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# Early chronic airway disease in young and middle-aged smokers

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ACADEMIC DISSERTATION

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**ABSTRACT**

Chronic obstructive pulmonary disease (COPD) is an under-diagnosed and life-threatening progressive disease permanently limiting normal breathing and evoking a severe disability. Common early symptoms of COPD include excessive sputum production and chronic cough. The primary cause of this disease in the western world is smoking. COPD diagnosis is confirmed by spirometry, which does not totally reveal reversible airway obstruction. Spirometry has been widely used in the assessment of disease severity, but it does not reflect either the activity or the progression of COPD. There is a need for more sensitive and specific tests to identify markers for early disease and disease progression.

This study, conducted mainly in the Northern Finland, aimed 1) to assess smoking habits and smoking cessation in healthy middle-aged and young adults, 2) to evaluate the prevalence of COPD and role of spirometry and prolonged respiratory symptoms in the diagnosis of COPD of smoking middle-aged adults, 3) to study the association of symptoms of chronic bronchitis with smoking habits in young adult males, and 4) to seek and identify new potential biomarkers related to early COPD using non-hypothesis driven unbiased proteomics.

A two-year prospective study included two visits in the Lapland Central Hospital to evaluate symptoms in "healthy" cigarette smokers, to screen new COPD cases using both Global Initiative for Obstructive Lung Diseases (GOLD) and national diagnostic criteria of COPD and to assess the success of smoking cessation using motivational counselling and pharmacological therapies. In addition, a quantitative cross-sectional questionnaire survey was conducted in 1163 male conscripts during their military service in Northern Finland. The association between cigarette smoking and prolonged respiratory symptoms, e.g. chronic cough and sputum production, were evaluated in this group of young smokers. Also their smoking habits, nicotine dependence and quit attempts were studied in detail. A lung tissue proteomic approach (two dimensional gel electrophoresis and mass spectrometry) was used to identify new COPD biomarkers.

Spirometry revealed COPD by GOLD criteria in 11.0% and by Finnish national criteria in 15.3% of originally asymptomatic chronic smokers. Further, chronic cough or sputum production was detected in 62.0% of the participants. After two years, 23.3% of adults had

succeeded in quitting smoking. In young adults, 46.5% were daily smokers, 27.4% were occasional smokers and 2.4% former smokers. The prevalence of self-reported chronic cough and sputum production was high both in daily smokers (40.7%) and occasional smokers (26.9%) as compared to non-smokers (12.0%). The majority (60.2%) of the daily young smokers had made quit attempts and 46.9% of them had used nicotine replacement therapy (NRT). Nearly all daily smokers (93.4%) felt themselves to be nicotine addicted to some extent.

Based on the lung tissue proteomics, surfactant protein A (SP-A) was one of the most highly elevated spots in the COPD lung compared to the control lung. In additional studies, SP-A levels were found to be elevated in the alveolar and bronchiolar cells and sputum in COPD compared to the controls. The plasma SP-A concentration was higher both in chronic smokers and in COPD cases as compared to the non-smokers, and declined significantly during 2-year follow-up in those who succeeded to quit smoking compared to those who continued to smoke.

To conclude there is remarkable burden of chronic bronchitis and COPD in the groups of chronic smokers who considered themselves as healthy. Motivational counselling seems to be successful, since over 23% of the adult smokers succeeded in stopping smoking. SP-A is a potential new biomarker for COPD although it needs further evaluation.

**TIIVISTELMÄ**

Keuhkoahtaumatauti (chronic obstructive pulmonary disease, COPD) on tavallisimpia kansantautejamme. Se on alidiagnosoitu, etenevä koko elimistöön vaikuttava pitkäaikaissairaus, joka voi haitata pysyvästi hengitystä ja joissakin tapauksissa johtaa pysyvään työkyvyttömyyteen. Länsimaissa tupakointi on tärkein sairauden riskitekijä. Vaikka huomattava osa keuhkoahtaumatautia potevista on täysin oireettomia, niin tupakoijien pitkittynyt yskä ja sitkeä limaneritys saattavat viitata joko krooniseen keuhkoputkitulehdukseen tai keuhkoahtaumatautiin. Keuhkoahtaumatauti varmennetaan spirometria -tutkimuksella, jossa todetaan avaavalla lääkkeellä pääosin laukeamaton ahtauttava uloshengityksen tuuletusvaje. Tätä spirometrisesti mittavaa uloshengityksen sekuntikapasiteettia (FEV1) käytetään yleisesti sairauden vaikeusasteiden kriteerien luokittelussa, vaikkei spirometria mittaa sairauden aktiivisuuden astetta eikä mahdollista sairauden monimuotoista etenemistä elimistössä. Nykyään keuhkoahtaumatautia selvittelevän tutkimuksen keskeisimpänä tavoitteena on löytää sensitiivisiä ja spesifejä testejä, joilla tunnistetaan sairauden varhaisvaiheita ja joita käytetään taudin etenemisen tunnistamiseen.

Tämän pääosin Pohjois-Suomessa tehdyn tutkimuksen kohdejoukkona olivat 30 - 77 vuoden ikäiset aikuiset ja 18 - 26 vuoden ikäiset varusmiehet. Tutkimuksen keskeisinä tavoitteina oli selvittää 1) keuhkoahtaumataudin esiintyvyyttä päivittäin tupakoivien aikuisten keskuudessa, 2) kroonisen keuhkoputkitulehduksen oireiden esiintyvyyttä tupakanpolttajien keskuudessa, 3) tutkittavien onnistumista tupakoinnin lopettamisessa ja 4) löytöihin perustuvan proteomiikan keinoin mahdollisia keuhkoahtaumatautiin liittyviä biologisia merkkiaineita.

Tutkimuksessa käytettiin keuhkoahtaumataudin toteamisessa sekä kansainvälisiä GOLD -kriteereitä että suomalaisia viitearvoja. Tullessaan tutkimukseen osallistujat täyttivät oirekyselylomakkeen. Samalla käynnillä he saivat halutessaan vieroitushoitajan henkilökohtaista kannustavaa tukea ja ohjausta mahdollisten vieroituslääkkeiden valintaan. Seurantakäynnillä kahden vuoden kuluttua samat mittaukset toistettiin ja lisäksi tehtiin spirometria -tutkimus. Tähän tutkimukseen osallistuneille 1163 varusmiehille tehtiin palvelusaikana kyselytutkimus. Kyselyn avulla kartoitettiin osallistujien tupakointitapoja, tupakoinnin lopettamista, nikotiiniriippuvuutta sekä pitkittyneen yskän ja liman erityksen esiintymistä. Kudosnäytteistä etsittiin proteiinieroja sekä kaksisuuntaisiin

elektroforeesigeeleihin perustuvalla proteomiikalla (two-dimensional gel electrophoresis, 2-DE) että massaspektrometriseen (MS) menetelmään perustuen, jolloin tavoitteena oli havaita keuhkoahtaumatautiin liittyviä biologisia merkkiaineita.

Spirometrialla varmennettiin 11,0 %:lla tutkittavista keuhkoahtaumatauti käyttäen kansainvälisiä kriteereitä. Suomalaisten kriteerien mukaan keuhkoahtaumatautia todettiin vastaavasti 15,3 %:lla tutkittavista. Tämän lisäksi 62 %:lla oli oirekyselyn mukaan pitkittynyttä yskää ja/tai limaneritystä. Seurantakäynnillä alun perin oireettomista keski-ikäisistä tutkittavista 23,3 % ilmoitti lopettaneensa tupakoinnin. Kaiken kaikkiaan 46,5 % varusmiehistä oli päivittäistupakoijia, 27,4 % satunnaistupakoijia ja 2,4 % tupakointinsa jo aiemmin lopettaneita. Pitkittynyttä yskää ja limaneritystä esiintyi eniten päivittäistupakoijilla (40,7 %) ja satunnais-tupakoijillakin selvästi useammin kuin tupakoimattomilla (26,9 % vrt. 12 %). Huomattava osa päivittäin tupakoivista varusmiehistä oli tehnyt lopetusyrityksiä (60,2 %) ja 46,9 % heistä ilmoitti käyttäneensä nikotiinikorvaustuotteita lopetusyrityksissään. Päivittäin tupakoivista varusmiehistä 71,4 % oli Fagerströmin kahden kysymyksen nikotiiniriippuvuustestin mukaan kohtalaisesti tai vahvasti nikotiiniriippuvaisia.

Keuhkokudoksen proteomiikkaan perustuvien tutkimusten mukaan surfaktantti proteiini A (SP-A) oli merkittävä mahdollinen keuhkoahtaumatautiin liittyvä biomerkkiaine, koska sen pitoisuus oli selvästi korkeampi verrattuna kontrollina toimineen keuhkokudoksen vastaavaan mittausarvoon. Kohonneita SP-A pitoisuuksia todettiin keuhkoahtaumatautia potevien ysköksissä verrattuna vastaaviin kontrollinäytteiden pitoisuuksiin. Pitkään tupakoineiden tai keuhkoahtaumatautia potevien tutkittavien plasman SP-A pitoisuus oli korkeampi kuin vastaava pitoisuus tupakoimattomilla. Plasman SP-A pitoisuus selvästi laski tupakointinsa kahden vuoden seuranta-aikana lopettaneilla verrattuna tupakointiaan jatkavien vastaaviin arvoihin.

Tutkimustulosten perusteella krooniseen keuhkoputkitulehdukseen viittaavat oireet ja keuhkoahtaumatauti ovat erittäin yleisiä itsensä oireettomaksi tuntevillakin pitkään tupakoineilla henkilöillä. Henkilökohtainen hoitajan ohjauskerta motivoivalla haastattelu-tekniikalla näyttäisi hyödylliseltä, sillä 23 % pitkään tupakoineista tutkittavista onnistui lopettamaan tupakointinsa. SP-A on mahdollinen keuhkoahtaumataudin biologinen merkkiaine, joskin lisätutkimuksia tarvitaan.

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**LIST OF ORIGINAL PUBLICATIONS**

This thesis is based on the following original articles:

I. Toljamo T, Kaukonen M, Nieminen P, Kinnula V.L. Early detection of COPD combined with individualized counselling for smoking cessation: A two-year prospective study. Scandinavian Journal of Primary Health Care 2010;28:41-46

II. Hamari A, Toljamo T, Nieminen P, Kinnula V.L. High frequency of chronic cough and sputum production with lowered exercise capacity in young smokers. Annals of Medicine 2010; 42(7):512- 520

III. Toljamo T, Hamari A, Nieminen P, Kinnula V.L. Young male daily smokers are nicotine dependent and experience several unsuccessful quit attempts. Scandinavian Journal of Primary Health Care 2012;30(3):183-188

IV. Ohlmeier S, Vuolanto M, Toljamo T, Vuopala K, Salmenkivi K, Myllärniemi M , Kinnula V.L. Proteomics of human lung tissue identifies surfactant protein A as a marker of chronic obstructive pulmonary disease. Journal of Proteome Research 2008;7(12):5125- 5132.

V. Mazur W, Toljamo T, Ohlmeier S, Vuopala K, Nieminen P, Kobayashi H, Kinnula V.L. Elevation of surfactant protein A in plasma and sputum in cigarette smokers. European Respiratory Journal 2011;38(2):277-284

Papers II and III will be included in the PhD thesis by Anna Hamari, University of Oulu.
Mazur and Toljamo had equal contributions in paper V.

**ABBREVIATIONS**

| | |
|---|---|
| AATD | Alfa1-antitrypsin deficiency |
| AAT | alpha- 1-antitrypsin |
| ANOVA | analysis of variance |
| ATS | American Thoracic Society |
| BAL,BALF | bronchoalveolar lavage fluid |
| BMI | body mass index |
| COPD | chronic obstructive pulmonary disease |
| CAT | COPD Assessment Test |
| CI | confidence interval |
| cm | centimetre(s) |
| CRP | C-reactive protein |
| sCRP | sensitive C-reactive protein |
| CT | computer tomography |
| 2-DE | two- dimensional gel electrophoresis |
| DSM–IV | Diagnostic and Statistical Manual of Mental Disorders |
| EBC | exhaled breath condensate |
| ERS | European Respiratory Society |
| ETS | environmental tobacco smoke |
| $FEV_1$ | forced expiratory volume in one second |
| FTND | Fagerström test for nicotine dependence |
| FVC | forced vital capacity |
| FEV% | $FEV_1$/ FVC x 100 (%) |
| GOLD | Global Initiative for Chronic Obstructive Lung Disease |
| GOLD criteria | diagnostic spirometry criteria for COPD diagnosis and classification |
| GOLD-U | GOLD unclassified |
| GWAS | genome-wide association study |
| HSI | Heaviness of Smoking Index |
| IEF | isoelectric focusing |
| IH | immunohistochemistry |
| IPF | idiopathic pulmonary fibrosis |

| | |
|---|---|
| IS | induced sputum |
| kg | kilogram(s) |
| m | metre(s) |
| MEF50 | maximal expiratory flow at 50% of FVC |
| MI | motivational interviewing technique |
| mMRC | modified Medical Research Council breathelessness scale |
| MS | mass spectrometry |
| ND | nicotine dependency |
| NO | nitric oxide |
| NRT | nicotine replacement therapy |
| PIGR | polymeric immunoglobulin receptor |
| PY | smoking in pack-years |
| RAGE | receptor for advanced glycation end-products |
| RNA | ribonuclein acid |
| SD | standard deviation |
| SGRQ | St George´s Respiratory Questionnaire |
| SNP | single-nucleotide polymorphism |
| SP-A | surfactant protein A |
| SP-A1 | surfactant protein A1 |
| SP-A2 | surfactant protein A2 |
| SP-B | surfactant protein B |
| SP-C | surfactant proteinC |
| SP-D | surfactant proteinD |
| spirometry | lung function test measuring expiratory flow volumes |
| SPSS | Statistical Package for the Social Sciences |
| TTF-1 | thyroid transcription factor 1 |
| WB | Western blotting |
| WHO | World Health Organization |

# 1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) has a permanent impact on normal breathing. It is a life-threatening lung disease characterized by mainly an irreversible airflow limitation and the destruction of the lung parenchyma. The primary cause of COPD is cigarette smoking; it is the main risk factor leading to systemic inflammatory consequences and the co-morbidities associated with this disease. COPD is defined by presence of a partly irreversible airway limitation, but it is a complex disease with many heterogenous pulmonary and extra pulmonary components that have not yet been wholly incorporated into the disease diagnosis. During recent years, the definition of COPD has been directed more and more to the importance of various sub-phenotypes and COPD heterogeneity (COPDGene 2012). Exacerbations and co-morbidities contribute to the overall severity and mortality of the disease in individual patients suffering from COPD (Pauwels et al. 2001, Mannino & Buist 2007, Rabe et al. 2007b)

In 2002, COPD was the fifth leading cause of death in the world, globally its prevalence and mortality has been estimated to increase so that it will become the third leading cause of death by the year 2020 (Ezzati & Lopez 2003, Mathers & Loncar 2006). The prevalence of COPD in population-based studies is estimated to be between 6 and 10% (Mannino et al. 2000, Pena et al. 2000). The prevalence of COPD in Finland has been assessed to range from 4.3 to 4.7% in males and from 1.4 to 1.5% in females (Vasankari et al. 2010). Importantly COPD is not increasing in Finland any longer, and the costs related to COPD are clearly less than predicted some years ago (Kinnula et al. 2011).

COPD is an under-diagnosed and progressive disease that begins many years before the diagnosis (Rabe et al. 2007a). Spirometry is the cornerstone of COPD diagnosis (Zielinski & Bednarek 2001). It is a safe, reliable, non-invasive, simple and non-expensive tool for detecting airway limitations and it is widely used in the management of obstructive diseases (Soriano et al. 2009, Celli & MacNee 2004). COPD is, however, a complex disease containing variable airway and parenchymal (emphysema) manifestations, which may largely differ not only at the cellular and molecular levels but also in different areas of the lung (Agusti et al. 2011, Miravitlles et al. 2012).

COPD will develop in 20-30% of all smokers, which evidently is indicating the extensive variability in disease susceptibility (Castaldi et al. 2010, Castaldi et al. 2011). Early lung growth, race, gender and even nutritional deficiency in childhood may explain some of the individual susceptibility to COPD (Stern et al. 2007). In Finland, the majority of adult smokers have started to smoke in their early adolescence. Thus, 23 - 28% of all young people at their twenties smoke daily (Rainio et al. 2009, Raisamo et al. 2011), although the trend of experimenting with tobacco products in adolescents and young adults has been on the decline during recent years. Today, 23% of adult men and 16% of adult women smoke daily in Finland (Helakorpi et al. 2011).

The most common symptoms of COPD are breathlessness, or a 'need for air', dyspnoea (difficult or labored breathing), excessive sputum production, and chronic cough. Persistent smokers may consider themselves as symptom-free and healthy, though they may have moderate or even severe COPD (Bednarek et al. 2008). In addition, young adult smokers generally do not complain of symptoms related to smoking. Nonetheless, the previous studies have shown that the presence of prolonged respiratory symptoms is associated with increased morbidity to COPD, even in subjects with normal lung function (Lindberg el al. 2005). On the other hand, young smokers may suffer from symptoms related to smoking even in their twenties, and want to quit (Urrutia et al. 2005). However, spirometry which is generally used for the diagnosis of COPD is not very sensitive; it is not specific and it does not reflect the disease activity or progression. Several biomarkers for COPD or its development have been proposed (Comandini et al. 2009) but so far none of them have been validated clinically or implemented in clinic for COPD diagnosis or evaluation.

This doctoral thesis assessed smoking habits and success in smoking cessation in middle-aged adults and in young adult males. The research project also evaluated the prevalence of early stage COPD and effects of early detection of airway limitation in the diagnosis of COPD in healthy symptom-free heavy smokers. Besides these, the association of prolonged respiratory symptoms with smoking habits in adult smokers was explored. In this work, also a lung tissue proteomics approach is employed to identify new possible biochemical markers related to COPD.

# 2. REVIEW OF THE LITERATURE

## 2.1 Definitions of COPD

COPD affects mainly the lungs, causing an airway limitation that is not fully reversible and parenchymal lung damage leading to a permanent impairment of normal breathing. The incidence of COPD is strongly associated with which disease definition being used. Chronic bronchitis simply means chronic cough and sputum production on most days of the month for at least three months in two consecutive years (British Medical Research Council 1960, 1965). Emphysema is defined histologically as an abnormal permanent enlargement of the air spaces distal to terminal bronchioles, accompanied by destruction of their walls, loss of elastic recoil and changes in gas diffusion (Heard et al. 1979). The diagnosis of emphysema has been partly renewed by including into the classification the results of CT-imaging (Thurlbeck & Muller 1994, COPDGene 2012).

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) strategy document COPD is defined as a common preventable and treatable disease; it is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles and gases (GOLD 2011). This GOLD definition is consistent with standards of the American Thoracic Society (ATS) and the European Respiratory Society (ERS). Spirometry results are essential components of COPD diagnosis. When spirometry is unavailable, clinical symptoms and signs, such as abnormal shortness of breath and increased forced expiratory time, can help in the diagnosis, although many patients with even severe COPD consider themselves to be asymptomatic. Smokers who have prolonged respiratory symptoms without any airway limitation often seek medical help only when the disease has already proceeded to its more severe stages (de Marco et al. 2007). There have been some efforts to develop COPD diagnostic questionnaire as a diagnostic tool to identify patients at increased risk of airflow limitation, but the results are disappointing so far (Kotz et al. 2008a).

## 2.2 Risk factors of COPD

Cigarette smoking is the main risk factor of COPD. Cigarette smoke contains over 4000 chemical compounds, and large amounts of those are highly reactive free radicals and oxidants (Church & Pryor 1985). Environmental tobacco smoke (ETS) contains chemically similar genotoxic and carcinogenic compounds e.g. alkenes, nitrosamines, aromatic and heterocyclic hydrocarbons and amines in mainstream and in sidestream smoke (Lofroth 1989, Bermudez et al. 1994). It has been estimated that COPD can develop in an individual with an over 20 pack-year smoking history. However, it is not only smoking exposure but also individual structural host factors and genetic background that evidently play an important role in triggering the disease.

Alpha-1-antitrypsin deficiency (AATD) is the only known genetic risk factor for COPD, especially in smokers (Ganrot et al. 1967, Ranes & Stoller 2005). The complex variability of the environment-gene interaction in susceptible smokers may lead to the development of COPD. Even decades ago a familial aggregation of lung function deterioration was observed in smokers as compared to non-smokers (Cohen et al. 1977, Khoury et al. 1986). Other common genetic variants predisposing smokers to COPD have been sought in genome-wide association studies (GWAS) and two single-nucleotide polymorphisms (SNPs) at the alpha-nicotinic acetylcholine receptor (CHRNA 3/5) locus may be involved in COPD (Pillai et al. 2009, Thorgeirsson et al. 2010). The interpretation of these findings in disease development is complicated by variable environmental factors e.g. changes in the smoking behavior of study populations.

Epigenetics refers to the study of heritable changes in gene expression caused by chemical modifications in a chromosome which alter gene transcription without changing the underlying sequence and thus having effects on whole organism phenotypes (Martino & Prescott 2011). Tobacco smoke is known to be able to induce epigenetic changes in gene expression and alter the COPD disease risk (Sakao & Tatsumi 2011).

Although smoking is the greatest risk factor for COPD in the western world, there are additional risk factors that can lead to the airway limitation, clinically significant small airway disease or COPD in nonsmokers. These risk factors include noxious particles, severe pollution, and inhalation of outdoor or indoor toxins (Isoaho et al. 1994, Pauwels et al. 2001)

such as biomass fumes from cooking and heating, ETS (Yin et al. 2007), and older age (Halbert et al. 2006). Other reported risk factors for COPD include the environment in fetal and infant life (Barker 1990), bronchial hyperresponsiveness (van den Berge et al. 2012), prior asthma diagnosis (Lange et al. 1998) or family history of asthma (de Marco et al. 2011), low education and low social status, pulmonary tuberculosis even in never smokers (Niroumand & Grossman 1998, Lamprecht et al. 2011, Lee et al. 2012). Finally, COPD is a heterogeneous disease (Agusti et al. 2011), and there are many factors e.g. gender, ageing of lung, co-morbidities and repeated child and adult respiratory infections which have controversial roles in the COPD development (Soriano & Rodriguez-Roisin 2011).

### 2.3 Pathogenesis of COPD

The pathogenesis of COPD is related to chronic, abnormal innate and adaptive inflammatory responses to inhalation of toxic particles (Hogg & Timens 2009). The lung possesses many protective mechanisms to maintain the equilibrium in the lower respiratory tract and airway epithelium i.e. mucus ciliary apparatus, antioxidant and oxidant detoxification enzymes and the innate inflammatory response controlled by chemokines and cytokines. Disruption of the protective mechanisms in conjunction with abnormalities due to inflammatory and repair processes ultimately leads to airway and parenchymal damage with local fibrosis (emphysema) and premature aging of the lung (Figure 1) (Keatings et al. 1996, Yamamoto et al. 1997, Hogg et al. 2004, Hogg 2008, Yao et al. 2012, Yang et al. 2012). According to Hogg and Timens (2009), the inflammatory process develops in the lungs of every smoker, and there is an association between the extent and severity of this tissue response and the severity of airflow limitation in COPD.

The histopathological features of COPD can vary between individuals and locally in the lung. Inflammatory exudates containing mucus occlude the airway lumen, and furthermore, there is a tissue-repair process which causes airway thickening and fibrosis between the epithelial surface and the muscle layer and narrows the lumen of the conducting airways less than 2 mm in diameter, increased numbers of microvessels, and the occurrence of mature lymphoid follicles in gas-exchanging tissue and around vessels (Hogg 2004, van der Strate et al. 2006, McDonough et al. 2011). This cascade is associated with nonspecific airway

hyperresponsiveness, which in turn leads to a rapid worsening in $FEV_1$ in COPD (Tashkin et al. 1996, Hogg et al. 2004).

Emphysematous destruction of the gas-exchanging tissue contributes to the airflow limitation by decreasing the elastic recoil pressure available to drive air out of the lung during forced expiration (Hogg & Timens 2009). It is not known why this tissue destruction begins in the respiratory bronchioles near to the small conducting airways, which are the major sites of obstruction in COPD.

Additional phenomena encountered in COPD include its systemic manifestations such as elevated levels of cytokines in the circulation, increased production of acute-phase proteins and their release from the liver such as C-reactive protein (CRP), highly sensitive C-reactive protein (sCRP) (Eagan et al. 2010) and release of leucocytes from the bone marrow into the circulation (Weiss et al. 1995, Chan-Yeung et al. 1988). The increased level of sCRP in serum was originally thought to be a biomarker of COPD, but is later shown to be a general marker of inflammation (Kinnula et al. 2009).

Oxidative stress is considered as one of the major mechanisms leading to the tissue damage/emphysema in COPD (Carp & Janoff 1978, Janoff et al. 1979). Cigarette smoke itself contains large amounts of oxidants and free radicals. These oxidants also worsen lung defences and inactivate the antioxidant enzymes present in the lung (Figure 1) (Kinnula et al. 1995, Rahman et al. 1996, Barnes 2009, Rahman & Kinnula 2012). The cascade of systematic inflammation contributes to and potentiates oxidative stress, for example, activating matrix metalloproteinases in the lung (Stockley et al. 2009, Sin & Vestbo 2009, Agusti et al. 2011).

It is not only the inflammatory cells in the peripheral lung, but also alveolar epithelial cells, may trigger the destruction of the alveolar walls in emphysema by releasing proteinases and inactivating the intrapulmonary proteinase inhibitors, and thus damaging alveolar septal structures e.g. extracellular matrix and respiratory bronchioles (Shapiro 1995)(Figure 1). However, with the exception of the emphysema provoked by alpha-1-antitrypsin deficiency, the predominant role of neutrophils in emphysematous lung has been recently questioned. Although none of the inflammatory cell types seems to predominate, the protease-antiprotease

imbalance (Figure 1.) is believed to be important in the pathogenesis of emphysema in COPD (Betsuyaku et al. 1999, Hogg 2002, Abboud & Vimalanathan 2008).

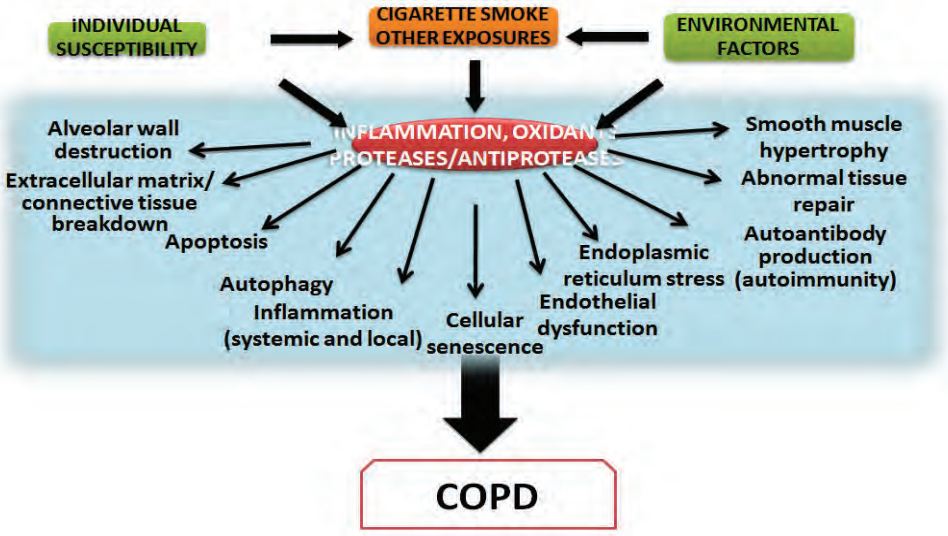


**Figure 1.** The development of COPD is associated with smoking/biomass smoke-induced oxidative stress. The illustration of the mechanisms involved in the pathogenesis of COPD by Rahman & Kinnula (2012).

### 2.4 Diagnosis of COPD

The internationally used GOLD classification for the definition of COPD has required only obstruction that the value of the post-bronchodilator forced expiratory volume in one second divided by forced vital capacity should be less than 70% ($FEV_1$/ FVC < 70%). From the year 2011, the updated version of the GOLD document also has included symptoms, quality of life, exacerbation frequency of the disease and co-morbidities (GOLD 2011). Recent international studies have also described a new sub-group of COPD patients i.e. unclassified disease in cases when the disease history appears typical for COPD but the spirometry reveals

nearly irreversible obstruction, decline of $FEV_1$ and also FVC e.g. the amount of air which can be forcibly exhaled from the lungs after taking the deepest breath possible, and thus due to this dynamic restriction, the ratio of $FEV_1$/FVC remains > 70% (Wan et al. 2011). Until now, the GOLD classification has been widely used in scientific publications. In Finland, the disease is defined by using Finnish reference values, not a strict $FEV_1$/FVC ratio (Viljanen et al. 1982, Sovijärvi et al. 2011). The classification of COPD severities are illustrated according to both the GOLD criteria and the Finnish national criteria in Table 1.

The heterogeneity (variable airway and/or emphysema) of COPD has been increasingly incorporated into COPD studies (Miravitlles et al. 2012, Martinez et al. 2012). Although COPD has many clinical phenotypes with variable stages of mainly irreversible airway limitation and parenchymal abnormalities (emphysema), the diagnosis, assessment and therapy of COPD patients is still today mainly confirmed by spirometry, which measures how deeply a person can breathe and how fast air can move into and out of the lungs (Rabe et al. 2007b, Agusti et al. 2011), even though the parameter obtained from spirometry, $FEV_1$, alone does not describe the complexity of the disease (Han et al. 2010). Nonetheless, with spirometry one can classify COPD into four severities though there is accumulating evidence that spirometry alone poorly reflects the disease severity (GOLD 2011, COPDGene 2012). A proportion of smokers may have lung function impairment characterized by a reduced $FEV_1$ but with a preserved $FEV_1$/FVC ratio, and these COPD patients are excluded from the GOLD classification; they are a heterogenous still poorly characterized patients, the so-called GOLD unclassified (GOLD-U) (Wan et al. 2011).

**Table 1**. Classifications of COPD severities. Classifications are based on post bronchodilator lung function.

| Criteria | Classification of COPD | | | |
|---|---|---|---|---|
| | **Stage I (mild)** | **Stage II (moderate)** | **Stage III (severe)** | **Stage IV (very severe)** |
| **GOLD 2007** | $FEV_1/FVC < 70\%$<br>$FEV_1 \geq 80\ \%$ | $FEV_1/FVC < 70\%$<br>$50\ \% \leq FEV_1 < 80\%$ | $FEV_1/FVC < 70\%$<br>$30\% \leq FEV_1 < 50\%$ | $FEV_1/FVC < 70\%$ and $FEV_1 < 30\ \%$ or $FEV_1 < 50\ \%$ plus chronic respiratory failure |
| **Finnish National** | $65\% \leq FEV_1 \leq 79\%$<br>$35\% \leq MEF_{50} \leq 61\%$ | $45\% \leq FEV_1 \leq 64\%$<br>$MEF_{50} \leq 34\%$ | $25\ \% \leq FEV_1 \leq 44\ \%$ | $FEV_1 \leq 24\ \%$ |

$FEV_1$isforced expiratory volume in one second, $MEF_{50}$ismid-expiratory flow, $FEV_1/FVC$ is forced expiratory volume in one second divided by forced vital capacity

Staging/grading criteria in the document of GOLD criteria omitted the earlier "at risk" Stage 0 i.e. those with chronic bronchitis but normal lung function ($FEV_1/FVC \geq 70\%$) subjects (Pauwels et al. 2001). However, prolonged cough and sputum production may precede by many years the appearance of airflow limitation, although not all smokers with cough and sputum production will develop any airway limitations (Fletcher & Peto 1977, Vestbo & Lange 2002, Mannino 2006).

### 2.5 Treatment strategies of COPD

According to GOLD Executive Summary, the primary aim in COPD is prevention, but there can be several different goals in the disease management, e.g. relief of symptoms, prevention of disease progression, improvements in exercise tolerance, elevating health status, preventing and treating complications, preventing and treating exacerbations, and reducing mortality (Rabe et al. 2007b). The management of COPD includes active treatment of its common co-morbidities e.g. cardiovascular diseases, osteoporosis, and depression. Pharmacologic treatment should be optimized individually aiming to decrease symptoms, frequency of exacerbations, and/or complications (Rabe et al. 2007b, Rabinovich & MacNee 2011). None

of the current medications can modify the long term decline in the lung function. According to Finnish Current Care guideline (2009), regular pharmaceutical therapy is generally needed for moderate to very severe symptomatic disease. It involves combinations of anticholinergics, beta-agonists, inhaled corticosteroids, and low dose theophylline. Antibiotics are given individually for COPD patients on an as-needed basis. In the national Current Care guidelines pneumococcal and influenza vaccinations are recommended, and practical suggestions are given about when to consider the addition of oxygen or non-invasive ventilation (Chronic Obstructive Pulmonary disease: Current Care guideline 2009).

In addition to medications, patient education and effective physiotherapy may limit the effects of exacerbations on the patient´s quality of life, decelerate further impairment of the disease, and improve exercise tolerance (Puhan et al. 2006, Johnston & Grimmer-Somers 2010, Keating et al. 2011, Puhan et al. 2011).

### 2.6 Prognosis of COPD

According to the latest World Health Organization (WHO) estimates, 65 million people had moderate to severe COPD in 2005, and 3 million people died of COPD (http://www.who.int). COPD is now the fourth most common cause of death, but may become the third leading cause of death worldwide by year 2030, and the burden of COPD may increase in the coming decades due to continued exposure to COPD risk factors and the aging of the world's population. In a Finnish 30-year study, the cumulative incidence of chronic bronchitis was 32% in current smokers (Pelkonen et al. 2006). Symptoms of chronic bronchitis have been documented to be associated with increased mortality (Annesi & Kaufmann 1986, Lindberg et al. 2005, Ekberg-Aronsson et al. 2005, de Marco et al. 2007, Medbo & Melbye 2008).

The early detection of new COPD cases and the motivation of the patient to give up smoking are of major importance in the prevention of COPD progression to greater severity. Screening with spirometry correlates with better diagnosis (Sims & Price 2012) but screening is still controversial and not widely accepted. The current evidence does not support uncontrolled screening of all smokers by spirometry due to difficulties in conducting the spirometry and the absence of evidence that spirometric testing achieves any prolonged changes in smoking habits or in the treatment of the COPD patients (Kotz et al. 2007b, Kotz et al. 2009a).

The phenotypes of COPD are defined as individual disease attributes that describe differences relating to clinically meaningful outcomes, and prognosis seems to depend very clearly on the phenotype of the disease and not only of the severity of airway limitation assessed in spirometry. Recently several studies and reviews (COPDGene 2012, Miravitlles et al. 2012) have emphasized the importance of different phenotypes such as exacerbator, overlap (COPD-asthma) and emphysema sub-phenotype. COPD patients with the chronic bronchitis phenotype are at a higher risk of exacerbations (Kim et al. 2011). The exacerbator phenotype is associated with increased mortality and frequent hospitalizations (Vestbo et al. 1996, Vestbo & Hogg 2006). Thus, the putative phenotypes of COPD differ not only in the clinical symptoms but also in their response to therapeutic and rehabilitation intervention and survival.

In all, the latest GOLD document contains new recommendations to assess symptoms by COPD Assessment test (CAT) or the modified Medical Research Council breathlessness scale (mMRC) and risk of exacerbation besides assessment of co-morbidities and the degree of airflow limitation using spirometry, although neither systemic inflammation, nor ”telehealth” or COPD/asthma overlap is mentioned in this document (Vestbo 2012). Further, an incidence rate of two or more exacerbationswithinthe last year or $FEV_1 < 50\%$ of predicted value are indicators of high risk of future exacerbations. All these assessments need to be combined for the purpose of improving management of COPD patient. The prognosis of co-morbidities like cardiovascular diseases, osteoporosis, respiratory infections, anxiety and depression, diabetes and lung cancer may significantly influence hospitalisation rate and mortality of COPD patients (GOLD 2011).

## 2.7 Smoking behavior

### *2.7.1 Smoking initiation*

Smoking is a complex behavior influenced by genetic, social and psychological factors and their interactions (Tobacco Dependence and Cessation: Current Care guideline 2012). Most adult daily smokers have initiated smoking at the age of 12 - 15 years (Raitasalo et al. 2012). Starting smoking at an early age increases the number of cigarettes smoked per day as an adult (Taioli E et al. 1991). The teen years are associated with an individual socialization

process and an elevated risk of smoking initiation, but smoking habits of other family members and parents´ socio-economic position are also important in whether or not adolescents start to smoke (Kemppainen et al. 2006, Rainio et al. 2008, McCool et al. 2011). In a recent systematic review, factors increasing risk among young adults to initiate smoking are exposure to smoking, boredom or stress while serving in the military, attending tobacco-sponsored social events while in college, and exposure to social norms and perceptions that encourage smoking (Freedman et al. 2012). A sedentary life-style in both adults and adolescents has been associated with smoking, whereas persistent physical inactivity in late adolescence proceeds into adulthood (Conway & Cronan 1988, Conway & Cronan 1992, Kujala et al. 2007). There is some evidence indicating linkage between depression and smoking (Fergusson et al. 2003, Korhonen et al. 2008). According to the self-medication hypothesis depressed subjects are more likely to start or continue smoking than the others (Murphy et al. 2003).

Young adults are more sensitive than adults to cigarette pricing policies, reductions in prices have encouraged smoking initiation in this age group (Zhang et al. 2006). On the other hand, taxation strategies and indoor-air legislation are believed to have reduced the smoking prevalence in the general population (Siahpush et al. 2009, Jemal et al. 2011).

### *2.7.2 Nicotine dependence*

Nicotine dependence as a term involves all the effects of nicotine on specific receptors in the brain. One of these effects is the release of dopamine, which acutely activates the reward system, often leading to the development of chronic relapsing withdrawal addiction (Watkins et al. 2000, Benowitz 2010). Twin, family and adoption studies have provided some evidence for genetic effects on smoking behavior and nicotine dependence (Korhonen & Kaprio 2012). Genome-wide association studies have revealed some nicotine dependence genes (Thorgeirsson et al. 2010, Liu et al. 2010).

Tobacco products are mostly used because of the presence of nicotine which is an addictive agent (Benowitz 2010). About 30% of ever-smokers and 50% of current smokers have been or are dependent on nicotine and this value did not remarkably differ by gender, age, or country (Hughes et al. 2006).

Nicotine dependence diagnosis is defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association 1994). However, the most commonly used measure of nicotine dependence is Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al. 1991). The FTND consists of 6 items scoring ranging 0 to 10. The shorter modified version of FTND is the Heaviness of Smoking Index (HSI) which has only two questions e.g. number of cigarettes each day and time to first cigarette in the morning with a range from 0 to 6. It is known that some young people can become addicted even from the first cigarette and occasional tobacco use in this age group can provoke early symptoms of dependence, increasing the risk of future smoking (DiFranza et al. 2007, Doubeni et al. 2010). Young adults may seriously underestimate how quickly nicotine addiction can develop (Centers for Disease Control and Prevention (CDC) 2006).

### *2.7.3 Smoking cessation*

#### *2.7.3.1 Predictors of smoking cessation*

It is very important to identify determinants of successful quitting behavior in order to increase cessation rates. The success rate in smoking cessation was 35% in COPD patients in the Lung Health Study with a 5 year follow-up (Anthonisen et al. 1994). According to the Finnish National Current Care guidelines for treatment of tobacco dependence and smoking cessation, the success rate in smoking cessation varies between 7-24% (Tobacco Dependence and Cessation: Current Care guideline, 2012).

Jardin & Carpenter (2012) examined predictors of quit attempts and 7-day point prevalence abstinence in a nationwide population-based randomized controlled trial with 849 smokers who were motivated to quit. The number of previous quit attempts significantly predicted future quit attempts. Motivational factors dominated in the prediction of success in quitting smoking over the short-term.

A literature search of systematic reviews and meta-analyses from Medline database using ‘predictors of smoking cessation’ and ‘young adult’ (with age limit 19-24 years) as keywords located one review. When the search was conducted without any age limits 18 studies were found. Most of these studies in young adults assessed smoking cessation rates in certain

patient groups e.g. chronic diseases, pregnancy, but only one study in COPD patients. A systematic review by Cengelli et al. (2012) reported that the following five factors predicted quitting: not having friends who smoke, not having intentions to smoke in the future, resisting peer pressure to smoke, being older at first use of cigarette and having negative beliefs about smoking.

Shelley et al. (2010) assessed the effectiveness of a tailored free nicotine patch program in 375 young adult smokers with a follow up of 4 months. Finally, the predictors of cessation included higher levels of self efficacy at baseline, not smoking while using the patch and concern about health risks. The nicotine replacement therapy (NRT) delivered free of charge by a local organization probably also helped smokers in quitting.

Additionally, higher education, familial support and especially marital status in men were factors favouring success in smoking cessation in Finnish middle-aged or older daily smokers (Broms et al. 2004). In a systematic review, Vangeli et al. (2011) examined predictors in success of smoking cessation in general adult population samples. They concluded that earlier quit attempts and measures of motivation to stop were highly predictive of quit attempts, but gender, age, and marital status and education were not consistently related to quit attempts or quit success.

There are many different outcome measurements used in randomized smoking cessation trials leading to difficulties in interpretation and comparison between trials. However, three commonly reported outcomes are 24-hour point prevalence, 7-day point prevalence and 30 – day prolonged prevalence as measures of self-reported abstinence in longitudinal cessation trials (Velicer & Prochaska 2004). West et al. (2005) suggested that there should be standardization of the success measurement e.g. trials with 6 to 12 months length of follow-up from the target quit date, self-report of smoking abstinence over the whole follow-up, biochemical verification of abstinence, and use of the intention-to-treat approach in data analysis.

*2.7.3.2 Smoking cessation interventions for COPD patients*

It has been emphasized that clinicians should at least encourage every smoker, also patients with COPD, to quit because the treatments are both clinically effective (Wu et al. 2006, Tonnesen et al. 2007) and cost-effective (Hoogendoorn et al. 2010). In Finland, the evidence-based guidelines recommend that all health professionals should assess smoking status of their patients and to offer advice to quit. All doctors and dentists are advised to offer brief counselling and targeted pharmacotherapies, and to refer quitters to local specialized centers in treatment of tobacco dependence when appropriate (Tobacco Dependence and Cessation: Current Care guideline 2012).

Strassmann et al. (2009) conducted a network meta-analysis in order to determine the order of the effectiveness of smoking cessation interventions to COPD patients. Cessation counselling with NRT had the greatest effect on prolonged abstinence rates versus usual care compared to that of counselling alone or counselling combined with an antidepressant. The behavioral interventions included here were individual or group setting, self-help material and telephone counselling (Strassmann et al. 2009). Five randomized controlled studies up to 2003 were included in a Cochrane systematic review to assess the effectiveness of smoking cessation interventions in individuals with COPD (van der Meer et al. 2003). They found evidence that a combination of psychosocial interventions and pharmacological interventions were superior to no treatment or to psychosocial interventions alone with a 6 month follow-up. There were no studies comparing psychosocial interventions with no treatment.

*2.7.3.3 Behavioral interventions in smoking cessation*

The Motivational Interviewing technique (MI) may assist smokers to quit (Lai et al. 2010, Hettema & Hendricks 2010). The MI technique is effective since it involves understanding, listening and empowering the patient, and thus avoiding a confrontational approach. In all, behavioral support (including brief advice and counselling) and pharmacotherapies (NRT, varenicline or bupropion) were found to be effective in smoking cessation when compared to controls receiving usual care or brief advice or less intensive behavioural support (Stead & Lancaster 2012).

In another Cochrane systematic review updated in 2008 included 30 trials with over 7000 subjects (Lancaster & Stead 2005). This review surveyed 21 trials and over 7000 participants aiming to assess the effects of individual counselling, which was found to be more effective compared to a minimal behavioral intervention in 18 trials. The measured outcome was smoking cessation at a follow-up of at least six months after the start of counselling. The authors reported that the intensive counselling was not any more effective than brief counselling.

*2.7.3.4 Pharmacological interventions in smoking cessation*

Two nationwide questionnaire surveys were conducted in the years 2000 and 2006 on smoking cessation practises of Dutch General Practitioners, chest physicians, cardiologists and respiratory nurses (Kotz et al. 2007a, Kotz et al. 2008b). Based on these studies, physicians preferred to conduct a short behavioral intervention like providing self-help materials for smokers to quit and referring these patients to respiratory nurses for more intensive counselling treatment. With respect to the available pharmacotherapies, most physicians recommended bupropion followed by nicotine patch, and nicotine gum. On the other hand, respiratory nurses preferred the use of intensive behavioral techniques to pharmacological aids, but nurses had also observed common problems in smokers´ motivation to quit. Thus, the authors suggested that there should be reimbursement of pharmacological aids and the simple tools should be devised to assess motivation to quit for use in patient evaluation. In addition, the nurses stressed that the majority of smokers relapse, make many quitting attempts and need repeated counselling interventions in combination with pharmacological aids before achieving success (Kotz et al. 2008b, Kotz 2008). Furthermore, confrontational counselling attempting to achieve changes in risk perceptions, health concerns and self-exempting beliefs seems to be more effective than “usual care” in the short term in convincing smoking COPD patients to quit, but further longitudinal research is needed to assess the effects over the long-term of repeated counselling (Kotz et al. 2009b).

One Cochrane review included 132 trials studying the efficacy of NRT in cessation with over 40,000 individuals in the main analysis in 2007; it concluded that NRTs (gum, transdermal patch, nasal spray, inhaler and sublingual tablets/lozenges) could increase the rate of quitting

by 50 - 70%. The effectiveness of NRT appeared to be largely independent of the intensity of additional support provided individually (Stead et al. 2008).

Eisenberg et al. (2008) compared the treatment effects of 7 approved pharmacologic interventions for smoking cessation based on a meta-analysis of randomized controlled trials. All included studies reported biochemically validated measures of abstinence at 6 and 12 months. The authors concluded that varenicline, bupropion and five different NRTs (nicotine nasal spray, patch, gum, inhaler and tablet) were all more efficacious than placebo at promoting abstinence at 6 and 12 months.

Self-help materials may be effective in preventing relapse in the long-term follow-up in initially unassisted quitters, and use of NRT, bupropion and varenicline appear to be effective in preventing relapse following an initial period of abstinence or an acute treatment period, but there is no evidence that behavioral support can prevent relapses (Agboola et al. 2010).

*2.7.3.5 Smoking cessation interventions for young adults*

Only a few systematic reviews or meta-analyses have been published about cessation interventions targeted to late adolescents and young adults (Grimshaw & Stanton 2006, Fagan et al. 2007, Villanti et al. 2010, Suls et al. 2012). The conclusion of these few studies seems to be that little progress in the smoking cessation programming for young adults has been achieved. A total of 24 smoking cessation interventions for young adults were registered into ClinicalTrials.gov database in October 2012 (http://www.clinicaltrials.gov).

At the moment, Cochrane Library includes six reviews on smoking cessation interventions of young people using “smoking cessation” and “young adults” (http://www.thecochranelibrary.com). These reviews assessed the results of trials evaluating the role of mass media, family, internet, mobile phone or role of incentives in smoking cessation and the effectiveness of different strategies in smoking cessation in young people aged less than 20 years; most of those studies were targeted at children and adolescents.

Fagan et al. (2007) investigated the variables associated with quitting behaviors among 7912 current, daily, and occasional young adult smokers in the United States. The authors found that nicotine dependence measures were significantly associated with quitting and intention to

quit among daily smokers, but in contrast sociodemographic factors may have been more important among nondaily smokers (Backinger et al. 2003, Fagan et al. 2007). It may be useful to use incentives in smoking cessation of some subgroups of smokers especially in the short term, although there is no evidence to recommend these practices into routine practice (Aveyard & Bauld 2011).

Villanti et al. (2010) conducted a systematic review of smoking cessation interventions aimed at young adults, and 12 randomized controlled trials and two non-randomized studies met the inclusion criteria. Pharmacologic interventions were not included in this review. They concluded that brief interventions with support via telephone and electronic media were promising ways to help smokers quit the habit.

Suls et al. (2012) investigated the efficacy of smoking-cessation programs in young adults. They concluded that young adults could benefit from existing evidence-based treatment methods for smoking cessation. Thus they proposed that it could be useful to focus resources on motivating young adults to seek cessation treatments.

*2.7.3.6 Summary of interventions in smoking cessation*

In conclusion, there are three first-line pharmacological treatments available for smoking cessation in adults. There is some evidence that combining counselling interventions with pharmacological aids may increase the success rate. Different behavioral interventions individually combined with drug treatments, mainly NRTs, seem to be more effective than counselling alone, especially in young adults with nicotine dependence (Tobacco Dependence and Cessation: Current Care guideline, 2012). The comparison of the results of smoking cessation trials is difficult due to many differences, for example, inclusion and exclusion criteria, outcome measurements, the length of follow-up, intensity and number of behavioral sessions, and use of pharmacological treatments.

## 2.8 Biomarkers of COPD

### *2.8.1 Definitions of biomarkers*

A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological process, pathological process, or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001), but in this work a biomarker is considered as a biochemical marker to find early disease using unbiased proteomics.

### *2.8.2 Characteristics of biomarkers in smoking and in COPD*

COPD biomarkers can be measured in sputum, lung biopsies or lung tissues, exhaled breath condensate (EBC), bronchoalveolar lavage fluid (BAL), and plasma (Day 1994). Sputum and lung tissue represent samples, which are derived directly from the lung. Induced sputum is nearly non-invasive and its processing has been standardized (Vignola et al. 2002, Djukanovic et al. 2002, Nicholas & Djukanovic 2009). Lung tissue can be obtained from surgical operations, but those results need to be confirmed by using non-invasive techniques. Exhaled air and EBC suffer from several problems and though exhaled air (nitric oxide, NO) has been widely accepted only for the evaluation of allergic asthma and EBC is laborious and non-standardized. Thus, neither exhaled air/NO nor measurement of biomarkers from EBC has been accepted for COPD studies. BAL requires invasive bronchofiberoscopy, which has specific problems especially with COPD e.g. airway collapse being a potential one. Plasma is very unreliable if that is the only available specimen source, since it reflects all systemic alterations, but plasma may be valuable in testing the findings discovered first from lung i.e. from the target organ. The pros and cons of the different sample types and techniques especially in using them in proteomic studies have been reviewed in detail recently (Kinnula et al. 2009).

Various molecular biomarkers have been reported that reflect oxidative stress, protease-antiprotease imbalance and inflammation (Table 2). The search for reliable COPD markers has involved both using non-hypothesis driven studies such as unbiased proteomics and studies on selected biomarker candidates e.g.by using microarray platforms (Pinto-Plata et al. 2007). Two proteomic studies were published from the group of Djukanovic who stated that

sputum could be evaluated using this technology (Nicholas et al. 2006, Nicholas et al. 2009). Other sputum proteomic studies listed COPD biomarker candidates, but did not investigate them in more detail (Casado et al. 2011) or only concentrated on the most significantly changed proteins, one of which was the polymerized immunoglobulin receptor (PIGR) (Ohlmeier et al. 2012). Two additional recent studies on lung tissue proteomics revealed the decline of receptor for advanced glycation end-product (RAGE) variants in COPD and pulmonary fibrosis and disappearance of hemoglobin subunits in pulmonary fibrosis but not in COPD (Ohlmeier et al. 2010, Ishikawa et al. 2010).

**Table 2.** Some of the **s**uggested candidates for biomarkers that have been reported to be associated with chronic obstructive pulmonary disease (COPD).

| **Biological sample** | **Biomarker** | **Outcome (if measured) e.g. FEV1 decline/mortality/ exacerbation rate** | **Study group** |
|---|---|---|---|
| Blood, Sputum, Clinical | Fibrinogen, sCRP, PARC,CCL-18,CC-16, neutrophil count, Bode index, CT scan | Future risk- systemic inflammation, therapeutic response, predictors of prognosis, systemic inflammation | Rosenberg & Kalhan 2012 |
| Blood | CRP, IL-6, procalcitonin, SAA, SP-D, micro ribonucleic acids, micro-RNAs, miRNAs | Pulmonary inflammation, exacerbation frequency, increased in smokers at risk prognosis, regulating gene expression-role not known in COPD | Agusti et al. 2011 |
| Sputum, exhaled breath condensate (EBC), blood | Neutrophil count, NO, PGP, N-α-PGP, SP-D, PARC, CCL-18, CRP, IL-6 | Prognosis, breakdown products of collagen, systemic inflammation | Yoon & Sin 2011 |
| Blood | Sirtuins, TNF-α, IL-6, increased production of growth factors and angiogenic factors | Ageing, insulin resistance, cachexia, depression, lung cancer | Barnes 2010 |
| Exhaled breath condensate | Reactive oxygen species, reactive nitrogen species, lipid peroxidation products, antioxidants, glutathione | Unknown clinical role | Rahman & Biswas 2004 |
| Blood (Sputum, EBC, Bronchoalveolar lavage (BAL), lung biopsies) | Alpha-1-antitrypsin SERPINA1, SERPINE2, EPHX1, SOD3, fibrinogen and CRP regulating IL-6, IL-1, TNF- α | Early disease and prognosis: α1-AT deficiency, risk of COPD, inhibiting oxidative stress, pathogenesis of early disease, systemic inflammation in stable COPD, future prognosis i.e. hospitalization, death | Dahl & Nordestgaard 2009 |
| Blood | CRP, MMP-9, PARC,MPIF-1, ACRP-30, s-ICAM-1, Eotaxin-2, IP-10,IL-1a,TNF-R1,IL-6, SAA, fibrinogen, SP-D,CC-16, Anti-elastin, N-acetyl-PGP | CRP, Fibrinogen – mortality, exacerbation, FEV1 decline - no data (possibly CC-16), no data concerning health status | Sin & Vestbo2009 |
| Induced sputum, lung biopsy, BAL, exhaled breath condensate, plasma | GRO-a, MCP-1, IL-8, CXCL9, CXCL10, CXCL11, CCL5, TNF-a, GM-CSF, VEGF, 8-isoprostane, nitrotyrosine-tagged cells, MMP-2, MMP-7, MMP-9, MMP-12, MMP-8, TIMP,NE, ECP, HNL, LTB4, hCAP-18, TLR2, PAR2,SPs/PIGR | Response to tobacco smoke, systemic inflammation in COPD, some variably or negatively associated to tobacco smoke or COPD | Comandini et al. 2009, Illumets et al. 2007, Louhelainen et al.2008, Mazur et al. 2009, Louhelainen et al. 2010, Illumets et al. 2011, Ohlmeier et al. 2012 |
| Blood | Chemo attractants I-TAC, eotaxin-2, MPIF-1, MCP-1, MIP-1b, IL-8, PARC, inflammation IL-15, IL-1ra, IL-17, TNFa, TNF R1, IFNc, IL-12, p40, IL-2Rc/destruction and repair TGFa, VEGF, AR, BDNF, | 24 serum markers of inflammation, tissue destruction, and repair related to lung function, exercise capacity, the BODE index, reported exacerbation frequency - clinical outcomes unknown | Pinto-Plata et al. 2007 |

| | | | |
|---|---|---|---|
| | bNGF, MMP-9, TIMP-1, Novel markers PAI-II, prolactin | | |
| Blood | Pneumoproteins SP-D, CC-16 | Mortality, health status in severe COPD | Jaw & Sin 2012, Sin et al. 2007 |
| Blood/sputum//BAL | TNF-α, IL-6, IL-8, IL-18, CRP, SAA, Fibrinogen | Systemic inflammation-cardiovascular disease or exacerbation rate | Gan et al. 2004 |

Abbreviations: SERPINE2 = Enzyme serine proteinase inhibitor E2;EPHX1 = Epoxide hydrolase 1; SOD3 = Superoxide dismutase 3; SAA = Serum amyloid A ; ACRP-30 = adipocyte complement–related protein of 30 kD; CC-16 = Clara Cell Secretory protein-16; CRPC-reactive protein; IL-1a = interleukin-1; IL-6 = Interleukin-6; IP-10 = Interferon-inducible protein 10; MMP = matrix metalloproteinase; MPIF = myeloid progenitor inhibitory factor; PARC/CCL-18 = pulmonary and activation-regulated chemokine; s-ICAM = soluble intercellular adhesion molecule; TNF-R1 = Tumour necrosis factor receptor-1;TNF-α = Tumour necrosis factor-α; PGP = proline-glycine-proline; CXCL9 = CX chemokine ligand 9; CXCL10 = CX; chemokine ligand 10; CXCL11 = CX chemokine ligand 11, CCL5 = C chemokine ligand 5; VEGF = Vascular endothelial growth; GRO-a = growth-related oncogene-alpha;MCP-1 = monocyte chemotactic protein-1factor; MMPs = Matrix etalloproteinases;TIMPs = tissue inhibitors of metallo-proteinases; NE = Neutrophil elastase;ECP = Eosinophil cationic protein; HNL = Huuman neutrophil lipocalin;LTB4 = Leukotriene B4; hCAP-18 = Human cationic antimicrobial protein-18; TLR2 = Toll-like receptor 2; PAR2 = Protease activated receptor 2;SPs = surfactant proteins; PIGR = polymeric immunoglobulin receptor ; AR = amphiregulin; bNGF = brain-derived neurotrophic factor; BDNF = b-nerve growth factor; IFNc = interferon c; IIL-1ra = interleukin 1receptor antagonist; IL-2Rc = interleukin 2 receptor gamma; I-TAC = interferon c-inducible T cell a chemoattractant; MIP-1b = macrophage inflammatory protein 1b; PAI-II = Plasminogen activator inhibitor II;

Pinto-Plata et al. (2007) selected 143 serum proteins, which empirically were thought to represent pathogenetic mechanisms in COPD and emphysema. In further studies, they selected 24 biomarkers such as cytokines and tissue metalloproteinases (MMP-7, MMP-8, MMP-9 and MMP-10) proteins of the protease-antiprotease cascade that can lead to emphysema. Their study was cross-sectional and thus the role of the potential COPD biomarkers remained unclear.

### *2.8.3 Surfactant protein A*

Surfactant protein A (SP-A) was the most significantly changed protein in the COPD lung tissue in non-biased proteomics conducted for this study. This protein is a hydrophilic surfactant protein that belongs to the collectin subgroup of the C-type lectin superfamily (Day 1994). The levels of this protein have also been recently found to be elevated in the circulating blood of chronic smokers (Kobayashi et al. 2008).

SP-A is generally expressed on mucosal surfaces, while SP-A RNA and protein seem to be restricted to the respiratory system and there, mainly in alveolar epithelium (Madsen et al. 2003, Ishikawa et al. 2011). The two types of non-ciliated epithelial cells in the peripheral

airways, Clara cells and the alveolar type II cells produce these lung collectins (Walker et al. 1986, Takahashi et al. 2000). Most SP-A levels that has been found in serum, are suggested to be synthesized mainly in the lung, although some expression of these proteins has been found in other organs e.g. gastrointestinal system. During the 1980´s, Japanese studies reported that the serum SP-A level is increased in patients with idiopathic fibrosis (IPF) and hypothesized that this may have reflected pathologic changes in lung. The concentration of SP-A is known to be higher in BAL fluid in patients with IPF as compared to normal subjects (McCormack et al. 1991), but in those studies, no other lung diseases have been investigated.

### 2.9 Summary of the literature

COPD is not only a treatable and preventable disease but once established it is irreversible and progressive. Thus early detection of the disease is crucial. Many smokers who have this disease experience no symptoms, and thus COPD is a common but predominantly undiagnosed disease. Spirometry is the golden standard for COPD diagnosis and assessment. The incidence of COPD depends on the diagnostic criteria being used, leading to different phenotypes with tailored pharmacotherapy and different prognosis. Furthermore, guidelines of COPD include different spirometry criteria ways to confirm diagnosis, and recently a GOLD-Unclassified (GOLD-U) COPD phenotype was characterized. In all, there is much debate not only about the best COPD definition, but also about the role of screening COPD.

Since 2006, GOLD Stage 0 has been dropped from the classification scheme. This Stage 0 consisted of smokers with normal lung function and symptoms of chronic bronchitis (chronic cough and/or sputum production). Persistent respiratory symptoms may be important indicators, independently of lung function, for all-cause mortality, although mechanisms to explain this phenomenon are still unknown. This link is more likely to exist, if smokers have also other respiratory symptoms, e.g. wheeze or dyspnoea. These symptoms are included in the assessment of exacerbation risk in COPD in the updated GOLD document (GOLD 2011). Symptoms related to smoking are not stable i.e. they may vary or disappear during time and after smoking cessation. A successful intervention may improve both the quality and prognosis of life in symptomatic cigarette smokers.

Smoking cessation is the most important intervention in primary and secondary prevention of COPD. It has been debated whether an approach which uses detection of early airway limitation and labeling mild COPD offers any advantage on the cessation rates in mainly asymptomatic smokers or whether this health strategy simply leads to increasing costs to society. From the smokers´ point of view, spirometry results may raise doubts of health damage due to smoking and thus contribute to stronger motivation in the quitting attempts. The results of many studies performed on the role of spirometry in screening and in smoking cessation are controversial mainly due to the differences in selection criteria of trials or in time schedules of the trials. In many smoking cessation trials, young adults have been a minority in a large study group of older adults or as late adolescents in cessation studies of school-aged teenagers.

Much research effort has been expended in attempting to characterize the mechanisms involved in COPD pathogenesis in order to discover new approaches for early diagnosis, but the only well known genetic risk factors for COPD so far are variations in the alpha-1-antitrypsin gene (AAT). A low level of AAT in plasma is indicative of an increased risk of disease development, especially in smokers. However this genetic variation is involved in only a very small number of COPD patients. Theoretically, as Dahl and Nordestgaard (2009) stated, biochemical markers in COPD should be able to demonstrate the association between a marker and COPD, as well as a change in the COPD risk with changes in the marker concentration or function. So far, only a few proteomic studies have been conducted on the lung tissues of COPD and no specific or sensitive biochemical marker suitable for clinical purposes has been discovered.

## 3. AIMS OF THE STUDY

The aim of this thesis was to evaluate the success in smoking cessation in adults and to identify the factors associated in successful cessation. Furthermore, it was intended to estimate the prevalence of early COPD in middle-aged heavy smokers who initially felt themselves symptom- free and healthy. In addition, this study aimed to detect biochemical markers of early COPD by unbiased proteomics and then further to test candidate biomarkers.

The specific aims of this thesis were

1. To assess smoking habits and smoking cessation in healthy middle-aged and young adults (studies I, III).

2. To evaluate the prevalence of COPD and the role of early detection of airway limitation and prolonged respiratory symptoms in the diagnosis of COPD of smoking middle-aged adults (study I).

3. To study the association of symptoms of chronic bronchitis with smoking habits in young adult males (study II).

4. To seek and to identify new potential biomarkers related to early COPD using non-hypothesis driven unbiased proteomics (studies IV,V ).

# 4. MATERIALS AND METHODS

This study consisted of three different study populations: 1) daily smoking adults aged 30-77 years old, 2) smoking young adult males aged 18-26 years and 3) the data consisted of tissue samples on which unbiased proteomics was conducted. The Ethics committees of Lapland Central Hospital and Helsinki University Hospital approved the study protocols, which are in accordance with the ethical standards of the Helsinki Declaration written in 1975. Written informed consent was obtained from all study subjects. Details of the materials and methods are provided in the separate publications (studies I-V).

## 4.1 Early detection of COPD combined with counselling for smoking cessation (I)

### *4.1.1 Participants*

A total of 584 smoking adults were recruited by newspaper announcements and they agreed to be followed up for their health status in a two-year prospective study. All study subjects were heavy smokers (median 29 pack years) but felt themselves asymptomatic and healthy without any chronic lung disease or allergies. The exclusion criteria consisted of subjects with all lung or other diseases, regular medication, risk factors for other lung diseases (such as allergies, infections, and exposures), any previous lung infection such as diagnosed pneumonia or bronchiectasiae, malignancy or viral infection during the last 2 months. There was a special emphasis to ensure the exclusion of asthma since in the detailed questionnaire, some of the smokers reported respiratory symptoms. In all, 5.5% of participants did not attend the follow-up visit i.e. 15 moved away from the region, 13 declined to participate in the follow-up visit for miscellaneous reasons, and four died (one pneumonia, two lung cancers, and one heart disease). Eight subjects were not able to perform spirometry as they were unable strictly to follow to instructions. Thus altogether 544 smoking subjects, 331 (60.8%) men and 213 (39.2%) women were included into this study. The study design included also 51 non-smoking over 40 years old healthy controls with normal lung function according to GOLD and national criteria for obstruction. Same information was also gathered from the controls.

### *4.1.2 Main outcome variables*

In this sub-study, the first primary outcome variable was the success in quitting smoking during a two-year prospective follow-up and the second primary outcome was the incidence of COPD diagnosis among symptom-free heavy smokers without any chronic diseases. The success in quitting was assessed in the personal interview by study nurse and was confirmed by measuring urine cotinine content. The COPD diagnosis was confirmed by detailed history, peak flow follow-up and spirometry before and after bronchodilatation were used to exclude asthma. Respiratory symptoms (chronic cough, sputum production) were assessed on every visit.

### *4.1.3 Explanatory variables*

Nicotine dependence (ND), smoking in pack-years (PY) and attitude to smoking cessation intervention were used as potential explanatory variables to explain success in smoking cessation. ND and PY were quantitative measurements of tobacco addiction in this study. Pack-year equals number of cigarettes smoked per day multiplied by number of years smoking then divided by 20. The attitude to smoking cessation intervention during motivational interview was used as a dependent variable with two classes: negative attitude (denial) vs. neutral or favour. Other covariates such as gender, age, body mass index (BMI), chronic cough with sputum production, use of pharmacological aids, and airway obstruction were also considered in the analyses. BMI ($kg/m^2$) was calculated as weight (kg) divided by length (m) multiplied length (m). Nicotine dependence was determined by modified FTND i.e. Heaviness of Smoking Scale (HSI) (Heatherton et al. 1989, Heatherton et al. 1991, Prokhorov et al. 2000). Nicotine dependence classified with HSI consists of two items scoring the time to the first cigarette of the day and the number of cigarettes smoked per day and it consists of four categories; mild (0-1), moderate (2), strong (3) and very strong (4-6) dependence (Heatherton et al. 1989).

### *4.1.4 Data collection*

The first visit included a standardized personal history, individualized smoking counselling by Motivational Interviewing technique (MI) and assessment of symptoms (chronic cough and

sputum). At the follow-up visit, the same analyses were repeated and the smoking status assessed. The same study nurse accomplished 30 minutes personal interview on both visits.

The study nurse had been trained in smoking cessation techniques including behavioral treatments in 2003. This 2-3 days tailored education was organized by Finnish Lung Health Association (Filha). All individualized counselling sessions took 30min in time and aimed to determine patient´s readiness to change by asking, informing and listening with MI technique. The evidence based effectiveness of brief behavioral interventions adapting the principles and techniques of MI to smoking cessation had been published (Dunn et al. 2001). Counselling was forwarded to each participant, because evidence based benefits were associated with individualized counselling in efforts to stop smoking (Lancaster & Stead 2005). NRTs and buprobion were offered to each participant if not contraindicated. Varenicline was not on the market at the start of this study.

Abstinence from smoking was verified by analyzing urine cotinine (Bruckertet al. 1992, Gorber et al. 2009). This is a semi-quantitative cotinine test with Accutest NicAlert Urine® (Nymox Co., Canada). This test results in 10 minutes displaying in cotinine equivalence for each level as 1-10 ng/ml (level 0), 10-30ng/ml (level 1), 30-100ng/ml (level 2), 100-200ng/ml (level 3), 200-500ng/ml (level 4), 500 -2000ng/ml (level 5) and over 2000 ng/ml is the equal to level 6. For urine test level 3 or above indicates the use of tobacco products. Measures of cotinine are shown to be better than exhaled CO content in discriminating smokers and nonsmokers and the tests are of choice for research protocols (Jarvis et al. 1987).

Respiratory symptoms and quality of life were measured by St George´s Respiratory Questionnaire (SGRQ) on both visits. Flow-volume-spirometry was conducted both before and after bronchodilatation (0.4mg salbutamol) only as part of the second visit. The diagnosis was based on the GOLD criteria with $FEV_1$ elevation < 12%, if a greater than 12% increase was observed after salbutamol administration, detailed history and peak flow follow-up (2 weeks) with sympathomimetic therapy were conducted to exclude asthma. COPD was defined according to GOLD criteria ($FEV_1/FVC$ < 70% after bronchodilatation) available from website http://www.goldcopd.com (Rabe et al. 2007b), and to Finnish national diagnostic criteria for obstruction using reference values where $FEV_1/FVC$ < 88% of predicted and $FEV_1$

< 80% of predicted are indicative for obstruction (Viljanen et al. 1982). Mid-expiratory flow (MEF50) was assessed from the flow-volume spirometry.

#### *4.1.5 Statistical methods*

The IBM SPSS Statistics software was used in all statistical analyses. Frequency and percentage distributions were used to demonstrate the results of the main outcome variables. The statistical significance of the differences in frequency distributions was evaluated by the chi-square test. Spearman's rank correlation coefficient was used to analyze the relationship between lung function and nicotine dependence variables. The mean values of age and BMI in subgroups of smokers were compared using one-way analysis of variance. Box plots were used to illustrate the findings. Since pack-years and HSI displayed a right-skewed distribution, Kruskal-Wallis test was used to demonstrate differences in medians of these variables between subjects who continued smoking compared with those who stopped. McNemar´s test was used to evaluate the significance of the changes in respiratory symptoms (prolonged cough, sputum production) between the baseline and the two-year control visit.

### 4.2 Smoking habits, prolonged respiratory symptoms and smoking cessation (II, III)

#### *4.2.1 Participants*

A cross-sectional study was undertaken with 1186 Finnish conscripts during their service time in the Light Infantry Brigade and Lapland Antiaircraft Regiment, part of the Northern Command of the Finnish Defence Forces in Northern Finland during autumn 2008 to spring 2009 with a high response rate (80%). Twelve women were excluded from the study due to their low number. In all, 33 former smokers were excluded as were11 subjects with unreliable or incomplete filling of the questionnaire. In the final sub-study II there were 1130 young males, and 614 (54.3%) of them were daily smokers, who were included into sub-study III. All male smokers felt themselves healthy.

### *4.2.2 Main outcome variables*

In sub-study II, the primary research question was to assess relationship between prolonged respiratory symptoms and smoking habits in young adult males. The symptoms of chronic bronchitis i.e. prolonged cough and or sputum production were self-reported in the questionnaire.

In sub-study III, the main outcome variable was the successful quitting of smoking in young male adults. The second main outcome variable was the use of pharmacological aids in smoking cessation in daily and in occasional smokers. All study subjects completed the standardized questionnaire with quitting attempts and aids used in smoking cessation retrospectively.

### *4.2.3 Explanatory variables*

In sub-study II, smoking habits were used as a primary explanatory variable to explain respiratory symptoms. Age, height, weight, BMI, education and pack-years were used as other potential covariates to explain prolonged respiratory symptoms in daily and occasional smokers. Exposure to environmental tobacco smoke was also considered as an explanatory variable to explain respiratory symptoms.

In sub-study III, age, education, age of initiating smoking, pack-years, variables of nicotine addiction i.e. nicotine dependence (HSI), number of quit attempts, self-assessment of nicotine addiction, intent to quit, and the first cigarette in the morning were used as potential explanatory variables to explain use of pharmacological aids in smoking cessation.

### *4.2.4 Data collection*

All participants were asked to fill a specific questionnaire voluntarily and anonymously during their service time. This questionnaire consisted about sex, height, weight, education, physical fitness, symptoms of cough and sputum, exposure to environmental tobacco smoke, detailed smoking habits and use of tobacco, and smoking cessation attempts. The questions about the smoking habits were based on a Finnish nationwide study questionnaire, The

Adolescent Health and Lifestyle Survey (Rimpelä et al. 2007). The subjects were classified as daily smokers, occasional smokers and non-smokers. A daily smoker was defined as a person who currently smoked at least one cigarette daily. An occasional smoker was defined as a subject who currently smoked less than one cigarette daily or reported having smoked over 50 cigarettes in the past, and at the time considered himself as a smoker. A non-smoker was a person who had smoked under 50 times in his life and did not consider himself as a smoker (Rimpelä et al. 2007). Nicotine dependence for daily smokers was calculated according to the Heaviness of Smoking Index (HSI) (Heatherton et al. 1991). Respiratory symptoms indicative of self-reported chronic bronchitis were assessed by the question "Have you ever had cough with sputum production on most days or nights for at least 3 months yearly?" as these symptoms may predict COPD development (Lindstrom et al. 2001, Kotaniemi et al. 2005, de Marco et al. 2007, Rabe et al. 2007b, de Marco et al. 2011).

### *4.2.5 Statistical methods*

Chi-square test was used to analyze the differences in respiratory symptoms. Since tobacco usage variables (daily usage, smoking years and pack-years) had a right-skewed distribution Kruskal-Wallis test was used to evaluate the statistical difference between respiratory symptoms groups. The binary logistic regression was used to evaluate the relationship between self-reported prolonged respiratory symptoms and smoking habits with potential confounders (BMI, age and education).

We compared the distribution of age, education, age of smoking initiation, nicotine dependence (HSI) and self-reported nicotine addiction in smoking cessation groups using cross-tabulation. We used confidence intervals (CI) to display the prevalence of main outcome variables. The statistical significance in frequency distributions between categorical variables and smoking groups was evaluated using chi-square test or Fisher´s test. Independent samples t-test was used to compare the distributions of the continuous variables between the daily and the occasional smokers.

The IBM SPSS Statistics software was used in all statistical analyses in sub-studies II-III.

### 4.3 Discovering and evaluating new biomarkers in COPD (IV, V)

#### *4.3.1 Samples*

##### *4.3.1.1 Lung tissues (IV)*

Samples of lung tissue specimen were collected by lung surgeons from solitary lung tumors or hamartomas or lung transplantations in Helsinki University Hospital. In all, there were 27 middle-aged patients, of whom seven had GOLD stage II-III (moderate - severe), seven with GOLD stage IV COPD (very severe), seven with AATD and seven non smoker controls. Four subjects of these controls were never smokers, two had quitted at least 2 years ago after having smoked 10-12 years. All patients with GOLD IV COPD or AATD have quitted at least 2 years ago before surgery. Patients with COPD and 7 subjects having AAT deficiency were using 5-10mg oral prednisolone and/or inhaled corticosteroids as regular medications, whereas none of the other subjects were receiving regular corticosteroid therapy. Tissues from 9 middle-aged patients having idiopathic pulmonary fibrosis (IPF) were used as a disease control in immunohistochemistry and 3 IPF patients in the 2-DE and Western blot analyses of the lung homogenates. IPF patients had histopathology of usual interstitial pneumonia (UIP) and they were never smokers except one.

##### *4.3.1.2 Induced sputum (IV, V)*

The sputum specimens and the blood samples (IV) were obtained from the subjects in sub-study I of Lapland Central Hospital and the patients of the Helsinki University Hospital. Details of subject characteristics are described in sub-study I. Altogether, 11 study subjects were healthy heavy smokers and 11 having GOLD stage II COPD. A group of 10 healthy never smoker controls in this sub-study are part of participants in sub-study I.

This study group (V) is a sample of all subjects in a trial described in detail in sub-study I. In all, 446 (255 men and 191 women) healthy daily smokers and 34 never smoker controls were included to this study. Sputum was induced from 91 smokers and 18 nonsmokers. A blood test was taken and the study nurse performed the induced sputum collection at both visits with each participant. The same procedures were repeated twice during the two-year follow-up.

### *4.3.2 Main research questions*

In sub-study IV, the primary research question was to compare healthy and diseased tissue profiles in non-hypothesis driven proteomics. The second primary research question was to identify these findings. The third research question was to investigate the association of these findings with smoking and COPD. Normal lung tissue, COPD lung, fibrotic (IPF) lung and induced sputum of non smokers, smokers and COPD patients were used as samples in this study.

In sub-study V, the first primary research question was to assess effects of daily smoking on plasma SP-A levels. The second primary research question was to assess effects of smoking cessation to plasma levels of SP-A during two-year follow-up. The third primary research question was to study SP-A levels in induced sputum of nonsmokers and smokers. The SP-A levels in plasma and in induced sputum were the main response variables.

In sub-study IV, several methodologies were used in research approach (two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), Western blotting and immunohistochemistry with morphometry) to study COPD specific biomarkers in lung /airway tissue compared to healthy controls, and the effects of smoking on these biomarkers.

In sub-study V, smoking status was the primary explanatory variable to explain plasma and sputum SP-A level. In the analyses age, BMI, pack-years, nicotine dependence (modified FTND i.e. HSI) and smoking cessation were used as other explanatory covariates to explain the plasma and the sputum SP-A levels. Lung function variables and prolonged cough with sputum production were also considered as explanatory variables.

### *4.3.3 Processing of the samples*

#### *4.3.3.1 Lung tissues (IV)*

The lung tissues for immunohistochemistry were fixed in 10% [v/v] buffered formalin and embedded in paraffin. Tissue specimens for 2-DE and Western blot analysis were snap-frozen in liquid nitrogen and kept in -80˚C.

*4.3.3.2 Sputum induction and sputum processing (IV,V)*

Sputum was induced by inhalation of 4.5% hypertonic saline as recommended by the European Respiratory Society taskforce (Vignola et al. 2002, Djukanovic et al. 2002, Kelly et al. 2002) and samples processed as previously described (Rytilä et al. 2006). The expectorated sputum samples were processed with dithioerythritol (DTE; Sigma, Munich, Germany) and phosphate-buffered saline. Suspensions were filtered through 70-µm nylon gauze and centrifuged at 400x g at 4˚C for 10 min. The supernatants and pellets were immediately frozen to -80˚C.

In sub-study V, the sample was smeared over glass slides, fixed and stained with Papanicolau stain, for the differential cell count, and at least 200 cells were counted by an experienced cytotechnologist and a pathologist blinded to the subject data. Detailed cell profiles were assessed for all sputum samples. Since SP-A is expressed in alveolar epithelium and bronchial epithelial cells of smokers, and in the tracheal epithelial cells, all samples i.e. also those containing epithelial cells, were included in the analysis. The samples were frozen at -80˚C.

*4.3.3.3 Plasma (V)*

Peripheral whole venous blood was collected into EDTA tubes, plasma was prepared by centrifugation for 10-15 min at 1,500 x g and stored at -80˚C until analyzed (V).

***4.3.4 Proteomics (IV)***

*4.3.4.1 Two-dimensional gel electrophoresis (2-DE)*

Frozen tissue from control, GOLD stage IV COPD and IPF lungs were processed as described earlier (Lehtonen et al. 2008). In brief, proteins were separated by isoelectric focusing (IEF) in immobilized pH gradient strips (pH 4-7, 18 cm, GE Healthcare) with the Multiphor II system (GE Healthcare) under paraffin oil for 55 kVh followed by SDS-PAGE in polyacrylamide gels (12.5%) overnight with the Ettan DALT II system (GE Healthcare). The gels were silver stained and analyzed with the 2-D PAGE image analysis software Melanie 3.0 (GeneBio), and reproducible changes in spot intensity (at least 2-fold) were marked in the

gel. The exact position (isoelectric point, molecular weight) of the SP-A spots was determined from our reference 2-D gel of human lung (pH4-7) with identified proteins.

*4.3.4.2 Protein identification using mass spectrometry (MS)*

For protein identification, excised spots were digested as described earlier (Lehtonen S et al. 2008). Peptide masses were measured with a VOYAGER-DE STR (Applied Biosystems), and proteins identified by full database search (Aldente database version 11/02/2008). Since the SP-A peptides in the mass spectrum matched to SP-A1 and SP-A2, the spot-specific minor peptides were also analyzed to reveal which SP-A protein might be present. These peptides were compared with the theoretical peptides specific for SP-A1 and SP-A2 calculated according to PeptideMass. It can be found on http://www.au.expasy.org/tools/peptide-mass.html.

### *4.3.5 Immunohistochemistry and morphometry (IV)*

The paraffin-embedded tissue sections were deparaffinised with xylene and rehydration was done in a graded series of ethanol. The sections were incubated in citrate buffer (pH6) in a microwave oven for 10 minutes to disclose the antigens. Endogenous peroxidase activity was eliminated with 0.3% hydrogen peroxidase. Histostain Plus Kit (Zymed Laboratories Inc., USA) was used for immunostainings according to the manufacturer´s instructions and with rabbit polyclonal SP-A antibody (AB3240, Chemicon, Temecula, USA) as the primary antibody at a dilution of 1:8000. Detection was performed with anti-rabbit secondary antibody horseradish peroxidase conjugated to SP-A and AEC chromogen (Zymed). Counterstaining was performed with Mayer- hematoxylin and mounted on glass slides. To determine the specificity of the staining series, negative control sections were treated with mouse isotype control (Zymed) or PBS. SP-A antibody and TTF-1 (Thyroid Transcription Factor 1.07-601, Upstate, Lake Placid) were used for sections in double staining. Digital images for morphometric analysis were taken using 200 x magnification and saved as Photoshop JPG files. The area of positively vs. negatively stained tissue was measured using Image Pro software (Media Cybernetics, UK).

### *4.3.6 Western Blot (IV)*

Western blot detection of SP-A was performed using standard procedures. Both tissue samples and all sputum supernatants were homogenized to obtain protein extracts. The samples were dissolved to 5% 2-mercaptoethanol in sample buffer (Bio-Rad, Hercules, CA). Twenty to 40 µg of protein was loaded per lane to polyacrylamide gels. Electrophoretic protein separation was done in a 12% polyacrylamide gel at 100V for one 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 Tris-buffered saline (TBS) containing 1% Tween. To verify if the new protein findings were dependent on the specificity of the antibody, two different SP-A antibodies were used, both of which showed similar results. The antibodies were mouse monoclonal SP-A antibodies (MAB3270 Chemicon, Temecula, CA or HYB-238 Santa Cruz Biotechnology Inc., Santa Cruz, CA, respectively). Sheep anti-mouse (IgG) horseradish peroxidase conjugated secondary antibody (Amersham Biosciences, U.K.) was used at 1:50 000 dilution. Immunodetection was performed with the chemiluminescent HPR-substrate immunodetection kit (Millipore, Billerica, MA). Membranes were exposed to X-ray film (Kodak, Rochester, NY). Since the expression of housekeeping proteins e.g. β-actin but possibly also others can vary in airway and parenchymal lung diseases including COPD (Glare et al. 2002, Ishii et al. 2006, Peltoniemi et al. 2006, Casado et al. 2007), the protein concentration was verified by measuring it in triplicate and equal loading was ensured by Ponceau S staining of the membranes (Sigma Aldrich, St. Louis, MO). Densitometric analysis for the Western blot bands was done with the Image I 1.37V software (National Institutes of Health, MD). Western blotting from the same control and COPD homogenates were also investigated for the expression of other three surfactants, namely SP-B, SP-C and SP-D (Sc-53137, Sc-13979, Sc-59695, Santa Cruz Biotechnology Inc., Santa Cruz, CA respectively).

### *4.3.7 Statistical methods*

The IBM SPSS Statistics software was used in all statistical analyses. In studies IV and V, statistical differences in continuous variables i.e. demographical characteristics, lung functions and SP-A levels between smoking groups were evaluated using one-way ANOVA or Kruskal-Wallis test. The pairwise comparisons were analysed using Tukey´s test or

Benjamini-Hochberg procedure (pairwise Mann-Whitney tests after Kruskal-Wallis test). Repeated measures analysis of variance was used to examine the change in plasma SP-A levels and the interaction between visits and smoking status (smokers vs. quitters). Pearson´s correlation coefficient and scatter plots were used to evaluate the associations between plasma SP-A levels and other continuous variables.

# 5. RESULTS

## 5.1 Smoking habits and smoking cessation in middle-aged adults (I)

### *5.1.1 Characteristics of subjects*

The basic characteristics of study subjects are shown in Table 3. The mean age and BMI in males and females did not differ significantly. The subjects were heavy smokers and the median of pack-years was 29.0, although the women had smoked less than men. The median of nicotine dependence (modified FTND) was 3.0, but 20% all women displayed weak nicotine dependence, and heavy dependence was more often present in men (Table 3). Men had more often graduated from high school than women, and more often were owners of business companies (13.9% vs. 5.2%). A total of 60% of women had completed vocational school, but in contrast 31.9% of them had completed only primary school.

**Table 3.** Basic characteristics of adult smokers by gender (Study I).

| Variables | Male<br>n = 331 | Female<br>n = 213 |
|---|---|---|
| Age; mean (SD) | 54.5 (9.1) | 52.4 (8.9) |
| BMI; mean (SD) | 27.7 (3.8) | 26.7 (4.3) |
| Age of smoking initiation: mean (SD) | 16.0 (3.3) | 17.2 (3.7) |
| Smoking in pack-years: median (lower and upper quartile) | 32.0 (25.0, 41.0) | 24.0 (18.0, 31.0) |
| HSI [1] | n (%) | n (%) |
| • weak (0-1) | 50 (15.2) | 43 (20.2) |
| • moderate (2) | 51 (15.5) | 32 (15.0) |
| • strong (3) | 118 (35.9) | 92 (43.2) |
| • very strong (4-6) | 110 (33.4) | 46 (21.6) |
| Education | | |
| • high school | 47 (14.2) | 17 ( 8.0) |
| • vocational school | 199 (60.1) | 128 (60.1) |
| • primary school | 85 (25.7) | 68 (31.9) |

[1]Data on HSI index is missing from two males.

### *5.1.2 Success in smoking cessation*

In all, 23.3% (n=127) of subjects stopped smoking, and 93 of them had stopped at the two years follow-up. In fact, 34 had quit before attending the first visit after reading the newspaper announcement or quit at the time of the baseline investigations. There were four statistically significant factors that predicted successful quitting i.e. positive attitude to the intervention, use of pharmacotherapy, older age, and higher BMI. Other factors such as the extent of airway obstruction, cough, gender, pack-years, or nicotine dependence displayed no

significant association with the ability to achieve successful cessation. Furthermore, the mean age of starting smoking had no impact on the success of smoking.

## 5.2 Smoking habits and smoking cessation in young adult males (III)

### *5.2.1 Characteristics of subjects*

A total of 614 male smokers aged 18-26 years completed the study questionnaire. In all, 85.2% (n=523) were daily smokers and 14.8% (n=91) were occasional smokers. The mean age of daily and occasional smokers did not differ (19.5 vs. 19.4 years). However, daily smokers had started to smoke earlier than occasional smokers (14.7 vs. 19.4 years), they had smoked for longer time than occasional smokers (4.8 vs. 3.4 years) and approximately one in three (63.1%) of them had smoked 3 or more in pack-years. Nearly all of them claimed to be aware of COPD, but 34 % of them considered COPD as a very severe pulmonary disease of elderly people.

### *5.2.2 Success in smoking cessation*

Nearly all daily smokers (93.4%) felt themselves to be nicotine addicted to some extent. Figure 2 shows the percentage of young daily smokers trying to quit smoking by nicotine dependence groups. The time delay after waking up in the morning to the first cigarette did not explain quitting attempts. A history of heavy smoking (over 6) in pack-years did not explain attempts of quitting smoking, because as many of those who had smoked less (under 3 pack-years) had made unsuccessful quit attempts in the past. The self-assessed nicotine dependence was related to the quit attempts: the more they felt addicted the more often they had tried to quit (Figure 2).

In all, 55.6% of the daily smokers had tried to quit, and as many as 20.2% of them had made at least three quit attempts, whereas 70% of the occasional smokers had not made any quit attempts. The self-assessed nicotine addiction of daily smokers differed from that of occasional smokers: over half of the occasional smokers (56.0%) who did answer this questionnaire felt themselves to be nicotine addicted at least to some extent.

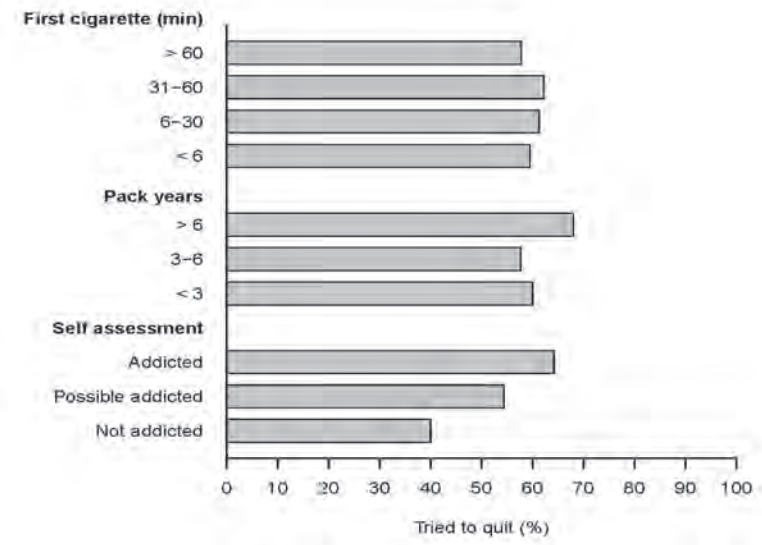


**Figure 2.** Percentage of young adults trying to quit smoking by nicotine dependence variables (N=523)

### *5.2.3 Use of pharmacological aids*

Figure 3 presents the percentage use of different types of NRT. The majority of daily smokers (52.8%) had not used any pharmacological aids in their quitting attempts, but if they had used something that had most often been nicotine gum (34%). Only two subjects had taken bupropion or varenicline. The study subjects did not admit having used community pharmacists´ advice or support. However, the more the daily smoker had made quit attempts, the more likely he had used NRT. As many as 40% of those who had made many quit attempts (five or more), had not included pharmacological aids in their strategies.

The descriptive analysis of use of any pharmacological treatment by nicotine dependence is illustrated in Figure 4. The stronger the self-assessed nicotine dependence, the more likely the subject had attempted to quit by using NRT (somewhat dependent 23.8% vs. severely dependent 48.9%). Furthermore, the age of initiating smoking, the first cigarette in the morning or education of daily or occasional smokers did not associate significantly with the use of NRT.

Though as many as 25% of occasional smokers reported themselves as being nicotine addicted, and only a few of them had tried NRT (n=12, 24%). Most of the occasional smokers tried to quit without NRT (80%), no matter how many quit attempts they had made.

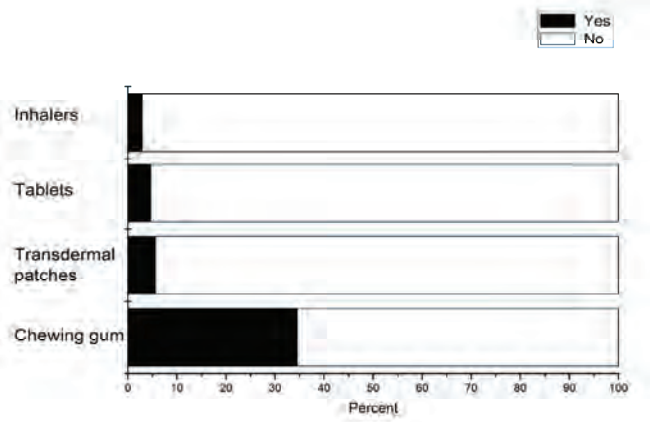


**Figure 3.** Distribution of the different types of nicotine replacement therapy (NRT) used by all young adult smokers.

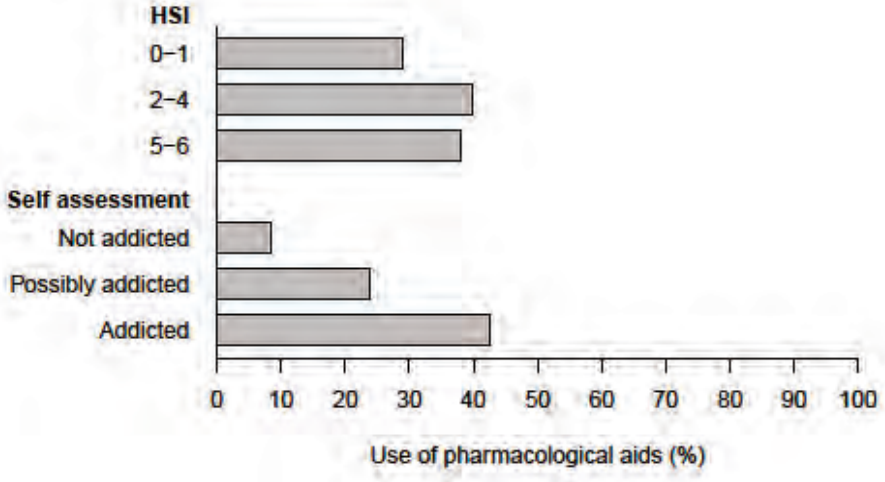


**Figure 4.** Percentage of daily smokers using pharmacological aids (NRT) by HSI and self-assessment of nicotine addiction (n=523).

### 5.3 Prevalence of COPD in healthy heavy smokers (I)

#### *5.3.1 Spirometry results*

All participants performed flow-volume spirometry with the bronchodilatation test during the second visit. In all, 25 smokers had 12% or ≥ 200ml increase after the bronchodilatator, and 17 of them were confirmed to have COPD, five were healthy and one subject with COPD fulfilled the asthma criteria as estimated from the PEF recordings. About one in every ten (11.0% n=60) of the subjects had $FEV_1/FVC < 70\%$ and 15.3% (n=83) had $FEV_1 < 80\%$ of predicted. MEF50 was reduced in 19.5% of the subjects. Three lung function parameters, $FEV_1/FVC$, $FEV_1\%$ of predicted and MEF50 showed a statistically significant correlation with pack-years. In all, these variables were not significantly associated with nicotine dependence. Spirometry revealed COPD by GOLD criteria in 11.0% and by national guidelines in 15.3%, mid-expiratory flow (MEF50) had significantly declined in 19.5%. COPD seems be common in heavy middle-aged smokers apart from diagnostic criteria used.

### 5.4 Prolonged respiratory symptoms in relation to smoking habits (I, II)

#### *5.4.1 Prolonged respiratory symptoms in middle-aged adults*

The majority, 62% (n=339), of subjects reported chronic cough or sputum production, in all 186 (34.2%) had both these symptoms. The presence of cough and/or sputum production did not correlate with pack-years. Furthermore, nicotine dependence or the presence of cough did not have any significant effect on the success of smoking cessation.

#### *5.4.2 Prolonged respiratory symptoms in young adults*

The prevalence of self-reported chronic cough and sputum production was high in daily smokers (40.7%) and occasional smokers (26.9%) when compared to non-smokers (12.0%). The difference between smoking subgroups remained highly significant after taking into account the potential confounders (BMI, age, education). The median of pack-years was 4.5 with symptoms, 3.0 with no symptoms, and 3.85 for those responding “I don´t know”. Self-

reported symptoms of prolonged cough and sputum production were very common both in young daily and in occasional smokers compared to non-smokers.

To summarise, middle-aged daily smokers are accustomed to their prolonged symptoms of chronic bronchitis without being concerned that this is one of the early signs of worsening health. Chronic respiratory symptoms may precede COPD development, and these symptoms are very common in young adult daily smokers.

## 5.5 SP-A as a biomarker of COPD (IV,V)

### *5.5.1 Results of the comparison of tissue and sputum in healthy and diseased subjects*

The 2-DE approach revealed nine spots localized in the same gel area within two "spot trains" which were only detectable in 2-D gels lung specimens from severe, GOLD stage IV COPD patients.

Furthermore, the MS analyses revealed that all changed spots corresponded to SP-A. Human SP-A consists of two isoforms, SP-A1 and SP-A2. In comparison with theoretical SPA peptide with PeptideMass, the spot-specific peptides were found to match to several peptides characteristic of SP-A2, suggesting that SP-A2 is affected in COPD.

Expression of SP-A was investigated by Western blot analyses of control lung, and lung of patients with various COPD severities (GOLD stage II – IV). These analyses revealed that SP-A is already elevated at an early stage of COPD. Additionally, lung tissue of AATD patients showed highly elevated SP-A levels when compared to control lung. Western blot analyses also revealed that other surfactant proteins, SP-B, SP-C, and SP-D were not affected by COPD.

The distribution of SP-A in lung tissue was further investigated by immunohistochemistry to clarify its role in COPD. SP-A was found to be expressed not only in the alveolar epithelium in non-smokers, but also in the bronchial epithelium near to the basement membrane in smokers and patients with COPD. Morphometrical quantification of SP-A positive areas

revealed a 3.7-fold elevation in stage II-III COPD, 3.4 -fold in stage IV COPD and 7.5-fold in AATD when compared to the control lung.

Additionally, analyses of 32 induced sputum specimen of COPD patients, current smokers and non-smokers by Western blot revealed that increased SP-A levels were detected in the sputum of COPD patients with the exception of some non-smokers and healthy smokers with sporadic elevated levels of SP-A.

Samples of IPF/ UIP lungs were studied by many different approaches (Western blot, 2-DE, and immunohistochemisty with morphometry) because of earlier studies with controversial results. However, SP-A levels were not elevated in the lung tissue of IPF/UIP patient.

In conclusion, highly elevated levels of SP-A were detected in the lung tissues of COPD patients, but not in IPF/UIP patients. The results were confirmed by several different approaches including 2-DE, Western blot and immunohistochemistry. In addition, elevated levels of SP-A were found also from induced sputum supernatants of stage II COPD patients.

### *5.5.2 Testing SP-A as a biomarker in COPD*

#### *5.5.2.1 Measurement of SP-A in plasma and sputum supernatant samples*

In sub-study V, S-PA levels were assessed using a commercially available EIA kit (SP-A test; Sysmex, Kobe, Japan) (Takahashi et al. 2000, Kobayashi et al. 2008) suitable for frozen serum and plasma. This test has undergone a thorough detailed evaluation and is approved as a clinically validated assay. The reference value for plasma is $\leq 43.8$ ng·mL$^{-1}$. The intra-assay coefficients of variation ranged from 4.5% to 6.2% and inter-assay coefficients of variation from 4.8% to 7.2% (Sysmex). Five concentration levels were used as standards for the analyses. No drifts of standard curves with time were seen. The method was adapted for the sputum supernatants without any major modifications.

*5.5.2.2 Plasma SP-A levels in smokers*

SP-A could be detected in all plasma samples. During the two year follow-up period, 23% (111/474) of study subjects succeeded in quitting smoking. There was no significant difference in plasma SP-A concentrations between healthy and asymptomatic smokers, or between males and females in non-smokers or all smokers. The plasma level of SP-A was elevated in smokers, but its level declined after smoking cessation during two-year interval as shown in Figure 5. On the other hand, there was an increasing trend towards SP-A elevation during the two year interval in those 324 subjects who continued smoking.

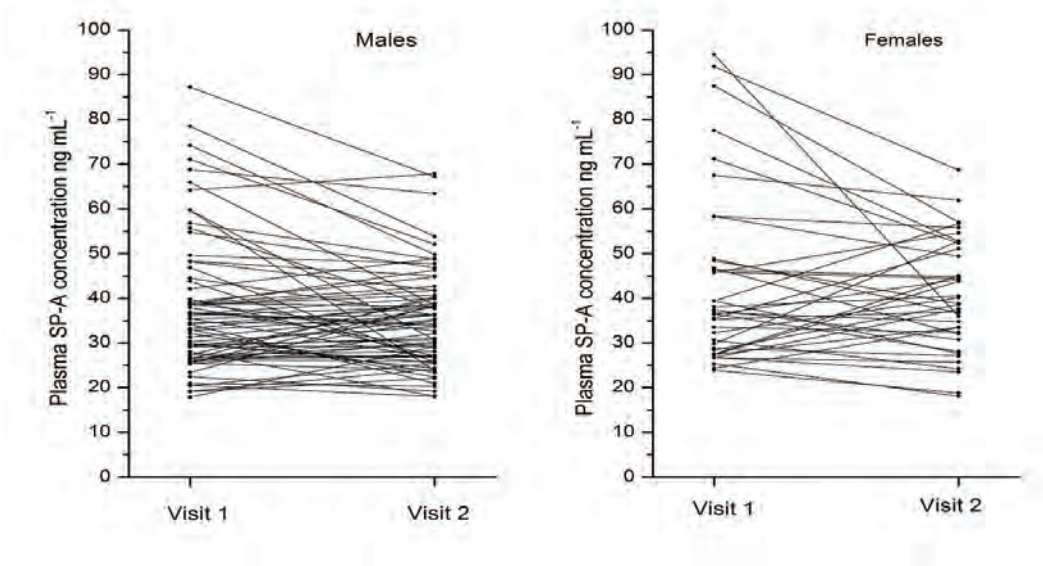


**Figure 5**. Distributions of plasma SP-A concentrations by gender during the first visit and repeated after smoking cessation two years later during the second visit. The total of 111 quitters was made up of 78 males and 31 females. Two cases with extreme high SP-A values are excluded.

*5.5.2.3 Sputum SP-A levels in smokers and in non-smokers*

The cell profile of the induced sputum samples revealed significantly higher numbers of neutrophils in both groups of smokers than in nonsmokers. There were no significant differences in cell counts between healthy and symptomatic smokers.

About one in three of the subjects (31/109) claimed that they experienced prolonged respiratory symptoms i.e. chronic cough and/or sputum production. SP-A levels were significantly higher in symptomatic smokers compared to nonsmokers. In contrast, the SP-A levels between healthy and symptomatic smokers did not differ significantly.

In conclusion, this was the first study on plasma and sputum SP-A from the same subjects. On the other hand, SP-A levels have not been previously studied in smokers with the emphasis on symptoms of chronic bronchitis. Plasma SP-A levels were high in daily smokers, and the absence or presence of respiratory symptoms in smokers had a significant effect on the sputum SP-A concentration, since the SP-A concentration is substantially elevated in the induced sputum supernatants of smokers with chronic bronchitis.

# 6. DISCUSSION

## 6.1 Summary of aims and results

The first aim was to assess the smoking habits and smoking cessation in healthy adults. One individualized 30 minute long counselling session using the MI-interviewing technique combined with pharmaceutical therapies achieved 23.0% success in smoking cessation over a two year period in daily smoking adults. The rate of success displayed a clear association with positive attitude of a smoker and with pharmacotherapy but not with the extent of airway obstruction. Currently, daily smoking is still very common among young adult males (> 45%) in northern Finland. Young adult smokers seem to be familiar with current pharmacotherapies, but they may need more professional counselling services aimed especially at this age group.

The second aim was to evaluate the prevalence of COPD, the role of early detection of airway limitation and the prevalence of symptoms of chronic bronchitis in a cohort of smoking adults. Screening revealed that over 10% of "healthy" adult smokers with over 20 pack-years smoking history had COPD. Furthermore, prolonged respiratory symptoms were very common, nearly half of daily smokers admitted suffering from prolonged cough and/or sputum production. Although in spirometry $FEV_1$/FVC, $FEV_1$% of predicted and MEF50 values all displayed a significant correlation with pack-years, these variables were not significantly associated with the level of nicotine dependence (HSI).

The third aim was to study the association of prolonged respiratory symptoms to smoking habits in young adult males. Prolonged cough and sputum production were very common already in young male smokers (40.7% of daily smokers, 26.9% of occasional smokers vs. 12.0% of non-smokers), thus posing a serious risk to their future health.

The fourth aim was to identify potential COPD biomarkers by non-hypothesis driven un-biased proteomics and then to characterize the proteins by several other technologies including Western blot, immunohistochemistry and morphometry. The levels of protein SP-A, but not other surfactant proteins, are elevated in COPD but not in the normal or fibrotic lung. Furthermore, current smokers have elevated plasma SP-A levels, and its level is highly

elevated in the induced sputum of smokers compared to nonsmokers. In addition, plasma SP-A levels are higher in individuals who continue to smoke compared to those who give up smoking. These results indicate that SP-A is linked to the pathogenesis of COPD and may be considered as a potential COPD biomarker.

### 6.2 Smoking habits and smoking cessation in healthy middle-aged adults

In the prospective part of this study, the majority of the smokers wanted to quit and a significant proportion of these chronic heavy smokers (23.3%) also succeeded in stopping smoking. Thus, the intervention conducted within the present 2-year follow-up study was successful in helping chronic smokers stop smoking. This study included one individualized counselling session by an experienced nurse combined with evidence based pharmacological therapies. It needs to be emphasized that the study subjects were recruited on the basis of newspaper announcements with a general discussion of health issues and the latest legal restrictions related to smoking (neither cessation counselling nor spirometry testing was mentioned). Therefore the study subjects do not represent the whole target population.

Although subjects felt themselves asymptomatic at baseline after over 20 years of smoking, they obviously were concerned about their health; moreover, those who succeeded in smoking cessation were more receptive to the face-to-face level anti-smoking information than those who continued smoking. In the present study, we did not evaluate symptoms of withdrawal and craving, which may be important in terminating quitting attempts and predicting relapse (Shiffman et al. 2004). Smokers in this study were very dependent on nicotine according to traditional measurements i.e. HSI and pack-years, but this did not seem to predict success in quitting attempt. This may be caused by effects of everyday life factors e.g. stressors, opportunities to smoke and alcohol use affect individual success in giving up smoking over a two year interval; perhaps they require more than one counselling intervention to gain assistance from pharmacotherapy. Some studies have shown that it is post-cessation depression, but not withdrawal symptoms, craving, or weight gain, that predicted relapse (Hughes 1992). Many relapses have been found to occur within 2 weeks after quitting (Kenford et al. 1994, Hughes et al. 2004), but even 10% of long-term abstainers relapse every year, thus it is debatable whether the two year follow-up was sufficient to assess the permanent quit rate (Baker et al. 2011). Based on the present study, neither the presence of

cough and sputum nor cumulative tobacco exposure appeared to have any major role in predicting success in smoking cessation.

So far, the evidence of effects of behavioral interventions on the long term quitting rate is more controversial (Kotz 2008). A meta-analysis conducted by Strassman et al. (2009) emphasized the importance of smoking cessation counselling in addition to the pharmacotherapy on the success of prolonged abstinence. Furthermore, Hajek et al. (2009) did not find any significant benefits of behavioral interventions for relapse prevention to reduce the proportion of recent quitters who return to smoking. Nonetheless, Kotz (2008) claimed, that tailored behavioral intervention alternatives could be better targeted and thus improve the quitting rates, if it was possible to identify different phenotypes of smokers. In this present longitudinal study, the smoking cessation counselling followed the technique of Motivational Interviewing (Dunn et al. 2001), and was combined with pharmacotherapy. This did seem to be an effective way to achieve success in smoking cessation in this selected population.

At the very beginning of this study, lung function data was not available. In contrast to many similar studies, the correlation with lung function values was obtained from a blinded examination of spirometry which was conducted at the 2-year follow-up control. In this respect, the results differ from many other studies such as those of Bednarek and co-workers (Bednarek et al. 2006), which conducted the spirometry at the very beginning of their study. Based on the results of the present study, smokers with COPD diagnosis did not succeed better in smoking cessation than others, which is in line with results obtained in another randomised controlled study with spirometry, where about 88% of smokers did not succeed in quitting at all or relapsed during 12 months follow up (Kotz et al. 2009a). This present study reveals the importance of motivation and one efficient individualized counselling combined with pharmacotherapy.

### 6.3 Smoking habits and smoking cessation in young adult males

The results revealed that young adult male daily smokers are more nicotine dependent than occasional smokers. In addition, the daily smokers had made several unsuccessful quit attempts without the benefit of NRT, which may be a reflection of the poor availability of

professional cessation services targeted at this age group. These results are in full agreement with a Finnish study (Rainio et al. 2009) in which 83% of late adolescent daily smokers described themselves as being addicted to smoking. In addition to daily smokers, there is also a considerable group of occasional smokers with a lower level of nicotine addiction, but the risk for this group to start daily smoking is unknown. The rapid development of nicotine dependence needs to be taken seriously and thus efficient anti-smoking campaigns should be targeted at young smokers, not only at adult chronic smokers with smoking histories lasting decades.

Young adult smokers may not be familiar with all available aids. There is one report suggesting that many smokers have used or planned to use a smoking cessation treatment, but only a minority who try to quit actually use the quit line or seek counselling. There are controversial results about whether uncontrolled use of NRT without any counselling can actually have harmful consequences (Stead & Lancaster 2007, Bentz 2009), but it is likely that a combination of several other methods in conjunction with NRT is most efficient and safe. Most young male smokers want to quit; they try to quit with the help of NRT but without counselling services and thus they are likely to relapse.

For example, young adults may prefer to access cessation services from social media sources and in this way they may not resemble older smokers, although evidence is still lacking for this proposal (Narhi & Helakorpi 2007, Vangeli & West 2008). There is insufficient evidence available to recommend that any specific behavioural intervention would especially help quitters or prevent relapse (Hajek et al. 2009). Prospective studies on smoking cessation need to be conducted before we are to obtain important information relevant to those young adults who have started to smoke.

### 6.4 Prevalence of COPD in middle-aged daily smokers

Though typical symptoms of COPD include chronic cough, sputum production and in more severe cases dyspnoea, still many of the smokers who consider themselves as being asymptomatic, may have COPD; this was also noted in this study. Based on earlier studies, as many as 31 - 45% of chronic smokers may have COPD, even though they have not received a

definite diagnosis (van Schayck & Chavannes 2003, Stratelis et al. 2004, Stratelis et al. 2008, Rytilä et al. 2008).

In this study over 10% of these subjects had COPD (at least Stage I) based on GOLD criteria, and even more if the national criteria were applied. The slightly higher numbers found by the national guidelines may be partly related to the dynamic restriction and reduced FVC in COPD, and therefore the ratio $FEV_1$/FVC is not necessarily below 70% (Wan et al. 2011). A high number of reduced MEF50 values were found in this study, but its role in COPD development is not known. Clinically, MEF50 may have a role in the differentiation between asthma and COPD (Goedhart et al. 2006).

In Finland, the National Programme for Chronic Bronchitis and Chronic Obstructive Pulmonary disease 1998-2007 was published in the late 1990´s (Laitinen & Koskela 1999), and also the Finnish evidence–based Chronic Obstructive Pulmonary Disease: Current Care guideline (2009) was published at that time. The main aims in that programme were not only to contribute to the diagnosis of COPD as an early disease and to encourage people to quit, but also to emphasize the role of primary health care in the diagnosis and treatment of COPD. Today, nearly two decades later, no further increases in COPD prevalence (Vasankari et al. 2010) have occurred even though the quality of diagnosis COPD has improved (Kinnula et al. 2011). Based on a recent Finnish study, the visits due to respiratory symptoms within primary health care have increased during the last ten years (Vasankari et al. 2011) and the use of spirometry has increased significantly. However, there was no increase in the numbers of COPD visits in that study, which could be a sign of the difficulties in recognizing COPD at the primary health care level.

This study confirms and further extends earlier findings that COPD is under-diagnosed in chronic smokers, especially among individuals who consider themselves as being healthy and asymptomatic. However, this two-year follow-up study is too short to have any power to determine whether small airway limitation leads to COPD.

### 6.5 Smoking related prolonged respiratory symptoms in middle-aged and young adults

One of the major results of the present study (I) was that there are numbers of chronic smokers who do not recognize any symptoms related to smoking but many (62%) experience

chronic cough or sputum production i.e. risk for COPD development (van Schayck et al. 2002). It is widely known that the risk of chronic bronchitis is increased with the amount of smoked and pack-years (Forey et al. 2011); even in non-smokers if these symptoms persist, they also point to the presence of chronic bronchitis. Furthermore, symptoms of chronic bronchitis are associated with increased morbidity and mortality (Pelkonen 2008, de Marco et al. 2011). In the present study, most of the participants had normal function in the spirometric evaluation and prolonged cough/sputum production, but neither prolonged respiratory symptoms nor obstruction were associated with the success in smoking cessation. In other studies, smokers with COPD have been able to quit smoking significantly more often than those with normal lung function (Stratelis et al. 2004). That study cannot be compared with the present results for many reasons e.g. major differences in study design. Here spirometry was conducted only once on the last visit and this could have had no effect on quit rate, whereas the earlier studies repeated spirometry more often during the follow up or conducted spirometry before the start of quitting attempt (Risser & Belcher 1990). In addition, as many as 45% of these heavy middle-aged smokers with normal lung function could have had emphysema if they had been examined with high resolution computered tomography (HRCT) (Stratelis et al. 2008)

Smoking habits in Northern Finland have been evaluated earlier; and it is known that almost ten years previously smoking was relatively common in the adult population (32%, 46 years of age) of this area (Kotaniemi et al. 2005), and in northern Sweden the corresponding value was 26% (Lindstrom et al. 2001). There are some differences in the definition of ever/current smokers, which need to be taken into consideration when the prevalence of smoking is being compared. Based on these earlier population studies, symptoms of chronic bronchitis were common in Northern Finland (25% sputum production, 11% productive cough) (Lindstrom et al. 2001). There is no safe level of smoking over the long term, not only moderate and heavy, but also former and light smokers may have a significant risk for suffering chronic bronchitis (Hukkinen et al. 2009).

Even today, smoking is very common in Northern Finland, almost every second young adult in this study (over 45%) was smoking on the daily basis; if also occasional smokers were included then the number rose to over 60%. Only a few studies have been conducted on young adult smokers, but it is clear that young age does not protect an individual from chronic bronchitis or COPD (de Marco et al. 2007, Vianna et al. 2008). There is some evidence for

the presence of early airway obstruction and small airway disease even in these young smokers (Gold et al. 1996). Moreover, chronic cough and sputum production represent early markers for future COPD development and increased mortality in subjects who were below fifty years of age (Guerra et al. 2009). In another large study (de Marco et al. 2004), 12% of young and middle aged (over 5000 individuals, aged between 20-44 years) smokers suffered from chronic cough and/or phlegm without airway limitation. In the study of de Marco et al. a considerable number of young people were already suffering from COPD (3.6%), and the presence of chronic cough or phlegm also significantly predicted COPD development (de Marco et al. 2007).

The present study shows that persistent chronic cough/sputum production are common both in middle-aged and young adult smokers. In this study, the average age to start smoking was 15 years, which is the age at which most adult smokers had usually began smoking.

### 6.6 Sputum/plasma SP-A changes in smokers as an early marker of lung injury

One goal was to identify early COPD by using unbiased proteomics in lung tissue. In the study, one protein, surfactant A (SP-A) was found to be promising COPD biomarker. This result was confirmed by Western blot, immunohistochemistry and morphometry. The levels of SP-A were not only elevated in the lung tissue in patients with very severe COPD, but also in those with moderate COPD in the lung, and in the induced sputum from subjects with Stage II COPD. There was extensive variability in the sputum samples, and thus more studies will be needed with large numbers of samples and a highly sensitive technique for SP-A, especially if one wishes to determine the two sub-forms, SP-A1 and SP-A2. The levels of other surfactant proteins (SP-B, SP-C, SP-D) were also tested, since there are studies which have hinted at an association of these proteins with COPD. However, no changes in any of their levels could be observed in tissue samples of COPD patients. Lung samples of IPF patients were used as a disease control, since SP-A levels are not elevated in the lung tissue of these patients. There was again variability and some tendency towards elevation in the lung tissue Western blot analysis, possibly due to the heterogeneity of the lung tissue damage in both COPD and IPF. Overall these results point to a specific role for SP-A in COPD, but the exact mechanisms through which SP-A regulation is disturbed are unclear. It also remains to

be determined whether some of the changes are associated with smoking alone or due to a combination of factors i.e. exposure to smoke and the lung damage caused by smoking.

The last sub-study investigated SP-A levels in plasma and confirmed the elevation of plasma SP-A in current smokers, in agreement with an earlier similar study on Japanese smokers (Kobayashi et al. 2008). The present study had a prospective follow-up design i.e. the smokers were followed for 2 years. Plasma levels of SP-A were higher in those who continued smoking compared to those who succeeded in quitting. This is the first study to examine plasma and sputum SP-A from the same subjects showing also that SPA levels are elevated in the induced sputum of smokers as compared to non-smokers. These findings are in agreement with the hypothesis that cigarette smoking evokes an exogenous stress reaction and possibly an early injury to the airways, and this is reflected by elevated levels of SP-A both in plasma and sputum supernatants in cigarette smokers (Kobayashi et al. 2008).

Some studies have been conducted into the levels of SP-A in plasma. The results of those studies have been inconsistent since they have reported either elevated or reduced plasma or serum SP-A concentrations in smokers. Based on two European studies, serum SP-A levels did not differ between non-smokers and smokers (Robin et al. 2002, Mutti et al. 2006). In contrast, two studies conducted on Japanese subjects revealed that the circulating SP-A levels were elevated in smokers (Kida et al. 1997, Nomori et al. 1998) and a subsequent Japanese study concluded that the serum SP-A concentrations were elevated in smokers, patients with COPD and also in subjects with some other lung disorders such as pulmonary embolism (Kobayashi et al. 2008). In those studies, SP-A was postulated to function as a circulating marker of increasing epithelial leakage in smokers (Robin et al. 2002) and/or a biomarker of lung injury (Kobayashi et al. 2008). Plasma/serum levels of SP-A may also reflect the permeability of the lung epithelium in several ways. Cigarette smoke has been shown to increase the permeability by smoke-mediated release of vasoactive neuropeptides and as well as causing the early destruction of the barrier between the alveolar epithelium and endothelium (Hulbert et al. 1981, Mason et al. 1983, Mason 1985). Some of these discrepancies might be related to the fact that both plasma and serum samples have been used. In the present study, plasma and serum levels of SP-A were compared but were found to be very similar.

SP-A was expressed not only in the alveolar epithelium of non-smokers, but also in the bronchial epithelium of smokers and patients with COPD. This difference between non-smokers and smokers/COPD is important since it can contribute to the SP-A levels detected in sputum specimens. A recent study on male smokers and patients with COPD (Vlachaki et al. 2010) yielded complex results i.e. evidence of either an abnormal production of SP-A in type II pneumocytes or an augmented SP-A turnover in the lungs of smokers without COPD as well as in patients with COPD. The level of SP-A in BAL fluid has been found to be lower in smokers compared to non-smokers (Honda 1996, Shijubo et al. 1998, Betsuyaku et al. 2004). The problem in assessing SP-A from BAL fluid samples in general is the invasiveness of this sampling procedure; for this reason it cannot be used in the early disease evaluation. Furthermore, in none of these previous studies was the SP-A concentration measured from the same subjects and simultaneously in their BAL fluid and serum, and none of the reports evaluated SP-A levels from induced sputum specimens.

In the present study, sputum SP-A levels were significantly higher in the majority of smokers as compared to non-smokers. Generally sputum SPA levels were not detectable or were very low in the non-smokers. In contrast, SP-A could be detected in the sputum of all smokers, although it displayed extensive variability. The reason for this finding remained unclear. The method has been tested and validated, and the samples were measured in duplicate. One explanation could be the nature of induced sputum: generally it consists of secretions derived mainly from the large, more proximal airways. Since it is known that SP-A is produced in the alveolar epithelium and concentrated in alveoli, the proportion of secretions originating from the more distal airways in sputum is unknown and may vary from person to person. In the present study, a detailed analysis of those subjects with extremely high and undetectable sputum SP-A levels did not indicate that these subjects suffered an even higher risk of developing COPD.

### 6.7 Strengths and limitations

The strengths of the present investigation include the carefully characterized groups of chronic smokers with no chronic diseases, no co-morbidities or no medications. Furthermore, some of these heavy smokers had abnormal lung function, and mild to moderate COPD was diagnosed to them. The subjects in the follow-up studies were carefully interviewed by the

same nurse, and the numbers of the drop outs were very low. The link between smoking cessation and its association with obstruction examined in this study was not subject to any counselling bias, because the spirometry was conducted at the two year follow-up examination at the end of the study. However, evidence from randomized controlled trials will be needed to assess the efficacy of the Motivational Interviewing technique in smoking cessation. SP-A was the most promising biomarker for COPD and this protein was tested using this same group of heavy smokers who were not taking any anti-inflammatory medications.

This experiment was not powered to assess the prevalence of COPD in healthy middle-aged smokers since it was directed at a selected group of subjects. One obvious limitation of sub-study II is that it was cross-sectional and no detailed individual information about allergies and asthma was available. It is worth noting however that during the conscription process, draftees with symptomatic asthma are quickly diagnosed and treated. In 2009, about 5% of the draftees in this cohort area were using asthma medication daily and considered themselves as symptom-free. In contrast, draftees with persistent symptomatic asthma are exempted from military service. Draftees with pollen allergy are enlisted into army in the winter and complete their service mostly before the start of the allergy season. Therefore it is unlikely that symptoms of asthma or pollen allergy impaired their performance in the fitness test. The study design (sub-study III) did not include any randomised prospective follow-up to confirm cessation attempt episodes.

The biomarker part of the study (sub-study IV) examined patients with very severe COPD, and it is likely that the profile of these components varies between cases of mild and severe disease. Even though the elevation of SP-A was confirmed also in mild-moderate COPD, its significance for early assessment of COPD and the progression of the disease was not evaluated. It is also unclear whether the elevation of SP-A levels in the plasma of smokers is a reflection of smoking related stress conditions associated with early lung injury in COPD. Spirometry has been widely accepted as an important predictor of COPD and its progression, but recent studies have reported a poor correlation of lung function values with COPD progression/activity. Therefore the determination of levels of SP-A and possibly other potential biomarkers that can be detected using even more sensitive methods could be very valuable. One further limitation of this sub-study is the fact that it did not classify the disease into airway or emphysema or to other sub-phenotypes. However, the first studies of this thesis

included chronic heavy smokers (sub-study I) or young adult male smokers (sub-studies II, III) who had not developed severe disease, and the same was true with the last experiment (sub-study V) which investigated sputum and plasma SP-A concentrations.

### 6.8 Clinical implications

Smoking rates in all age groups have been declining in Finland during recent years, but this trend has not been so obvious in all parts of the country and especially so in young adults in their twenties. Finland is aiming to be a totally smoke-free country by the year 2040. Much work still needs to be done, to implement the newly updated Tobacco Dependence and Cessation: Current Care guideline (2012) for treating smoking cessation into everyday clinical practice concerning also young adult smokers. Further, it may be significant whether tobacco addiction is handled as a syndrome or as a common chronic disease. It does seem that there are different phenotypes and these may be susceptible to more individualized therapies. Thus, more research will be needed to identify these clinical phenotypes, which could benefit from targeted treatments and have their own distinct prognosis.

The longitudinal study design was an advantage and as far as is known, this is the first to involve one individualized counselling session on smoking cessation plus recommending pharmacotherapies for quitting and then correlating the results from blinded examination of spirometry with the success of quitting smoking in subjects with earlier unknown airway limitation.

SP-A seem to be a promising candidate as a COPD biomarker, but further unbiased and hypothesis -driven studies will be needed to validate this marker before it can be brought into clinical practice. In the near future, COPD specific biochemical markers are supposed to be useful not only for early diagnosis of COPD, but hopefully also in prevention of smoking and in smoking cessation.

## 7. CONCLUSIONS

This non-randomized longitudinal study assessed success in smoking cessation in motivated healthy middle-aged adults with the combination of pharmacological treatment and personal counselling support without spirometry results. The results of the present study are in line with earlier studies about the equivocal role of spirometry in improving smoking cessation rates. Airway limitation in spirometry was not significantly associated to success in smoking cessation. Complementary well-design randomized controlled studies in real-world settings are needed.

One efficient individualized counselling session combined with pharmacotherapy seemed to be helpful in achieving successful smoking cessation. This study emphasised the importance of motivation and a positive attitude in smoking cessation, because those who succeeded to quit smoking were more receptive to the anti-smoking intervention information than those who continued smoking. Furthermore, smoking young adult males seem to make numerous quit attempts supplemented by the uncontrolled use of pharmacotherapy.

Significant numbers of initially healthy asymptomatic middle-aged smokers confirmed that they suffered prolonged cough and/or sputum production indicating that screening of COPD could be targeted to symptomatic smokers, since COPD is common in this group. However, the symptoms of chronic bronchitis may predict a poor clinical outcome even in smokers with normal lung function and in patients with mild COPD. Furthermore, this study identified a very high frequency of young adult smokers with chronic cough and sputum production; this may represent a significant risk for their future health.

Finally, new possible biochemical markers were studied by lung tissue proteomics with the goal being to find biomarkers connected to lung injury in heavy smokers. It is clear that the discovery and clarification of COPD-specific biochemical markers would be very useful not only for early diagnosis of COPD, but hopefully also in promoting prevention of smoking and in encouraging smoking cessation in future. This study represents one of those few proteomic studies conducted with lung tissue from COPD patients aiming to identify new biochemical markers of early disease. SP-A may be involved in the pathogenesis of smoking induced lung

diseases and it possibly represents a meaningful biomarker for early COPD. Further studies will be needed to confirm the role of SP-A.

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