University of Helsinki
and
Department of Medicine, Pulmonary Division,
Helsinki University Central Hospital
Helsinki, Finland

Non-invasive biomarkers of oxidative stress
and cell defence in COPD and asthma;
specific emphasis on smoking cessation

Noora Louhelainen

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine, University of Helsinki,
for public examination in Biomedicum Helsinki 1,
Haartmaninkatu 8, Helsinki, on September 14th 2012, at 12 noon

Helsinki 2012
Supervised by

Professor Vuokko Kinnula, MD, PhD
Department of Medicine, Pulmonary Division
Helsinki University Central Hospital and University of Helsinki
Helsinki, Finland

Docent Witold Mazur, MD, PhD
Department of Medicine, Pulmonary Division
Helsinki University Central Hospital and University of Helsinki
Helsinki, Finland

Reviewed by

Docent Anne Pietinalho, MD, PhD
Chief physician at Raasepori Health Care Center
Tammisaari, Finland
and
Finnish Lung Health Association (FILHA)
Helsinki, Finland

Docent Leo Pekka Malmberg, MD, PhD
Skin and Allergy Hospital
Helsinki University Central Hospital
Helsinki, Finland

Opponent at the Dissertation

Docent Tuula Vasankari, MD, PhD
Finnish Lung Health Association (FILHA)
Helsinki, Finland

ISBN 978-952-10-8170-5 (PDF)
http://ethesis.helsinki.fi
Unigrafia
Helsinki 2012
CONTENTS

LIST OF ORIGINAL PUBLICATIONS ................................................................. 6
ABBREVIATIONS ............................................................................................ 7
ABSTRACT ........................................................................................................ 9
INTRODUCTION ............................................................................................... 12
REVIEW OF THE LITERATURE ...................................................................... 15
1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AND ASTHMA .... 15
   1.1 Classification and clinical features of COPD and asthma ....................... 15
   1.2 Inflammatory profiles of COPD and asthma ..................................... 16
   1.3 Epidemiology and etiology of COPD and asthma ............................... 17
   1.4 Diagnosis of COPD and asthma ......................................................... 18
   1.5 COPD/asthma overlapping ................................................................. 19
   1.6 Effects of smoking cessation in the airways of COPD and asthma ......... 22
   1.7 Treatment strategies for COPD and asthma ....................................... 22
   1.8 Prognosis of COPD and asthma ......................................................... 24
2. PATHOGENESIS OF COPD ......................................................................... 25
   2.1 Chronic inflammation ......................................................................... 25
      2.1.1 Key inflammatory cells ................................................................. 26
         2.1.1.1 Neutrophils ............................................................................ 26
         2.1.1.2 Macrophages ......................................................................... 26
         2.1.1.3 Lymphocytes ......................................................................... 27
         2.1.1.4 Eosinophils ........................................................................... 28
   2.2 Oxidant/antioxidant balance ............................................................... 28
   2.3 Protease/antiprotease balance ............................................................. 32
3. PATHOGENESIS OF ASTHMA ................................................................. 32
4. NEED FOR SENSITIVE AND SPECIFIC BIOMARKERS FOR EARLY SMOKING RELATED LUNG DISEASE ............................................................. 33
5. BIOMARKER REQUIREMENTS IN COPD AND ASTHMA ...................... 34
6. PROBLEMS IN MEASURING OXIDATIVE STRESS IN SMOKING ASTHMATICS ................................................................. 34
7. BIOMARKERS OF OXIDANT BURDEN IN COPD AND ASTHMA .......... 36
   7.1 Oxidant markers .................................................................................. 36
      7.1.1 Oxidant generating enzymes ....................................................... 36
      7.1.2 Nitric oxide metabolites ............................................................... 37
         7.1.2.1 Fractional exhaled nitric oxide (FeNO) ................................... 37
         7.1.2.2 3-Nitrotyrosine .................................................................... 39
         7.1.2.3 S-Nitrothiosols ..................................................................... 39
      7.1.3 Other exhaled markers of oxidative stress ..................................... 40
         7.1.3.1 Hydrogen peroxide (H$_2$O$_2$) .................................................. 40
         7.1.3.2 Carbon monoxide (CO) .......................................................... 40
         7.1.3.3 Volatile organic compounds (VOCs) ....................................... 41
      7.1.4 Markers of lipid peroxidation ...................................................... 41
         7.1.4.1 8-isoprostane .......................................................................... 41
         7.1.4.2 4-hydroxy-2-nonenal (4-HNE) ............................................... 43
      7.1.5 Antioxidants and antioxidant enzymes ....................................... 44
   7.2 Matrix metalloproteinases (MMPs) ...................................................... 44
7.3 Other potential markers................................................................. 45
  7.3.1 C-reactive protein (CRP) ............................................................ 45
  7.3.2 Fibrinogen ................................................................................. 46
  7.3.3 Panels of serum markers ............................................................ 47
  7.3.4 Tumour necrosis factor-α (TNF-α) ............................................. 47
8. NON-BIASED PROTEOMICS .............................................................. 48
  8.1 Proteomic studies on COPD/smokers ............................................ 48
    8.1.1 Surfactant protein A (SP-A)....................................................... 49
    8.1.2 Surfactant protein D (SP-D)....................................................... 50
    8.1.3 Polymeric immunoglobulin receptor (PIGR) .............................. 51
    8.1.4 Receptor for advanced glycation end products (RAGE) .......... 52
    8.1.5 Serum amyloid A (SAA)........................................................... 52
    8.1.6 Lipocalin-1 .............................................................................. 53

AIMS OF THE STUDY ........................................................................ 54
MATERIALS AND METHODS ................................................................ 55
1. MATERIALS .................................................................................. 55
  1.1 Patient/subject characteristics ..................................................... 55
    1.1.1 Study I ..................................................................................... 55
    1.1.2 Studies II and III ................................................................. 56
    1.1.3 Study IV ................................................................................ 56
    1.1.4 Study V ............................................................................... 57
2. LUNG FUNCTION MEASUREMENT .................................................. 57
3. EXHALED NO MEASUREMENT ....................................................... 57
4. EXHALED BREATH CONDENSATE (EBC) COLLECTION ............... 58
5. PROCESSING OF THE SAMPLES ...................................................... 58
  5.1 Sputum ....................................................................................... 58
    5.1.1 Sputum induction ................................................................. 58
    5.1.2 Sputum processing ............................................................... 58
    5.1.3 Sputum processing for proteomics ........................................ 59
  5.2. Tissue ....................................................................................... 59
    5.2.1 Paraffin blocks for immunohistochemistry ............................ 59
    5.2.2 Tissue homogenates for Western blotting ............................ 59
  5.3 Plasma ....................................................................................... 59
6. METHODS ..................................................................................... 61
  6.1 Sputum staining for cell differential counts (Studies I, II, III) ........ 61
  6.2 Sputum proteomics (Study V) ..................................................... 61
    6.2.1 Two-dimensional Difference Gel Electrophoresis (2D-DIGE) .... 61
    6.2.2 Protein identification using mass spectrometry (MS) .......... 61
  6.3 Western blot analysis (Study V) .................................................. 62
  6.4 Enzyme immunoassays (EIA)/Enzyme-linked immunosorbent assay (ELISA) (Studies I, II, III, IV, V) ........................................ 62
  6.5 Immunohistochemistry/morphometry (Study V) ......................... 62
7. STATISTICAL ANALYSES ................................................................ 63

RESULTS ............................................................................................ 64
  1. FeNO, SPUTUM EOSINOPHILS AND 8-ISOPROSTANE IN EARLY MILD ASTHMA .... 64
  2. EFFECTS OF SMOKING CESSATION ON OXIDANT MARKERS IN SPUTUM AND EXHALED AIR (STUDIES II, III) ............................................. 65
     2.1 Study II ................................................................................ 65
LIST OF ORIGINAL PUBLICATIONS


ABBREVIATIONS

2-D DIGE  two-dimensional difference gel electrophoresis
4-HNE  4-hydroxy-2-nonenal
AIDS  acquired immune deficiency syndrome
AMP  adenosine monophosphate
ANOVA  analyses of variance
ATS  American Thoracic Society
B  regression coefficient
BAL  bronchoalveolar lavage
BMI  body mass index
CAD  coronary artery disease
CI  confidence interval
CO  carbon monoxide
CRP  C-reactive protein
COPD  chronic obstructive pulmonary disease
DTE  dithioerythritol
EBC  exhaled breath condensate
ECP  eosinophilic cationic protein
ECSOD  extracellular superoxide dismutase
EIA  enzyme immunoassay
ELF  epithelial lining fluid
ELISA  enzyme-linked immunosorbent assay
EPO  eosinophil peroxidase
ERS  European Respiratory Society
FeNO  fractional exhaled nitric oxide
FEV1  forced expiratory volume in one second
FOXO3  Forkhead box class O3
FVC  forced vital capacity
GC  gas chromatography
GCL  glutamate cysteine ligase
GINA  the Global Initiative for Asthma
GOLD  the Global initiative for chronic Obstructive Lung Disease
GSH  glutathione
HDAC  histone deacetylase
HIV  human immunodeficiency virus
HLA  human leukocyte antigen
HPLC  high-performance liquid chromatography
HRP  horseradish peroxidase
ICS  inhaled corticosteroid
Ig  immunoglobulin
IL  interleukin
iNOS  inducible nitric oxide synthase
IPF  idiopathic pulmonary fibrosis
MGG  May-Grünwald-Giemsa
MMP  matrix metalloproteinase
MMRC  Modified Medical Research Council
MPO  myeloperoxidase
MS  mass spectrometry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NHLBI</td>
<td>National Heart, Lung, and Blood Institute</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>NF-E2-related factor 2</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDE-4</td>
<td>phosphodiesterase-4</td>
</tr>
<tr>
<td>PEF</td>
<td>peak expiratory flow</td>
</tr>
<tr>
<td>PIGR</td>
<td>polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAA</td>
<td>serum amyloid A</td>
</tr>
<tr>
<td>SC</td>
<td>secretory component</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SELDI-ToF</td>
<td>surface enhanced laser desorption/ionization time-of-flight</td>
</tr>
<tr>
<td>SGRQ</td>
<td>St Gerorges Respiratory Questionnaire</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SP</td>
<td>surfactant protein</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterized by airway obstruction that is mostly irreversible. The typical features of COPD are chronic inflammation in the small airways, with varying degrees of chronic bronchitis and/or emphysema. In the Western world, long-term smoking is the major risk factor for COPD. The pathogenesis of smoking related airway disease such as COPD has been postulated to be highly associated with oxidative stress and protease/antiprotease imbalance.

The diagnosis of COPD is based on spirometry, but the major problem is that the diagnosis is often delayed since during its early stages, clear symptoms such as dyspnea and coughing are lacking, even though the deterioration of lung function has already started. The differential diagnosis of COPD from asthma is especially difficult in smokers. There is an urgent need to develop reliable biomarkers that would detect early COPD, and which could differentiate the sub-phenotypes of COPD as well as COPD from asthma and finally be useful in developing treatment strategies for different phenotypes and used in monitoring disease activity and progression.

The first goal of this study was to compare different potential markers in various sample types that reflect oxidative stress in the lung, how they are related to each other, and to find the most specific markers for the early detection and monitoring of COPD and asthma and differentiation of these diseases as well as the disease from the condition of the so-called “healthy smoker”. Another goal was to examine the effects of smoking cessation on the best potential markers of oxidative and protease burden. Recent lung proteomic studies have indicated that SP-A could be potential marker for COPD; sputum proteomics was used to find other biomarkers for early COPD.

Induced sputum specimens were obtained from asthmatic children and adults, patients with COPD exacerbation, smokers with chronic bronchitis/COPD, smoking asthmatics, asymptomatic smokers and non-smoking healthy controls. Plasma samples were collected from young non-smokers and smokers and from middle-aged/elderly smokers and non-smokers and subjects with stable stage I-III COPD. Sputum and plasma samples and lung tissue specimens were taken from non-smokers, smokers and smokers with mild to moderate COPD.
Inflammatory cell counts were evaluated from sputum cytospins. Nitrotyrosine was assessed by immunohistochemistry by counting nitrotyrosine positive cells in cytospins. FeNO measurements were performed using a chemiluminescence analyzer. The levels of 8-isoprostane, MMP-7, -8, -9, TIMP-1, SP-A and SP-D were determined by EIA/ELISA methods. Induced sputum was analyzed by cysteine-specific two-dimensional difference gel electrophoresis (2-D DIGE) and PIGR was further studied in sputum, lung tissue and plasma by Western blot, immunohistochemistry/image analysis and/or ELISA.

FeNO levels were elevated in mild asthma and eosinophils were elevated in asthmatic adults and patients with COPD exacerbation when compared to healthy controls. No significant differences were detected in sputum 8-isoprostane levels between subjects with mild asthma and healthy controls, but in patients with COPD exacerbation, the 8-isoprostane levels were greatly increased when compared to healthy subjects. In the smoking cessation studies, smokers had elevated levels of all of the investigated markers at the baseline compared to non-smokers. Neutrophil counts increased after 3 months of quitting of smoking in the group of chronic bronchitis/COPD but at 6 months neutrophils had returned to the levels of non-smokers. 8-Isoprostane declined significantly but the levels of FeNO, nitrotyrosine and MMP-8 did not change significantly during 3 months after smoking cessation, although after 6 months, the levels of MMP-7, -8 and TIMP-1 decreased to the levels of non-smokers and only MMP-9 remained elevated at 6 months of smoking cessation. However, the symptoms declined significantly already after 3 months of smoking cessation. The plasma levels of SP-A, SP-D, MMP-9, TIMP-1 and the ratio of MMP-9/TIMP-1 were increased by long-term smoking and COPD, and SP-A correlated with the extent of airway limitation. In proteomic screening of sputum, PIGR was found to be elevated in smokers and in mild to moderate COPD, and this was confirmed in lung tissue and plasma specimens.

To conclude, it seems that sputum 8-isoprostane is not sensitive enough in detecting oxidative stress in mild recent asthma while in COPD it may be useful. After smoking cessation, the symptoms decline significantly already after a few months but the oxidant and protease burdens seem to persist in the airways for months. SP-A and PIGR
can be proposed as representing new promising markers in smoking related chronic inflammation and COPD.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an important disease globally (Menezes et al. 2005, Xu et al. 2005) and it is predicted that it will cause even more disability and death and generating enormous healthcare costs (Sullivan et al. 2000). COPD is characterized by chronic inflammation of the peripheral airways, chronic bronchitis, destruction of the lung tissue and systemic manifestations (MacNee 2005, Pauwels et al. 2001, Rabe et al. 2007). The diagnosis of COPD is often delayed since in its early stages, there is a lack of clear symptoms. Changes in lung function are not seen until the mainly irreversible lung damage is already extensive. In addition, even though spirometry can be used in the assessment of disease severity, it does not reflect its activity.

Cigarette smoking is the main risk factor for COPD (Fletcher and Peto 1977, Rabe et al. 2007). Cigarette smoke contains billions of reactive oxygen species (ROS) (Church and Pryor 1985), which trigger a major inflammatory response in the airways and activate many proteases (Kawikova et al. 1996, Rahman and MacNee 1998, Wood et al. 2003). Oxidant/antioxidant and protease/antiprotease imbalances further contribute to chronic airway inflammation causing damage to lung tissue and obstruction of the airways (Dhami et al. 2000, Janoff and Carp 1977, Wright and Churg 2008).

The differentiation of COPD from asthma is challenging especially in smokers since smoking modifies the typical asthmatic inflammation towards the characteristics that can be seen in smoke-related airway inflammation and pathology. Smoking asthmatics are often excluded from experimental studies and not many studies have compared potential non-invasive markers in samples taken from patients with COPD and asthma. Only a few studies exist on the effects of smoking cessation on airway inflammation/pathology. At present, there is no valid method for detecting early COPD although biomarker research is vigorously seeking non-invasive tools for disease detection, differentiation of sub-phenotypes and the disease of “healthy” smokers and COPD from those with asthma and for disease follow-up. Fractional exhaled nitric oxide (FeNO) has been widely used in detecting allergic asthma, but the corresponding sensitive/specific markers for early COPD have still to be discovered. Proteomic screening of sputum has offered a new non-hypothesis driven approach for biomarker
research and has already revealed several potential markers for COPD, which require further investigation (Ishikawa et al. 2010, Ohlmeier et al. 2010, Ohlmeier et al. 2008).

Several studies exist about FeNO in asthma, which appears to be sensitive in detecting oxidative/nitrosative stress in asthmatic airways and to correlate with the extent of eosinophilic inflammation and with several markers of asthma control (Kharitonov and Barnes 2001b, Sippel et al. 2000, Smith et al. 2005). The levels of FeNO are not changed in stable COPD (Kharitonov and Barnes 2001a, Rytia et al. 2006) but increase during exacerbations (Maziak et al. 1998). FeNO is not however specific to asthmatic inflammation (Kharitonov and Barnes 2001a) and generally decreases in smokers (Maziak et al. 1998, Rytia et al. 2006), so it is not reliable for the diagnosis of asthma in smokers.

8-Isoprostane has been proposed to represent a reliable marker for oxidative stress in lung diseases (Montuschi et al. 2004). Elevated 8-isoprostane levels have been reported in exhaled breath condensate (EBC) and sputum samples from asthmatics (Baraldi et al. 2003a, Montuschi et al. 1999) and COPD patients and from the sputum of smokers (Kinnula et al. 2007, Kostikas et al. 2003, Montuschi et al. 2000).

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have an important role not only in tissue repair but also in the pathogenetic processes in both COPD and asthma (Kelly and Jarjour 2003, Parks and Shapiro 2001). Oxidants present in cigarette smoke contribute to MMP activation (Kinnula 2005a). COPD patients have increased levels of sputum MMP-1, -8, -9 and -12 (Beeh et al. 2003, Culpitt et al. 2005, Demedts et al. 2006, Elkington and Friedland 2006, Vernooy et al. 2004).

Polymeric immunoglobulin receptor (PIGR) is proposed to be a potential regulator of specific immune defence and inflammation (Wines and Hogarth 2006). Decreased PIGR expression in the airway epithelium from subjects with severe to very severe COPD (Stage III-IV) patients have been reported (Pilette et al. 2001, Polosukhin et al. 2011), but the role of PIGR in the development of COPD or in early COPD is unknown.

The present study investigated potential non-invasive oxidant markers in different sample types e.g. sputum, plasma, exhaled air and lung tissue in order to find markers for early diagnosis of COPD and to differentiate “healthy” smokers from the disease. In study I, the value of sputum and EBC 8-isoprostane was assessed in recently diagnosed children and adults with mild asthma. In studies II and III the effects of smoking cessation on sputum neutrophils, 8-isoprostane, nitrotyrosine, matrix metalloproteinases (MMP-7, -8, -9), TIMP-1 and FeNO was investigated in asymptomatic smokers and patients with chronic bronchitis/COPD or asthma. In study IV, the potential effects of ageing and smoking to the levels and relationships between plasma SP-A, SP-D, MMP-9 and TIMP-1 were determined. In study V, non-hypothesis driven non-biased proteomics was used to seek new potential markers from sputum for early COPD. Based on these findings PIGR was chosen for further investigations and to study if the elevation of PIGR in sputum from smokers and patients with COPD could be observed also in lung tissue and plasma samples.
REVIEW OF THE LITERATURE

1. Chronic obstructive pulmonary disease (COPD) and asthma

1.1 Classification and clinical features of COPD and asthma

According to Global Initiative for Chronic Obstructive Lung Disease (GOLD) Guidelines (Pauwels et al. 2001) COPD is defined as a disease state characterized by airflow limitation that is not fully reversible and usually is progressive. Characteristic features of COPD include chronic inflammation in the small airways, with varying degrees of chronic bronchitis and destruction of the lung parenchyma (emphysema). Due to the tissue destruction, there are also abnormalities in gas diffusion (MacNee 2005, Pauwels et al. 2001, Rabe et al. 2007). It varies whether the patient has one or several of these conditions. COPD is also characterized by exacerbation phases often triggered by viral and/or bacterial infections when the patients usually suffer from increased cough, sputum, dyspnea and they may require treatment with antibiotics and/or steroids and be hospitalized (Celli and MacNee 2004a).

Cigarette smoke induces mucous gland enlargement and goblet cell hyperplasia leading to increased mucus secretion and cough, the main symptoms of chronic bronchitis (Hogg 2004a). Chronic inflammation in the small airways is associated with several abnormalities including smooth muscle hypertrophy, excess mucus secretion, oedema and infiltration of several inflammatory cells. It has been postulated that airway remodeling with fibrosis plays a crucial role in the narrowing of the small airways leading to airway obstruction in COPD patients (Hogg et al. 2004b, Wright 1995). Emphysematous lung is characterized by enlarged and/or destroyed airspaces and airway walls and also by the local presence of fibrotic lesions especially in the small airways and in some areas of lung parenchyma (Snider et al. 1985).

COPD is increasingly being accepted as a systemic disease since it exhibits also many systemic co-morbidities (GOLD 2011) i.e. cardiovascular diseases, persistent systemic inflammation, nutritional abnormalities and weight loss, exercise intolerance,

In the Global Initiative for Asthma (GINA) Guidelines (GINA 2009), asthma is defined as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation evokes an associated increase in airway hyperresponsiveness leading to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, especially at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that often is reversible, either with treatment or spontaneously.

1.2 Inflammatory profiles of COPD and asthma

There are many studies and reviews on the inflammatory profile in COPD and asthma (Chanez et al. 1999, Gibson et al. 1999, Jeffery 1999, Keatings and Barnes 1997, Kirby et al. 1987, Loh et al. 2005, NHLBI/WHO 2002, Saetta 1999, Tetley 2005, van Aalderen et al. 1999, Walters and Gardiner 1992). COPD is characterized by airway inflammation with neutrophil predominance (Bosken et al. 1992) and periods of acute exacerbations (Fletcher and Peto 1977) with further worsening of lung function and more severe airway inflammation (Gompertz et al. 2001). Exacerbations are often triggered by viral and/or bacterial infections, which increase the numbers not only of neutrophils but also eosinophils and activated macrophages in the airways (Rahman and MacNee 2000, Saetta et al. 2001).

In asthma, airway inflammation is present in all forms, even in mild (Battaglia et al. 2005) and asymptomatic cases (Bousquet et al. 2000, Montuschi et al. 2004) and it is present in both central and peripheral airways (Hamid et al. 1997). A strong association is seen between fractional exhaled nitric oxide (FeNO) and skin prick test scores, total IgE levels (Ho et al. 2000) and eosinophilic airway inflammation (Silvestri et al. 1999, Piacentini et al. 1999, Payne et al. 2001, Jones et al. 2001). Neutrophil accumulation is a typical feature also in asthma exacerbations (Fahy et al. 1995, Kim et al. 2000). An overlapping in the inflammatory profiles and also in oxidant markers is seen especially in acute asthma and COPD exacerbation.
Cigarette smoke induces macrophages and polymorphonuclear leukocytes to produce more ROS, leading to the recruitment of further leukocytes into the airways (Hoidal and Niewoehner 1982, Niewoehner et al. 1974). It appears that in smokers with asthma there are features of both accelerated and suppressed inflammatory responses, but generally, the cell profile seems to change to the situation seen in COPD. In these individuals the levels of sputum eosinophils are decreased (Chalmers et al. 2001), possibly partly due to the exogenous NO in cigarette smoke that is known to trigger apoptosis of activated eosinophils (Assreuy et al. 1993) whereas the numbers of neutrophils are elevated (Chalmers et al. 2001). Many asthmatic smokers display mixed features of typical asthmatic and smoke-related airway inflammation and pathology. Symptoms and lung function tests are only indirect ways to assess the disease severity in both COPD and asthma and they do not reveal the extent of the underlying airway pathology. Smoking alone has several effects on the airways and there is a need to clearly separate the effects of smoking from the disease and its progression.

1.3 Epidemiology and etiology of COPD and asthma

The prevalence of COPD is approximately 10% in the general population (Menezes et al. 2005, Xu et al. 2005) but it is projected to increase globally as smoking frequencies rise and the population ages (Murray and Lopez 1997, Feenstra et al. 2001) generating even higher healthcare costs (Sullivan 2000). A recent study reported a COPD prevalence of 5% in Finland and it showed an association with low economic status (Kanervisto et al. 2011) but the incidence of COPD does not seem to be increasing anymore (Vasankari et al. 2010) and in fact both hospitalization and costs due to COPD are declining in Finland (Kinnula et al. 2011). Importantly, in global terms, COPD is an increasing cause of chronic disability and is indeed estimated to become the fifth commonest cause of chronic disability throughout the world by 2020 (Lopez 2006). The Global Burden of Disease studies ranked COPD in 1990 as the sixth commonest cause of death worldwide and it is predicted that it will have risen to third place by 2020 (Lopez 2006). In addition, the World Health Organization predicted that COPD will rise from its current ranking as fifth commonest reason of death to be the fourth commonest by 2030 behind only cerebrovascular disease, ischemic heart disease and HIV/AIDS (Mathers and Loncar 2006).
Especially in the Western world, COPD is strongly associated with cigarette smoking (Culpitt and Rogers 2000, Scanlon et al. 2000); 90% of COPD patients are smokers. There are older studies suggesting that only 10%-15% of smokers develop COPD (Fletcher and Peto 1977). However, the results from more recent large epidemiological studies indicate that the risk may be nearly 50% (Lundback et al. 2003). Not all smokers develop clinically significant COPD suggesting that there must be environmental and genetic factors which can modify the risk for each individual (Fletcher and Peto 1977, Smith and Harrison 1997). The best documented genetic risk factor is a severe deficiency of α1-antitrypsin (Stoller and Aboussouan 2005). Globally other risk factors include environmental and indoor biomass smoke, occupational dusts, chemicals and pollution (Rabe et al. 2007, Viegi et al. 2001). The symptoms generally emerge in middle-age but can also become earlier since smoking is being initiated earlier and earlier (13-15 years old) and the development of COPD requires a minimum of 10-15 years smoking history.

Worldwide, the prevalence of asthma can range from 1% up to 18% in the populations in different countries (Masoli et al. 2004, Urrutia et al. 2007); in Finland, the prevalence is approximately 6% and it is increasing but the burden of asthma in terms of hospital days and chronic disability has decreased considerably (Haashtela et al. 2006). Asthma is a complex inflammatory disorder caused by the interaction of several environmental factors and multiple genes (Umetsu et al. 2002, von Mutius 2009). Risk factors for asthma include history of allergies and/or atopy, a family history of asthma, a low birth weight and exposure to tobacco smoke or pollution/environmental toxins (Robays et al. 2009, Pichavant et al. 2008, Haby et al. 2001). Exposure to tobacco smoke increases the risk of asthma especially when exposure occurs during pregnancy and in early childhood (Haashtela et al. 2008, Elliot et al. 2003, OEHHA 2005) but also in adults (Jaakkola and Jaakkola 2002, Jaakkola et al. 2003, Larsson et al. 2003).

1.4 Diagnosis of COPD and asthma

Spirometry is essential for COPD diagnosis; according to GOLD criteria the post-bronchodilator FEV1/FVC is below 0.70 in COPD (GOLD 2011). The characteristic symptoms of COPD include chronic and progressive cough, dyspnea and sputum
production and usually the patient has a long smoking history or other prolonged exposure to risk factors. Coughing and sputum production may occur many years before the development of airflow limitation offering a unique opportunity to identify smokers who are at risk of developing COPD (Rabe et al. 2007). On the other hand, subjects with COPD may consider themselves asymptomatic without any signs of the disease (Toljamo et al. 2010). Most patients only tend to seek help when lung function deterioration has already started since there are no significant symptoms in the early stages of COPD.

In asthmatic patients, the clinical diagnosis is indicated by symptoms such as episodic breathlessness, wheezing, cough and chest tightness (Levy et al. 2006). An atopic history, episodic symptoms after allergen exposure, seasonal variability of symptoms and asthmatic individuals in the family may also be helpful diagnostic tips. Significant bronchial variability is a characteristic of asthma and is defined as a reduction in the post-exercise peak expiratory flow (PEF) and/or forced expiratory volume in 1 second (FEV1) of ≥ 15% of the pre-exercise value (ATS 2000), or in spirometry, improvement of FEV1 ≥ 12% and at least 200ml after a bronchodilating drug or a provocative concentration of histamine causing a 15% fall in FEV1 (PD15FEV1) < 0.4mg in histamine challenge test (Sovijarvi et al. 1993, ATS 2000), or ≥ 20% diurnal variation in the PEF values and/or at least ≥ 15% improvement in PEF after a dose of bronchodilator during home recording.

1.5 COPD/asthma overlapping

COPD and asthma both are chronic inflammatory lung diseases and in both conditions, inflammation is associated with structural alterations in the airways (Jeffery 2004, Guerra 2005). Despite their distinct clinical features, a considerable overlap in symptoms and pathogenesis is seen. This can lead to a phenotypic overlap or a mixed syndrome with characteristics of both diseases (Kim and Rhee 2010). Several studies have reported partial reversibility after short-term and long-term bronchodilator administration in COPD patients (Mahler et al. 1999, Donohue et al. 2002). The current guidelines also depict a fixed or irreversible component to the airway obstruction in some asthma patients (GINA 2009).
Alveolar inflammation and emphysema are characteristics of COPD but alveolar inflammation has also been detected in asthma though it is classically considered as a chronic inflammatory disease of the airways (Bousquet et al. 1996). The “Dutch hypothesis” postulates that asthma and COPD are simply different expressions of a single disease entity (Vestbo and Prescott 1998), but the “British hypothesis” states that asthma and COPD are separate clinical entities with similar symptoms (Lange et al. 1998), although the general consensus is that similar pathogenetic mechanisms are involved in both diseases (Kim and Rhee 2010).

The differentiation between the inflammatory profiles of asthma and COPD may be challenging in some cases. Classically, asthma is associated with a prominence of eosinophils, CD4+ lymphocytes and macrophages whereas increases in neutrophils, macrophages and CD8+ T-cells are seen in the airways of COPD patients (Kim and Rhee 2010). The differentiation of asthma from COPD is especially difficult in smokers. In smokers aged 30 to 40 years, clinical and pathologic manifestations resembling early-stage COPD may already be present, indicative of mixed or intermediary condition (Boulet et al. 2006). The prevalence of active smoking in asthmatic individuals is as common as in the general population; in Finland for example as many as 30% of asthmatics are smokers (Haahtela et al. 2006). Smoking asthmatics are frequently excluded from experimental studies and thus there is limited data about the airway pathology of smoking asthmatics. Asthmatics who smoke show higher numbers of neutrophils in BAL fluid and lung tissue (Wenzel et al. 1997, Louis et al. 2000). Conversely, in COPD exacerbations, tissue eosinophilia is common (Saetta et al. 1994).

Overall, an overlap in clinical features is often seen in COPD and asthma, but they are considered as distinct conditions whose differences affect both management and prognosis. The assessment of the patient’s clinical history, current symptoms, lung function tests and usage of radiology and cellular markers of inflammation may help in differentiating COPD from asthma (Kim and Rhee 2010).
Table 1. The main classical differences between COPD and asthma.
In reality, there is extensive overlapping in these diseases.

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>ASTHMA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Airflow limitation</strong></td>
<td>Usually progressive, mostly irreversible</td>
<td>Widespread but variable, usually reversible</td>
</tr>
<tr>
<td><strong>Airway hyperreactivity</strong></td>
<td>Mild to no</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Bronchodilator response</strong></td>
<td>Mild to no</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Corticosteroid response</strong></td>
<td>Mild to no</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Long-term smoking history</strong></td>
<td>Usually</td>
<td>No</td>
</tr>
<tr>
<td><strong>Association with atopy</strong></td>
<td>No</td>
<td>Often</td>
</tr>
<tr>
<td><strong>Onset (typical)</strong></td>
<td>Middleage/older</td>
<td>Childhood/Middleage</td>
</tr>
<tr>
<td><strong>Location of inflammation in the lung</strong></td>
<td>Small airways, lung parenchyma, alveolar region</td>
<td>Airways</td>
</tr>
<tr>
<td><strong>Parenchymal destruction</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Diffusion capacity</strong></td>
<td>Normal/worsened</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Extrapulmonary manifestations</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Main inflammatory cells</strong></td>
<td>Neutrophils, macrophages, CD8+</td>
<td>Eosinophils, mast cells, CD4+</td>
</tr>
<tr>
<td><strong>Prognosis</strong></td>
<td>Increased disability and morbidity</td>
<td>Usually good</td>
</tr>
</tbody>
</table>

1.6 Effects of smoking cessation in the airways of COPD and asthma

Smoking cessation is the only effective way to reduce COPD morbidity, hospital admissions (Godtfredsen et al. 2002) and COPD progression (Rabe et al. 2007). However, the effects of smoking cessation programs and guidance in order to break of a habit of smoking are limited and success rates vary around 5-15% (Kaper et al. 2005, Stratelis et al. 2006, Tashkin et al. 2001). It has been reported that as the disease becomes more severe, giving up smoking has only a marginal effect on its progression (Scanlon et al. 2000). Smoking is also an important contributor to asthma pathogenesis. Smoking asthmatics tend to have more severe disease than non-smoking asthmatics, their inflammatory features are different to those individuals with typical asthma and their symptoms and inflammation are rather resistant to corticosteroid therapy (Siroux et al. 2000). Several cross-sectional studies have been conducted on smokers, ex-smokers and patients with COPD, but only a few longitudinal studies have assessed the long-term effects of smoking cessation (Chaudhuri et al. 2006, Willemse et al. 2005). It is not known in detail how the airway inflammatory processes change after smoking cessation in chronic smokers with or without COPD or asthma.

One study analyzed pooled data from three bronchial studies and concluded that several airway inflammatory cells, including CD4+ and CD8+ lymphocytes were rather similar in current and ex-smokers (Gamble et al. 2007). Evidence of ongoing inflammation has been documented via the increased levels of neutrophils and eosinophils in BAL fluid and sputum and eosinophilic cationic protein (ECP) in sputum from COPD patients who do not currently smoke (Lapperre et al. 2006). The persistence of elevated sputum neutrophils and lymphocytes even after one year of smoking cessation has been reported (Willemse et al. 2005). However, in asthmatics, sputum neutrophils are reduced after six weeks of quitting of smoking (Chaudhuri et al. 2006).

1.7 Treatment strategies for COPD and asthma

Pharmacologic therapy is used to prevent and control symptoms, to reduce the severity and frequency of deterioration phases and to improve health status and exercise tolerance. However, there are no medications for COPD that would prevent the long-term decline in lung function (Anthonisen et al. 1994, Burge et al. 2000, Pauwels et al.
Short-acting bronchodilators (e.g. short-acting beta2-agonists and anticholinergics) are recommended as short-term therapy in COPD patients and treatment with long-acting bronchodilators (e.g. anticholinergics, beta2-adrenergic agonists, theophylline) using one or more of these medications to control symptoms is usually recommended in COPD patients in whom short-acting beta2-agonists do not provide sufficient relief (GOLD 2011). Inhaled corticosteroid treatment (ICS) is used in combination with a long-acting bronchodilator in patients with severe to very severe COPD (stages III-IV) who have severe or frequent exacerbations (GOLD 2011). However, COPD patients exhibit a poor response to corticosteroids and it has been shown that there may even be active resistance against the anti-inflammatory actions of steroids (Barnes 2006). Oral corticosteroids and antibiotics are used mainly in COPD exacerbations.

Roflumilast is the newest medication in COPD treatment. It is a phosphodiesterase-4 (PDE-4) inhibitor that reduces inflammation by inhibiting the breakdown of intracellular cyclic AMP (Giembycz and Field 2010), which in turn is an important intracellular signal for a diverse array of cytokines, hormones, neurotransmitters and medications that stimulate membrane-bound adenyl cyclase (Bender and Beavo 2006). It has been reported that roflumilast can reduce exacerbations especially in patients with severe to very severe COPD and that it improves FEV1 in patients treated with salmeterol or tiotropium (Calverley et al. 2009, Fabbri et al. 2009).

Controlled oxygen support may be used in advanced cases of COPD (Calverley 2003, GOLD 2011) and some highly selected COPD patients may benefit from surgery: lung volume reduction, bullectomy or even lung transplantation (Pauwels et al. 2001).

In contrast to COPD, anti-inflammatory drugs, such as ICS are used successfully in asthma therapy (Juniper et al. 1990). Other medications commonly used in asthma include leukotriene modifiers (Dicpinigaitis et al. 2002, Barnes and Miller 2000) that are alternatives to ICS in mild asthma (Leff et al. 1998), long-acting inhaled β2-agonists combined with ICS (Lazarus et al. 2001) and rapid-acting inhaled β2-agonists for quick relief of bronchospasm (GINA 2009). Smokers with asthma often show an impaired response to asthma medications, leading to impaired asthma control.
1.8 Prognosis of COPD and asthma

If diagnosed early enough and if the patient quits smoking, COPD prognosis is relatively favourable. However, if smoking continues, then the decline in lung function accelerates and may eventually lead to respiratory failure and/or premature death. A recent study reported high variability in the rate of decline in FEV1 in COPD patients over a period of 3 years; in more than half of the COPD patients the rate of decline in FEV1 was no greater than is observed in subjects without lung disease and increase in the rate of decline in FEV1 was related to current smoking, emphysema and bronchodilator response (Vestbo et al. 2011). The 5-year mortality rate of patients with COPD varies from 40 to 70% depending on the disease severity and the major causes of death are COPD itself, lung cancer and cardiovascular diseases (Nishimura and Tsukino 2000). The symptoms do not closely reflect spirometric changes and they seem to predict exacerbation only in some individuals (Seemungal et al. 2000). COPD exacerbations are in turn important events in terms of disease progression, morbidity and mortality. Many COPD patients never recover completely from an exacerbation. It is clear that the two main solutions in preventing the increase of already high burden of COPD throughout the world would be a way of detecting COPD early enough that symptoms have not appeared and better methods of encouraging these patients to give up smoking.

After several years of smoking cessation, the decline rate in lung function becomes similar to that of never smokers (Anthonisen et al. 1994). However, the lung function lost during the time the patient smoked never fully recovers and COPD patients might develop additional symptoms and progress to a more severe disease even after many years of smoking cessation (Kohansal et al. 2009). Although the rate of decline in FEV1 is a good marker of disease progression and mortality (Anthonisen et al. 1986), it does not adequately reflect all the systemic manifestations of the disease.

The BODE index has been developed as a practical tool for measuring a patient’s degree of lung impairment and to show how the disease affects the entire body negatively by using factors that might predict death (Celli et al. 2004). BODE measures body mass index (BMI), airway obstruction (measured by FEV1), dyspnea (measured by the MMRC scale) and exercise tolerance (measured by the 6-minute walk test). The
higher the BODE index, the higher is the risk of death (Celli et al. 2004). In general practice, such complex estimates are not useful, and simple measures for assessing the quality of life are being developed (Jones et al. 2009a, Jones et al. 2009b).

Most children with mild intermittent asthma outgrow their asthma although symptoms might return as episodic at an older age (Koh and Irving 2007). In most asthma patients, the clinical manifestations of asthma (symptoms, exercise tolerance, impairment of lung function and need for rescue medication) can be controlled with appropriate treatment and avoidance of allergens and the prognosis is good in these cases (GINA 2009). When asthma is not treated, or the patient continues to smoke despite the asthma, remodeling of the airways might occur, leading to irreversible fibrosis in the airways and loss of lung function.

2. Pathogenesis of COPD

The dominant features in COPD are varying degrees of chronic bronchitis and emphysema, with some degree of overlapping with asthma, with the common phenotype being airflow obstruction. Pathological changes are found in the proximal and peripheral airways, lung parenchyma and pulmonary vasculature (Hogg 2004). Long-term cigarette smoking is the main risk factor for COPD but also occupational exposures to dusts and chemicals as well as genetics contribute to COPD pathogenesis. Therefore, the interactions of host factors with the environment are linked to pathologic changes and clinical symptoms generating the pathologic triad of COPD: chronic inflammation, oxidative stress and protease-antiprotease imbalance (Fischer et al. 2011). Together these mechanisms result in mucous cell metaplasia and hyperplasia, hypersecretion of mucous, airway fibrosis, smooth-muscle alterations, and lung tissue destruction (Wright and Churg 2008).

2.1 Chronic inflammation

Long-term tissue damage and acute inflammation induced by cigarette smoke or other noxious particles contribute to the switch to a chronic, persistent inflammation of the lung (Oudijk et al. 2003). Neutrophils, macrophages, T-lymphocytes and also
eosinophils and mast cells have all been linked to chronic bronchitis or COPD (O'Donnell et al. 2006).

2.1.1 Key inflammatory cells

2.1.1.1 Neutrophils

Neutrophils are the main inflammatory cells at the site of acute inflammation and they are recruited to the airways and lung parenchyma from the circulation via adhesion molecules allured by chemotactic factors (Barnes et al. 2003). Nicotine itself may also act as a chemoattractant for neutrophils (Totti et al. 1984). Neutrophils secrete many proteases, such as neutrophil elastase, proteinase-3, cathepsin G and MMPs -8 and -9 which are potent mucus stimulants and contribute to alveolar destruction and tissue damage (Barnes 2004, Barnes et al. 2003). Increased numbers of activated neutrophils can be found in bronchial biopsies, sputum and BAL fluid of smokers (Willemsen et al. 2005) and COPD patients (Keatings et al. 1996, Lacoste et al. 1993) but only a relatively minor elevation in the airways and parenchyma (Finkelstein et al. 1995). The neutrophil numbers in both bronchial biopsies and induced sputum correlate with COPD disease severity (Di Stefano et al. 1998, Keatings et al. 1996) as well as with the rate of decline in lung function (Stanescu et al. 1996).

2.1.1.2 Macrophages

Macrophages are phagocytic for bacteria and thus have an important role in host defence. Macrophages appear also to have a crucial role in the pathophysiology of COPD (Shapiro 1999). The numbers of macrophages are increased in airways, lung parenchyma, sputum and BAL fluid in COPD patients (Barnes et al. 2003, Di Stefano et al. 1998, Saetta et al. 1993, Tetley 2002) and those numbers correlate with the severity of COPD (Di Stefano et al. 1998). The increase in the numbers of macrophages might be due to the increased recruitment of monocytes from the circulation in response to monocyte-selective chemokines (Capelli et al. 1999, Traves et al. 2002), but also due to increased prolonged survival of macrophages in smokers and COPD patients (Tomita
et al. 2002). Cigarette smoke stimulates macrophages to release inflammatory mediators, such as TNF-α and IL-8, chemotactic factors, proteolytic enzymes and ROS (Punturieri et al. 2000, Russell et al. 2002). Corticosteroids seem to be rather ineffective in suppressing inflammation, including the elevations in the proteases, cytokines and chemokines encountered in COPD patients (Culpitt et al. 1999, Keatings et al. 1997). In COPD patients and to some extent also in smokers without COPD, the macrophages are resistant to the actions of the corticosteroids, which may be explained by the major decline in the expression of histone deacetylase (HDAC) (Ito et al. 2001), which normally switches off inflammatory genes and thus reduces cytokine release (Ito et al. 2000).

2.1.1.3 Lymphocytes

The numbers of T-lymphocytes, especially CD8+ but also CD4+, are increased in lung parenchyma and in both central and peripheral airways of patients with COPD (Finkelstein et al. 1995, Majo et al. 2001, O'Shaughnessy et al. 1997, Saetta et al. 1999). The role of T-cells in the pathophysiology is still not clear, but the numbers of T-lymphocytes correlate with the amount of alveolar destruction and the severity of airflow obstruction (Majo et al. 2001). CD8+ cells can trigger cytolysis and apoptosis of alveolar epithelial cells by releasing perforins, TNF-α and granzyme-B (Hashimoto et al. 2000). The mechanisms by which T-cells accumulate in the airways and lungs of COPD patients are still unclear but homing seems to require the presence of some kind of triggering factor and after which there is adhesion and selective chemotaxis (Barnes et al. 2003). The numbers of CD8+ and T-helper type 1 CD4+ cells are increased in the circulation of COPD patients (de Jong et al. 1997, Kim et al. 2002, Majori et al. 1999). This suggests that there might be some kind of chronic stimulation via antigens presented via the HLA Class 1 pathway. Dendritic cells can migrate to regional lymph nodes from the airways and stimulate the proliferation of T-lymphocytes (Barnes et al. 2003). CD8+ cell numbers are commonly elevated in airway infections and it may be that the chronic colonisation by bacterial and viral pathogens in the airways of COPD patients is responsible for this inflammatory response (Cosio and Majo 2002). In addition, protease-induced lung damage may reveal normally isolated autoantigens or it
may be that cigarette smoke itself causes injury to airway epithelial cells making them antigenic (Cosio and Majo 2002).

2.1.4 Eosinophils

Eosinophils are most commonly seen in asthma. However some studies have reported increased numbers of eosinophils and eosinophil cationic protein (ECP) in the sputum and BAL fluid from stable COPD patients (Brightling et al. 2005, Fujimoto et al. 1999, Lacoste et al. 1993, Pizzichini et al. 1998), with a further increase occurring during COPD exacerbations in which the increase is at least partially related to viral infections (Papi et al. 2000, Rohde et al. 2004, Saetta et al. 1994). The presence of eosinophils in COPD patients presumes that a response will be obtained to corticosteroids and may indicate the coexistence of asthma (Brightling et al. 2000, Papi et al. 2000). Eosinophils generate ROS, which in turn leads to an increased inflammatory response producing asthma-like symptoms.

2.2 Oxidant/antioxidant balance

Reactive oxygen species (ROS) and nitrogen species (RNS) are produced endogenously by several cell types, the most potent generators being eosinophils and neutrophils which are abundant both in COPD and asthma (Kinnula 2005b, Kinnula et al. 1995). The burden of ROS/RNS is increased further by exogenous factors, mostly cigarette smoke and environmental agents. One puff of cigarette smoke contains billions of free radicals (Church and Pryor 1985). These ROS have multiple effects i.e. activation of proteases, elevation in mucus secretion, evoking airway smooth muscle contraction, airway hyperresponsiveness, and transcription of many inflammatory genes (Kawikova et al. 1996, Rahman and MacNee 1998). The major sites of oxidative attack in COPD are the small airways, lung parenchyma and alveolar region. Outside the lung, tissue oxidative stress can have systemic effects when ROS leak into the circulation or indirectly by inducing stress-related products e.g. cytokines (MacNee and Rahman 2001).
The oxidants present in cigarette smoke stimulate alveolar macrophages to further produce ROS (O$_2^•$ and •OH) worsening the vicious cycle by releasing many potent mediators, some of which attract neutrophils and other inflammatory cells into the lungs of COPD patients (GOLD 2011) which then can generate even more ROS via the NADPH oxidase system. Similarly, elevated xanthine oxidase levels found in BAL fluid and plasma from COPD patients can increase O$_2^•$ and lipid peroxide levels (GOLD 2011). Therefore, oxidants may play a key role in initiating or triggering the underlying inflammation seen in pathogenesis of COPD.

Many oxidants are unstable and have very short half-lives and therefore their measurement is difficult. Hydrogen peroxide (H$_2$O$_2$) is formed from superoxide mainly by inflammatory cells and directly involved in several reactions. NADPH oxidase is one of the key superoxide generating enzymes that is expressed both in phagocytic and non-phagocytic cells (Jones et al. 2000). Endogenous NO is derived from L-arginine by at least three different isoforms of NOS (Nathan and Xie 1994). Two of these forms are expressed constitutively (NOS1, NOS3) but the inducible form (iNOS, NOS2) is activated by inflammatory cytokines. Inducible NOS is responsible for generating the majority of NO seen in inflammatory states, being induced by cytokines and expressed in inflammation e.g. neutrophils and in non-phagocytic cells (Barnes and Belvisi 1993). When ROS are generated, they immediately attack membrane lipids leading to the generation of lipid peroxidation products like isoprostanoids, malondialdehyde and 4-HNE. These markers have been widely investigated in assessing oxidative stress in the lung (Kharitonov and Barnes 2001a, Paredi et al. 2002) but a major limitation with most of these end products is their poor specificity and reproducibility in different diseases that are related to oxidative stress and that they have been generally assessed only in moderate/severe COPD.

The oxidant burden in COPD is not only associated with cigarette smoke/environmental oxidants and activation of several oxidant generating enzymes. The imbalance is further worsened due to decline of the major antioxidant defence in the lung. Complex antioxidant machinery of the lung contains various low molecular weight antioxidants, metal binding proteins, mucus glycoproteins and several specific antioxidant enzymes (Kinnula 2005a and 2005b, Kinnula and Crapo 2003, Kinnula et al. 1995). Generally
many protective antioxidant enzymes are first induced by cytokines and oxidative stress, which leads to protection but the same enzymes are subsequently inactivated due to persistent severe oxidative stress by complex oxidation/nitrosylation and proteolytic reactions (Kinnula and Crapo 2003). It has been reported also that the thiol antioxidant glutathione (GSH) and its biosynthesizing key enzyme, glutamate cysteine ligase (GCL), are decreased in the airways and macrophages of smokers when compared to non-smokers (Harju et al. 2002). Furthermore, a deficiency of the major parenchymal superoxide scavenging enzyme extracellular superoxide dismutase (ECSOD) leads to emphysema (Yao et al. 2010) and this enzyme declines in the lung of severe COPD (Regan et al. 2011). Additionally, a deficiency of the major regulator/inducer of several antioxidant/detoxification enzymes, nuclear factor erythroid-derived 2-like 2 (Nrf2) and both Sirtuin 1 and Forkhead box class O 3a (FOXO3) leads to emphysema in mice and they also disappear in severe COPD (Hwang et al. 2011). Overall, there is limited data about biomarkers of oxidative stress in COPD/asthma, and little is known about the relative importance of the various antioxidant pathways and specific oxidant biomarkers in COPD.
**Figure 1.** Cigarette smoke and several inflammatory cells lead to generation of ROS/RNS in the lung that cause cell and tissue injury. $O_2\cdot:-$: superoxide; $H_2O_2$: hydrogen peroxide; $NO\cdot$: nitric oxide; iNOS: inducible nitric oxide synthase; ONOO$^-$: peroxynitrite; GSH: glutathione; SODs: superoxide dismutases (MnSOD, ECSOD, CuZnSOD); MPO: myeloperoxidase; HOCl: hypochlorous acid; OH$: $hydroxyl$ radical; $H_2O$: water; GPXs: glutathione peroxidases; CAT: catalase.

Modified from Kinnula and Crapo 2003 and Jaber et al. 2005.
2.3 Protease/antiprotease balance

Smoking-induced emphysema is thought to be mediated partly by the prolonged and direct inhibition of antiproteases in the lung tissue (Dhami et al. 2000, Janoff and Carp 1977). Proteases are important factors in pulmonary defence but “spilling” of these enzymes can lead to host tissue damage if the inactivation mechanisms are blocked. Cigarette smoke or exposure to other smokes can inactivate endogenous antiproteases (Cavarra et al. 2001) and trigger an acute pulmonary response that activates alveolar macrophages and contributes to the influx of neutrophils into lungs (Fischer et al. 2011). Long-term smoke exposure causes the continued accumulation of neutrophils, macrophages and CD8+ T cells in the lungs (Shapiro 2002). Neutrophils and macrophages secrete several proteinases, which act by activating each other or inhibiting their endogenous inhibitors, e.g. MMPs degrade α1-antitrypsin and neutrophil elastase inhibits tissue inhibitors of MMPs (Fischer et al. 2011, Shapiro 2002). These enzymes cleave the extracellular matrix, generating chemotactic peptide fragments from elastin and collagen for macrophages and neutrophils thus aggravating the inflammatory cell accumulation and lung destruction (Fischer et al. 2011, Hunninghake et al. 1981, Weathington et al. 2006).

3. Pathogenesis of asthma

Airway inflammation in asthma involves a very complex interaction of several cells, mediators, cytokines and chemokines. Immunologic and nonimmunologic environmental factors are major triggers of asthma, including viral infections, air pollutants such as ozone and cigarette smoke and other cellular mediators (Dworski 2000). These agents cause activation and influx of inflammatory cells leading to generation of inflammatory mediators, which converge to generate ROS, which are associated also with the pathogenesis of asthma by evoking bronchial hyperreactivity as well as stimulating histamine release from mast cells and mucus secretion from airway epithelial cells (Dworski 2000). Thus, while oxidative stress due to smoking is a direct causative factor for COPD leading to a further burden of oxidative stress, it is usually a secondary effect in asthma subsequent to prior antigenic/allergic challenge of the immune cells in lungs. In asthma, the airways are the major site of action, this being
characterized by reversible airflow obstruction, airway hyper-responsiveness/ hyper-reactivity, and chronic inflammation due to the influx of eosinophils and activation of inflammatory cells such as macrophages, neutrophils, lymphocytes, and mast cells. Airway smooth muscle contraction, increased airway reactivity and secretions, increased vascular permeability and increased generation of chemoattractants are the major features of the asthmatic response (Hamid et al. 1997). Persistent inflammation, epithelial damage and/or aberrant tissue repair mechanisms may lead to structural changes of the airway wall, referred to as remodeling (Jeffery 2001, Mauad et al. 2007, Broide 2008). Characteristic structural changes include subepithelial fibrosis, smooth muscle hypertrophy/hyperplasia, increased deposition of extracellular matrix protein, goblet cell hyperplasia, mucus gland hypertrophy and increased angiogenesis (Mauad et al. 2007, Broide 2008). The exact immunologic and inflammatory mechanisms leading to airway remodeling are still incompletely understood but it is likely that several immune and inflammatory cells and mediators are involved in mediating these changes (Humbles et al. 2004, Fulkerson et al. 2006).

4. Need for sensitive and specific biomarkers for early smoking related lung disease

It is challenging to evaluate oxidative stress in COPD disease progression. The symptoms do not correlate with spirometric changes and the symptoms only predict exacerbation in some patients (Seemungal et al. 2000). Spirometry is not very sensitive in finding mild disease and does not actually reflect the disease activity. The diagnosis of COPD is often delayed, since in its early stages, symptoms are lacking. Many COPD patients never recover completely from an exacerbation, i.e. these events are important when it comes to disease progression, morbidity and mortality. COPD is not simply one disease but instead contains many phenotypes with varying degrees of airway and emphysematous changes, meaning that the lung pathology is very complex. All the phenotypes are however treated in the same way since no specific biomarkers for the differentiation of the disease subtypes or their progression have been identified. Small airway inflammation with fibrosis and alveolar destruction are the main pathophysiologic events in COPD (Barnes et al. 2006) but at present there is no biomarker in clinical practice, which is able to differentiate these conditions from each other. Further studies are needed aimed at finding reliable biomarkers that would detect
early COPD, differentiate the phenotypes of COPD and identify markers that can be used in the assessment of disease progression. There are many studies examining the significance of FeNO in asthma (Alving et al. 1993, Byrnes et al. 1997, Kharitonov and Barnes 2001b, Kharitonov et al. 1994, Lundberg et al. 1996, Nelson et al. 1997, Persson et al. 1994) but fewer studies on the other oxidant markers.

Several markers of oxidant burden have been suggested to associate both with the pathogenesis and disease progression of asthma (Barnes 1990) and COPD (Repine et al. 1997). However, given the complexity of oxidative stress and the inflammatory cascade and the interaction between these two processes, it is unlikely that measurement of any single molecule in the EBC, sputum or biological fluids will be able to provide adequate information about disease progression.

5. Biomarker requirements in COPD and asthma

Biomarker research is focusing on developing non-invasive tools for disease detection and follow-up. Only totally non-invasive, easily repeatable and practical techniques offer these possibilities. The biomarkers themselves will require to have several characteristics if they are to be of any use. In addition to sensitivity and specificity, the biomarker will need to be cost-effective, stable with minimal day to day or diurnal variability and easy to access and analyze. A good biomarker should also provide possibilities for early diagnostics, differential diagnosis and monitoring response to therapy and for the evaluation of disease progression. It is likely that one biomarker can never fulfill these requirements, instead panels or various combinations of biomarkers might be the answer and these panels may well be different if they are intended for the differential diagnosis of the disease or for following its progression.

6. Problems in measuring oxidative stress in smoking asthmatics

It is problematic to assess the importance of oxidant-related biomarkers especially in smoking asthmatics, since smoking per se has significant effects on the levels of FeNO and 8-isoprostane. It is also unclear whether these oxidant markers can predict disease development in follow-up periods of years or decades. Furthermore, the half-lives of
free radicals and oxidants are short, why their direct evaluation is often impossible or difficult.

The airway pathology of smoking asthmatics is still poorly understood since this group is frequently excluded from experimental studies. Asthma and cigarette smoking potentiate the effects of each other leading to impaired asthma control, more severe symptoms (Althuis et al. 1999), increased need for rescue medication (Gallefoss and Bakke 2003), accelerated deterioration in lung function (Lange et al. 1998) and reduced therapeutic response to corticosteroids (Chalmers et al. 2001, Chaudhuri et al. 2003). There is evidence that the time of initiation of smoking might influence the asthma phenotype: atopy-associated asthma seems to develop before the start of smoking (Raherison et al. 2003). Differential diagnosis from COPD is challenging since smoking modifies the typical asthmatic inflammation and asthmatic smokers often have features of typical asthmatic and smoke-related airway inflammation and pathology. Both accelerated and suppressed inflammatory responses are also seen in smokers with asthma. Sputum eosinophils decrease (Chalmers et al. 2001) perhaps due to exogenous NO present in cigarette smoke which can increase the apoptosis of activated eosinophils (Assreuy et al. 1993) and an elevation in neutrophils is seen (Chalmers et al. 2001). Remodeling of airways may also be more severe in asthmatic smokers (Carroll et al. 2000). Several markers of oxidative /nitrosative stress are increased even in “healthy” smokers.
Table 2. The main differences in non-smokers and smokers with asthma.
In smokers with asthma, both asthma and COPD diagnosis can occur simultaneously.

<table>
<thead>
<tr>
<th></th>
<th>NON-SMOKER</th>
<th>SMOKER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main inflammatory cells</strong></td>
<td>Eosinophils</td>
<td>Neutrophils</td>
</tr>
<tr>
<td><strong>Asthma control</strong></td>
<td>Good with appropriate medication</td>
<td>Impaired</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td>Usually in control with appropriate medication</td>
<td>Often not in control</td>
</tr>
<tr>
<td><strong>Lung function</strong></td>
<td>Normal/near to normal</td>
<td>Accelerated worsening</td>
</tr>
<tr>
<td><strong>Need for rescue medication</strong></td>
<td>Rare</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Response to corticosteroids</strong></td>
<td>Good</td>
<td>Reduced</td>
</tr>
<tr>
<td><strong>Inflammation in the airways</strong></td>
<td>Typical asthmatic</td>
<td>Mixed features of asthmatic and smoke-related</td>
</tr>
<tr>
<td><strong>Airway remodeling</strong></td>
<td>Variable</td>
<td>More severe</td>
</tr>
<tr>
<td><strong>Prognosis</strong></td>
<td>Good</td>
<td>Increased morbidity and mortality</td>
</tr>
</tbody>
</table>

7. Biomarkers of oxidant burden in COPD and asthma

Oxidative/nitrosative stress has generally been assessed by evaluating oxidant producing enzymes and their activation, by analyzing markers of protein modifications such as protein nitration and carbonylation, by measuring exhaled gaseous metabolites related to oxidative/nitrosative stress and by detecting decline/inactivation of small molecular weight antioxidants or antioxidant enzymes.

7.1 Oxidant markers

7.1.1 Oxidant generating enzymes

Inducible NOS (iNOS), eosinophil peroxidase (EPO) and myeloperoxidase (MPO) represent most widely studied oxidant generating enzymes (Kinnula 2005b). Inducible
NOS is the most efficient of the NO synthases in producing NO during inflammation and its main location is the airway epithelium but has also been detected in inflammatory cells (Barnes and Belvisi 1993). Elevated levels of iNOS in sputum have been reported in patients with asthma (Sugiura et al. 2003) but in COPD the iNOS levels have been variable (Ichinose et al. 2000, Rytila et al. 2006). The amount of EPO is elevated in asthma but MPO, which is expressed in neutrophils and monocytes (Kim et al. 2001) is increased in COPD with the levels being higher in COPD than those encountered in asthma (Metso et al. 2001). A rather comparable increase in the number of MPO positive cells is seen in the induced sputum of chronic non-symptomatic and symptomatic smokers with normal lung function who may be at risk of developing COPD (Rytila et al. 2006). High variability with large standard deviations has been reported in different investigations into sputum MPO concentrations (Kim et al. 2001). However, EPO and MPO might have potential significance in differentiating asthma from COPD but these have been evaluated only in a few investigations (Metso et al. 2002a and 2002b). EPO leads to the formation of hypobromous acid (HOBr) and MPO to hypochlorous acid (HOCl).

7.1.2 Nitric oxide metabolites

7.1.2.1 Fractional exhaled nitric oxide (FeNO)

Increased FeNO levels have been widely reported in adults (Kharitonov et al. 1994, Persson et al. 1994) and children (Byrnes et al. 1997, Lundberg et al. 1996, Nelson et al. 1997) with asthma, even in individuals with mild or even no symptoms (Alving et al. 1993, Kharitonov and Barnes 2001a). FeNO levels correlate with eosinophilic airway inflammation and can predict a decline in asthma control when repeated longitudinal measurements are taken (Kharitonov and Barnes 2001b, Liu and Thomas 2005, Smith et al. 2005). The changes in serial FeNO have a higher predictive value than single measurements (Jatakanon et al. 2000, Kharitonov and Barnes 2001b, Leuppi et al. 2001). In addition, FeNO is associated with several markers of asthma control, such as asthma symptoms, dyspnea score, daily use of medication, and reversibility of airflow obstruction (Sippel et al. 2000). An elevation in FeNO levels can be seen before any significant decline in airway hyperresponsiveness, elevation of sputum eosinophil
levels or changes in lung function parameters (Jatakanon et al. 2000, Kharitonov et al. 1996a). The main origin of the increased FeNO levels in asthma is the lower airways (Kharitonov et al. 1996b). The determination has been shown to be highly reproducible in healthy and asthmatic adults and children (Kharitonov et al. 2003). The measurement of exhaled NO at different flows reveals that the increase in peripheral NO is often related to disease severity (Brindicci et al. 2005). Recent ATS guidelines recommend that FeNO measurements could be used as an additional tool for detecting eosinophilic airway inflammation i.e. phenotyping asthma, predicting the likelihood of corticosteroid responsiveness and to monitor the anti-inflammatory effect (ATS 2011).

However, exhaled NO is somewhat non-specific to asthmatic inflammation; its levels increase in viral infections (Kharitonov and Barnes 2001a) and generally decline in smokers (Maziak et al. 1998, Rytila et al. 2006). There is also some disagreement on whether changes in serial FeNO are significant evidence of loss of asthma control or simply due to errors in measurements or to the natural variability of airway inflammation over time (Kharitonov 2004). It might be that FeNO is not always related to asthma severity or airway inflammation (Kharitonov and Barnes 2000). FeNO measurements have remained, despite these problems, as one of the most important parameters in the evaluation, treatment and monitoring patients with asthma and airway disease with eosinophilic inflammation.

In contrast to asthma, the FeNO levels in stable COPD do not differ significantly from controls (Kharitonov and Barnes 2001a, Rytila et al. 2006). However in COPD exacerbations, FeNO levels increase (Maziak et al. 1998) most likely due to increased oxidative stress, especially in cases where there is coexistent asthma and/or the presence of eosinophils (Papi et al. 2000). The increased FeNO levels in unstable COPD compared both with stable smokers or ex-smokers with COPD are related especially to the presence of eosinophils (Maziak et al. 1998). Since cigarette smoke decreases FeNO levels (Kharitonov et al. 1995, Robbins et al. 1996) by down-regulating NOS3 (Su et al. 1998) and consuming NO, it could be speculated that FeNO is not reliable marker for assessing asthma in smokers. FeNO might have importance in the differentiation of asthma, eosinophilic bronchitis and COPD but it has only a minor role in evaluating COPD.
7.1.2.2 3-Nitrotyrosine

The combination of NO and superoxide anion (O$_2^-$) in the airways leads to the formation of peroxynitrite (ONOO$^-$) which reacts with tyrosine residues of proteins to form a stable product, nitrotyrosine (Ischiropoulos et al. 1992). Nitrotyrosine can be formed not only by iNOS activation but also by MPO (Davis et al. 2001). Nitrotyrosine can be detected in exhaled breath condensate (EBC). Its levels are elevated in asthmatic children and adults and decreased in response to corticosteroid treatment (Baraldi et al. 2006, Bodini et al. 2006, Hanazawa et al. 2000). Increased nitrotyrosine immunoreactivity has also been reported in the lung biopsies of asthmatics (Saleh et al. 1998). It has been postulated that nitrotyrosine might play a major role in the pathogenesis of airway remodeling (Kharitonov and Barnes 2001a) and it may contribute to airway obstruction and hyperresponsiveness and epithelial damage in asthma (Saleh et al. 1998). Abundant iNOS and nitrotyrosine positive sputum cells have also been seen in COPD patients when compared to healthy smokers with a negative correlation to FEV1 (Ichinose et al. 2000). However, also in the sputum samples of smokers without airway obstruction and also in some samples of non-smokers one can detect increased levels of nitrotyrosine (Rytila et al. 2006). Although both HPLC technique and GC/MS are sensitive and provide similar nitrotyrosine levels in the EBC, they are not able to differentiate healthy controls from asthmatics, suggesting that nitrotyrosine may not be a selective marker for oxidative stress in asthma (Celio et al. 2006). It is also likely that nitrotyrosine is not useful in differentiating between asthmatic and cigarette smoke-related airway disease.

7.1.2.3 S-Nitrothiosols

Nitric oxide can be trapped by thiol containing biomolecules such as glutathione (GSH) to form S-nitrothiosols. These compounds are increased in the EBC of smokers and patients with asthma (Corradi et al. 2001) and the levels decrease with corticosteroid treatment in asthma (Kharitonov et al. 2002). However, the increase in the levels of the S-nitrothiosols in EBC is transient and most likely not sensitive enough to be of use in the diagnostic assessment of airway disease (Franklin et al. 2006).
7.1.3 Other exhaled markers of oxidative stress

In addition to FeNO, several other gaseous markers suggesting increased oxidative stress in the airways have been studied both in asthma and COPD.

7.1.3.1 Hydrogen peroxide (H$_2$O$_2$)

Exhaled H$_2$O$_2$ has been detected in patients with stable COPD and during exacerbation phases (Dekhuijzen et al. 1996, Kharitonov 2004) with the levels being related to the disease severity and also in steroid-naïve asthmatics (Kharitonov et al. 2002). In healthy smokers, macrophages release more H$_2$O$_2$ than the cells from non-smokers (Baughman et al. 1986, Guatura et al. 2000, Nowak et al. 1996). Exhaled H$_2$O$_2$ concentrations are higher in smoking than non-smoking asthmatics or controls, though there is high variability (Horvath et al. 2004). In addition, cigarette smoke evokes to an acute elevation in exhaled H$_2$O$_2$ levels in patients with asthma, suggesting that smoking causes additional release of ROS in the airways (Horvath et al. 2004). Comparison of the H$_2$O$_2$ results from different laboratories is difficult since there are several techniques to measure H$_2$O$_2$, all with their own limitations (Van Hoydonck et al. 2004). Overall, H$_2$O$_2$ is relatively unstable and its analysis is not sensitive or specific enough to permit the diagnosis, differential diagnosis or monitoring of COPD or asthma.

7.1.3.2 Carbon monoxide (CO)

Carbon monoxide (CO) is produced from the degradation of heme by hemeoxygenase or non-heme-related release from xenobiotics and bacteria. Approximately 85% of CO is exhaled (Barnes et al. 2006, Kharitonov and Barnes 2001a). Several pathological conditions other than airway inflammation can contribute to CO formation. It has been reported that exhaled CO levels are elevated in stable asthma and decrease near to the normal values by treatment with inhaled corticosteroids (Barnes et al. 2006, Horvath et al. 1998, Zayasu et al. 1997). Cigarette smoke has marked effects on exhaled CO, complicating the assessment in COPD. Although the analysis of CO is straightforward, the levels of CO are too variable for the clinical assessment of asthma and COPD, their diagnosis or follow-up.
7.1.3.3 Volatile organic compounds (VOCs)

Many volatile hydrocarbons exist, but only a few of them have been analyzed, one of the most extensively investigated has been ethane. Increased exhaled ethane levels have been detected in patients with mild steroid-naïve asthma when compared to steroid-treated asthmatics and healthy controls (Paredi et al. 2000a). It has also been reported that COPD patients have elevated exhaled ethane levels that correlate with the degree of airway obstruction (Paredi et al. 2000b). However, exhaled ethane levels increase also due to physical (Leaf et al. 1997) and mental stress and smoking (Habib et al. 1995) perhaps due to the high concentrations of hydrocarbons and direct oxidative damage. Cigarette smoke contains ethane and in healthy smokers, a transient elevation of the exhaled ethane concentration has also been reported (Habib et al. 1995). In addition, measurement of ethane is demanding and difficult to use in clinical practice (Barnes et al. 2006, Larstad et al. 2002). The relative concentrations of the aldehydes are also different and do not correlate with each other when measured in EBC and induced sputum (Corradi et al. 2004). Several VOCs exist and combinations i.e. VOC profiles can be developed and assayed with modern small gas chromatographic techniques. The technique of the so-called electronic nose has been found to be a promising method in discriminating the exhaled breath of patients with asthma from controls but it seems that it is less accurate in distinguishing asthma severity (Dragonieri et al. 2007).

7.1.4 Markers of lipid peroxidation

7.1.4.1 8-isoprostane

Oxidative damage to lipids leads to the production of isoprostanes, which are prostaglandin analogues produced mainly by free radical-induced peroxidation of arachidonic acid (Morrow et al. 1990). The best-characterized isomer is 8-isoprostaglandin F$_{2a}$ (8-isoprostane), which is considered to reliably reflect the degree of oxidative stress in vivo– it is a stable metabolite and specific for lipid peroxidation (Cracowski et al. 2002, Janssen 2001, Morrow and Roberts 2002). Dietary fats do not have any significant effect on the 8-isoprostane concentrations (Gopaul et al. 2000). 8-
Isoprostane has been proposed to represent a useful tool in exploring oxidative stress in lung diseases with high reliability (Montuschi et al. 2004). Several studies have reported that 8-isoprostane may be an important mediator of oxidative stress and pulmonary oxygen toxicity (Janssen 2001) and form part of a common pathway leading to airway obstruction (Paredi et al. 2002). Antioxidants may be able to scavenge ROS but it seems that they fail to decrease the rate of lipid peroxidation (Anderson et al. 1988, Habib et al. 1999) perhaps due to the fact that only the first step of the complicated metabolic pathway of lipid peroxidation is initiated by free radicals (Morrow et al. 1990).

Most studies have used commercial EIA (enzyme immunoassay) kits to detect 8-isoprostane in EBC, this technique shows a good correlation with GC/MS (Antczak et al. 2002, Baraldi et al. 2003a, Battaglia et al. 2005, Biernacki et al. 2003, Kostikas et al. 2003, Montuschi et al. 2000 and 1999, Simpson et al. 2005, Van Hoydonck et al. 2004), however there are some contradictory findings (Bodini et al. 2004, Rahman 2004, Simpson et al. 2005, Van Hoydonck et al. 2004). Increased EBC 8-isoprostane levels have been reported in children (Baraldi et al. 2003a and 2003b, Mondino et al. 2004, Shahid et al. 2005) and adults (Antczak et al. 2002, Battaglia et al. 2005, Montuschi et al. 1999) with asthma and COPD (Biernacki et al. 2003, Ko et al. 2006, Kostikas et al. 2003, Montuschi 2000). The concentrations increase with asthma severity (Kharitonov and Barnes 2001a, Montuschi et al. 1999) and decline after treatment of acute asthma exacerbations though remaining higher than in healthy controls (Baraldi et al. 2003b). Smoking has been reported to cause an acute 50% increase in EBC 8-isoprostane levels within 15 minutes (Montuschi et al. 2000). No correlations have been found between EBC 8-isoprostane levels and age, sex, or history of smoking in pack-years (Montuschi et al. 2000). Furthermore, 8-isoprostane levels have not been found to associate with the dyspnea score, neutrophil count or lung function parameters (Montuschi et al. 2000).

Major limitations still exist in the detection of 8-isoprostane in the EBC, the concentrations are variable and often remain below the detection limit in the analysis (Van Hoydonck et al. 2004). This might be explained by the extensive dilution that occurs from water vapour during condensation and the low concentrations that are close to the detection limits of the EIA measurements (Effros et al. 2005). Several
confounding factors such as smoking, diurnal variation, age, alcohol consumption, caffeine and possibly also a diet rich in antioxidants have to be taken also into consideration. Many studies have also reported a major variation in the levels of 8-isoprostane in non-smokers and smokers, suggesting that the commercial assay itself is variable. When one examines the earlier and more recent studies, then it seems that EBC 8-isoprostane may not be either a very sensitive or a specific biomarker of oxidative stress given the variability in its measurement.

The levels of 8-isoprostane are higher in sputum than in EBC (Mazur et al. 2009, Simpson et al. 2005). Sputum 8-isoprostane levels are elevated in COPD and cigarette smokers, but do not clearly differentiate healthy smokers from symptomatic smokers i.e. those who are at risk of developing COPD (Kinnula et al. 2007). It is important that 8-isoprostane was elevated in smokers without COPD reflecting oxidative stress already due to smoking alone, but in a cross-sectional setting, its relevance for COPD development could not be evaluated. The level of 8-isoprostane in sputum is elevated also in adults with stable asthma when compared to healthy subjects, increase further with disease severity and decrease significantly after treatment of an acute asthma exacerbation. However there is a wide variability in the 8-isoprostane concentrations between and also within groups (Paredi et al. 2002). Overall 8-isoprostane is elevated in the sputum samples of smokers, subjects with COPD and asthma.

7.1.4.2 4-hydroxy-2-nonenal (4-HNE)

During lipid peroxidation, aldehydes are generated endogenously and they may be involved in many pathophysiological events associated with oxidative stress in cells and tissues (Gutteridge 1995). 4-HNE is a highly diffusible and reactive end-product of oxidative stress-induced lipid peroxidation and can attack targets far from the original site of free radical generation (Esterbauer et al. 1991). It has been postulated that the degree of formation of 4-HNE in response to smoking may also contribute to the development of enhanced airspace inflammation in COPD (Rahman et al. 2002). 4-HNE has been detected in lung biopsies of COPD patients (Rahman et al. 2002) but also in induced sputum samples of chronic smokers, irrespective of the lung function parameters or symptoms compared to non-smokers (Rytiala et al. 2006). It seems that
the presence 4-HNE might indicate that oxidative damage has occurred, but it may not provide any specific insight into the pathogenesis of the airway disease and most likely it will not prove to be a sensitive or specific biomarker for assessing the severity of COPD or asthma.

7.1.5 Antioxidants and antioxidant enzymes

The oxidant/antioxidant equilibrium is disturbed in COPD and asthmatic patients due to the chronic inflammation, activation of inflammatory cells and/or oxidant producing enzymes and also because of the changes in the antioxidant defence of the lung. It is believed that the major low molecular weight antioxidant of human airway secretions is GSH (Cantin et al. 1987) but its maintenance in the epithelial lining fluid (ELF) is still poorly understood. Free GSH can be measured but most of the GSH is bound to proteins and this is difficult to measure. The levels of GSH have been investigated in airway secretions and BAL but it seems that they are neither sensitive nor specific for COPD or asthma.

The first line defence enzymes against superoxide radicals include superoxide dismutases (SODs) like manganese SOD (MnSOD) and it has been suggested that MnSOD and extracellular SOD (ECSOD) may be impaired or changed in the airways of asthmatics and COPD patients (Comhair et al. 2005, Regan et al. 2011). Enzymes related to GSH synthesis/homeostasis have an important role in the antioxidant defence of human lung (Cantin et al. 1987) and have been found to decrease towards COPD severity. Numbers of studies suggest that combined profiles of antioxidant and detoxification enzymes and their post-translational modifications like oxidation, thiolation and S-nitrosylation may be potential indicators of the severity of oxidative stress in the airways.

7.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a large family of proteolytic enzymes (over 30 MMPs have been characterized) that degrade the components of the extracellular matrix (Ohbayashi 2002). MMPs are involved in pulmonary remodeling processes in both
COPD and asthma (Kelly and Jarjour 2003, Parks and Shapiro 2001). Oxidative stress, mainly cigarette smoke associated oxidants, can trigger MMP activation (Kinnula 2005a). MMPs can be measured in sputum specimens rather easily. Significant differences have been reported in the MMP profiles in COPD and asthma i.e. COPD patients have elevated levels of MMP-1, -8, -9 and -12 (Beeh et al. 2003, Culpitt et al. 2005, Demedts et al. 2006, Elkington and Friedland 2006, Vernooy et al. 2004) when compared to asthmatics, non-smokers and non-symptomatic smokers and these changes may reflect different pathogenesis of these diseases (Culpitt et al. 2005). MMPs might have significance as additional biomarkers in COPD and or/asthma development and also in evaluating different COPD phenotypes since the emphysematous type of COPD most likely displays a different profile of MMPs than the disease with airway predominance.

7.3 Other potential markers

7.3.1 C-reactive protein (CRP)

Pulmonary inflammation in COPD is associated with elevated acute phase protein levels in plasma and it is possible that these proteins could be useful in predicting risk of future COPD events (Dahl and Nordestgaard 2009). C-reactive protein is an acute phase plasma protein synthesized in response to general inflammatory episodes (Black et al. 2004, Pepys and Hirschfield 2003). CRP is produced mostly in hepatocytes but also in adipocytes (Ouchi et al. 2003) and cultured coronary artery smooth muscle cells (Calabro et al. 2005). Although a major increase in CRP levels is seen in response to infection or tissue injury, a minor increase in CRP levels has been considered as a possible marker of disease in systemic conditions i.e. sensitive CRP (sCRP) (Ridker 2001) and sCRP has indeed been detected by immunofluorescence in atherosclerotic plaques from human coronary arteries (Zhang et al. 1999).

One study showed correlation between sCRP levels and pack-years of smoking and also in predicting mortality in patients with mild to moderate COPD in the short-term, although this correlation becomes weaker with time (Man et al. 2006). The same study demonstrated that high sCRP is associated with a yearly decrease in forced expiration
volume of habitual smokers and they suggested that combining the results of the sCRP assay and forced expiration volume would help to predict outcome and therefore enable early intervention. Dahl and co-workers measured sCRP levels in subjects with spirometry-defined airway obstruction i.e. subjects with a high risk of clinical COPD and during 8 years of follow-up, they found that subjects with higher sCRP levels at baseline had an increased cumulative incidence of COPD hospitalization and death (Dahl et al. 2007). However, in another study although there were significant differences in sCRP levels in the COPD patients, there was no difference in sCRP levels between smokers and non-smokers (Pinto-Plata et al. 2006). In a recent cohort study, sCRP was higher in COPD patients than in ex-smokers or non-smokers but showed wide variability over the 3 months of follow-up (Dickens et al. 2011).

Most studies that have investigated sCRP levels in former smokers suggest that levels decrease slowly after smoking cessation (Hastie et al. 2008, Wannamethee et al. 2005, Lowe et al. 2001) indicating that the tissue damage caused by smoking takes some time to recover (Yanbaeva et al. 2007). A few longitudinal studies following sCRP levels before and after smoking cessation have also been undertaken (Crook et al. 2000, Hammett et al. 2007). In the study of Crook and co-workers (Crook et al. 2000) sCRP levels did not change significantly after 1 year and in the study of Hammett et al. (Hammett et al. 2007) after 6 weeks of smoking cessation. Since sCRP has been connected not only to COPD but also to coronary artery disease (CAD) and atherosclerosis, it is clear that it is not a specific marker for COPD. Overall, the studies both on COPD and CAD suggest that sCRP is one marker of systemic inflammation in both disorders.

7.3.2 Fibrinogen

Fibrinogen is one of the acute phase proteins and its value in predicting future COPD has been evaluated in a few studies (Dahl et al. 2001, Gan et al. 2004). In the study of Dahl and co-workers, individuals with higher baseline plasma fibrinogen levels had reduced lung function, increased cumulative incidence of COPD hospitalization and an increased risk for COPD hospitalization during 6 years of follow up (Dahl et al. 2001). Gan et al. also reported a clear difference in plasma fibrinogen between COPD patients
and controls (Gan et al. 2004). However, it has been claimed that fibrinogen has limited value in clinical practice due to its low predictive value for COPD (Dahl et al. 2001). A recent study measured several blood biomarkers in patients with COPD, ex-smokers and non-smokers at baseline and at 3 months and found that fibrinogen was one of the biomarkers that were significantly higher in COPD patients when compared to ex-smokers and non-smokers (Dickens et al. 2011). The same study reported that fibrinogen was the most repeatable biomarker and exhibited a weak correlation with the dyspnea score, 6-minute walk distance and exacerbation rate in COPD patients. In conclusion, fibrinogen may be a useful biomarker according to studies conducted so far, but these will require further confirmation.

7.3.3 Panels of serum markers

One study investigated a platform of several potential COPD biomarkers in a hypothesis driven study in the serum of patients with severe COPD and controls using novel microarray platform (PMP) technology (Pinto-Plata et al. 2007). They identified 30 biomarker clusters for COPD and from the 19 best predictive clusters, 2-3 biomarkers were selected based on their pathophysiological profile and the statistical significance of the correlations towards clinically important end points was calculated. They found a good correlation between the selected panel of 24 biomarkers and FEV1, carbon monoxide transfer factor, 6-minute walk distance, BODE index and exacerbation frequency. They concluded that PMP technology may be useful in identifying potential biomarkers in COPD and that it may be possible to differentiate patients with COPD from smokers and non-smokers using panels of selected serum markers. In summary, this study used a group of COPD patients including all phenotypes with severe disease in one cross-sectional study why the most promising markers identified in that study will require future investigations.

7.3.4 Tumour necrosis factor-α (TNF-α)

TNF-α is the most widely studied cytokine of the TNF super family in COPD and was originally described as a factor produced by the endotoxin stimulated macrophages that causes hemorrhagic necrosis of tumors (Carswell et al. 1975). Increased levels of TNF-
α have been reported in induced sputum and lung biopsy of COPD patients (Keatings et al. 1996, Mueller et al. 1996, Gan et al. 2004). A recent study investigated TNF-α serum levels in healthy heavy smokers and healthy non-smokers (Petrescu et al. 2010). They reported significantly higher TNF-α serum levels in smokers when compared to non-smokers, and in addition a further increase of TNF-α serum levels in patients who had daily smoke exposure from smoking more than 1 pack/day. Thus, it has been claimed that the serum level of TNF-α may be a useful biomarker for the selection of heavy smokers with a high risk to develop smoke induced COPD (Petrescu et al. 2010). TNF-α is however one marker of systemic inflammation, and may thus also be associated with other diseases in addition to COPD.

8. Non-biased Proteomics

8.1 Proteomic studies on COPD/smokers

Non-biased proteomics offers non-hypothesis driven approach to study disease biomarkers to airway diseases compared to healthy controls and/or to “healthy” smokers. This is particularly important in COPD where the activity of the disease is more difficult to evaluate when compared to asthma. Proteomics can be conducted for example from sputum, lung tissue, BAL or plasma/serum samples. BAL suffers from major problems for example its invasiveness and high dilution. Instead, serum contains several “contaminant” proteins, such as albumin and reflects systemic manifestations of other diseases as well. Sputum and lung tissue proteomics offer an interesting way to evaluate both airway and lung tissue protein profiles and can be assessed in more detail after the identified proteins have been characterized from both sputum and plasma samples. The most widely used proteomic technologies are two-dimensional gel electrophoresis (2DE), and the two-dimensional Difference Gel electrophoresis (2D-DIGE) method representing a new and sensitive way to evaluate especially cysteine containing proteins; both of these techniques are usually combined with mass spectrometry to identify the changed proteins (Kinnula et al. 2009). To date, only a few proteomic studies have been conducted in the lung tissues of COPD patients (Ishikawa et al. 2010, Ohlmeier et al. 2008 and 2010).
Nicholas et al. investigated an induced sputum sample from one smoker using the combination of 2D gel analysis and GeLC-MS/MS and represented the first extensive survey of the proteome, with a total of 191 human proteins detected (Nicholas et al. 2006). Another pilot-study studied induced sputum proteome profiles from healthy smokers, smokers with chronic bronchitis and smokers with COPD using CapLC-ESI-Q/TOF-MS technique (Casado et al. 2007). A total of 203 human proteins were identified and clear differences were seen in the protein expression profiles related to the COPD phenotype and cigarette smoking illness severity. That particular study did not compare specific proteins between diseased and healthy samples, but assessed how often certain proteins could be detected, for example in COPD vs control specimens. It is possible that the proteomic approach might be useful in spotting specific and sensitive biomarkers associated to specific COPD stages and phenotypes.

8.1.1 Surfactant protein A (SP-A)

The initial proteomic studies reported highly elevated SP-A levels in the lung tissue samples of COPD patients (Ohlmeier et al. 2008). The finding is not surprising considering that SP-A is greatly associated with the protection of the lung. Pulmonary surfactant is a mixture of phospholipids and proteins formed mainly by type II pneumocytes (Kishore et al. 2006). SP-A and SP-D are members of the collectin family and have important and unique roles in pulmonary defence against inflammation/oxidative stress (Mason et al. 1998, Whitsett 2005). Surfactants have also been found to regulate the protease/antiprotease balance through different pathways, it has been proposed that SP-A might even regulate MMP-9 production and function (LeVine et al. 2000, Ramadas et al. 2009, Vazquez de Lara et al. 2003, Wert et al. 2000). The exact mechanism leading to increased serum levels of surfactant proteins is unresolved but currently the most widely accepted hypothesis is that they are translocated from the lung into the circulation due to the changes in the alveolar-capillary permeability (Hermans and Bernard 1999). Elevated plasma/serum SP-A levels have been earlier reported in smokers and patients with COPD in several studies (Behera et al. 2005, Kida et al. 1997, Kobayashi et al. 2008, Nomori et al. 1998, Robin et al. 2002). A proteomic study of pulmonary tissue conducted by Ohlmeier et al. revealed increased SP-A in COPD but not in the fibrotic lung (Ohlmeier et al. 2008).
addition, a recent study reported elevated levels of both plasma and sputum SP-A in smokers without COPD when compared to non-smokers (Mazur et al. 2011). The same study investigated the effect of smoking cessation on plasma SP-A levels and found that after 2 years of follow-up, the SP-A level was significantly higher in those individuals who continued smoking compared to the quitters.

Some opposite findings also exist about the circulating SP-A levels in smokers and/or COPD determined in serum (Mutti et al. 2006), lung tissue (Vlachaki et al. 2010) or BAL (Betsuyaku et al. 2004, Honda et al. 1996). However, some of the above mentioned studies on SP-A had been examined from BAL, which is somewhat problematic since it is invasive and also the risk of collapse of the airways in COPD, or by non-quantitative immunohistochemical technique.

Age and genotype of the patient have been reported to influence the SP-A levels (Floros 2001, Tagaram et al. 2007) and some SP-A alleles might increase the risk for development of COPD (Guo et al. 2001). In the study of Ohlmeier and co-workers, SP-A was confirmed to represent SP-A2 (Ohlmeier et al. 2008) but it is unclear if SP-A1 and SP-A2 are related to smoking alone or if they can predict disease progression.

8.1.2 Surfactant protein D (SP-D)

In the proteomic study of Ohlmeier et al. (Ohlmeier et al. 2008) no change in SP-D levels were detected, neither could any change be seen between non-smokers, smokers or subjects with COPD could be seen in the lung tissue by Western blot analyses. However SP-D has been suggested to represent a marker for COPD and that it can especially predict COPD exacerbations (Lomas et al. 2009). The relationships between SP-D concentrations in serum and BAL are different in smokers or COPD patients when compared to allergic diseases like asthma (Winkler et al. 2011). In smokers and especially COPD patients have reduced levels of SP-D in BAL but elevated levels of SP-D in serum (Lomas et al. 2009, Winkler et al. 2011). It has been reported also that COPD patients receiving inhaled corticosteroid treatment have higher SP-D levels in BAL when compared to steroid-naïve COPD patients (Sims et al. 2008) and that treatment with oral corticosteroids decreases the serum SP-D levels in COPD patients...
(Lomas et al. 2009). Winkler et al. reported that pulmonary and serum SP-D levels correlated even with the degree of airway obstruction in smokers and that cigarette smoke can disrupt SP-D’s quaternary structure which may impair its immunological function and increase the leakage of SP-D from the lung into the circulation (Winkler et al. 2011). However, in the study of Kobayashi and co-workers, the serum SP-D level was not changed in smokers (Kobayashi et al. 2008). In asthma or allergen induced airway inflammation, elevated levels of SP-D have been reported in both serum (Koopmans et al. 2004) and BAL (Erpenbeck et al. 2006). Overall, the role of SP-D in COPD has remained unclear.

8.1.3 Polymeric immunoglobulin receptor (PIGR)

The defence mechanisms of the lung include both cellular and humoral immune components. Secretory immunoglobulin A (SIgA) is the predominant Ig isotype in mucosal secretions and it acts as a scavenger i.e. prevents absorption and adherence of bacteria and viral agents (Pilette et al. 2001). Epithelial cells express specific receptors for polymeric Igs (pIgA and IgM) allowing their active transport into the mucosal lumen (Brandtzaeg et al. 1996). The polymeric Ig receptor (PIGR) can exist in several variants with different functions: full-length PIGR is associated with the transcytosis of IgA to mucosal surfaces while the cleaved form (secretory component, SC) together with polymeric IgA or as free form acts as a scavenger for environmental and microbial antigens (Kaetzel 2005, Wines and Hogarth 2006). PIGR is suggested to be a potential regulator of specific immune defence and inflammation (Wines and Hogarth 2006). SC/PIGR regulation is complex and dependent on several factors associated with COPD (Kaetzel 2005, Pilette et al. 2001, Ratajczak et al. 2010).

At present, only a few studies have investigated the role of PIGR in COPD (Pilette et al. 2001, Polosukhin et al. 2011). Polosukhin et al. revealed a decline in the PIGR level in abnormal epithelium and airway remodeling areas in severe to very severe COPD (GOLD III-IV) (Polosukhin et al. 2011). In that particular study, total PIGR instead of SC was investigated. In addition, decrease of PIGR expression in airway epithelium was reported by Pilette and et al. in patients with very severe COPD (Pilette et al. 2001). No studies have evaluated PIGR and its possible role in mild to moderate COPD.
8.1.4 Receptor for advanced glycation end products (RAGE)

The receptor for advanced glycation end products (RAGE) is a transmembrane receptor belonging to the immunoglobulin superfamily (Neeper et al. 1992). Soluble RAGE (sRAGE) is an isoform of RAGE lacking transmembrane and cytosolic domains which acts as deception receptor for RAGE ligands in the extracellular matrix and it is postulated to be protective against inflammation and cell injury (Park et al. 1998).

Smith et al. investigated sRAGE in plasma of COPD patients and reported that circulating sRAGE is lower in COPD when compared to healthy controls and shows a significant correlation to the degree of airway obstruction (Smith et al. 2011). Another study also reported significantly lower plasma sRAGE levels in COPD patients when compared to the control group including ex-smokers and current smokers and also a good correlation between sRAGE levels and the severity of emphysema (Miniati et al. 2011). RAGE was also reported to be over-expressed in the airway epithelium and in the airway smooth muscle of smokers with COPD (Ferhani et al. 2010).

Ohlmeier and co-workers investigated different RAGE isoforms in idiopathic pulmonary fibrosis (IPF) and COPD from lung tissue and BAL samples using 2-DE, MS and Western blotting (Ohlmeier et al. 2010). This study revealed a decrease of the full-length-RAGE (FL-RAGE) and its C-terminal processed variant (cRAGE) in the lung tissues of IPF and COPD patients, but endogenous secretory RAGE (esRAGE) was decreased only in IPF and unchanged in COPD. Another study also reported a decrease in RAGE levels in the lung tissue samples of IPF patients (Englert et al. 2008). Based on these studies, RAGE was not chosen for further investigations since it is not specific for COPD.

8.1.5 Serum amyloid A (SAA)

Serum amyloid A (SAA) is an acute phase protein induced by inflammatory mediators like IL-6, IL-1β and TNF-α and the levels rise during exacerbation phase of COPD (Sapey and Stockley 2006). SAA is secreted from the liver and of the four members of
the human SAA family, SAA1 and SAA2 are induced during inflammation or in response to tissue damage (Bozinovski et al. 2008).

The study of Bozinovski et al. claimed that SAA was a novel candidate biomarker for COPD exacerbation by proteomic screening with SELDI-ToF (Bozinovski et al. 2008). Further measurements were conducted using the ELISA assay, which detects induced SAA1 and SAA2. They reported that the elevation of SAA is associated with an exacerbation severity and that it is more sensitive than CRP alone or combined with dyspnea.

8.1.6 Lipocalin-1

Lipocalin-1 is an innate defence molecule against microbes and has been shown to be a biomarker in tear fluid for several conditions including exposure to cigarette smoke (Xu and Venge 2000). Its main role is thought to be epithelial defence in the airways since its structure is similar to other antimicrobial proteins in the lipocalin superfamily and is widespread in the respiratory tract (Nicholas et al. 2010).

In the recent sputum proteomic study of Nicholas et al. the levels of lipocalin-1 were elevated in healthy smokers when compared to non-smokers and they speculated that it may be due to either direct effects of cigarette smoke or an increased risk of smokers for bacterial infections (Nicholas et al. 2010). Surprisingly, in the same study, lipocalin-1 was significantly reduced in the sputum of smoking COPD patients when compared to healthy smokers perhaps evidence of deficiencies in the innate epithelial defence, which might expose COPD patients to infectious exacerbations. They also reported a good correlation between lipocalin-1 and airway obstruction. However, there are no studies examining the role of lipocalin-1 in other lung diseases so it may not be sensitive or specific for COPD.
AIMS OF THE STUDY

The present study investigated potential non-invasive markers that reflect oxidative stress and cell defence in the airways of patients with COPD, asthma and smokers in order to find specific and sensitive markers for early diagnosis and differentiation of “healthy” smokers from the disease using different sample types. Another main topic was to assess the persistence of oxidative/nitrosative stress in the airways after smoking cessation.

The aims of this study were

- to assess the value of sputum and EBC 8-isoprostane in recently diagnosed children and adults with mild asthma
- to compare oxidant markers in different sample types, such as sputum, plasma, exhaled air and lung tissue
- to study the effects of smoking cessation on sputum neutrophils, 8-isoprostane, nitrotyrosine, matrix metalloproteinases (MMP-7, -8, -9), TIMP-1 and FeNO in asymptomatic smokers and patients with chronic bronchitis/COPD or asthma.
- to determine whether smoking and ageing affect the levels and relationships between plasma SP-A, SP-D, MMP-9 and TIMP1, which were selected based on previous studies on COPD suggesting that these markers may predict COPD, its development and/or progression
- to seek new potential markers from sputum for early COPD using non-hypothesis driven non-biased proteomics
MATERIALS AND METHODS

1. Materials

1.1 Patient/subject characteristics

The main characteristics of the study subjects are shown in Table 3.

1.1.1 Study I

Newly diagnosed asthmatic children, adults and healthy controls were included. Sputum samples, exhaled breath condensates (EBCs), FeNO and spirometry results of these study subjects were collected and received from the Helsinki University Central Hospital, Department of Allergology. The diagnosis of asthma was based on symptoms and on the bronchodilatation in spirometry, diurnal variation in PEF (peak expiratory flow) and/or significant improvement in PEF after a dose of bronchodilating medicine during home recording. In some cases, significant bronchial reversibility measured in exercise challenge test or histamine challenge test was used. The atopic status was assessed by skin prick tests for common aeroallergens and if there were one or more positive reactions, the patient was given a diagnosis of allergic asthma and the rest were considered as being non-allergic. Most of the subjects had allergic asthma. All patients started medication after the induced sputum had been collected.

Patients with COPD exacerbation were included as a positive disease control for the 8-isoprostane determinations. All the patients had airway obstruction with FEV1 of <80% predicted and the ratio of FEV1 to forced vital capacity (FVC) was <70 with no significant reversibility (GOLD criteria) (Pauwels et al. 2001) measured before the deterioration phase. All the patients had a history of chronic progressive symptoms and a long-term smoking history and half of them were still smoking. During exacerbations, the patients exhibited worsened lung function and the major symptoms increased. All patients or their parents gave full informed consent and the Ethics Committee of Helsinki University Hospital approved the study protocol.
1.1.2 Studies II and III

In these prospective studies, subjects were recruited from three different smoking cessation clinics: Helsinki University Central Hospital (studies II, III), Southampton University Hospital (studies II, III) and the “Quitters” specialist smoking cessation service” of the Southampton and South West Hampshire Region (study II). The groups examined included asymptomatic smokers, smokers with chronic bronchitis (Stage 0 COPD in earlier GOLD classification), smokers with varying severity of COPD, smoking asthmatics and non-smoking controls. Patients were advised to terminate smoking and the effects of smoking cessation on airway oxidative stress markers were investigated. In study II, fractional exhaled nitric oxide (FeNO), sputum 8-isoprostane, nitrotyrosine and matrix metalloproteinase-8 (MMP-8) were measured at baseline and 1 and 3 months after successful cessation. Symptoms improvement was measured using the St Georges Respiratory Questionnaire (SGRQ). In study III, sputum neutrophils, matrix metalloproteinases (MMP-7, -8, -9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were measured at baseline and at 3 and 6 months after cessation. Smoking cessation was confirmed by frequent exhaled carbon monoxide analyses. Lung function was measured using spirometry. All subjects gave full informed consent. The Ethics Committees of the Helsinki University Hospital and the Southampton University Hospital approved the studies.

1.1.3 Study IV

Plasma samples were collected from young non-smokers and smokers who were military draftees in the Northern Command of the Finnish Defence Forces (Hamari et al. 2010) and from middle-aged/elderly subjects who had been contacted from the Division of Pulmonary Medicine, Lapland Central Hospital (Toljamo et al. 2010). The study population included young healthy smokers and non-smoking healthy controls, middle-aged/elderly healthy smokers and non-smoking controls and patients with stable stage I-III COPD. All COPD cases were newly diagnosed. The diagnosis of COPD was defined according to the GOLD criteria (Pauwels et al. 2001). Lung function parameter tests included flow volume spirometry and single breath diffusion capacity. None of the subjects had any diagnosed disease or any medications.
1.1.4 Study V

Sputum specimens of non-smokers, smokers and smokers with COPD were obtained from the area of Lapland Central Hospital (Toljamo et al. 2010). COPD was defined according to GOLD criteria (Pauwels et al. 2001) and only mild to moderate (Stage I-II) cases were included. Smokers had a smoking history for at least 20 years, considered themselves as healthy and were not taking any medications. The study protocol was approved by the ethical committee of Lapland Central Hospital with written consent being obtained from each of the subjects.

Lung tissue specimens were collected by lung surgery from patients treated in Helsinki University Central Hospital. Control tissues were obtained from lung surgery e.g. from hamartomas. The Ethics Committee of the Helsinki University Central Hospital approved the study and all patients received written information and gave their consent to use the samples.

Plasma samples were received from non-smokers, smokers and smokers with COPD (stage I-II) from the area of Lapland Central Hospital. Smokers had a smoking history at least 20 pack-years, were healthy and were not taking any medications. The study protocol was approved by the ethical committee of Lapland Central Hospital with written consent being obtained from each of the subjects.

2. Lung function measurement

Standard spirometric measurements (Medikro M 904, Kuopio, Finland) were performed according to ATS/ERS recommendations (ATS/ERS 2005b). Reference values for Finnish population were used (Viljanen et al. 1982).

3. Exhaled NO measurement

The FeNO measurements (Niox, Aerocrine AB, Sweden) were performed according to ATS guidelines (ATS 1999). Expiratory airflow was 50 ml/s against a flow resistor. An exhalation time of 10 sec was used as a default, but in children less than 12 years old,
the exhalation time was allowed to be reduced to 6 sec, if needed. The mean value from a 3-second period from the end-exhaled NO plateau was recorded. At least three successive FeNO measurements were performed and the mean value was used for the analysis.

4. Exhaled breath condensate (EBC) collection

Exhaled breath condensates (EBCs) were collected according to the instructions of ATS/ERS; during tidal breathing using noseclip and saliva trap with a defined cooling temperature and collection time (ATS/ERS 2005a).

5. Processing of the samples

5.1 Sputum

5.1.1 Sputum induction

A standard procedure of induction was conducted using 4.5% hypertonic saline given at 5-min intervals for a maximum of 20 min according to the guidelines of the European Respiratory Society’s Task Force (Djukanovic et al. 2002) in all centres.

5.1.2 Sputum processing

Sputum samples were processed immediately after induction. All sputum macroscopically free of salivary contamination was selected and treated with dithioerythritol (DTE, Sigma, Germany) and phosphate-buffered saline. The suspensions were filtered through 70-μm nylon gauze and centrifuged. The supernatant was stored at -80°C for biochemical analyses and immunoassay. The pellet was resuspended and the cell viability was studied by Trypan blue exclusion test. Coded cytospins were stained using May-Grunwald-Giemsa (MGG) method to obtain cell differential counts. At least 300-400 were counted on each slide. The slides were frozen at -20°C for further assays. The same researcher (Paula Rytilä) was involved in the
processing of the samples in Southampton and Helsinki. The same technician (Tiina Marjomaa) was involved in the teaching of how to process sputum in Helsinki and Rovaniemi.

5.1.3 Sputum processing for proteomics

For proteomics sputum supernatants processed as above were used. Protein concentrations were analyzed by the DC Protein Assay kit – method (Bio-Rad, Hercules, CA).

5.2. Tissue

5.2.1 Paraffin blocks for immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigens were retrieved by heating the sections in citrate buffer and endogenous activity was blocked using hydrogen peroxidase.

5.2.2 Tissue homogenates for Western blotting

Frozen tissue samples were homogenized in cold phosphate buffered saline (PBS) and adjusted to standard μg/μl protein concentration. Protein concentrations were analyzed by the DC Protein Assay kit – method (Bio-Rad, Hercules, CA).

5.3 Plasma

Peripheral venous blood was collected into EDTA tubes. Plasma was prepared by centrifugation for 10-15 min at 1500 × g and stored at -80 C until analysed.
Table 3. Main clinical characteristics of the subjects of all studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects in final analyses</th>
<th>Male/female</th>
<th>Age yrs</th>
<th>Pack years</th>
<th>FEV1 (%pred)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthmatic children</td>
<td>23</td>
<td>15/8</td>
<td>10</td>
<td>93</td>
<td>10</td>
</tr>
<tr>
<td>Asthmatic adults</td>
<td>14</td>
<td>2/12</td>
<td>38</td>
<td>84</td>
<td>10</td>
</tr>
<tr>
<td>Healthy children</td>
<td>13</td>
<td>5/8</td>
<td>11</td>
<td>101</td>
<td>8</td>
</tr>
<tr>
<td>Healthy adults</td>
<td>15</td>
<td>6/9</td>
<td>40</td>
<td>5</td>
<td>103</td>
</tr>
<tr>
<td>COPD exacerbation</td>
<td>11</td>
<td>7/4</td>
<td>72</td>
<td>35</td>
<td>43</td>
</tr>
<tr>
<td><strong>Study II</strong></td>
<td>Baseline/1mo/3mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic smokers</td>
<td>25</td>
<td>11/4</td>
<td>41</td>
<td>22</td>
<td>98</td>
</tr>
<tr>
<td>Smokers with chronic bronchitis/COPD stage I-IV</td>
<td>21</td>
<td>7/14</td>
<td>56</td>
<td>39</td>
<td>77</td>
</tr>
<tr>
<td>Smokers with asthma</td>
<td>15</td>
<td>5/10</td>
<td>42</td>
<td>22</td>
<td>96</td>
</tr>
<tr>
<td>Non-smoking controls</td>
<td>33</td>
<td>27/6</td>
<td>58</td>
<td>4</td>
<td>99</td>
</tr>
<tr>
<td><strong>Study III</strong></td>
<td>Baseline/3mo/6mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic smokers</td>
<td>25</td>
<td>10/15</td>
<td>42</td>
<td>23</td>
<td>97</td>
</tr>
<tr>
<td>Smokers with chronic bronchitis/COPD stage I-II</td>
<td>21</td>
<td>7/14</td>
<td>56</td>
<td>39</td>
<td>79</td>
</tr>
<tr>
<td>Smokers with asthma</td>
<td>15</td>
<td>6/9</td>
<td>42</td>
<td>22</td>
<td>99</td>
</tr>
<tr>
<td>Non-smoking controls</td>
<td>30</td>
<td>23/7</td>
<td>56</td>
<td>4</td>
<td>104</td>
</tr>
<tr>
<td><strong>Study IV</strong></td>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young non-smokers</td>
<td>36</td>
<td>34/2</td>
<td>20</td>
<td>-</td>
<td>97</td>
</tr>
<tr>
<td>Young smokers</td>
<td>51</td>
<td>50/1</td>
<td>20</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Middle-aged non-smokers</td>
<td>40</td>
<td>12/28</td>
<td>5</td>
<td>-</td>
<td>105</td>
</tr>
<tr>
<td>Middle-aged smokers</td>
<td>64</td>
<td>40/24</td>
<td>52</td>
<td>30</td>
<td>95</td>
</tr>
<tr>
<td>COPD stage I-II</td>
<td>44</td>
<td>35/9</td>
<td>61</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td><strong>Study V (PIGR)</strong></td>
<td>FEV1/FVC %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum/plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy non-smokers</td>
<td>7/36</td>
<td>59/56</td>
<td>-</td>
<td>88/86</td>
<td></td>
</tr>
<tr>
<td>Healthy smokers</td>
<td>72</td>
<td>53</td>
<td>29</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>COPD stage I-II</td>
<td>7/42</td>
<td>63/61</td>
<td>44/39</td>
<td>64/60</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as means.

* This group includes 2 ex-smokers who had stopped smoking ≥ 20 years before the study and 1 current smoker
** This group includes 6 ex-smokers who had stopped smoking ≥ 10 years before the study
*** This group includes 12 ex-smokers who had stopped smoking ≥ 20 years before the study
**** This group includes 9 ex-smokers who had stopped smoking ≥ 20 years before the study
6. Methods

6.1 Sputum staining for cell differential counts (Studies I, II, III)

The cytospin slides were stained using May-Grunwald-Giemsa (MGG) method to obtain cell differential counts. At least 300-400 cells were counted on each slide. If the samples had less than 70% of squamous epithelial cells they were accepted for further assessments.

6.2 Sputum proteomics (Study V)

6.2.1 Two-dimensional Difference Gel Electrophoresis (2D-DIGE)

Purified sputum (5 µg) from non-smokers, smokers, and smokers with COPD (Stage II) was labelled with the “CyDye DIGE Fluor labeling kit (“saturation DIGE”; GE Healthcare, Piscataway, NJ, USA) according to the manufacturer’s protocol. Proteins were separated by isoelectric focusing in immobilized pH gradient strips (pH 4-7, 24 cm; GE Healthcare) with the Multiphor II system (GE Healthcare) followed by SDS-PAGE in polyacrylamide gels (12.5%) overnight with the Ettan DALT II system (GE Healthcare). Fluorescence signals were detected with a Typhoon 9400 (GE Healthcare) and 2-D gels analyzed with Delta2D 4.0 (Decodon, Greifswald, Germany).

6.2.2 Protein identification using mass spectrometry (MS)

For protein identification, excised spots were digested and peptide masses were measured with a VOYAGER-DE™ STR (Applied Biosystems, Foster City, CA, USA). Proteins were identified by full database search (Aldente database version 11/02/2008, [http://au.expasy.org/tools/aldente/] with the following parameters: 20 ppm; 1 missed cut; [M+H]; +CAM; +MSO). The expected spot position in the 2D-gel according to the known protein sequence was calculated with the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).
6.3 Western blot analysis (Study V)

The sputum and tissue samples were loaded to polyacrylamide gels. Electrophoretic separation of proteins was done at 100V for 1 hour and the protein bands were transferred to nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). Membranes were blocked overnight in 10% skimmed milk in Tween-TBS. The membranes were probed with anti- PIGR antibody followed by secondary antibody treatments. Ponceau S staining was used to standardize the loading of the lung homogenate. For the Western blot of the sputum supernatants, equal volumes of the supernatants were loaded. A chemiluminescent HRP-substrate immunodetection kit (Millipore, Billerica, MA) was used for immunodetection. Membranes were exposed to X-ray film (Kodak, Rochester, NY).

6.4 Enzyme immunoassays (EIA)/Enzyme-linked immunosorbent assay (ELISA) (Studies I, II, III, IV, V)

Sputum (Studies I, II) and EBC (Study I) 8-isoprostane concentrations were determined by a specific EIA kit (Cayman Chemical, Ann Arbor, MI, USA) with standard curves. Sputum/plasma MMP-7, MMP-8, MMP-9 and TIMP-1 (Studies II, III, IV) concentrations were analyzed by commercial ELISA kits (Amersham Biosciences, Cardiff, UK) according to the manufacturer’s instructions. Plasma SP-A and SP-D levels (Study IV) were measured by commercially available EIA/ELISA kits (SP-A test Kokusai-F kit, Sysmex, Kobe, Japan; SP-D kit Yamasa EIA kit, Yamasa Co.,Chiba, Japan). PIGR in plasma was analyzed by ELISA (E91074Hu, Uscn Life Science Inc., Burlington, NC, USA; according to the manufacturer’s instructions.

6.5 Immunohistochemistry/morphometry (Study V)

NovoLink polymer detection system (RE7150-CE, Novocastra Laboratories ltd, Newcastle Upon Tyne, UK) was used for immunostaining according to the manufacturer's instructions. Detection was performed with anti-goat secondary antibody. Negative control sections were treated with mouse isotype control (Zymed Laboratories, San Francisco, CA, USA) or PBS. Digital images of lung tissue sections
with normal looking lung histology by excluding the damaged areas, assessed by an experienced pathologist, were taken using 200 x magnification and saved as Photoshop JPG files. The areas of positively vs. negatively stained tissue were measured using Image Pro software (Media cybernetics, UK)

7. Statistical analyses

All the statistical analyses were performed using the SPSS (versions 10.0, 15.0 and 18.0) software program (SPSS Inc., Chicago, IL, US). The data are given as median and range or means together with standard deviation (SD). Data for all groups was analyzed by Kruskall-Wallis test and differences between individual variables from two groups were analyzed by the Mann-Whitney U-test, the analyses of variance (ANOVA) or t-test. All differences between two time points were analyzed by the non-parametric Wilcoxon signed rank test. Correlations between variables were determined using the Spearman rank correlation test or with the Pearson correlation coefficient. Linear multivariate regression analysis was used to study the independent effect e.g. the effect of age on plasma SP-A levels. Receiver operating characteristic (ROC) curves were used to analyze if plasma SP-A has a predictive capability to distinguish the healthy subjects from the patients. P-value $\leq 0.05$ (Studies I, II, III, V) or $p < 0.01$ (Study IV) was considered as statistically significant.
RESULTS

1. FeNO, sputum eosinophils and 8-isoprostane in early mild asthma (Study I)

FeNO and sputum eosinophils were increased in mild asthma

FeNO levels were increased in asthmatic children and adults compared to healthy children (p<0.001) and healthy adults (p=0.025). Only 10/23 asthmatic and 8/13 healthy children and 10/14 asthmatic and 9/14 healthy adults could produce suitable sputum specimens so the sputum biomarkers could not be analyzed from every participant. The percentages of sputum eosinophils did not change in asthmatic children compared to healthy children (p=0.52) but were highly significantly elevated in asthmatic adults compared to healthy adults (p<0.0001). Further, patients with COPD exacerbation (n=11) had a higher percentage of eosinophils than healthy controls (p=0.005). A good correlation was found between FeNO and eosinophils (R=0.65, p<0.0001) calculated between all groups.

Sputum 8-isoprostane was not elevated in mild early asthma

Sputum 8-isoprostane levels overlapped widely between the healthy and asthmatic subjects and there was extensive variation in the 8-isoprostane levels within the study groups. 8-Isoprostane was detectable in all healthy children and adults but the levels did not differ from those in asthmatic children (p=0.90) or adults (p=1.00) with recent asthma. No correlations were found between 8-isoprostane levels and FEV, FEV1/FVC or sputum eosinophils. Patients with COPD exacerbation, which were used as positive controls, had significantly elevated sputum 8-isoprostane levels when compared to healthy adults (Figure 2).
2. Effects of smoking cessation on oxidant markers in sputum and exhaled air (Studies II, III)

2.1 Study II

Oxidative markers were elevated in smokers at the baseline
The baseline values of the FeNo, sputum 8-isoprostane, nitrotyrosine and MMP-8 were significantly lower (p<0.001 for each marker) in non-smokers (n=33) than in smokers. However, the markers did not differ significantly between the asymptomatic smokers (n=25), smoking asthmatics (n=15) or smokers with chronic bronchitis/COPD (n=21).

Symptoms decreased in smokers who succeeded to quit smoking
Of the total of 61 subjects, only six smokers with bronchitis or COPD (one with previous GOLD Stage 0 and five with Stage I-II COPD), six subjects with asthma and three asymptomatic smokers succeeded in quitting smoking for 3 months. Symptoms assessed by the St Georges Respiratory Questionnaire (SGRQ) improved significantly in these subjects (Table 4). The final analyses were conducted on the quitters who also had produced good quality sputum specimens (n=15). Spirometry values (FVC, FEV1,
FEV1/FVC) did not change significantly after smoking cessation when compared to baseline in any of the groups.

**Table 4.** Symptoms score decreased significantly (a decrease of more than four points) in the quitters assessed by the St Georges Respiratory Questionnaire (SGRQ).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>1 mo</th>
<th>3 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms score</td>
<td>47</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>Activity score</td>
<td>39</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Impact score</td>
<td>19</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Total score</td>
<td>30</td>
<td>10</td>
<td>21</td>
</tr>
</tbody>
</table>

The data are shown as means. Symptoms score addresses the frequency of respiratory symptoms, activity score measures disturbances to daily physical activity and impact score covers a range of disturbances of psycho-social function. Total score summarises the impact of the airway disease on overall health status.

**Neutrophils increased in chronic bronchitis/COPD after smoking cessation**

In the group of chronic bronchitis/COPD, the percentage of sputum neutrophils increased significantly after 3 months of smoking cessation (p=0.046) but in the asymptomatic smokers and asthmatics, the cell counts remained similar.

**Only 8-isoprostane declined significantly after smoking cessation**

The results of the subjects who succeeded in quitting of smoking were combined into one analysis. Sputum 8-isoprostane declined significantly during the follow-up at 3 months (p=0.035), but levels still remained significantly higher than in non-smokers. The levels of FeNO, nitrotyrosine and MMP-8 did not change significantly during the 3 months after smoking cessation.

2.2 Study III

Of the total 61 subjects, 17 smokers stopped smoking for 3 months and nine managed for 6 months. Of these 17 individuals, six were asymptomatic smokers, four had mild asthma and seven were suffering from chronic bronchitis or COPD (one with previous GOLD Stage 0 and six with Stage I i.e. mild COPD). Four asymptomatic smokers, four
asthmatic individuals and one subject with COPD succeeded in quitting smoking for six
months. In the MMP analyses, these three groups were assessed as a single group.

**Inflammatory markers were increased in smokers at the baseline**
In the smokers, baseline induced sputum neutrophils, MMP-7, -8, -9 and TIMP-1 were
significantly higher compared to non-smokers (n=30) (p=0.021, p=0.014, p=0.001,
p=0.02, p=0.006, respectively).

**Neutrophils decreased to the levels of non-smokers after 6 months of smoking
cessation**
The sputum neutrophil numbers increased significantly at 3 months after smoking
cessation (p=0.035), but at 6 months the difference from non-smokers was no longer
statistically significant.

**Sputum MMP-9 remained elevated after 6 months of smoking cessation**
The individual levels of MMP-7, -8 and TIMP-1 at 3 months after cessation did not
change significantly from the corresponding baseline levels but after 6 months, the
levels decreased significantly near to the levels of non-smokers compared to the
baseline (p=0.032, p=0.001, p=0.04, respectively). In contrast, the individual levels of
MMP-9 increased significantly from the baseline at 3 months after cessation (p=0.009)
and did not differ significantly at 6 months after the cessation when compared to the
baseline levels in these same individuals (p=0.069), and the differences were even more
significant when compared to the non-smokers in a cross-sectional evaluation
(p=0.017) (Figure 3).
3. Differences in potential markers related to smoking and COPD in young and middle-aged/older subjects in plasma samples (Study IV)

Plasma SP-A increased with age and was elevated in smokers and patients with COPD

Studies on plasma SP-A levels were based on a recent proteomic study by Ohlmeier and coworkers (Ohlmeier et al. 2008). This particular study was focused on plasma SP-A, since the number of the sputum samples was not representative. Recently the levels of SP-A were found to be elevated in a small number of samples analyzed by the Western blot technique. In this particular study, plasma levels of SP-A by EIA increased with age in non-smokers and smokers (p < 0.0001 and p < 0.0001). Older smokers (n=64) had higher levels than young smokers (n=51) which might be related to their longer smoking history. In the young age group, there was no significant difference between non-smokers (n=36) and smokers. In the older age group, the plasma concentration of SP-A was higher in smokers when compared to non-smokers (n=40) (p < 0.0001). Importantly, plasma SP-A levels were also higher in COPD patients (n=44) compared to older smokers (p = 0.009), and non-smokers (p < 0.0001) (Figure 4). The linear regression analysis revealed that age (regression coefficient (B) = 6.01, standard error (SE) = 2.54, p = 0.019), cigarette smoking (B = 0.45, SE = 0.07, p
< 0.001) and COPD (B = 17.08, SE = 3.67, p < 0.001) had independent effects on the SP-A level.

**Figure 4.** The mean plasma levels of SP-A in young non-smokers (YNS), young smokers (YS), middle aged/elderly (old) non-smokers (ONS), middle aged/elderly (old) smokers (OS) and patients with stable stage I-III COPD. Plasma SP-A increased with age in non-smokers and smokers (p<0.0001 and p<0.0001). Plasma SP-A was higher in OS when compared to ONS (p<0.0001) and was also higher in COPD when compared to OS (p=0.009) and ONS (p<0.0001).

**Plasma SP-D did not change with age but was increased in older smokers and in COPD**

The mean levels of plasma SP-D were similar in young and old non-smokers and did not change with age. Furthermore, there was no difference between young non-smokers and smokers. In the older group, plasma SP-D levels were higher in smokers and patients with COPD when compared to non-smokers (p = 0.012 and p < 0.0001) but there was no significant difference between COPD and smokers. COPD patients had even higher levels of plasma SP-D (B = 64.72, SE = 19.53, p = 0.001) when adjusted for age in the linear multivariate regression analysis.

**Plasma MMP-9 did not change with age but was elevated in older smokers and in COPD**

Protease/antiprotease imbalance and especially MMP-9 has been widely associated with COPD and its pathogenesis (Beeh et al. 2003, Gadek and Pacht 1990, Ilumets et al. 2007). The mean plasma levels of MMP-9 were very similar in young and old non-
smokers and there was no significant difference between young non-smokers and smokers. Older smokers and COPD patients had significantly higher plasma MMP-9 levels when compared to old non-smokers (p < 0.0001 and p=0.033). Linear regression analysis revealed that both age (B = 0.44, SE = 0.19, p = 0.022) and cigarette smoking (B = 26.0, SE = 6.91, p < 0.0001) had independent effects on the plasma MMP-9 level.

**Plasma TIMP-1 declined with age and MMP-9/TIMP-1 increased in smokers and COPD with long-term smoking history**

The plasma level of TIMP-1 declined with age (p = 0.03). No significant difference was detected between young non-smokers and smokers. In the older group, the TIMP-1 plasma levels were higher in smokers and COPD patients than in non-smokers, but no significant difference was seen between smokers and the COPD subjects. The ratio of plasma MMP-9 to TIMP-1 did not change with age. In the young group, no difference was seen between non-smokers and smokers. In the older group, the plasma MMP-9/TIMP-1 ratio was higher in smokers than in non-smokers indicating a significant protease/antiprotease imbalance in favor of proteases. The linear regression analysis confirmed the independent effect of smoking on MMP-9 (B = 16.18, SE = 6.66, p = 0.018) and MMP-9/TIMP-1 (B = 4.01, SE = 0.15, p = 0.014).

**Especially plasma SP-A was related to age, smoking and lung function**

Clear positive linear correlations were observed between plasma SP-A levels and age and pack years and negative correlation between SP-A and lung function and some correlations were observed also with SP-D. The levels of SP-A correlated also with those of SP-D (r=0.36, p <0.0001). The plasma MMP-9 levels correlated only with age and pack years.

**Receiver Operating Characteristic (ROC) curve analysis is most accurate for SP-A**

ROC curve analysis was used to evaluate the sensitivity, specificity and diagnostic accuracy of plasma levels of SP-A, SP-D, MMP-9 and TIMP-1 in distinguishing patients with COPD from the control subjects. The areas under the ROC curve were as follows: for SP-A: 0.845 (95% confidence interval (CI), 0.787 to 0.902, p < 0.001); for SP-D: 0.734 (95% CI, 0.636 to 0.883, p < 0.001); for MMP-9: 0.551 (95% CI, 0.450 to
0.652, \( p = 0.320 \) and for TIMP-1: 0.664 (95% CI, 0.516 to 0.813, \( p = 0.051 \)). Based on this study, SP-A appeared to represent the most promising marker for COPD.

4. Proteomics revealed elevated PIGR levels in sputum, lung tissue and plasma in smokers and COPD patients (Study V)

Sputum samples from non-smokers, smokers and smokers with COPD revealed 15 altered proteins

Sputum samples were screened using 2D-DIGE method in order to find smoking- and/or-COPD-dependent changes. Significant changes (\( p \leq 0.05 \)) including mainly COPD-dependent alterations were detected and further investigated by mass spectrometry. “Healthy” smokers displayed no specific changes when compared to non-smokers but the signals for zinc-\( \alpha \)-2-glycoprotein, long palate lung and nasal epithelium carcinoma-associated protein and transthyretin were significantly elevated in both smokers and COPD whereas that of \( \alpha \)-1-antitrypsin was elevated in COPD indicative of its protective role against COPD (Eriksson et al. 1985). The polymeric immunoglobulin receptor (PIGR) was elevated in COPD and was selected for more detailed investigation since its role in COPD pathogenesis is poorly understood (Pilette et al. 2001, Polosukhin et al. 2011).

Cleaved PIGR form (secretery component, SC) was elevated in sputum samples of COPD patients

Mass spectrometry analyses of the PIGR spots indicated that sputum PIGR is present in its SC form based on the detected amino acids. As observed in 2-DE, sputum PIGR was detected by Western blot at 86 kDa with the level being elevated in COPD when compared to non-smokers (\( p = 0.01 \)) indicating the presence of full (83 kDa) or mature (81 kDa) PIGR whereas the expected size of the SC is 64 kDa. Since PIGR can exist also as a glycosylated form altering its migration in the gel, this was investigated by PNGase F treatment. The deglycosylation resulted in a shift from 86 kDa to 60 kDa. These findings verify the presence of glycosylated SC.
PIGR/SC is localized in specific lung cells and elevated in subjects with COPD compared to smokers and non-smokers

PIGR in lung tissue specimens was investigated by Western blot and immunohistochemistry. As in sputum, PIGR was detected by Western blot in tissue homogenates only in one band at 86 kDa, evidence for the presence of glycosylated SC. However, there were no significant differences in PIGR between the groups detected by Western blot of the lung tissue in contrast to sputum PIGR. Therefore PIGR was further investigated by immunohistochemistry and it was found to be extensively expressed in the epithelium and alveolar macrophages. Immunoreactivity of PIGR was further quantified by image analysis. Elevated PIGR levels were detected in the alveolar-interstitial area in smokers and even more in COPD when compared to non-smokers (all p <0.0001). A significant difference was also observed between “healthy smokers” and subjects with COPD (p<0.0001). PIGR levels were also elevated in normal looking bronchial epithelium in smokers and patients with COPD when compared to non-smokers (Figure 5).

Plasma PIGR elevation in smokers and smokers with COPD correlated with airway obstruction

Plasma samples from non-smokers, smokers, and smokers with COPD were investigated by ELISA to confirm the role of PIGR as a potential local or systemic inflammatory regulator. Smokers and smokers with COPD possessed significantly higher PIGR levels in plasma as compared to non-smokers (p<0.0001 and p<0.0001). Plasma PIGR levels correlated significantly with the extent of airway obstruction (correlation co-efficient r = 0.240, p = 0.006).

Sputum amylase and cystatin-S were decreased in COPD compared to the smokers and non-smokers

Proteomics revealed decreased levels of amylase and cystatin-S in COPD when compared to the smokers and non-smokers. However, there was no difference in plasma amylase levels between groups when evaluated using ELISA.
Figure 5. PIGR was widely expressed in the bronchial epithelium. Specific localization of PIGR (brown color) is indicated by arrows. A) Non-smoker B) Smoker C-D) COPD.
DISCUSSION

1. FeNO and sputum eosinophils were superior to 8-isoprostane in detecting early mild asthma

Fractional nitric oxide (FeNO) is one of the few markers used in the clinical assessment and which can help in the diagnosis of asthma (Smith et al. 2005). Elevated FeNO levels have been documented widely in adults (Kharitonov et al. 1994, Persson et al. 1994) and children (Byrnes et al. 1997, Lundberg et al. 1996, Nelson et al. 1997) with asthma, including mild and asymptomatic conditions (Alving et al. 1993, Kharitonov and Barnes 2001a) and the results of our study are in agreement with previous observations. FeNO is also sensitive to steroid treatment in asthma (Smith et al. 2005). Standardized measurements (ATS 1999) of FeNO correlate with eosinophilic airway inflammation and can predict the decline in asthma control (Kharitonov and Barnes 2001b, Liu and Thomas 2005). Our study also found a good correlation between FeNO and sputum eosinophils. Sputum eosinophils were elevated in asthma, confirming that eosinophilic inflammation is a key feature of asthma.

8-Isoprostane has been suggested to be a specific marker for lipid peroxidation (Janssen 2001) and to be sensitive for evaluating oxidative/nitrosative stress in the airways. Increased levels of 8-isoprostane have been previously measured in sputum samples of smokers and mild stable COPD (Kinnula et al. 2007) and moderate/severe asthma when compared to healthy controls (Wood et al. 2005), although levels have been highly variable. Our study was the first to measure 8-isoprostane in sputum specimens with FeNO in asthmatic children and adults. We found a wide overlap in the 8-isoprostane levels between the healthy and asthmatic subjects and also a wide variation within study groups. Wood and co-workers (Wood et al. 2005) reported elevated sputum 8-isoprostane levels in asthmatic adults but there was also overlapping between groups and the patients had moderate/severe asthma. Most of our subjects had mild asthma that might explain this difference, but it is also possible that commercial EIA method for 8-isoprostane may not be sensitive enough to assess mild oxidative stress. COPD patients, who were used as positive controls in 8-isoprostane analyses, had significantly elevated sputum 8-isoprostane levels when compared to asthmatic and healthy adults which is in agreement with the previous findings (Kinnula et al. 2007). This might reflect the
increased oxidative stress boosted by neutrophils and their activation, which is a typical feature in airway inflammation in COPD (Hogg et al. 2004, Peleman et al. 1999).

The power of this present investigation is reduced by two factors i.e. that patient material remained small and only some of the subjects could produce representative sputum specimens so the 8-isoprostane could only be analyzed from these subjects. This was also a cross-sectional study so longitudinal studies on the reproducibility of 8-isoprostane in stable asthma are needed.

Overall, it appears that while FeNO is sensitive in detecting oxidative stress even in mild asthma, sputum 8-isoprostane concentrations are highly variable and overlapping and do not detect mild asthma which might be due to low oxidant burden in the airways of asthma patients or due to the low sensitivity of the EIA method. In this particular study, an attempt was made to assess 8-isoprostane also in the EBC samples from mild asthmatics but most of the values were below the detection limit even though the samples had been concentrated. There was also a high variability both in and between the groups and no significant differences were seen between groups. However, the oxidant burden is high in the lungs of COPD patients and 8-isoprostane might be a better marker in this disease.

2. Oxidant and protease burden persists in the airways for several months after smoking cessation

Most of the previous smoking cessation studies have been cross-sectional and the real effects of smoking cessation on airway remodeling processes/tissue destruction have remained unclear. These present studies (II and III) revealed that oxidative stress can persist for months after the patient has stopped smoking. In study II, the neutrophil counts increased in COPD after three months of smoking cessation which is in agreement with previous results (Willemse et al. 2005). It seems that study III was the first prospective study to examine how smoking cessation influences the levels of sputum MMPs. It was found that sputum MMP levels generally decline slowly after smoking cessation. However, the levels of MMP-9 remained elevated in smokers at six months after giving up smoking for unknown reasons.
Smoking cessation is the most effective therapeutic intervention in smokers, especially in patients with COPD and asthma (Scanlon et al. 2000). Cessation success rates are generally low to moderate despite major efforts in developing efficient cessation programs and drugs to ease the withdrawal process (Aveyard et al. 2007, Gorecka et al. 2003, Lancaster and Stead 2005). In the present studies, 25% (Study II) and 15% (Study III) of the smokers succeeded in quitting smoking at least for the duration of the follow-ups. This remarkable number of dropouts was anticipated when one considers the well-known difficulties of quitting smoking. However, these success rates were rather good and close to the published results of programs involving COPD patients who had received active counseling (Stratelis et al. 2006).

Smoking cessation reduces COPD morbidity, hospital admissions (Godtfredsen et al. 2002) and COPD progression (Rabe et al. 2007). It is known that after smoking cessation, the decline in lung function suppresses slowly not until after 2-3 years (Burchfiel et al. 1995, Townsend et al. 1991). Smoking causes very similar airway pathology in subjects with COPD and asthma; the typical features are the recruitment of increased numbers of neutrophils into the airways and elevated oxidative stress (Chalmers et al. 2001). This was found in studies II and III. Smoking asthmatics suffer from greater symptom severity and increased exacerbation frequency (Siroux et al. 2000), accelerated decline in lung function (Ulrik and Lange 2001) and impaired response to corticosteroids (Chalmers et al. 2002, Chaudhuri et al. 2003, Pedersen et al. 1996). Smoking asthmatics are also a group which is often excluded from asthma studies, in order to avoid a major confounding factor i.e. the effects of cigarette smoke.

Cross-sectional studies have reported elevated inflammatory indices in terms of neutrophils, eosinophils and macrophages in ex-smokers when compared to never smokers (Bhowmik et al. 2000, Rutgers et al. 2000, Turato et al. 1995, Yamamoto et al. 1997), and a shift from CD4+ (T-helper) to CD8+ (T-suppressor) predominance in heavy smokers and COPD patients (Costabel et al. 1986, Gamble et al. 2007). Only a few prospective longitudinal studies have assessed long-term effects of smoking cessation (Chaudhuri et al. 2006, Willemse et al. 2005). There is published evidence that inflammation may persist in the airways after smoking cessation for at least for one or two years but it is unclear how long the inflammation actually continues, even if it is completely irreversible.
2.1 Study II

**Sputum 8-isoprostane declined significantly but the levels of FeNO, sputum MMP-8 and nitrotyrosine remained elevated after 3 months of smoking cessation**

In the group of bronchitis/COPD the percentage of sputum neutrophils increased significantly after 3 months of smoking cessation. Although the subject number remained low and these results have to be interpreted with caution, this in agreement with the study of Willemse et al (Willemse et al. 2005), which reported high neutrophil counts one year after smoking cessation in asymptomatic smokers and COPD patients. This would seem to suggest that neutrophilic inflammation remains long after subjects have stopped smoking.

Smoking alone increases levels of sputum 8-isoprostane when compared to non-smoking controls (Kinnula et al. 2007). In the present study, there was extensive variability in the baseline levels of 8-isoprostane, especially in the group of asthmatics. When the results from the quitters in the three groups were combined, the levels of sputum 8-isoprostane in the individual subgroups declined significantly after smoking cessation, but still remained higher than in non-smokers. Overall, these results suggest that there is a clear tendency but no significant or immediate decline in the oxidative stress within the first months after smoking cessation when evaluated by sputum 8-isoprostane.

Previous studies have shown that FeNO levels decline in smokers (Kharitonov and Barnes 2001a, Rytila et al. 2006) but in stable COPD, FeNO does not seem to differ from normal values (Balint et al. 2001, Corradi et al. 1999, Kharitonov and Barnes 2001a, Rutgers et al. 1999, Rytila et al. 2006). Since cigarette smoke decreases FeNO levels it could be speculated that FeNO would increase after smoking cessation. In this study, FeNO was highly variable in asthma, which might be caused by the severity of the disease or the extent of smoking but also the different doses of inhaled corticosteroids used during the study. FeNO values were also quite variable in the group of patients with bronchitis/COPD and did not change after smoking cessation. Since all the asthma patients were using inhaled corticosteroids but most of the COPD patients did not, it is difficult to compare these groups. However, the fact that FeNO levels did not change significantly suggests that oxidative/nitrosative stress continues.
after 3 months of smoking cessation. It is good to keep in mind also that FeNO 
regulation is complex and many of the reactions associated with its changes in smoking 
asthmatics and COPD are still partly unclear.

Nitrotyrosine has been proposed to play a major role in the pathogenesis of airway 
remodeling (Kharitonov and Barnes 2001a). There is evidence that nitrotyrosine 
positive sputum cells are elevated in healthy smokers when compared to never smokers 
(Rytila et al. 2006) with further increase in severe COPD (Ichinose et al. 2000). In the 
present study, there was no change in the number of nitrotyrosine positive sputum cells 
after smoking cessation at 3 months in the combined group which is in agreement with 
the results of other markers of oxidative/nitrosative stress. These results indicate that 
smoking cessation does not lead to any immediate changes in the oxidant burden in 
asthma or COPD.

Matrix metalloproteinases degrade the components of the extracellular matrix 
(Ohbayashi 2002) and are involved in the pulmonary remodeling processes in asthma 
and COPD (Kelly and Jarjour 2003, Parks and Shapiro 2001). MMPs including MMP- 
8, MMP-9 and MMP-12 have been linked to COPD (Elkington and Friedland 2006). 
Smoking and oxidative stress might enhance MMP activation i.e. it can be speculated 
that MMP levels might decrease after smoking cessation. In this present study sputum 
MMP-8 in the combined group showed some tendency to decline but extensive MMP-8 
variation was seen in individuals after 3 months of smoking cessation and the levels of 
MMP-8 remained much higher than in non-smokers during the follow-up. These 
findings are in agreement with the results of Ilumets et al. (Ilumets et al. 2007) where 
the levels of MMP-8 were higher in subjects with chronic bronchitis compared to 
asymptomatic smokers. These results suggest that there is a persistent imbalance in 
protease/antiprotease cascade lasting at least for months after smoking cessation.

The finding that symptoms were alleviated already one month after smoking cessation 
despite the persistent neutrophilic airway inflammation and oxidative stress is 
important. This suggests that the clinical improvement might not correlate with 
objective assessment of asthma/COPD. It is possible that in subjects who managed to 
quit smoking, the assessment of symptoms was influenced by a positive perception of 
smoking cessation. It also might be that these particular investigated markers may not
have real clinical importance in COPD since the complex mechanisms involved in COPD pathogenesis are still poorly understood.

2.2 Study III

**Sputum MMP-9 remained elevated after 6 months of smoking cessation while other markers declined to the levels of non-smokers**

In this study, at the baseline setting, smokers with or without mild airway disease had neutrophil predominance and elevated levels of several MMPs in induced sputum samples when compared to non-smokers. This is in agreement with the previous studies reporting elevation of the levels of MMP-8, -9 and TIMP-1 in smokers and patients with COPD (Elkington and Friedland 2006, Ilumets et al. 2007, Vernooy et al. 2004). There is also evidence that the disease itself, not only smoking, results in elevated levels of MMPs (Babusyte et al. 2007, Demedts et al. 2006, Deshmukh et al. 2008). Subgroups were combined which was justified for several reasons i.e. the subjects were smokers without airway disease or the disease was mild. The phenomenon that smoking asthmatics develop airway neutrophilia as is the case in smokers and subjects with COPD, was found also in this study. The MMP levels were overlapping in smokers as expected. In addition, the differentiation of smoking asthmatics from patients with COPD and symptomatic smokers from mild COPD is indeed a major challenge since there is overlapping in the cell profiles and the diseases (Chalmers et al. 2001). The levels of MMP-9 increased from the baseline during the 3-6 months after smoking cessation, suggesting that even if an individual stops smoking, the extracellular matrix breakdown can proceed for several months. Sputum neutrophil proportions increased at 3 months after smoking cessation but at 6 months, the difference from non-smokers was no longer significant. This could be interpreted that the MMP-9 elevation is not dependent on the numbers of neutrophils per se but might reflect increased MMP-9 release since neutrophils are an important source of MMP-9. There are somewhat controversial reports about whether elevated levels of MMP-9 are associated with increased MMP-9 activity. The studies of our laboratory have demonstrated that elevated levels of MMP-9 are associated with increased MMP-9 activity in smokers, patients with Stage 0 COPD as well as during COPD exacerbations (Ilumets et al. 2007 and 2008). However, in the study of Lowrey et al. (Lowrey et al. 2008) sputum MMP-9
levels but not MMP activity was elevated in smokers with COPD when compared to smokers without COPD. It is important to remember that the present results are only preliminary and further investigations are needed about the role and significance of MMP-9 after smoking cessation.

All of the other markers; MMP-7, -8 and TIMP-1 though exhibiting extensive variability, did return to the levels of non-smokers after 6 months of smoking cessation, suggesting that the repair of tissue damage may have been initiated. This is in agreement with the study of Willemse et al. (Willemse et al. 2005) where the values of the inflammatory markers like macrophages and IL-8 decreased significantly in asymptomatic smokers after one year of quitting smoking. This seems to be the first study showing MMP-7 elevation due to smoking. Macrophages are the main source of MMP-7 and their expression is also upregulated in pulmonary epithelial cells in the presence of chronic infection, suggesting that MMP-7 might contribute to pulmonary immunity (Dunsmore et al. 1998) and that chronic inflammation in the airways decreases after smoking cessation.

There are some important factors that have to be taken into consideration when evaluating these results. The most important limitation is the modest success of the smoking cessation in both studies (II and III). There were also differences in the success rates between groups; these may have been due to differences in smoking cessation programs, the use of smoking cessation aids and the intensity of counselling. Also, the quitters in the groups of asymptomatic smokers, smokers with asthma and smokers with chronic bronchitis/COPD were assessed as a single group, so the results cannot be generalized to any specific group. In study II, one limitation is the variability of the subjects and medications within the groups; subjects with symptoms of chronic bronchitis were included in COPD group and the use of inhaled steroids differed between groups. In addition, the levels of the oxidant markers varied within each group in both studies. Sapey et al. (Sapey et al. 2008) reported also extensive variability in sputum inflammatory/oxidant markers obtained from COPD patients. Unfortunately, these reduce the statistical power of our analyses.

The present studies suggest that there is no major immediate decline in the oxidant burden after smoking cessation in COPD or asthma. Since there was extensive
variability between individuals in the measured markers, it is possible that some of these markers may predict which patients might develop further lung damage in contrast to those patients in whom the disease processes may arrest. Although these results are important, further investigations with larger numbers of subjects with different phenotypes of asthma and COPD are needed.

3. Age affects to smoking related markers

It was decided to investigate plasma levels of SP-A, SP-D, MMP-9 and TIMP-1 in young and middle aged/elderly smokers and in patients with COPD. These potential markers were selected since previous studies have suggested that these markers may predict COPD, its development and/or progression (Beeh et al. 2003, Kobayashi et al. 2008, Ohlmeier et al. 2008, Sin et al. 2007). The main goal was to study whether smoking and ageing affect the levels and relationships between plasma SP-A, SP-D, MMP-9 and TIMP-1 since only a few studies have investigated the effect of age on these potential biomarkers.

It was found that SP-A appeared to be the most promising marker for COPD; its levels were elevated after long-term smoking and a further increase was seen in COPD when compared to chronic smokers. There was also a good correlation between age, pack-years and airway obstruction. Whether SP-A can be used as a marker for COPD or its development remains still unclear. No difference was seen in any of the investigated markers between young non-smokers and those smokers with relatively short smoking histories, normal spirometry results and no medications.

The main finding was that plasma SP-A levels were elevated in smokers and in COPD; these are in agreement with previous studies though there are several controversial findings as well. Kobayashi et al. reported elevated plasma/serum SP-A in Japanese smokers and patients with COPD and many studies have also reported elevated SP-A levels in circulating blood in smokers when compared to non-smokers (Behera et al. 2005, Kida et al. 1997, Mazur et al. 2011, Nomori et al. 1998, Robin et al. 2002). The finding that circulating levels of SP-A are elevated in smokers independently of the symptoms (Mazur et al. 2011) may indicate that damage has already occurred in the
lung or alternatively the elevation may be simply due to smoking itself. SP-A has also been shown to be elevated in the lung tissue of COPD patients (Ohlmeier et al. 2008). Some studies have presented opposite findings about circulating SP-A levels in smokers and/or COPD measured in serum (Mutti et al. 2006), lung tissue (Vlachaki et al. 2010) and BAL (Betsuyaku et al. 2004, Honda et al. 1996).

There are controversial results about the significance of plasma SP-D in COPD as well. One study found that serum SP-D is significantly elevated in COPD exacerbations when compared to smokers and non-smokers (Lomas et al. 2009). However, some studies have reported that the serum SP-D level does not change in smokers/COPD (Kobayashi et al. 2008) and several BAL studies have indicated that the SP-D level is reduced in smokers (Betsuyaku et al. 2004, Honda et al. 1996, More et al. 2010) and in COPD (Sims et al. 2008). In this study, there was no difference in plasma SP-D levels between the young and old smokers and non-smokers nor was any difference between old smokers and COPD. This in agreement with the study of Ohlmeier et al. (Ohlmeier et al. 2008) showing no significant change in lung tissue SP-D in COPD patients. In conclusion, there was slight variability in plasma SP-D levels and perhaps some elevation due to smoking but no significant changes were seen as in the case with SP-A.

The amount of MMP-9 was increased in long-term smokers which is in agreement with the previous studies (Ilumets et al. 2007, Lim et al. 2000). Apparently, this was the first study to investigate the levels of MMP-9 and TIMP-1 in different age groups of non-smokers and smokers. We found no difference in plasma MMP-9 or TIMP-1 between young non-smokers and smokers but old smokers and COPD patients had significantly higher MMP-9 and TIMP-1 levels when compared to old non-smokers. These results suggest that only long-term smoking (>10 years) increases both plasma levels of MMP-9 and the ratio of MMP-9/TIMP-1, perhaps reflecting the greater systemic inflammation. However, several other MMPs and TIMPs exist i.e. the MMP-9/TIMP-1 ratio alone does not really reflect the overall protease/antiprotease balance in the lung and it is still unclear if the changes in MMPs and in the protease/antiprotease balance predict COPD in the longitudinal perspective.
One important limitation of this study was the fact that this was a cross-sectional study, so the value of these biomarkers in COPD will require further prospective investigations. Another weakness was that in the young age group, most of the subjects were men. In the group of older subjects, the plasma SP-A level was significantly higher in women than in men but no difference was seen between genders in the levels of SP-D, MMP-9 or TIMP-1. Strengths of this study include the fact that none of the smokers or non-smokers had any other exposures and did not use any medications. COPD cases were newly diagnosed so they were not using any medications and were otherwise healthy.

4. Proteomics reveals new potential markers for smoking and COPD

In this study 15 altered proteins were identified in sputum samples of non-smokers, smokers and smokers with COPD. PIGR was selected for more detailed investigation since it is postulated to be a potential regulator of specific immune defence and inflammation (Wines and Hogarth 2006). The 2-D DIGE method was used which is a highly sensitive detection of cysteine-containing proteins and an internal standard for improved statistical analysis. The most important finding was that PIGR was elevated in smokers and in individuals with mild to moderate COPD and this elevation was not confined to lung cells but also detected in plasma suggesting that PIGR might play a role in the systemic inflammation associated with smoking and COPD.

At the time of this study, only a few sputum proteomic studies on COPD had been conducted and these studies identified only four proteins; zinc-α-2-glycoprotein, β-microseminoprotein (isoform PSP94), cystatin S and transthyretin (Casado et al. 2007, Nicholas et al. 2010). Nicholas and co-workers found no change in zinc-α-2-glycoprotein in smokers/COPD while in the present study, this protein was elevated. In that same study, transthyretin levels were elevated in COPD as was found also in this present study. In both of the earlier studies, PSP94 levels were elevated in COPD but in this study, this protein was detectable only in COPD as compared to non-smokers. The differences in these studies might be explained partly by the differences in the study groups and the variations in the sputum protein levels in smokers. In addition a different proteomic approach was adopted.
The elevation in PIGR was first observed in sputum using 2-D DIGE but importantly now the elevated levels of PIGR could be confirmed by Western blot and immunohistochemistry/image analysis in lung tissue and by ELISA in plasma samples from smokers and COPD patients. Furthermore, PIGR levels were greatly elevated in the normal looking alveolar region in COPD when compared to smokers and non-smokers. This suggests that the PIGR alteration might be associated with smoking and COPD, which is actually not surprising since PIGR has a role in mucosal immunity (Kaetzel 2005, Wines and Hogarth 2006).

PIGR exists in several variants with different functions: full-length PIGR is associated with the transcytosis of IgA to mucosal surfaces while the cleaved form (secretory component, SC) together with polymeric IgA or as free form scavenges for environmental and microbial antigens (Kaetzel 2005, Wines and Hogarth 2006). The IgA levels did not differ significantly in smokers or patients with COPD in sputum according to 2-D DIGE assay so this suggests that PIGR is present as the free form rather than in a complex with IgA. In addition the finding that SC was glycosylated, which is required for free SC to bind to host and pathogenic factors (Kaetzel 2005), suggests that PIGR was present in its free form since binding of IgA to a specific SC domain is not dependent in glycosylation (Hamburger et al. 2004). Glycosylated SC has been shown to inactivate interleukin-8 (IL-8) (Marshall et al. 2001 and 2004), which is one of the many inflammatory cytokines associated with COPD (Aaron et al. 2010, Eickmeier et al. 2010). It could be proposed that the elevation of SC in mild to moderate COPD might represent a mechanism to decrease inflammation in the airways. In agreement with the present results, elevated SC levels have been reported in the sputum samples from subjects with asthma and cystic fibrosis (Marshall et al. 2004) further support for a potential involvement of SC in lung defence and inflammation.

Polosukhin and co-workers reported that PIGR levels decreased in abnormal epithelium and airway remodeling areas in severe to very severe COPD (GOLD III-IV) (Polosukhin et al. 2011). In that study, no current smokers were included and total PIGR was investigated instead of SC. Pilette et al. also reported decreased SC expression in airway epithelium but again in very severe COPD (Pilette et al. 2001). These results and the present findings suggest that PIGR is initially elevated by smoking, inflammation and in early COPD but declines towards the end stage disease in the injured epithelium. SC/PIGR regulation is also complex and dependent on
several of the factors that have been associated with COPD (Kaetzel 2005, Pilette et al. 2001, Ratajczak et al. 2010) and this might explain part of these differences.

In summary, in the present study PIGR levels were elevated not only in sputum but also in lung tissue specimens and plasma samples of smokers and patients with mild to moderate COPD. A good correlation was also seen between the elevated plasma PIGR levels and airway obstruction in smokers and subjects with early stage COPD suggesting that PIGR might be involved in the systemic effects of this disease. Further longitudinal studies will be needed to clarify the role of PIGR in COPD with systemic inflammation.
CONCLUSIONS

Different potential markers that reflect oxidative/nitrosative stress in the lung were investigated in sputum, plasma and tissue samples in order to identify the most specific markers for early detection and monitoring of COPD and asthma and to permit the differentiation of the disease from the condition of the “healthy smoker”. The effects of smoking cessation on oxidative/nitrosative markers were also assessed.

FeNO levels were significantly elevated in newly diagnosed children and adults with mild asthma compared to healthy controls. Sputum eosinophil numbers were also significantly elevated in asthmatic adults and in asthmatic children there was a non-significant trend towards eosinophil increase, when compared to healthy controls. Patients with COPD exacerbation had higher eosinophil levels than healthy controls. The levels of FeNO and eosinophils correlated with each other, confirming the key roles of FeNO and eosinophils in asthma.

Sputum 8-isoprostane levels were very variable and there was overlapping in the groups of patients with recent mild asthma and healthy subjects, while in patients with COPD exacerbation, the sputum 8-isoprostane levels were significantly elevated when compared to healthy subjects. These results suggest that sputum 8-isoprostane may not be sensitive in reflecting oxidant burden in mild asthma, but in COPD, where the oxidant burden is high, 8-isoprostane may be useful.

Smokers had increased levels of all investigated markers in the smoking cessation studies (II and III); sputum 8-isoprostane, nitrotyrosine, neutrophil counts, MMP-7, -8, -9, TIMP-1 and FeNO when compared to non-smokers. After 3 months of smoking cessation, sputum neutrophils increased in patients with chronic bronchitis/COPD (Study II) and in the combined group of quitters (Study III) but at 6 months, the difference from non-smokers was no longer significant. The sputum 8-isoprostane level decreased significantly after 3 months but FeNO, nitrotyrosine and MMP-8 did not change during the 3 months of follow-up. However, the levels of MMP-7, -8 and TIMP-1 decreased after 6 months of smoking cessation although sputum MMP-9 remained elevated after 6 months of quitting smoking, and this may contribute to the lung damage typically encountered in COPD.
Plasma level of SP-A appeared to represent the most promising marker for COPD among those markers that had been included in this study, since it was elevated in COPD and long-term smokers and correlated with lung function and pack-years. Long-term smoking increased also the plasma levels of SP-D, MMP-9, TIMP-1 and the ratio of MMP-9/TIMP-1 but there were no correlations with the extent of airway obstruction, in contrast to the situation with SP-A. According to ROC curve analysis, plasma SP-A was the most sensitive and specific marker for COPD.

Proteomic screening of sputum and lung tissue samples revealed PIGR elevation in smokers and patients with early stage COPD. The altered PIGR was characterized as the glycosylated secretory component pointing to a role of PIGR/SC in the regulation of the inflammation present in COPD pathogenesis. PIGR was elevated also in the plasma of smokers and patients with COPD and the increase correlated with the extent of airway obstruction, indicating that this protein may be involved in the systemic effects associated with COPD.
FUTURE PERSPECTIVES

To date, there are no validated and reliable markers for early COPD diagnosis. Of various markers, FeNO has been the most widely used and shown to have clinical significance in asthma, e.g. in differential diagnosis and monitoring. Although it is increasingly recognized that COPD has more than one phenotype (for example airway or emphysema predominant disease), all the patients are treated similarly since these different phenotypes have not generally been evaluated separately in any clinical trial so far and there is no specific biomarker which can differentiate between these phenotypes. Combining the existent knowledge of oxidant markers in sputum specimens and expanding the methodology to new technologies such as proteomics will be needed to clarify disease mechanisms and develop novel therapies as well as being able to predict the prognosis with accuracy.
ACKNOWLEDGEMENTS

The present study was carried out at the Department of Medicine, Pulmonary Division, Helsinki University Central Hospital and University of Helsinki. I have been privileged to work with many skilled people and I wish to express my gratitude to all of those who helped me in this study.

This study was financially supported by the Ida Montin Foundation, Pulmonary Association Heli, Finnish Anti-tuberculosis Association Foundation, and funding of the Helsinki University Hospital (EVO).

I owe my deepest gratitude to my supervisor, Professor Vuokko Kinnula MD, PhD, who introduced me to the world of clinical research and guided and encouraged me tirelessly throughout this project. I wish to thank you for all the excellent professional help, endless support and understanding, for the unforgettable congresses and for providing me the opportunity to undertake both clinical work and research. You always had confidence in me even at times when I myself had none and felt uncertain in succeeding in this project. I am so grateful that I had the luck and privilege to have you as my supervisor.

I am deeply grateful also to my other supervisor, Docent Witold Mazur, MD, PhD for always finding time to help me. His knowledge, constant encouragement, experience and enthusiastic attitude towards science have been invaluable for me and for the completion of this study.

I express my gratitude to Docent Paula Rytilä, MD, PhD for being my supervisor at the beginning of my project. I wish to thank her for her excellent help and guidance, her ever optimistic and encouraging attitude and for sharing her expertise with me.

My warmest appreciation goes to Docent Leo Pekka Malmberg MD, PhD and Docent Anne Pietinalho MD, PhD, the official reviewers of this thesis, for their accurate and constructive criticism and for providing valuable and insightful comments and suggestions that improved the thesis.
I owe sincere gratitude to Docent Tuula Vasankari, MD, PhD, for accepting the role of opponent at the Dissertation.

Even McDonald is thanked for excellent linguistic revision of the final version of this thesis.

I wish to express my gratitude to all my co-authors and other collaborators of the published manuscripts: Yasushi Obase MD, PhD, Docent Mika Mäkelä MD, PhD, Professor Tari Haahtela MD, PhD, Docent Anna Pelkonen MD, PhD, Professor Ratko Djukanovic MD, PhD, Harri Stark MD, PhD, Helen Illmets MD, PhD, Tuula Toljamo MD, Pentti Nieminen PhD, Associate Professor Nobuhisa Ishikawa MD, PhD, Associate Professor Hideo Kobayashi MD, PhD, Steffen Ohlmeier PhD, Anna Linja-Aho, Mikko Rönty MD, PhD, Ulrich Bergmann PhD, Katri Vuopala MD, PhD, Associate Professor Irfan Rahman BSc, MSc, PhD, Kaisa Salmenkivi MD, PhD and Docent Marjukka Myllärniemi MD, PhD.

My special thanks go to Tiina Marjomaa for always helping and assisting me in the laboratory despite her busy schedule. Your expertise as a technician has been extremely valuable, and your organizational ability is truly amazing; you always had ten things going on at the same time with no problem! I also want to thank Kirsi Vuorinen PhD, Outi Leppäranta, Minna Vuolanto, Sari Nummijoki, Jing Gao MD and Eva Sutinen for their friendship, willingness to help and for creating a nice working atmosphere.

I owe profound gratitude to all of the participating subjects of this study.

I am extremely grateful to my parents Jarmo and Leena. You have always been there for me and helped me in every possible way and your never-ending support has allowed me to follow my dreams. I am happy for inheriting my love to sports from you; I believe that it is indeed the thing that has kept me insane. Special thanks go to my dad, my own computer consult, for always helping me with my frequent problems with my Mac (which was driving me mad many times) and for helping me with numerous figures. You really saved my day many times and I can’t thank you enough for that.
I also wish to thank my friends for giving me something else to think and for understanding especially during this last busy year.

Finally, my love, Erkki Ebeling, I thank you for always listening to me, taking care of me and for making me smile every day. I wish to thank you also for being patient with my mood swings and long evenings in the “research chamber”. I truly appreciate you for understanding me during these crazy years. Your endless love and support gave me strength to complete the thesis.

Helsinki, July 2012

Noora Louhelainen
REFERENCES


Bleecker ER (2004) Similarities and differences in asthma and COPD. The Dutch hypothesis. Chest 126: 93S-95S; discussion 159S-161S


Elliot JG, Carrol NG, James AL, Robinson PJ (2003) Airway alveolar attachment points and exposure to cigarette smoke in utero. Respir Crit Care Med 167: 45-49


100


Hamid Q, Song Y, Kotsimbos TC, Minshall E, Bai TR, Hegele RG, Hogg JC (1997) Inflammation of small airways in asthma. *J Allergy Clin Immunol* **100**: 44-51


101


Kaper J, Wagena EJ, Willemsen MC, van Schayck CP (2005) Reimbursement for smoking cessation treatment may double the abstinence rate: results of a randomized trial. *Addiction* **100**: 1012-1020


Kharitonov SA, Barnes PJ (2001b) Does exhaled nitric oxide reflect asthma control? Yes, it does! *Am J Respir Crit Care Med* **164**: 727-728


Kharitonov SA, Chung KF, Evans D, O'Connor BJ, Barnes PJ (1996b) Increased exhaled nitric oxide in asthma is mainly derived from the lower respiratory tract. *Am J Respir Crit Care Med* **153**: 1773-1780


104


OEHHHA (Office of Environmental Health Hazard Assessment) California Environmental Protection Agency (2005) Health effects assessment for environmental tobacco smoke. www.oehha.ca.org


Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE (2002) Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. Thorax 57: 590-595


