, XI, elastin, entactin, gelatin, aggregan, fibronectin, laminin, pro-MMP-1, pro-MMP-7, pro-MMP-8, pro-MMP-9, pro-MMP-13</td>
</tr>
<tr>
<td>MMP-26 (matrilysin-2)</td>
<td>28/unknown</td>
<td>Collagen type IV, gelatin, fibronectin, fibrinogen</td>
</tr>
<tr>
<td><strong>Membrane-type MMPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Transmembrane type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-14 (MT1-MMP)</td>
<td>66/60</td>
<td>Pro-MMP-2, -9, -13, collagen I, II, III, gelatin, aggregan, fibronectin, laminin</td>
</tr>
<tr>
<td>MMP-15 (MT2-MMP)</td>
<td>68/62</td>
<td>Pro-MMP-2, gelatin, fibronectin, laminin</td>
</tr>
<tr>
<td>MMP-16 (MT3-MMP)</td>
<td>64/55</td>
<td>Pro-MMP-2, laminin, fibronectin</td>
</tr>
<tr>
<td>MMP-24 (MT5-MMP)</td>
<td>63/45</td>
<td>Pro-MMP-2, proteoglycan, collagen type I, laminin, fibronectin</td>
</tr>
<tr>
<td><strong>GPI-anchored</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-17 (MT4-MMP)</td>
<td>57/53</td>
<td>Pro-MMP-2, gelatin, fibrin, fibronectin</td>
</tr>
</tbody>
</table>
The basic molecular structure of most MMPs is similar i.e.: 1) a signal sequence, to the direct secretion from the cell, 2) N-terminal pro-domain (propeptide) with a free cysteine residue that maintains the latency of the zymogen by direct coordination with the zinc atom in the active site of the catalytic domain, blocking the access of the catalytic site to the substrate, 3) the catalytic domain including the zinc binding motif, 4) the hinge region, which is often proline rich, 5) C-terminal domain (hemopexin-like domain), implicated in macromolecular substrate recognition and binding, and inhibition by TIMPs (Baragi et al. 1994, Birkedal-Hansen 1995, Parks and Shapiro 2001).

MMPs also share a similar gene arrangement so, that at least eight of the known human MMP genes (MMP-1, -3, -7, -8, -10, -12, -13 and -20) are located on chromosome 11 and the other known MMP genes on chromosomes 1, 8, 12, 14, 16, 20 and 22 (Mattei et al. 1997, Shapiro 1998).

Elevation/activations of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-12 have been found to occur both in experimental emphysema and human COPD (Finlay et al. 1997, Hautamaki et al. 1997, Beeh et al. 2003a, Selman et al. 2003, Culpitt et al. 2005, Demedts et al. 2006, Elkington and Friedland 2006). Before the current studies, the levels of MMP-8, MMP-9 and MMP-12 had not been compared in non-smokers, healthy smokers and symptomatic smokers (Stage 0 COPD, who probably have risk for COPD development) nor during COPD exacerbation. Due to the direct
effects of cigarette smoking on many signalling cascades, accumulation of the inflammatory cells, their activation and increased oxidative stress, it is highly likely that there is also a significant increase/activation of MMPs in chronic smokers, even in those without airway limitation.

MMP-8
MMP-8 (collagenase-2, neutrophil collagenase) is the major member of the interstitial collagenase subgroup of the MMP family and is mainly synthesised in maturating PMNs in the bone marrow, stored in intracellular granules and released in response to extracellular stimuli (Owen and Campbell 1999, Sorsa et al. 2004). PMN-type MMP-8 is highly glycosylated and secreted by activated PMNs in a latent 75- to 80-kD form. During PMN degranulation latent MMP-8 isoform is converted to a 65-kD active form (Hasty et al. 1986, Ding et al. 1997, Balbin et al. 1998). The less glycosylated non-PMN-type MMP-8 is secreted by various mesenchymal lineage cells; bronchial epithelial cells, macrophages, monocytes (Prikk et al. 2001), fibroblasts and endothelial cells (Hanemaaijer et al. 1997), chondrocytes (Cole et al. 1996), also by malignant epithelial cells as the 55-kD latent isoform and is converted to a 45-kD active form during activation (Moilanen et al. 2002, 2003). Pro-MMP-8 can be activated by reactive oxygen species, chymotrypsin, cathepsin G and other MMPs (Goldberg et al. 1992, Sorsa et al. 1992, Crabbe et al. 1994). In addition, MMP-8 appears to be present on the surface of activated PMN cells, which accounts for its stability in the extracellular environment in the presence of tissue inhibitors (TIMPs) (Owen et al. 2004). MMP-8 can cleave all three interstitial collagens and it is the most efficient proteinase in the degradation of type I collagen in humans (Weglus et al. 1981, Jeffrey 2001), cleaving also nonmatrix components such as serpins, bradykinin, substance P and angiotensin I (Knauper et al. 1993, Diekmann and Tschesche 1994). It is thought to have a role in the tissue remodelling processes during inflammation, especially in chronic diseases characterized by activation of polymorphonuclear cells (Power et al. 1994).

MMP-9
MMP-9 (gelatinase B) is secreted by PMNs, alveolar macrophages, eosinophils, mast cells, T-lymphocytes, keratinocytes and several transformed cell lines (Mainardi et al. 1984, Ohno et al. 1997). PMN-derived MMP-9 differs from the MMP-9 expressed
by other cell types in two ways: mature PMN cells do not synthesize MMP-9 de novo and pro-MMP-9 is not released from activated PMN complexed to tissue inhibitor of metalloproteinases-1 (TIMP-1) (Pugin et al. 1999). PMNs release MMP-9 in three different forms. Latent pro-MMP-9 is released as a 120 kD complex with a 29 kD neutrophil gelatinase-B associated lipocalin (NGAL) and 220 kD dimeric forms of the molecule (Triebel et al. 1992). In addition, MMP-9 is secreted in a glycosylated monomeric 92 kD form that is cleaved into 82- to 68-kD active forms (Sorsa et al. 1997) which degrade type IV, V, VII and X collagens, elastin, gelatine, laminin, fibronectin, proteoglycans (Senior et al.1991, Birkedal-Hansen 1995, Jeffery 1998, Shapiro 1998, Opdenakker et al. 2001, Pirila et al. 2001). In addition, MMP-9 degrades serine protease inhibitors and regulates other members of the protease cascade, further evidence supporting the theory that there is a protease/antiprotease imbalance in the pathogenesis of inflammatory lung diseases including COPD (Lim et al. 2000, Atkinson and Senior 2003). Owen et al. (2003) have shown that in addition to the soluble form of MMP-9, the enzyme can be expressed on the cell surface of PMNs where it retains its activity despite the presence of TIMPs in extracellular environment, tipping the balance of proteases/antiproteases in favour of proteases and extracellular matrix degradation, and development of lung injury.

MMP-12
MMP-12 (macrophage metalloelastase or macrophage elastase) is classified as a stromelysine-like group (Nagase and Woessner 1999) and is secreted as proenzyme (54 kD) that is activated by N-terminal processing to a short-term 45 kD form that is further reduced to a 22 kD mature form by C-terminal cleavage between the catalytic and hemopexin-like domains (Shapiro et al. 1993). MMP-12 is expressed primarily in alveolar macrophages, is essential for macrophage migration and has the capacity to hydrolyze a large spectrum of extracellular matrix components, with the exception of interstitial collagens (Shapiro 1998, Wang et al. 2000, Warner et al. 2001, Lanone et al. 2002, Molet et al. 2005, Demedts et al. 2006). MMP-12 can also cleave a variety of non-ECM proteins including plasminogen and latent tumor necrosis factor α, and has a potential role in developing acute or chronic lung injury, especially COPD (Chandler et al. 1996, Cornelius et al. 1998). Hautamaki et al. (1997) have reported that MMP-12 knock-out mice were protected from the development of
emphysema despite long-term smoke exposure, whereas the wild-type mice developed alveolar space enlargement.

**TIMP**

Tissue inhibitors of matrix metalloproteinases (TIMPs) are the 21-34 kDa specific endogenous inhibitors of MMPs that are widely distributed in tissue and body fluids (Birkedal-Hansen et al. 1993, Russell et al. 2002a, Lambert et al. 2004). They have similar inhibitory functions towards all MMPs with some variability, for example TIMP-1 is a poor inhibitor of some membrane-type matrix metalloproteinases (MT-MMPs) and MT1-MMP is inhibited by TIMP-2 and -3, but not TIMP-1 (Will et al. 1996, Stetler-Stevenson 2008). TIMP-1 is the main inhibitor of MMP-8 and -9, but also MMP-12. TIMPs inhibit the catalytically active enzyme and regulate the MMP activation process by delaying the conversion of proMMPs into their active forms. TIMPs also control the autocatalytic activation of many proMMPs, producing complexes with proenzymes (DeClerck et al. 1991, Howard et al. 1991, Lambert et al. 2004). TIMP-1 forms preferential complexes with proMMP-9, TIMP-2 and -4 and proMMP-2 (Goldberg et al. 1989). The balance between MMPs and TIMPs is necessary for the maintenance of normal physiological conditions in tissues (Ryan et al. 1996).

**Neutrophil elastase**

Neutrophil elastase (NE) is a serine protease that is stored mainly in azurophilic granules in neutrophils and inhibited by α1-antitrypsin in the lung parenchyma (Janoff et al. 1977, Senior et al. 1977, Damiano et al. 1986, Saetta et al. 2001, Barnes et al. 2003). NE may play a role in degenerative and inflammatory diseases by cleaving the collagen IV and elastin of the ECM. Neutrophil-derived proteinases, especially NE, are known to be associated with airway obstruction, emphysema and mucous gland hypersecretion in COPD (Janoff et al. 1977, Senior et al. 1977, Damiano et al. 1986, Shapiro and Ingenito 2005). NE reduces the ciliary beat frequency of the human respiratory epithelium in vitro (Smallman et al. 1984), which is consistent with the reduced mucociliary clearance and the damage and subsequent repair occurring in the bronchi of COPD patients (Currie et al 1987). In addition, NE enhances oxidative stress (Aoshiba et al. 2001), causes mucus gland hyperplasia and
epithelial cell metaplasia (Suzuki et al. 1996) and acts as secretagogue (Voynow et al. 1999), contributing to the development of chronic bronchitis in COPD.

3.3.3. Surfactant protein-A and -D in COPD

Pulmonary surfactant is a complex mixture of the lipids and proteins that cover the surface of the alveolar epithelium and which is essential for normal lung function (Haagsman and van Golde 1991). Pulmonary surfactant, or the components of surfactant, play a significant role in bronchiolar stability, innate host defence, and the regulation of the inflammatory processes in the lung (Whitsett 2005).

Surfactant protein (SP)-A and SP-D are collagen-like glycoproteins belonging to the collectin class of C-type lectins. They are synthesised in alveolar type II cells and nonciliated bronchiolar cells of the distal pulmonary epithelium. Both surfactant proteins play important roles in pulmonary immunity, surfactant homeostasis and the regulation of oxidant and inflammatory stress in the lung (Mason et al. 1998, Whitsett 2005).

SP-A is a glycoprotein, synthesized by alveolar epithelial type II and Clara cells and it is known to be involved in lung defence mechanisms (McCormack and Whitsett 2002, Kishore et al. 2006). SP-A is the most abundant surfactant protein in the alveolar space, playing roles in the structure, metabolism, and function of surfactant (Uthaisangsook et al. 2002). In addition, SP-A regulates immune cell functions, including cell proliferation, expression of cell surface markers, cytokine production and the generation of oxidative activity (Phelps 2001). SP-A has a low molecular weight (30 kDa) and it leaks into the bloodstream if smoking has caused an epithelial injury and this is thought to occur before the changes in pulmonary function tests (McCormack 1998).

Non-biased proteomic studies on human lung revealed SP-A to be substantially elevated in lung tissues, lung cells and tissue homogenates obtained from subjects with COPD as compared to the control lung, and the findings could be confirmed in sputum specimens (Ohlmeier et al. 2008, Mazur et al. 2011). Several other studies have revealed increased SP-A levels in the serum of smokers, COPD patients and pulmonary fibrosis (Whitsett 2005, Kishore et al. 2006) while in some studies the levels of SP-A were decreased in bronchoalveolar lavage (BAL) fluid of smokers.
and COPD patients (Honda et al. 1996, Fujishima et al. 1999, Betsuyaku et al. 2004). Overall, those studies suggest that circulating SP-A is generally related to the lung damage, and is not specific to COPD, but whether sputum SP-A is associated with smoking/COPD will require future investigations.

SP-D is a large hydrophilic protein with a structure of a triple-helical collagen region and a C-terminal homotrimERIC lectin or a carbohydrate recognition domain. SP-D is formed in the endoplasmic reticulum of type II pneumocytes and the secretory granules of Clara cells (Mori et al. 2002).

Elevated SP-D levels have been found in smokers and COPD patients’ serum samples (Mutti et al. 2006, Lomas et al. 2009) and they have been reported to be reduced by corticosteroid therapy (Sin et al. 2008, Lomas et al. 2009). Some other studies have found decreased SP-D levels in BAL fluid from smokers and COPD patients (Honda et al. 1996, Betsuyaku et al. 2004, Sims et al. 2008). However, the role of SP-A and SP-D in COPD, and their role compared to some other potential COPD biomarkers such as MMPs have remained unclear.

4. Diagnosis of COPD

COPD should be considered in any patient who has symptoms of chronic cough, sputum production, dyspnoea and/or a prolonged history of exposure to risk factors for the disease, such as cigarette smoke. Since there are no significant clinical symptoms in the early stages of the disease, most patients only seek medical help when there are already signs of ventilatory abnormalities.

Spirometry is the standard tool for diagnosing and monitoring COPD. Post-bronchodilator (e.g. salbutamol 400 µg) ratio of forced expiratory volume in one second/forced vital capacity (FEV₁/FVC) <0.7 in combination with FEV₁ <80% of predicted value confirms the presence of airflow limitation that is not fully reversible (Pauwels et al. 2001, GOLD 2010). The GOLD criteria classify COPD into four stages based on the spirometry results (Table 1). Even the early stages of the disease with normal lung function parameters (FEV₁/FVC>0.7) possess inflammatory changes, lung functional and structural abnormalities in the airways and lung.
parenchyma (Hogg 2004, Nagelmann et al. 2011). However, there are no good diagnostic methods for early COPD.

In addition to spirometry, the severity of dyspnoea can be assessed by the Medical Research Council Questionnaire for Assessing the Severity of Breathlessness. Body mass index (BMI) values <21 kg/m2 are also associated with increased mortality. BMI and dyspnoea have proved to be useful in predicting outcome such as survival, and should thus be evaluated in all patients (GOLD 2010). Physical examination is important component of patient care, but it is rarely diagnostic in COPD (Kesten and Chapman 1993).

5. Treatment strategies for COPD

The treatment objectives for COPD include slowing the accelerating decline in lung function; relieving symptoms, such as shortness of breath and cough; improving daily lung function; reducing exacerbations; and improving quality of life (Pauwels et al. 2001).

While many medications are available to treat COPD, no drug has demonstrated effectiveness in halting the progression of the disease. The most important and effective treatment for COPD is smoking cessation and this should be regarded as a primary and specific intervention since at present, this is the only effective way to slow down the disease progression (Culpitt and Rogers 2000, Scanlon et al. 2000, Anthonisen et al. 2005). Other treatment options depend on the general condition of the patient, the severity of the disease and the presence of co-morbidities.

Pharmacological therapy for COPD can reduce symptoms, increase exercise capacity, reduce the number and severity of exacerbations and improve health quality, but no treatment has been shown to significantly modify the rate of decline of lung function (Anthonisen et al. 2005). Short-acting bronchodilators, both beta-agonists and anticholinergics are the mainstay of medication therapy for COPD, providing rapid relief of symptoms. Long-acting bronchodilators are used for moderate to severe COPD and inhaled corticosteroids are recommended for those patients with moderate to severe COPD who often experience worsening symptoms (exacerbations) or have experienced recurrent hospitalization due to COPD. At present, once daily administered inhaled long-acting bronchodilators are available,
i.e. tiotropium and indacaterol (Tashkin et al. 2008, Donohue et al. 2010). Systemic corticosteroids are used to relieve airway inflammation and constriction, mainly during exacerbations. Antibiotics are beneficial during periods of exacerbation. Combining different bronchodilators (β-agonists, anticholinergic drugs and methylxanthines) achieves a greater change in spirometry and symptoms than single agents alone. Although there is limited evidence that regular treatment with long-acting β2-agonists and inhaled glucocorticosteroids can decelerate the decline of lung function (Calverley and Rennard 2007, Celli et al. 2008). The newest medication in COPD treatment is the phosphodiesterase-4 (PDE-4) inhibitor (rofumilast) that reduces inflammation through the inhibition of breakdown of intracellular cyclic AMP, but has no direct bronchodilator effect. It has been shown that rofumilast reduces the exacerbations of COPD and improves the FEV₁ in patients treated with salmeterol or tiotropium (Fabbri et al. 2009).

In addition to medical therapy, long-term oxygen therapy improves survival, exercise, sleep and cognitive performance in individuals who have hypoxemia. COPD patients with dyspnoea, reduced exercise tolerance or impaired health status obtain benefit from pulmonary rehabilitation (Spruit and Wouters 2007, Wijkstra and Wempe 2011). Despite receiving the best available care, lung function in the COPD patient can be expected to worsen over time. In some cases highly selected COPD patients may benefit from surgical treatment: bullectomy, lung volume reduction or lung transplantation (Pauwels et al. 2001). In addition to the medical therapies, regular exercise/training and individualized rehabilitation are of major importance.

6. Prognosis of COPD

COPD prognosis may vary with individual conditions and the progression of the disease. When COPD is diagnosed at an early stage and the patient undertakes lifestyle changes such as smoking cessation, adhering to a healthy diet and exercising regularly, the prognosis may be favorable. The five year survival rates for people with advanced COPD are approximately 26%, but increasing age, BMI and domestic oxygen use are strong predictors of mortality within 5 years (Martinez et al. 2006, Chung et al. 2010).
It is apparent that detecting early mild disease and preventing the disease progression with efficient antismoking campaigns are the key means to prevent the high burden of severe COPD in the community. At present, biomarkers have been assessed in urine, blood, sputum, bronchoalveolar lavage fluid, skin and exhaled breath condensate, mostly in moderate or severe COPD and very often by using invasive techniques. None of those postulated markers have been utilized successfully in the early diagnosis of COPD.
AIMS OF THE STUDY

The present study attempted to find sensitive non-invasive markers for early COPD and to clarify the role of oxidative/nitrosative stress, the proteinase/antiproteinase imbalance and surfactant proteins in the pathogenesis of COPD.

The aims of the present study were

- to investigate whether smoking increases the levels of oxidant markers in the induced sputum, and to assess if there are any differences in these biomarkers between non-symptomatic smokers with normal lung function values and symptomatic smokers who are at risk for experiencing COPD development

- to evaluate the significance of fractioned exhaled NO (FENO) in smokers and its association with sputum oxidative/nitrosative stress and airway inflammation

- to study whether induced sputum and/or plasma matrixmetalloproteinases (MMP-8, -9, -12) and tissue inhibitor of MMPs (TIMP-1) can differentiate non-symptomatic smokers from symptomatic smokers

- to characterize the association of MMP-8, MMP-9, MMP-12, TIMP-1 with the oxidative stress and inflammatory profile of the airways

- to study the role of neutrophil elastase (NE), MMP-8 and MMP-9 in COPD, and during its exacerbation and recovery period

- to compare and evaluate the levels between circulating SP-A, a marker identified from recent proteomic study on COPD, SP-D, MMP-9 and TIMP-1 in young and elderly smokers and subjects with COPD.
MATERIALS AND METHODS

1. Subjects

All subjects including non-smokers, smokers and subjects with COPD in studies I-V provided written consent for their participation. The studies were approved by the Ethics Committee of Helsinki University Hospital (www.hus.fi/clinicaltrials) and Ethics Committee of Lapland Central Hospital (4th June 2003 and 31st October 2006). Clinical symptoms were assessed with the St George’s Respiratory Questionnaire (Jones et al. 1992). Each subject underwent spirometry with the bronchodilator test and an assessment of total lung capacity and diffusion capacity. The diagnosis and staging of COPD were based on the clinical symptoms and GOLD criteria with post-bronchodilator forced expiratory flow in one second (FEV₁)/ forced vital capacity (FVC) <70% with the post-bronchodilator effect (FEV₁ after 400 µg of inhaled salbutamol) less than 10% (Pauwels et al. 2001).

Exclusion criteria included allergies, asthma, a history of respiratory disease other than COPD, or respiratory infection within 8 weeks preceding the study. None of the subjects were allowed to smoke during the 12 hours before collection of the specimens.

The main characteristics of the study populations are shown in Table 4.

Study I

The study population consisted of 22 current smokers: 13 healthy non-symptomatic smokers, 9 smokers with chronic symptoms such as cough and sputum production but with normal lung function parameters (Stage 0 COPD), with an average smoking history of 41 pack years. Twenty-two non-smokers were recruited into the control group: 11 never smokers and 11 ex-smokers who had stopped smoking at least 20 years earlier (less than 15 pack years) with no history of lung disease. None of the subjects had airway obstruction (postbronchodilator FEV₁/FVC > 70%) or significant reversibility (less than 10% reversibility in FEV₁ after 400 µg of inhaled salbutamol). One smoker with chronic symptoms was taking an inhaled steroid and two had been
prescribed a short-acting bronchodilator as a relief medication. None of the other subjects were using any medications.

**Study II**

The study population comprised a total of 58 subjects: 11 never smokers, 11 ex-smokers (at least 20 years from quitting of smoking with a smoking history of less than 15 pack years), 13 “healthy” smokers without any respiratory symptoms, 9 GOLD Stage 0 COPD patients with chronic symptoms (cough and phlegm), and 14 patients with Stage I-III COPD. Never smokers, ex-smokers, asymptomatic smokers and those with Stage 0 COPD had no medication except for two individuals with Stage 0 COPD who had been prescribed a short-acting $\beta_2$-agonist. All 14 subjects with Stage I-III COPD (100%) were using inhaled short-acting bronchodilators, 11 (79%) were receiving long-acting bronchodilators and 9 (64%) were taking inhaled steroids.

**Study III**

This study population consisted of 23 smokers with Stage 0 COPD, an average smoking history of 37 pack-years, 23 non-symptomatic smokers who had smoked for 24 pack-years and 32 non-smoking healthy controls. Twelve of the non-smoking subjects were former smokers (mean 15.7 pack-years) who had stopped smoking at least 20 years before the study; all of the others were never smokers. For comparison, 10 stable Stage I-III COPD patients (mean 47 pack-years, $\text{FEV}_1$ 62 % of predicted, DLCO/VA 66 % of predicted) were included. 

Three subjects with Stage 0 COPD were using short-acting $\beta_2$-agonists as well as inhaled corticosteroid therapy. Eight Stage I-III COPD patients had been prescribed inhaled steroids, nine were using long-acting $\beta_2$-agonists and five patients were being treated with anticholinergics. All had been given short-acting $\beta_2$-agonists as a rescue medication. None of them were regular users of oral steroids.
Study IV

The study population included a total of 85 subjects. Ten patients with Stage II-III COPD were examined during COPD exacerbation within 24-72 hours after admission to the hospital and 8 of them one month after treatment. The exclusion criteria included evidence of pneumonia in the chest X-ray, malignancies, major cardiac diseases, asthma, allergies, respiratory insufficiency, and all systemic and other lung diseases other than COPD. The regular medications of the COPD patients with exacerbation consisted of inhaled corticosteroid for 6 patients, long-acting β2-agonists for 5 patients, short-acting β2-agonists for all and anticholinergics for 5 subjects. During the COPD exacerbation, all patients received systemic corticosteroid and antibiotic treatment. Fifteen patients with stable Stage I-III COPD, 28 smokers with normal lung function values and 32 non-smoking healthy non-smokers were recruited into the control groups. Twelve of the non-smoking subjects were ex-smokers, who had stopped smoking at least 20 years earlier, all others were never-smokers. Thirteen subjects with stable COPD were using inhaled steroids and long-acting β2-agonists, eight subjects anticholinergics and all subjects with stable COPD were prescribed with short-acting β2-agonists. None of the subjects with stable COPD were on oral steroid treatment.

Study V

The study population included 51 young (age < 25 years) healthy smokers and 36 non-smoking healthy controls who were military draftees in the Northern Command of the Finnish Defence Forces, and 40 middle-aged/elderly non-smoking healthy controls, 64 “healthy” smokers and 44 subjects with stable COPD (Stages I-III) who had been contacted from the Division of Pulmonary Medicine, Lapland Central Hospital. None of the subjects displayed any symptoms and they considered themselves as healthy. They had no other environmental exposures, no other diagnosed diseases and were taking no medications.
2. Samples and data collection

2.1. Pulmonary function tests

Flow-volume spirometry was conducted at the clinic with a pneumotachograph-based spirometer connected to a computer (Medikro M 904, Kuopio, Finland). At least three acceptable curves were recorded. The results of the following variables were taken from the envelope curve: FEV<sub>1</sub>, FVC, FEV<sub>1</sub>/FVC, MEF50, MEF25 and Finnish reference values were used (Viljanen et al. 1982a, 1982b). The pulmonary diffusing capacity for carbon monoxide (DLCO) and static lung volumes were measured with the single breath method according to the European Respiratory Society. The measurements for the exacerbation group were conducted during the stable phase at one month after the exacerbation.

2.2. Sputum induction and processing

Sputum was induced by inhalation of an aerosol of hypertonic saline (4.5%) generated by an ultrasonic nebulizer (Fuchs Medical, USA) as described and recommended by the European Respiratory Society’s Task force (Djukanovic et al. 2002). Before and during the procedure, the subjects were asked to rinse their mouths and blow their noses to minimize contamination with saliva. No premedication was used for healthy smokers or controls, whereas 200µg salbutamol (GSK) was used for COPD patients before induction. To ensure patient safety the PEF measurements were conducted before and after each 10 min inhalation of hypertonic saline. If the PEF value decreased more than 10%, then induction was interrupted and the subject was treated with inhaled salbutamol. The subjects were instructed to cough up sputum into a sterile container whenever they felt that sputum might be expectorated or at least after each 10 min inhalation period. Sputum plugs were separated from saliva and were treated as described by Rytia et al. (2000). Briefly, sputum was treated by adding an equal volume, based on the weight of the sample, of dithioerythritol (DTE, Sigma, Germany) or phosphate-buffered-saline (PBS). The samples were mixed for 30 minutes and homogenized sputum was centrifuged at 4500 rpm (400g) for 10 min to separate the supernatant from the cell pellet. The
supernatant was frozen at -80°C for biochemical and immunological analyses. PBS was added to the cell pellet and cell viability was assessed by Trypan blue in a Burker chamber. Cytocentrifuge slides were prepared by Cytospin at 450 rpm for 6 min. One slide from each patient was stained with May-Grunwald-Giemsa-staining (Merck, Germany) to differentiate macrophages, neutrophils, eosinophils, lymphocytes, and squamous epithelial cells. Four hundred cells were counted from each slide. In the further investigations, only those samples having less than 70% of squamous epithelial cells were accepted. The slides were frozen at -20°C. The samples from patients during COPD exacerbation were collected 24-72 hours after the hospital admission, which means that every patient had already been on antibiotic and systemic steroid therapies for at least 24 hours.

2.3. Plasma specimens

Plasma samples were collected from each individual at the same visit when the sputum induction was conducted. Plasma samples were centrifuged and frozen immediately at –80°C.

2.4. NO measurements (Study I)

Exhaled NO (FENO) was measured in a chemiluminescence analyser (Sievers Model 270B NOA, Sievers Instruments Boulder, CO, US) with a PC and software devised for the purpose (Rouhos et al. 2005) according to the ATS guidelines (ATS 1999). Expiratory airflow was 50ml/s and the subjects exhaled against a flow resistor (Hans Rudolph, Model No.7100R, 200 cmH2O/l/s). The mean value was recorded from a 3-second period from the end-exhaled NO plateau and in the analysis, the mean value from at least three successive FENO measurements were included.
3. Studies conducted on the sputum cells, supernatants and plasma samples

3.1. Enzyme-Linked Immunosorbent Assay (ELISA)/Enzyme Immunoassay (EIA) (Studies I, II, III, IV, V)

For the measurement of sputum and plasma MMP-8, MMP-9, TIMP-1, lactoferrin and eosinophil cationic protein (ECP) (studies I, III, IV, V) commercially available ELISA kits (Amersham Biosciences, Cardiff, UK; Calbiochem and Pharmacia Diagnostics AB, Uppsala, Sweden) were used according to the manufacturers’ instructions. The detection limits were: 0.032 ng/ml for MMP-8, 0.6 ng/ml for MMP-9, 1.25 ng/ml for TIMP-1, 1.6 ng/ml for lactoferrin and 0.5 µg/ml for ECP.

Free 8-isoprostane (study II) concentrations in induced sputum specimens were measured using a commercial enzyme immunoassay (EIA) kit according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, Mich., USA). The assay has been validated with gas chromatography-mass spectroscopy (GC-MS) (r=0.95) (Montuschi et al. 1999, Wood et al. 2005). The detection limit for the assay was 5 pg/ml.

MMP-12 (study III) measurements from induced sputum and plasma samples were made in collaboration with Professor Guy Bruselle using a custom-made ELISA, as previously described (Demedts et al. 2006). The detection limit for MMP-12 was 0.05 ng/ml.

In study V, SP-A and SP-D levels were measured with commercially available EIA/ELISA kits (SP-A test Kokusai-F kit, Sysmex, Kobe, Japan; SP-D kit Yamasa EIA kit, Yamasa Co., Chiba, Japan) as described (Takahashi et al. 2000, Kobayashi et al. 2008). The detection limits were 1.0 ng/ml for SP-A and 17.2 ng/ml for SP-D, respectively.

3.2. Immunocytochemistry for sputum cells (Studies I, III)

Induced sputum cytospin samples were treated for 40 min at room temperature for fixation and permeabilisation with Ortho Permeafix (Ortho Diagnostic Systems Inc., UK) (MMP-8, MMP-9, MMP-12, TIMP-1, iNOS and 4-HNE) or with formalin (nitrotyrosine and MPO). The endogenous peroxidase activity was blocked with
0.3% hydrogen peroxide in PBS at room temperature. For immunostaining, Zymed ABC Histostain-Plus Kit (Zymed Laboratories Inc.) was used according to the manufacturer’s instructions. The samples were incubated with polyclonal rabbit anti-human MMP-8 (Study III) (Hanemaaijer et al. 1997), MMP-9 (Study III) (NeoMarkers, Fremont, CA), TIMP-1 (Study III) (Chemicon, Temecula, CA), iNOS (Study I) (Santa Cruz Biotechnology, US), MPO (Study I, III) (LabVision Corp., Fremont, US), nitrotyrosine (Study I, III) (Upstate Lake Placid, NY, US) and 4-HNE (Study I) (Calbiochem, San Diego, US) or monoclonal MMP-12 (Study III) (R&D Systems Inc., Minneapolis, US) antibody, and negative control samples with Zymed Rabbit or Mouse (MMP-12) Isotype Control and PBS overnight at 4°C. For the testing of the specificity of the method, we used a positive control i.e. stained a control slide known to be positive. Zymed Broad spectrum antibody (Zymed Laboratories Inc., South San Francisco, CA, USA) as the secondary antibody and AEC (Zymed Laboratories Inc., South San Francisco, CA) for visualization was used for all antibodies except MPO. For MPO, Dako rabbit secondary antibody was used (Dako Cytomation, Glostrup, Denmark). Subsequently, the sputum samples were stained with Mayer’s haematoxylin. The immunoreactivities were expressed as the percentage of positive cells (from 400 cells in every cytospin) and as the total number of positive cells in the specimen (iNOS, MPO, nitrotyrosine, and 4-HNE) or as the percentage or a total number of positive macrophages and neutrophils (MMP-8, MMP-9, MMP-12 and TIMP-1).

### 3.3. Western blot analysis (Study III)

The molecular forms of MMP-9 in induced sputum were assessed by Western blot analysis. Sputum supernatants were lyophilized (total protein content 40 µg per well in 15 µL volume). After electrophoresis on 12% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), the loaded samples were blotted into nitrocellulose membranes (Schleicher & Shuell, Dassel, Germany). The membranes were first treated with polyclonal rabbit anti-human MMP-9 (Calbiochem, Darmstadt, Germany), followed washing with 0.1% TTBS and treatment with the secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). The membranes were developed and quantification was carried out using the Bio-
Rad Model GS-700 Imaging Densitometer and Analyst™ program (Sorsa et al. 1997).

3.4. Gelatinase assay (Study III)

Gelatinolytic activity of MMP-9 was assessed by zymography using 8% SDS-PAGE containing 1 mg/ml gelatine (Sigma, St. Louis, MO) as the substrate. Before electrophoresis, the sputum supernatants were lyophilized (60 µg protein) and incubated with Laemmli’s sample running buffer for 2 h at room temperature. After electrophoresis, the gels were processed for the detection of 92 kD MMP-9 (Calbiochem, Darmstadt, Germany) and stained with 0.1% Coomassie Brilliant Blue R250 as described by Sorsa et al. (1997). The gelatinolytic activity was visualized as clear bands against a blue background and quantified densitometrically (Sepper et al. 1994).

3.5. Measurement of serine proteinase activity (Study IV)

Neutrophil elastase-like activity in the induced sputum specimens was measured by monitoring the changes of optical density at 405 nm spectrophotometrically at 37°C using synthetic 1mM N-succinyl-Ala-Ala-Val- paranitroanilide (SAAVNA) (Sigma, St Louis, USA) as substrate (Bieth et al. 1974, Ingman et al. 1993). Briefly, 15 µl of sputum sample were incubated with 1 mM SAAVNA in Tris-HCl buffer, pH 7.8 containing 0.2 M NaCl and 1 mM CaCl₂ at 37°C in flat bottom 96 well polystyrene ELISA plates (A/S Nunc, Roskilde, Denmark) (Ingman et al. 1993). The absorbance at 405 nm was measured at 30 min intervals with Labsystems Multiskan PLUS (Labsystems, Helsinki, Finland) and corrected for background. The activity was expressed as international units (IU) (Bieth et al. 1974, Ding et al. 1996, Tervahartiala et al. 1996).
3.6. MMP-8 immunofluorometric assay (Study IV)

MMP-8 levels were determined by a time-resolved immunofluorometric assay (IFMA) (Hemmila et al. 1984, Holopainen et al. 2003). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labeled using europium-chelate (Hemmila et al. 1984). The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl$_2$, 50 µM ZnCl$_2$, 0.5% BSA, 0.05% sodium azide and 20 mg/l diethylenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for one hour, followed by incubation for one hour with tracer antibody. Enhancement solution was added and after 5 min fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The specificity of monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8.

4. Statistical analysis

The results are given as mean ± the standard error of the mean (SEM) or as means together with standard deviation (SD). All statistical analyses were performed with the SPSS 10, 15 and 18 software programs (SPSS Inc., Chicago, IL). As the data were not normally distributed, non-parametric tests were used for all comparisons in studies I-IV. Data for individual variables from the several groups were first analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U-test. In Study IV, all differences within sets of paired data were analysed by the non-parametric Wilcoxon signed rank test. In order to correlate the results of clinical parameters, cellular profiles and analyses the Spearman rank correlation coefficient was used. A p-value of <0.05 was considered to be statistically significant.

In study V, the analyses of variance (ANOVA) and t-test for independent groups were used to evaluate the statistical significances between the study groups. In order to study the independent effect of age, smoking status and COPD to SP-A, SP-D, MMP-9 and TIMP-1, linear multivariate regression analysis was used. Correlations between the variables were determined with the Pearson correlation coefficient. SP-A, SP-D, MMP-9 and TIMP-1 levels were further analyzed for their predictive
capability to distinguish patients with COPD from the control subjects according to receiver operating characteristic (ROC) curves. Due to the pair-wise comparisons, a p-value of <0.01 was considered statistically significant.
RESULTS

1. Characteristics of subjects

**Characteristics of non-smokers, healthy smokers and subjects with Stage 0 COPD (Studies I-III)**

The clinical characteristics of all subjects are shown in Table 4. If the group of non-smokers was divided into two subgroups, i.e. never smokers and ex-smokers, none of these characteristics differed significantly. When the group of smokers was divided into two subgroups, i.e. “healthy” smokers without any respiratory symptoms and Stage 0 COPD patients with symptoms, healthy smokers were younger than Stage 0 COPD and had smoked less pack years than Stage 0 COPD. All smokers had normal airway function according to GOLD criteria (post bronchodilator FEV$_1$/FVC >0.7), but healthy smokers tended to have better lung function i.e. post bronchodilator FEV$_1$/FVC than stage 0 COPD (Table 5).

**Characteristics of non-smokers, smokers and subjects with stable Stage I-III COPD and patients with Stage II-III COPD during exacerbation and after a one-month recovery period (Study IV)**

In all of the study groups, most of the subjects were male. COPD patients tended to be older when compared to smokers and non-smokers. There were no significant differences in numbers of smoked pack years in stable COPD and during its exacerbation (Table 4). COPD patients, especially those experiencing an exacerbation, had lower lung function values when compared to smokers and non-smokers (Table 5).

**Characteristics of young and middle-aged non-smokers, smokers and COPD patients (Study V)**

Most subjects in all study groups, except the middle-aged non-smokers, were male. Middle-aged subjects had higher BMI values and longer smoking histories. COPD
patients had smoked the most pack years. All smokers and non-smokers in both age
groups had normal lung function values according to GOLD criteria. Staging was
based on GOLD classification. The detailed characteristics are shown in Tables 4 and
5.

Table 4. Clinical characteristics of the subjects of all studies

<table>
<thead>
<tr>
<th>Study I</th>
<th>Subjects n</th>
<th>Male/female</th>
<th>Age yrs</th>
<th>BMI</th>
<th>Pack-years</th>
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<tbody>
<tr>
<td>Never-smokers</td>
<td>11</td>
<td>9/2</td>
<td>59</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Ex- smokers</td>
<td>11</td>
<td>9/2</td>
<td>56</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>Healthy smokers</td>
<td>13</td>
<td>10/3</td>
<td>53</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>COPD stage 0</td>
<td>9</td>
<td>7/2</td>
<td>64</td>
<td>29</td>
<td>55</td>
</tr>
<tr>
<td>Study II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
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<td>18/4</td>
<td>58</td>
<td>26</td>
<td>7*</td>
</tr>
<tr>
<td>Healthy smokers</td>
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<td>9/4</td>
<td>53</td>
<td>27</td>
<td>30</td>
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<tr>
<td>COPD stage ≥1</td>
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<td>7/7</td>
<td>58</td>
<td>27</td>
<td>44</td>
</tr>
<tr>
<td>Study III</td>
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<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>32</td>
<td>26/6</td>
<td>56</td>
<td>15.7**</td>
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<td>12/11</td>
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<tr>
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<tr>
<td>COPD stage ≥1</td>
<td>10</td>
<td>7/3</td>
<td>63</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Study IV</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>32</td>
<td>21/11</td>
<td>57</td>
<td>7**</td>
<td></td>
</tr>
<tr>
<td>Healthy smokers</td>
<td>28</td>
<td>21/7</td>
<td>56</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>COPD stage ≥1</td>
<td>15</td>
<td>9/6</td>
<td>63</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>COPD exa</td>
<td>10</td>
<td>5/5</td>
<td>61</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Study V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young non-smokers</td>
<td>36</td>
<td>34/2</td>
<td>19.5</td>
<td>24.1</td>
<td>-</td>
</tr>
<tr>
<td>Young smokers</td>
<td>51</td>
<td>50/1</td>
<td>20.0</td>
<td>24.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Middle-aged non-smokers</td>
<td>40</td>
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<td>26.5</td>
<td>-</td>
</tr>
<tr>
<td>Middle-aged smokers</td>
<td>64</td>
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<td>52.1</td>
<td>27.7</td>
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<td>44</td>
<td>35/9</td>
<td>61.3</td>
<td>26.9</td>
<td>39.8</td>
</tr>
</tbody>
</table>

Data is shown in means.
* This group includes 11 ex-smokers who had stopped smoking at least 20 years before the study
** This group includes 12 ex-smokers who had stopped smoking at least 20 years before the study
Table 5. Lung function (post-bronchodilator) characteristics of the subjects

<table>
<thead>
<tr>
<th>Study</th>
<th>FVC (l)</th>
<th>FVC % predicted</th>
<th>FEV₁ (l)</th>
<th>FEV₁ % predicted</th>
<th>FEV₁/FVC</th>
<th>MEF 50 % predicted</th>
<th>MEF 25 % predicted</th>
<th>Diffusion capacity (%)</th>
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<tr>
<td><strong>Study I</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never-smokers</td>
<td>4.6</td>
<td>98</td>
<td>3.6</td>
<td>97</td>
<td>79</td>
<td>87</td>
<td>98</td>
<td>94</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>5.0</td>
<td>102</td>
<td>4.4</td>
<td>111</td>
<td>87</td>
<td>131</td>
<td>151</td>
<td>98</td>
</tr>
<tr>
<td>Healthy smokers</td>
<td>4.7</td>
<td>95</td>
<td>3.8</td>
<td>96</td>
<td>81</td>
<td>85</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
<td>COPD stage 0</td>
<td>3.4</td>
<td>82</td>
<td>2.6</td>
<td>77*</td>
<td>76</td>
<td>57</td>
<td>53</td>
<td>80</td>
</tr>
<tr>
<td><strong>Study II</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>4.6</td>
<td>100</td>
<td>3.7</td>
<td>100</td>
<td>80</td>
<td></td>
<td></td>
<td>96</td>
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<tr>
<td>Healthy smokers</td>
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<td>95</td>
<td>3.8</td>
<td>96</td>
<td>80</td>
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<tr>
<td>COPD stage 0</td>
<td>3.4</td>
<td>82</td>
<td>2.6</td>
<td>77*</td>
<td>76</td>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>COPD stage ≥1</td>
<td>2.9</td>
<td>80</td>
<td>1.7</td>
<td>57</td>
<td>57</td>
<td></td>
<td></td>
<td>58</td>
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<tr>
<td><strong>Study III</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>4.7</td>
<td>101</td>
<td>3.8</td>
<td>104</td>
<td>83</td>
<td>99</td>
<td>120</td>
<td>104</td>
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<tr>
<td>Healthy smokers</td>
<td>4.2</td>
<td>97</td>
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<td>81</td>
<td>88</td>
<td>109</td>
<td>96</td>
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<tr>
<td>COPD stage 0</td>
<td>4.1</td>
<td>87</td>
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<td>85</td>
<td>79</td>
<td>71</td>
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<tr>
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<td>1.7</td>
<td>59</td>
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<td>23.4</td>
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<tr>
<td>Non-smokers</td>
<td>4.8</td>
<td>101</td>
<td>4.0</td>
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<td>89</td>
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<td>87</td>
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<td>82</td>
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<tr>
<td>COPD stage ≥1</td>
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<td>76</td>
<td>1.6</td>
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<td>63</td>
<td>1.3</td>
<td>43</td>
<td>54</td>
<td></td>
<td></td>
<td>51</td>
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<tr>
<td><strong>Study V</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young non-smokers</td>
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<td>4.8</td>
<td>97</td>
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<td></td>
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</tr>
<tr>
<td>Young smokers</td>
<td>5.5</td>
<td>4.8</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>87</td>
</tr>
<tr>
<td>Middle-aged non-smokers</td>
<td>3.8</td>
<td>3.2</td>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>Middle-aged smokers</td>
<td>4.0</td>
<td>3.3</td>
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<td>83</td>
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<tr>
<td>COPD stage ≥1</td>
<td>3.8</td>
<td>2.3</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61</td>
</tr>
</tbody>
</table>

Data is shown in means.

* In studies I and II, most of the subjects in Stage 0 group had airway restriction measured by spirometry, but normal FEV₁/FVC (>0.7). However, they had no other diagnosed diseases and according to the GOLD guideline (Pauwels et al. 2001) COPD could not be diagnosed.
2. Cell profile (Studies I, II, III, IV)

The inflammatory profile of induced sputum was measured in studies I-IV (Table 6). In all studies, the smokers had a higher number and larger percentage of neutrophils when compared to non-smokers. When the group of smokers was divided into the subgroups (smokers without symptoms vs. smokers with chronic symptoms but normal lung function parameters (GOLD Stage 0), the levels of neutrophils were very similar, while in the group of non-smokers, ex-smokers (at least 20 years from quitting) tended to have higher levels of neutrophils than never-smokers. Subjects with stable Stage I-III COPD had higher neutrophil levels than non-smokers (Study II, III, IV) and these values increased further during the exacerbation (Study IV) and declined in the recovery period (Figure 2).

The numbers or percentages of macrophages were not significantly different between the groups or subgroups, but smokers and COPD patients with or without exacerbation tended to have a lower percentage of macrophages when compared to non-smokers.
Table 6. Sputum cell profiles (Studies I-IV)

<table>
<thead>
<tr>
<th></th>
<th>Neutrophils (%)</th>
<th>Macrophages (%)</th>
<th>Neutrophils x 10^6/g</th>
<th>Macrophages x 10^6/g</th>
</tr>
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<tbody>
<tr>
<td><strong>Study I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never-smokers</td>
<td>17</td>
<td>63</td>
<td>0.07</td>
<td>0.26</td>
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<tr>
<td>Ex-smokers</td>
<td>42</td>
<td>37</td>
<td>0.25</td>
<td>0.22</td>
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<tr>
<td>Healthy smokers</td>
<td>44</td>
<td>47</td>
<td>0.49</td>
<td>0.50</td>
</tr>
<tr>
<td>COPD stage 0</td>
<td>64</td>
<td>33</td>
<td>0.70</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Study II</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>30</td>
<td>47</td>
<td>0.16</td>
<td>0.24</td>
</tr>
<tr>
<td>Healthy smokers</td>
<td>44</td>
<td>47</td>
<td>0.50</td>
<td>0.53</td>
</tr>
<tr>
<td>COPD stage 0</td>
<td>64</td>
<td>33</td>
<td>0.70</td>
<td>0.34</td>
</tr>
<tr>
<td>COPD stage ≥1</td>
<td>65</td>
<td>27</td>
<td>0.83</td>
<td>0.33</td>
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<tr>
<td><strong>Study III</strong></td>
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<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>31</td>
<td>53</td>
<td>0.45</td>
<td>0.93</td>
</tr>
<tr>
<td>Healthy smokers</td>
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<td>46</td>
<td>0.99</td>
<td>1.04</td>
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<td>COPD stage 0</td>
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<td>1.48</td>
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<tr>
<td>COPD stage ≥1</td>
<td>73</td>
<td>22</td>
<td>2.87</td>
<td>0.75</td>
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<td></td>
</tr>
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<td>0.52</td>
<td>2.06</td>
</tr>
<tr>
<td>Healthy smokers</td>
<td>55</td>
<td>38</td>
<td>1.20</td>
<td>1.08</td>
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<tr>
<td>COPD stage ≥1</td>
<td>60</td>
<td>31</td>
<td>2.55</td>
<td>0.79</td>
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<tr>
<td>COPD exa</td>
<td>83</td>
<td>9</td>
<td>2.78</td>
<td>0.33</td>
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</table>

Data is shown in means.

**Figure 2.** Cell differential counts in induced sputum from non-smokers (NS), healthy non-symptomatic smokers (HS), Stage 0 subjects (Stage 0), stable Stage I-III COPD and patients with COPD exacerbation (COPDexa) and its one (COPD exa1) and two (COPD exa2) month’s recovery period. The results from the two-month recovery period are not shown in article IV because of the small number (n = 4) in this patient group. This data includes all of the patients examined in articles I-IV.
3. Oxidative/nitrosative stress in smokers

3.1. Markers of increased oxidative stress in smokers (Study I)

The levels or expression of inducible nitric oxide synthase (iNOS), myeloperoxidase (MPO), lactoferrin, eosinophil cationic protein (ECP), nitrotyrosine and 4-hydroxy-2-nonenal (4-HNE) were investigated from the induced sputum to assess whether they differed between the asymptomatic healthy smokers and those who had Stage 0 COPD. Fractioned exhaled NO (FENO) was measured to identify smoking and COPD related changes separately.

3.1.1. The expression of oxidative stress markers in induced sputum of non-smokers and smokers

iNOS, MPO, nitrotyrosine and 4-HNE immunoreactivity in induced sputum of smokers and non-smokers were evaluated by immunocytochemistry. The percentage and the number of iNOS, MPO, and nitrotyrosine positive cells in induced sputum (Figure 3) were higher in “healthy” smokers than in non-smokers (p = 0.08 (%) and p = 0.004 (total), p = 0.01 (%) and p = 0.003 (total), p = 0.01 (%) and p=0.003 (total)), with no significant differences between the subgroups of never smokers vs. ex smokers and non-symptomatic smokers vs. Stage 0 COPD. Four representative sputum samples showed 4-HNE immunopositivity in cigarette smokers when compared to non-smokers as a marker of ongoing lipid peroxidation (p = 0.03 (total)).
**Figure 3.** The total number of nitrotyrosine, iNOS and MPO positive cells in induced sputum of non-smokers (NS), healthy non-symptomatic smokers (HS) and GOLD Stage 0 (Stage 0).

3.1.2. ECP, lactoferrin and FENO in smokers

Induced sputum ECP level in non-smokers was 26 (20) µg/ml whereas in smokers it was 109 (245) µg/ml, but the difference between these groups was not statistically significant (p = 0.266). In addition, there were no significant differences in ECP levels between the subgroups (never-smokers vs. ex-smokers p = 0.831 and non-symptomatic smokers vs. Stage 0 p = 0.699).

Sputum lactoferrin levels were increased in smokers when compared to non-smokers (p = 0.02), but the difference between never-smokers and ex-smokers (p = 0.669) and non-symptomatic smokers and Stage 0 COPD was not significant (p = 0.17).

Smokers had significantly lower FENO levels i.e. 11 (6.7) ppb as compared to non-smokers 23 (10.0) (p < 0.0001). The level of FENO did not differ between the subgroups i.e. never smokers vs. ex smokers (p = 0.193) or healthy smokers vs. Stage 0 COPD (p = 0.744).
3.1.3. Correlations between oxidative stress markers, inflammatory cell profile and lung function values

Since the regulation of lowered FENO in cigarette smokers is poorly understood, it was decided to correlate the levels of FENO with the markers of oxidative/nitrosative stress. A significant negative correlation was found between FENO and total number of neutrophils (r = -0.367, p = 0.02), FENO and total number of iNOS positive cells (r = -0.503, p = 0.005), FENO and total number of MPO positive cells (r = -0.547, p = 0.008) and FENO and total number of nitrotyrosine positive cells (r = -0.424, p = 0.03). In addition, there was a significant inverse correlation between FENO and BMI (r = -0.320, p = 0.03), total number of iNOS positive cells and MEF 50 (r = -0.567, p = 0.006) and a positive correlation between iNOS and total nitrotyrosine (r = 0.435, p = 0.038).

Since FENO is associated with atopy, asthma and reversibility, its association with eosinophils was evaluated. The percentage of eosinophils in induced sputum was 1.0 (0.7-3.2) % in non-smokers and 0.8 (0.3-1.3) % in smokers. No eosinophilia was found in any of the subjects. However, significant positive correlations were observed between sputum eosinophils and ECP (r = 0.661, p < 0.0001), eosinophils and iNOS (r = 0.409, p = 0.02), and negative correlation between eosinophils and MEF 25 (r = -0.374, p = 0.05).

3.2. 8-isoprostane as a marker of oxidative stress (Study II)

The levels of 8-isoprostane were analyzed from the induced sputum to specify the local oxidant burden in the airways of smokers. 8-Isoprostane levels were higher in healthy smokers as compared to non-smokers (p = 0.005) but the levels did not differ between healthy smokers and Stage 0 COPD or between never-smokers and ex-smokers. 8-Isoprostane levels were significantly increased in stable Stage I-III COPD compared to non-smokers (p < 0.0001) and healthy smokers (p = 0.02).

Sputum 8-isoprostane levels correlated with smoking history as evaluated by pack years (r = 0.56, p = 0.001), with total number of sputum neutrophils (r = 0.37, p =
0.02) and with pulmonary function tests (FEV₁/FVC: r = -0.66, p < 0.0001; FEV₁: r = -0.48, p = 0.006).

Three subjects (33%) in Stage 0 COPD and six patients (43%) in Stage I-III COPD group had stopped smoking (mean time from quitting 2 and 3.6 years). However, no significant differences in the levels of 8-isoprostane could be found between current and ex-smokers in these COPD-patients groups.

If all the subjects were divided into two groups, non-smokers and current smokers, the levels of sputum 8-isoprostane were significantly higher in current smokers (p = 0.001). In all, 64% of the COPD group were using inhaled steroids, but there were no significant differences in 8-isoprostane levels between subjects taking inhaled steroids and those without these drugs.

4. Matrix metalloproteinases as non-invasive markers for chronic obstructive pulmonary disease

Different MMPs, TIMP-1 and neutrophil elastase were evaluated from non-symptomatic and symptomatic smokers (Stage 0 COPD) (Study III) and during COPD exacerbation and the one month recovery period (Study IV) to further characterize the association between these markers and the differences in the inflammatory profiles of lung (non-symptomatic and symptomatic smokers with normal airflow parameters and severe COPD including its exacerbation and recovery period).

4.1. Matrix metalloproteinases in smokers and early stages of COPD (GOLD Stage 0) (Study III)

MMP-8, -9 and -12 and TIMP-1 levels in induced sputum and plasma samples were measured and compared in order to assess whether they differ between non-symptomatic healthy smokers and smokers who exhibit chronic symptoms i.e. Stage 0 COPD.
4.1.1. Increased sputum and plasma levels of matrix metalloproteinases in non-symptomatic and symptomatic chronic smokers (Stage 0 COPD)

Sputum MMP-8 levels significantly differed between smokers with chronic symptoms (Stage 0 COPD) and non-symptomatic smokers and non-smokers (p = 0.02 and p < 0.0001). MMP-9 levels were also higher in Stage 0 COPD and non-symptomatic smokers than in non-smokers (p < 0.0001 and p = 0.01), but the difference between non-symptomatic smokers and Stage 0 patients was not significant (p = 0.062). MMP-12 levels were higher in Stage 0 group than in non-smokers (p = 0.04), but there were no significant differences between non-symptomatic smokers and those at Stage 0 (p = 0.14). The TIMP-1 levels did not differ between the subgroups.

We also measured MMP-8 and MMP-9 levels in induced sputum in Stage I-III COPD (n=10) to confirm earlier findings. Both MMP-8 and -9 were significantly elevated in COPD compared to healthy smokers (p < 0.0001 and p = 0.01) (Figure 4).

**Figure 4.** MMP-8, MMP-9 and MMP-12 protein levels (ng/ml) in the induced sputum of non-smokers (NS), healthy non-symptomatic smokers (HS), GOLD Stage 0 (Stage 0) and Stage I-III COPD. The levels of MMP-8, -9 and -12 were significantly higher in both groups of smokers and COPD compared to non-smokers, but only MMP-8 could differentiate HS from those with Stage 0.
In induced sputum there were no significant difference in the MMP-8/TIMP-1, MMP-9/TIMP-1 or MMP-12/TIMP-1 ratios between Stage 0, smokers and non-smokers. In plasma, smokers exhibited higher MMP-9 concentrations than non-smokers (p = 0.04), but there were no significant differences between smokers without or with symptoms (Stage 0) (p = 0.62). MMP-12 levels in plasma were significantly higher in the Stage 0 group when compared to non-symptomatic smokers and non-smokers (p = 0.008). Plasma MMP-8 and TIMP-1 levels did not differ between the subgroups. In plasma, the MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios were substantially higher in healthy smokers when compared to non-smokers (p = 0.009 and p = 0.02) and MMP-12/TIMP-1 ratio was higher in Stage 0 compared to non-symptomatic smokers and non-smokers (p = 0.04 and p = 0.005).

4.1.2. Immunocytochemistry of MMPs and TIMP-1 in induced sputum cytospins

Immunocytochemical staining was performed to determine MMP-8, MMP-9, MMP-12 and TIMP-1 cell-localisation in induced sputum cytospin slides. MMP-8, MMP-9 and TIMP-1 proteins were localized predominantly in neutrophils and macrophages. MMP-12 expression was mainly detected in macrophages but could also be found in some neutrophils. Weak MMP-8 and TIMP-1 immunoreactivity was found in some ciliated epithelial cells. Immunocytochemical reactivity was similar in healthy smokers and subjects with Stage 0 COPD, whereas the expression in non-smokers was very weak.

4.1.3. MMP-9 activity by zymography and Western blotting

Zymography and Western blotting were used to evaluate MMP-9 gelatinolytic activity and to identify the molecular forms of MMP-9, demonstrating comprehensive variability. Zymography revealed gelatinolytic activity in sputum supernatants corresponding to MMP-9 (92 kD pro- and 77-82 kD active MMP-9) both in healthy smokers and those at Stage 0 with no major difference between the groups. In agreement with the results obtained in zymography, the degree of activation of MMP-9 calculated from the bands obtained from Western blot analysis (Sorsa et al 1997)
was variable (14% increase in the postulated MMP-9 active form in smokers compared to the non-smokers, n= 16).

4.1.4. Correlations of MMPs and TIMP-1

In order to characterize the association between MMP-8, MMP-9, MMP-12, TIMP-1 and inflammatory profile of the lung these MMP levels, assessed by ELISA, were correlated with sputum neutrophils and macrophages. There was a positive correlation between the levels of these MMPs and the total number and percentage of neutrophils (MMP-8: r = 0.70, p = 0.0001 and r = 0.48, p = 0.0002; MMP-9: r = 0.66, p < 0.0001 and r = 0.63, p < 0.0001; MMP-12 r = 0.50, p < 0.0001 and r = 0.19, p = 0.129; TIMP-1: r = 0.36, p = 0.002 and r = 0.32, p = 0.004). Sputum MMP-8 and MMP-9 levels displayed an inverse correlation with the percentage of macrophages (r = - 0.26, p = 0.02 and r = - 0.41, p = 0.0003). MMP-12 levels correlated positively with the total number of macrophages (r = 0.39, p = 0.008).

MMPs are activated by many different factors including oxidative stress; therefore the levels of lactoferrin by ELISA and myeloperoxidase- (MPO) and nitrotyrosine-positive cells by immunocytochemistry were evaluated. Induced sputum lactoferrin levels positively correlated with sputum MMP-8, MMP-9 and MMP-12 (r = 0.63, p < 0.0001; r = 0.50, p = 0.002; r = 0.53, p = 0.008). These correlations were not found in the plasma levels of these enzymes. In addition, sputum MMP-8 and MMP–9 positively correlated with the levels of MPO positive cells (r = 0.55, p = 0.004 and r = 0.49, p = 0.01) but not with nitrotyrosine.

The levels of various MMPs were correlated with the lung function values of all smokers with or without chronic symptoms to evaluate the significance of these MMPs in the early stages of COPD (GOLD Stage 0). There was a correlation between all MMPs and lung function values (Table 7).
Table 7. Correlations between MMPs and lung function values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MMP-8</th>
<th>MMP-9</th>
<th>MMP-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁% pred</td>
<td>r = -0.42</td>
<td>r = -0.39</td>
<td>r = -0.32</td>
</tr>
<tr>
<td></td>
<td>p = 0.0007</td>
<td>p = 0.002</td>
<td>p = 0.03</td>
</tr>
<tr>
<td>FVC% pred</td>
<td>r = -0.25</td>
<td>r = -0.25</td>
<td>r = -0.32</td>
</tr>
<tr>
<td></td>
<td>p = 0.05</td>
<td>p = 0.05</td>
<td>p = 0.03</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>r = -0.36</td>
<td>r = -0.36</td>
<td>r = -0.13</td>
</tr>
<tr>
<td></td>
<td>p = 0.005</td>
<td>p = 0.004</td>
<td>p = 0.36</td>
</tr>
<tr>
<td>MEF50% pred</td>
<td>r = -0.35</td>
<td>r = -0.35</td>
<td>r = -0.27</td>
</tr>
<tr>
<td></td>
<td>p = 0.005</td>
<td>p = 0.005</td>
<td>p = 0.06</td>
</tr>
<tr>
<td>MEF25% pred</td>
<td>r = -0.44</td>
<td>r = -0.46</td>
<td>r = -0.3</td>
</tr>
<tr>
<td></td>
<td>p = 0.0004</td>
<td>p = 0.0003</td>
<td>p = 0.04</td>
</tr>
</tbody>
</table>

4.2. Neutrophil proteinases in COPD exacerbation (Study IV)

The levels of neutrophil elastase (NE), MMP-8 and -9 from induced sputum were investigated during COPD exacerbation and after a one-month recovery period to ascertain the role of these proteinases in COPD exacerbations.

4.2.1. Neutrophil elastase, MMP-8 and -9 in sputum samples

The levels of MMP-8 and MMP-9 and the activity of neutrophil elastase (NE) in induced sputum were higher during the COPD exacerbation than in stable COPD (p = 0.02, p = 0.02 and p = 0.01), smokers (p < 0.001, p = 0.003 and p = 0.001) and healthy controls (p < 0.0001, p < 0.0001 and p < 0.0001).

From the 10 COPD patients with exacerbations, only eight could come to the control visit, and even some of them could not expectorate enough sputum for all analyses. Despite those drop outs, the levels of NE and MMP-8 were significantly higher
during the COPD exacerbation than after the one month recovery period (NE: p = 0.03 and MMP-8: p = 0.04) (Figure 5).

**Figure 5.** The mean values for neutrophil elastase (A), and MMP-8 and MMP-9 (B) in non-smokers, healthy smokers, stable Stage I-III COPD and patients with COPD exacerbation (COPDexa) and its one (COPD exa1) and two (COPD exa2) months recovery period. The results at the two-month recovery period are not shown in the article IV because of the low number (n = 4) of the patients in this group. However, there was a clear decline in the levels of these enzymes during the recovery period.
4.2.2. Neutrophil proteinases correlated with sputum cells and lung function parameters

There was a significant positive correlation between NE, MMP-8 and MMP-9 and the percentage of sputum neutrophils (r = 0.59, p < 0.0001, r = 0.58, p < 0.0001; r = 0.57, p < 0.0001).

MMP-8 and MMP-9 levels displayed an inverse correlation with the extent of airway obstruction (FEV₁/FVC) (r = -0.31, p = 0.01 and r = -0.41, p = 0.003) and NE and MMP-9 negatively correlated with the diffusion capacity (DLCO) (r = -0.35, p = 0.006 and r = -0.43, p = 0.0002).

5. Age and smoking affect plasma biomarkers (Study V)

The levels of circulating SP-A, SP-D, MMP-9 and TIMP-1 were measured from young and middle-aged/older smokers and non-smokers, and COPD patients to determine whether there were some age related alterations in the levels of these proteins in healthy non-smokers and smokers, and whether they might be suitable as markers in the early detection of cigarette smoke-induced airway inflammation and the early assessment of COPD.

5.1. Surfactant protein A

The concentration of plasma SP-A increased with ageing both in smokers and non-smokers (p < 0.0001 and p < 0.0001). In addition, long-term cigarette smoking increased the SP-A level (p < 0.0001), whereas short-term smoking had no significant effect on the SP-A levels in the young age group.

The COPD group had higher SP-A levels compared to middle-aged/elderly smokers and non-smokers (p = 0.009 and p < 0.0001). Importantly, the linear regression analysis confirmed the independent effect of age, cigarette smoking and COPD (p = 0.019, p < 0.001 and p < 0.001) on the SP-A level. The plasma SP-A levels most significantly correlated with age, pack years, lung function values, especially with
FEV₁/FVC and also with SP-D (r = 0.58, p < 0.0001; r = 0.40, p < 0.0001; r = 0.46, p < 0.0001 and r = 0.36, p < 0.0001).

5.2. Surfactant protein D

The level of plasma SP-D did not change with age or smoking. The middle-aged/elderly group of smokers and smokers with COPD had higher levels of SP-D when compared to non-smokers (p=0.012 and p<0.0001) with no significant difference between smokers and COPD. When age was adjusted in the linear multivariate regression analysis, then the SP-D levels of the COPD group were even higher (p=0.001). The plasma SP-D level correlated with age, pack years and lung function values (FEV₁/FVC) (r = 0.27, p = 0.0008; r = 0.26, p = 0.009 and r = 0.31, p = 0.0001).

5.3. Matrix metalloproteinase -9

The plasma level of MMP-9 did not change with age or short-term smoking. In the older age group the levels were higher in smokers and COPD patients compared to non-smokers (p<0.0001 and p=0.033). The linear regression analysis confirmed that both age and cigarette smoking (p = 0.022 and p < 0.0001) had independent effects on the MMP-9 level. Some correlations could be detected between plasma MMP-9 and age, pack years, and BMI.

5.4. TIMP-1 and MMP-9/TIMP-1

The concentration of plasma TIMP-1 decreased with age (p=0.03), but short term smoking did not affect the level of TIMP-1. Middle aged/elderly smokers and the COPD group had higher levels of TIMP-1 than non-smokers of that same age group (p<0.001 and p=0.001), but there was no significant difference between smokers and individuals with COPD. The ratio of MMP-9 to TIMP-1 did not change with age or short-term smoking, while long-term smoking in the older group elevated the MMP-
TIMP-1 ratio \( p<0.0001 \) suggesting a significant protease/antiprotease imbalance in favour of proteases.

The linear regression analysis revealed that smoking had an independent effect on MMP-9 and MMP-9/TIMP-1 \( p = 0.018 \) and \( p = 0.014 \).

**Figure 6.** SP-A, SP-D, MMP-9 and TIMP-1 protein levels (ng/ml) in plasma samples of young non-smokers (YNS), young smokers (YS), middle-aged/elderly (old) non-smokers (ONS) and smokers (OS), and stable Stage I-III COPD.

5.5. **SP-A may be the promising marker for COPD**

The receiver-operating characteristic (ROC) curve analysis was performed to assess the sensitivity, specificity and diagnostic accuracy of plasma concentrations of SP-A, SP-D, MMP-9 and TIMP-1. These graphs revealed that plasma SP-A had the best diagnostic accuracy for COPD: the area under the ROC curve: 0.845 (95% confidence interval (CI), 0.787 to 0.902, \( p < 0.001 \)). In addition, SP-D showed good diagnostic accuracy: 0.734 (95% CI, 0.636 to 0.883, \( p < 0.001 \)) while MMP-9 and TIMP-1 curves did not show any ability to differentiate COPD patients from controls.
DISCUSSION

COPD is a disease characterized by poorly reversible progressive airflow limitation. It is associated with chronic airway inflammation with neutrophil predominance (Bosken et al. 1992), and periods of acute exacerbations (Fletcher and Peto 1977) that vary in frequency, worsening airway inflammation and impairing lung function (Gompertz et al. 2001) finally culminating in severe COPD. The major risk factor for the development of COPD is cigarette smoking, which has many effects on the airways and there is urgent need to clearly differentiate the effects of smoking from those of the disease and its progression. Since there are no significant clinical symptoms in the early stages of the disease, most patients only seek medical help when substantial ventilatory abnormalities have been developed.

The GOLD classification of COPD from the year 2001 has included a group of symptomatic subjects with normal $\text{FEV}_1/\text{FVC}>0.7$. This group was called Stage 0, a term which has been subsequently deleted from the GOLD classification, and was termed as smokers who have a risk for suffering COPD (Pauwels et al. 2001). The present study was intended to investigate the effects of smoking alone and the Stage 0 on the levels of oxidant markers, MMPs and TIMPs in induced sputum specimens and plasma samples and to compare the values to those from non-smokers. Additional studies were conducted to evaluate the effect of age and the value of recently reported potential COPD markers using plasma samples obtained from chronic smokers i.e. subjects from 20 years of age to their sixties. The present study confirmed that even early stages of the disease when the subjects have normal lung function parameters ($\text{FEV}_1/\text{FVC}>0.7$) and only mild symptoms, such as cough and sputum production (Stage 0 COPD), are associated with inflammatory changes, the oxidant burden and protease/antiprotease imbalance in the airways. However, most of the changes can be even detected in chronic smokers who have normal lung function values and who consider themselves as being totally asymptomatic. In addition, several potential markers of early COPD /lung injury correlate significantly with age, which need to be taken into consideration when the potential value of these compounds are being evaluated. Moreover, several compounds which were first detected in the sputum specimens were found to be changed also in the systemic circulation, emphasizing the systemic manifestations of smoking.
1. The potential markers of COPD and their significance in the early diagnosis of this disease (Studies I, II and III)

Markers of airway inflammation and oxidative stress have been mainly investigated in moderate/severe chronic obstructive pulmonary disease (COPD) or during disease exacerbation. They have not been compared in healthy non-symptomatic smokers or in smokers who have chronic symptoms i.e. Stage 0 COPD.

1.1. The expression of oxidative stress markers in symptomatic and non-symptomatic smokers

Oxidative/nitrosative stress has been proposed to be involved in the development of COPD (Kharitonov and Barnes 2001, Montuschi et al. 2004, Kinnula 2005, Rahman and Adcock 2006). One puff of a cigarette smoke contains billions of free radicals (Church and Pryor 1985), which stimulate alveolar macrophages to further produce reactive oxygen species (ROS) and to release a number of mediators, some of which attract neutrophils and other inflammatory cells into the lungs of COPD patients. Many markers of oxidative/nitrosative stress such as nitrotyrosine, MPO, and 4-HNE are known to be associated with COPD (Ichinose et al. 2000, Aaron et al. 2001, Rahman et al. 2002, Barczyk et al. 2004) and some markers, for instance FENO and iNOS have been found to exhibit extensive variability in COPD. However, little attention has been paid to the expression of these markers in those smokers with symptoms, but without airway obstruction (Stage 0) or in “healthy” asymptomatic smokers.

In the present study the expression of iNOS, nitrotyrosine, MPO and 4-HNE were assessed by immunocytochemistry that is a widely used laboratory technique that allows the evaluation of whether or not cells in a particular sample express the antigen in question. However, there are many problems that can influence the sensitivity or specificity of this method. The specificity can be tested by using a positive control, or staining a control slide known to be positive, as was done in this study. Negative control staining was carried out by substituting antibodies with PBS, non-immune mouse or rabbit serum, or isotype control. It is also possible that positive immunocytochemical staining occurs even though the protein is in an inactive form.
since it measures only immunoreactivity (Burry 2010). Immunohisto-cytochemistry remains also a poor alternative compared to standardized quantitative analyses of proteins. As a single method for assessing these enzymes, immunocytochemistry could be considered as weak, but since induced sputum showed marked positivity for all these oxidative/nitrosative stress markers in symptomatic and non-symptomatic smokers with normal lung function parameters when they were compared with healthy non-smoking controls, this points to better reliability with this method. There were no significant differences between those two groups of smokers.

Cigarette smoke can cause increased levels of NO and ROS either directly or endogenously via iNOS activation and recruitment of inflammatory cells. The present study detected an increased expression of iNOS in the inflammatory cells in the sputum of smokers with normal lung function when compared to non-smokers, indicating that smoking had induced up-regulation of iNOS and that this may be involved in the smoking-induced airway inflammation and modulation of NO production.

Increased immunoreactivity of nitrotyrosine has been found in biopsies of different respiratory disorders like asthma (Saleh et al. 1998, MacPherson et al. 2001), idiopathic pulmonary fibrosis (Saleh et al. 1997), respiratory distress syndrome (Haddad et al. 1994) and in severe COPD (Ricciardolo et al. 2005) pointing to the involvement of nitrosative stress in the pathogenesis of COPD and in the progressive deterioration of lung function. In addition, iNOS and nitrotyrosine positive cells have been described as being present in the sputum of moderate-severe COPD (Ichinose et al. 2000). In the present study, the numbers of nitrotyrosine positive cells were significantly increased already in smokers with no airway limitation accompanied with or without symptoms.

MPO positive cells were increased in both groups of smokers without airway limitation when compared to non-smokers, suggesting that MPO-dependent nitrotyrosine formation could be involved in the development of the airway inflammation encountered in chronic smokers. In smokers with normal lung functions, the numbers of nitrotyrosine and MPO positive cells correlated with the numbers of neutrophils but not with those of macrophages, indicative of a major role
for neutrophils in mediating nitrosative stress in the airways. As expected, MPO positively correlated with lactoferrin, a protein that is located in neutrophils. Similarly, the numbers of 4-HNE positive cells, a marker of lipid peroxidation, which has been earlier detected in lung biopsies of COPD patients (Rahman et al. 2002), were higher in both groups of smokers as compared to non-smokers.

These data suggest the involvement of oxidative and nitrosative stress being reflected in several different markers such as iNOS, nitrotyrosine, MPO and 4-HNE in smoking-induced airway inflammation even in "healthy" asymptomatic smokers as well as in symptomatic smokers. This study was cross-sectional, and based on the data, the role of oxidative/nitrosative stress in the pathogenesis and progression of COPD remained unclear. However, it can be concluded that there is significant oxidant burden in smokers’ lung before the development of disease, which in turn can contribute to persistent alterations in the levels of redox signaling molecules and cytokines found in these same individuals.

In addition, lactoferrin levels, measured by ELISA, were increased in smokers compared to non-smokers with no differences between non-symptomatic smokers and those with Stage 0 COPD indicating that none of these markers can differentiate "healthy" smokers from Stage 0 subjects. One important finding from these studies was that all these markers of oxidant burden were elevated already in “healthy” smokers who had no COPD. This finding is new since previous studies have associated these markers mainly to moderate-very severe COPD.

The values of fractional exhaled nitric oxide (FENO) and inducible nitric oxide synthase (iNOS) have been found to be increased in asthmatic/eosinophilic inflammation, but being highly variable in COPD, and thus it has been difficult to interpret their role in the development and progression of COPD (Maziak et al. 1998, Corradi et al. 1999, Rutgers et al. 1999, Ichinose et al. 2000, Maestrelli et al. 2003, Ricciardolo 2003, Sugiura et al. 2003, Smith et al. 2005). In the present study, the FENO value was lower in smokers compared to non-smokers, but there were no significant differences between the non-symptomatic smokers and those with Stage 0 COPD. Previous observations have shown that cigarette smoke decreases the levels of FENO (Kharitonov et al. 1995, Robbins et al. 1996) by down-regulating NO synthase
(Su et al. 1998) and consuming NO. In the present study, there was a significant inverse correlation between the FENO value and induced sputum iNOS, nitrotyrosine and MPO positive cells and neutrophils, but not macrophages, and this differs from the situation in patients with moderate/severe COPD (Silkoff et al. 2001). This finding emphasizes how complex role FENO plays in COPD. Furthermore, these results confirm the fact that low FENO values are associated with neutrophilic inflammation and increased production of iNOS and MPO by neutrophils as markers of oxidative/nitrosative stress. FENO displayed a significant correlation with BMI, suggesting that it may be related to the structure of the airways and this may differ depending of subject’s height and body weight. It seems that FENO does not have a significant role in COPD development and diagnosis, but has a role in the differential diagnosis of COPD from eosinophilic airway inflammation, especially asthma. In the present study, no eosinophilia was found in any subjects.

8-Isoprostane has been proposed to be one of the most promising and sensitive markers of oxidative stress in vivo and since induced sputum is probably the most sensitive non-invasive way of assessing oxidative stress in the airways, this explains why 8-isoprostane was selected for additional investigations on “healthy” smokers, smokers with Stage 0 COPD and individuals with moderate COPD. Earlier studies have found that the levels of 8-isoprostane are elevated in exhaled breath condensate or sputum in patients with asthma and during its exacerbations, bronchiectasis (Wood et al. 2005), stable COPD and COPD exacerbation (Biernacki et al. 2003, Ko et al. 2006), interstitial lung diseases (Montushi et al. 1998), cystic fibrosis (Collins et al. 1999, Montuschi et al. 1999, Wood et al. 2001), pulmonary hypertension (Cracowski et al. 2001), acute respiratory distress syndrome (Carpenter et al. 1998) and in infants with respiratory failure (Goil et al. 1998). The present study was the first in which the concentration of 8-isoprostane has been evaluated in the induced sputum of COPD, Stage 0 COPD and in “healthy” smokers. Similarly as the other oxidant markers, levels of 8-isoprostane were elevated already in smokers with or without chronic symptoms when compared to non-smokers, but there was no significant difference between the levels found in non-symptomatic smokers and those with Stage 0 COPD. Sputum samples of subjects with stable Stage I-III COPD had higher levels of 8-isoprostane than smokers with Stage 0 COPD, and there was a significant inverse correlation between the 8-isoprostane concentration and the extent of airway
obstruction. These results further confirm the hypothesis that smoking alone increases the local oxidant burden in the airways, and that this is further elevated in COPD. All in all, 8-isoprostane levels could not differentiate healthy smokers from those who probably are at risk of developing COPD in the future.

Despite the fact that all these oxidative/nitrosative stress markers were determined from different specimens (induced sputum supernatant, cytospin slides of induced sputum, exhaled breath) by different methods (immunocytochemistry, ELISA, FENO measurements with a chemiluminescence analyser), the results are similar adding more credibility to this finding. These results suggest that all tested markers of oxidative/nitrosative stress can be detected in the sputum of smokers with normal lung function parameters, but none of them could differentiate non-symptomatic smokers from smokers with chronic symptoms i.e. subjects at risk of developing COPD. These enzymes evidently contribute to the persistent stress condition and to the regulation of amounts of cytokines and defence molecules in the lung. It can be speculated that these alterations are associated with enhanced airway defence since many detoxifying enzymes are known to be induced by oxidants and cytokines.

1.2. Matrix metalloproteinases as specific markers of early COPD

The imbalance in the ratio of proteinases/antiproteinases has been postulated as being an important contributor to the pathogenesis and progression of COPD (Barnes et al. 2003, Calverley and Walker 2003). MMP activation and inhibition are tightly regulated by their specific endogenous inhibitors. However, their role in COPD onset is not clear. In this study, the levels of MMPs and TIMP were evaluated in order to assess whether they can differentiate smokers with chronic symptoms, but normal lung function parameters i.e. Stage 0 COPD individuals who are at risk of developing COPD in the future (Pauwels et al. 2001, Willemse et al. 2005, Mannino 2006, Stavem et al. 2006) from healthy non-symptomatic smokers; and whether they are elevated already in asymptomatic smokers compared to non-smokers.
The main finding was that the levels of sputum MMP-8 and plasma MMP-12 could differentiate subjects with Stage 0 COPD from non-symptomatic smokers indicating that they may function as sensitive biomarkers for COPD development, however, the statistical power of this finding needs additional conformation because of the relatively small number of subjects. Other results were in line with previous studies showing elevated levels of MMP-8 (Segura-Valdez et al. 2000, Culpitt et al. 2005), MMP-9 (Lim et al. 2000, Kang et al. 2003) and MMP-12 (Russell et al. 2002a, Demedts et al. 2006) in the sputum samples of moderate-severe COPD and/or smokers when compared to non-smokers. Since MMP-9 levels in both groups i.e. asymptomatic and symptomatic smokers were very similar, MMP-9 activation was further investigated by zymography and Western blot analysis, which showed comprehensive variability in all groups. This study indicated that the levels of MMPs, especially MMP-8 and MMP-9 were highly significantly correlated with lung function, especially with small airflow values suggesting that MMPs have clinical significance in COPD development. These findings confirm previous results in the significance of MMPs in the pathogenesis of COPD but also indicate that the levels of several MMPs are already elevated in non-symptomatic healthy cigarette smokers without airway obstruction.

It has previously been demonstrated that COPD is a systemic disease and MMPs are associated with other smoking related systemic diseases (Nakamura et al. 1998, Jones et al. 2003, MacCallum 2005, Wouters 2005), therefore the plasma levels of these enzymes were also assessed. This study found that plasma levels of MMP-9 and -12 were elevated in smokers as compared to those in non-smokers, and plasma MMP-12 could even differentiate non-symptomatic smokers from those at risk of developing COPD. Due to the cross-sectional evaluation and small numbers of the subjects in each subgroup of the subjects, the actual role of these MMPs as potential systemic markers for COPD development remained unclear.

Earlier studies have shown that the level of TIMP-1, a major endogenous inhibitor of MMP-8 and -9, is elevated in COPD (Beeh et al. 2003a, Owen et al. 2003, Culpitt et al. 2005, Higashimoto et al. 2005). In this study, no significant differences could be observed in the values of sputum ratios of MMP-8/TIMP-1, MMP-9/TIMP-1 or MMP-12/TIMP-1 while these ratios in plasma were significantly higher in smokers.
compared to non-smokers suggesting that TIMP-1 has a protective role in preventing tissue destruction.

The major sources of the MMP-8 and MMP-9 found in human lung are neutrophils (Cataldo et al. 2001, Barnes et al. 2003, Beeh et al. 2003a, Takafuji et al. 2003, Vernooy et al. 2004, Culpitt et al. 2005, Gueders et al. 2005) and alveolar macrophages (Russell et al. 2002a, Vernooy et al. 2004) and these previous findings were confirmed also in the present study. MMP-12 is expressed by macrophages (Churg et al. 2003, Bracke et al. 2005, Molet et al. 2005), and in agreement, this study could detect MMP-12 immunoreactivity in macrophages, but surprisingly, also in some neutrophils. The present findings support the role of neutrophils in cigarette smoking induced airway inflammation as all the levels of MMPs correlated with the numbers of neutrophils and markers of neutrophil activation. Neutrophils are thought to play an important role in the chronic inflammatory reaction that is present in the airways of COPD patients (Barnes et al. 2003). In line with previous observations, a significant increase in the numbers of neutrophils was found in the induced sputum of symptomatic smokers as compared to non-smokers.

The activation of matrix metalloproteinases can be triggered by increased oxidative stress (Nelson and Melendez 2004) and in agreement this study found significant correlation between MMP-8 and MMP-9 and oxidative stress markers (MPO and lactoferrin) that are released by neutrophils. Importantly, all measured MMPs significantly correlated with the total number of neutrophils suggesting that especially neutrophil derived oxidants may activate the tissue destructive MMPs and initiate remodelling processes already in the lungs of healthy cigarette smokers. However, there was no correlation between the degree of immunopositivity of MMPs and nitrotyrosine immunoreactivity highlighting the complexity of the relationship between smoking, oxidative stress and the balance/imbalance of proteinases/antiproteinases.

The strength of this study is that the levels of MMPs and TIMP were analyzed both in sputum and in plasma, providing the information about the inflammation and proteinase/antiproteinase balance in airway as well as in the circulation. In addition, the levels/expressions of sputum MMPs and TIMP-1 were assessed by ELISA that
assesses the quantitative levels of these enzymes in biological material, as well as by immunocytochemistry that confirms the localization of the protein in different cells. Furthermore, the MMP-9 results were confirmed by Western blot and zymography, which makes it possible to determine the molecular weight and relative amount of a protein, and its gelatinolytic activity. In this study we had carefully selected groups of non-smokers and smokers with or without symptoms. Half of the non-smokers were never-smokers and all ex-smokers had stopped smoking at least 20 years before the study. There were no significant differences in sputum MMP-8, -9 and -12 levels between these two groups of non-smokers. However, since the whole number of non-smokers was relatively small (n = 32), the statistical power of these results remains weak. Nevertheless, these results suggest that smoking secession is an effective way to slow down the disease progression and that influence of cigarette smoking on these MMPs is reversible. However, there are other studies that have reported that inflammation, oxidative stress and protease/antiprotease imbalance continues for months after smoking cessation (Louhelainen et al. 2010, Willemse et al. 2005).

2. Changes in inflammatory profile and levels of proteases during COPD exacerbation and its recovery period (Study IV)

There is recent evidence suggesting that exacerbations may have an important impact on COPD progression and the decline in lung function (Donaldson et al. 2002). COPD exacerbations are associated with airway inflammation leading to the belief that neutrophil derived proteinases may have a role in COPD progression. Recent studies have shown that the levels of neutrophil elastase (NE), MMP-8 and MMP-9 are elevated in COPD (Janoff et al. 1977, Senior et al. 1977, Damiano et al. 1986, Segura-Valdez et al. 2000, Beeh et al. 2003b, Barnes 2004) and MMP-9 levels even increase during COPD exacerbation as compared to the situation prior to the exacerbation (Mercer et al. 2005). The present study confirmed our presumption showing increased levels of NE, MMP-8 and also MMP-9 detected during COPD exacerbation as compared to the situation in individuals with stable disease, smokers and healthy non-smokers. Since the sputum was gathered 24-72 hours after the admission to hospital, patients had been on antibiotic and systemic corticosteroid treatment for at least 24 hours, which suggests that the values of these enzymes at the
exacerbation were probably even higher. Importantly, the values of NE and MMP-8 were significantly reduced during the one-month recovery period and we believe that the decline of these enzymes could have been even greater if the monitoring period had been longer. It has been demonstrated that NE may directly cleave MMPs, resulting in their activation (Okada et al. 1988, Zhu et al. 2001). This has led to the speculation that the increased MMP-8 and MMP-9 activity may be directly related to the NE that is present in the neutrophils of the induced sputum of COPD patients, but also to the reactions of possible viral or bacterial infections occurring during the exacerbation.

Regardless of the small number of patients in the COPD exacerbation group and recovery phase, poor standardization and variability of the symptoms in the acute exacerbation, there was a significant increase in NE and MMP-8 levels in acute exacerbation compared to stable COPD, and a reduction in the levels of these enzymes during the recovery phase compared to disease exacerbation, suggesting the role for neutrophil-derived proteinases in COPD progression.

3. Age related alterations and value of plasma surfactant proteins (SP) and MMP-9 in healthy non-smokers and smokers (Study V)

Surfactant proteins and the protease/antiprotease imbalance have been suggested to associate with COPD. Since COPD is a systemic disease, the levels of these markers were measured from plasma samples of healthy non-smokers and smokers in different age groups, and compared with the corresponding values in COPD patients. The results of the current study suggest that plasma SP-A level may be a promising marker for COPD as it displayed age related changes and a significant elevation due to smoking, and also, exhibited a significant correlation with airway obstruction. Previous studies have depicted increased levels of SP-A in circulating blood of smokers as compared to non-smokers (Kida et al. 1997, Nomori et al. 1998, Robin et al. 2002, Behera et al. 2005, Kobayashi et al. 2008) and our results were in line with these studies showing increased levels of plasma SP-A in long-term smokers and even higher levels in COPD patients confirming the role for SP-A in the pathogenesis of smoking induced lung disease. There are also some controversial findings where SP-A levels have found to be reduced in smokers` serum (Greene et al. 2002, Mutti et al.
lung tissue (Vlachaki et al. 2010) and BAL (Honda et al. 1996, Fujishima et al. 1999, Betsuyaku et al. 2004). These kinds of differences in SP-A values between serum and BAL have also reported in idiopathic interstitial pneumonia (IIP), where SP-A levels in BAL of IIP patients were lower and in serum higher compared to those in healthy subjects (McCormack et al. 1991, Kuroki et al. 1993, Abe et al. 1995, Honda et al. 1995). Since smoking is known to increase the airway epithelial permeability (Jones et al. 1980, Mason et al. 1983), this may explain the elevated levels of SP-A in plasma of smokers and COPD patients, while the lower level of SP-A in BAL fluid of smokers could be caused by leakage of SP-A from the alveolar space into blood vessels. However, short-term smoking in young healthy subjects did not affect the levels of plasma SP-A making it unclear whether SP-A levels can be used as a marker for COPD development or whether these changes are related to long-term smoking alone.

Only a few studies have investigated the effect of age on the levels of potential plasma biomarkers. There are some studies which have detected higher serum SP-A values in older subjects compared to younger (Abe et al. 1995, Nomori et al. 1998) being in line with this study, where SP-A levels in middle-aged/elderly non-smoking subjects were higher than those in younger ones. Interestingly, it has been found that also ageing alone or the combined effects of long-term smoking and emphysema decrease the level of SP-A in BAL (Betsuyaku et al. 2004) leading to the speculation that the elderly have increased airway epithelial permeability.

Serum SP-D has been shown to predict an increased risk of exacerbations of COPD and has been proposed to be a powerful biomarker for smoking (Lomas et al. 2009). There are studies that have detected no significant changes in serum or lung tissue SP-D of smokers/COPD (Kobayashi et al. 2008, Ohlmeier et al. 2008) or that SP-D levels in BAL of smokers/COPD are even decreased (Honda et al. 1996, Betsuyaku et al. 2004, Sims et al. 2008, More et al. 2010). In the present study, the levels of plasma SP-D were elevated due to long-term smoking with no differences between middle-aged/elderly smokers and COPD patients or young and older non-smokers. This finding suggests that the cigarette smoke had induced an increase in alveolar-capillary permeability and the escape of SP-D from airways into the circulating blood i.e. plasma SP-D could be used as a biomarker for smoking related airway inflammation,
but further studies in this field are required.

The balance between the proteases/antiproteases is thought to play a key role in COPD development (Barnes 1999, Beeh et al. 2003a, Nagase et al. 2006). The levels of MMP-9 have been found to be increased in sputum of COPD patients (Beeh et al. 2003a, Culpitt et al. 2005) and already in chronic smokers (Lim et al. 2000). In addition, few studies have evaluated the levels of MMP-9 and TIMP-1 in circulating blood of COPD patients (Cataldo et al. 2001, Higashimoto et al. 2005), however, the levels of plasma MMP-9 and TIMP-1 have not been compared in different age groups of smokers and non-smokers. The present study found that long-term smoking increased the levels of MMP-9 and MMP-9/TIMP-1 in plasma, suggesting that an imbalance of MMP-9 and TIMP-1 may play a key role not only in local, but also in the systemic inflammatory process. Nonetheless, the level of plasma MMP-9 did not change with age whereas the TIMP-1 level decreased in middle-aged older subjects as compared to younger ones, indicating that a chronic imbalance of MMP-9 and TIMP-1 may be an important factor in the development of poor lung function in the elderly. This study also observed that short-term smoking (<10 years) does not affect the levels of circulating SP-A, SP-D, MMP-9 or TIMP-1.

As most of the subjects in young age group were men, it was decided to compare the levels of these proteins in middle aged/elderly group and it was found that only the level of SP-A was higher in middle aged/elderly women as compared to the men (p = 0.012) highlighting the importance of gender differences. The strengths of this study were that none of the subjects in any of the groups had any other exposures or chronic diseases and that COPD patients were not taking any medications as the COPD had been newly diagnosed.
CONCLUSIONS

Sputum and/or plasma markers of oxidative/nitrosative stress, proteinase/antiproteinase imbalance and/or surfactant proteins were hypothesized to present potential markers for the risk of COPD development and for early COPD.

Levels of nitrotyrosine, iNOS, MPO, 4-HNE and lactoferrin were higher in current cigarette smokers compared to non-smokers pointing to an increased oxidant burden already in the airways of smokers with normal lung function values. Induced sputum 8-isoprostane levels were significantly increased in smokers and especially in subjects with stable Stage I-III COPD, and correlated with the severity of airway obstruction. However, none of these markers could differentiate healthy smokers from symptomatic smokers with normal lung function values i.e. those individuals who are at risk of developing COPD (GOLD Stage 0).

The FENO value was significantly lower in smokers compared to the corresponding situation in non-smokers, with no differences being detected between healthy smokers vs. symptomatic smokers. The reduced FENO value was significantly associated with neutrophilic inflammation and the elevated oxidant burden.

Sputum MMP-8 levels and plasma MMP-12 concentrations could differentiate symptomatic smokers from non-symptomatic chronic smokers; the statistical power of this finding remained poor and will require further confirmation.

Neutrophils and macrophages were the main sources of MMP-8, MMP-9 and TIMP-1, but also some ciliated epithelial cells expressed MMP-8 and TIMP-1 immunoreactivity. MMP-12 was primarily localized in macrophages, but also in some neutrophils. The levels of all these MMPs correlated with the numbers of neutrophils, and MMP-8 and MMP-9 with markers of neutrophil activation (MPO, lactoferrin) suggesting that especially neutrophil derived oxidants may stimulate the tissue destructive MMPs already in lungs of smokers who are not yet experiencing any airflow limitation.

COPD was associated with an accumulation of neutrophils and elevation and/or
activation of NE, MMP-8 and MMP-9 in induced sputum. During COPD exacerbations, the neutrophilic inflammation and the burden of neutrophil-derived proteinases in the airways were further increased pointing to a role for these enzymes in COPD exacerbation and probably also in COPD progression.

The circulating SP-A level may well be as a promising marker for COPD since it was increased by long-term smoking, COPD and the increase significantly correlated with the extent of airway obstruction. Long-term smoking increased also the levels of plasma SP-D, MMP-9, TIMP-1 and MMP-9/TIMP-1 but based on the ROC curve analysis, plasma SP-A appeared to be the most sensitive inductor.
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82
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110


