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Pulmonary manifestations in smoking-related diseases

Clinical studies with emphasis on chronic obstructive pulmonary disease and rheumatoid arthritis

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Pulmonary manifestations in smoking-related diseases

Clinical studies with emphasis on chronic obstructive pulmonary disease and rheumatoid arthritis

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ABSTRACT

Smoking is a risk factor for a number of diseases including chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA). Cigarette smoke initiates an inflammatory response which leads to structural changes in the airways and in the lung parenchyma. The present work was undertaken in order to shed light on pulmonary manifestations of two common smoking-related diseases, COPD and RA.

A retrospective review on bronchoalveolar lavage (BAL) constituents, encompassing 132 smokers with normal lung function and 44 ex-smokers, was performed. Two hundred and ninety- five neversmokers served as reference group. The median (5-95 percentile) cell concentration in smokers were 382.1 (189.7-864.3) X 106 /L which was higher compared to the neversmokers. The majority of cells were alveolar macrophages (median 96.7%; range 73.2-99.6%, lymphocytes (2%; range 0.2-26%) and neutrophils (0.6%; range 0-6%). Cell concentration was positively correlated to cumulative smoking history.

One hundred and five patients with newly-diagnosed RA, (70% ACPA+), underwent high resolution computer tomography (HRCT) examination and a sub group of 23 patients also performed bronchoscopy and BAL. A group of 43 non-diseased smokers and never smokers were examined as control. Parenchymal lung abnormality on HRCT was found in 63% of ACPA+ compared to 37% ACPA- RA patients, 30% control regardless of smoking status. The level of ACPA was higher in BAL fluid than sera in ACPA+ RA patients.

Forty smokers with normal lung function, (mean 35 pack-years), 40 healthy never-smokers, and 40 COPD-patients of GOLD, I-II, (38 PY), performed HRCT. In addition BAL was performed. Percentage of pixels between -750- -900 HU (%HDS) was calculated. Lung density was increased in smokers (44.0% ± 5.8%) compared to never smokers (38.3 ± 5.8%), $p<0.001$. Cell concentration in BAL was positively correlated to lung density in smokers ($r=0.50$, $p<0.001$). Females had denser lungs than males.

Regional air trapping was assessed on expiatory HRCT on 40 never-smokers, 40 smokers and 40 COPD-patients. Emphysema, micronoduli, bronchial wall thickening was determined on inspiratory HRCT. Air trapping index (AI) was quantified as the ratio of mean lung attenuation at expiration and inspiration. Regional air trapping was present in 63% of smokers and 45% of never smokers. Smokers with visible regional air trapping had an AI of 0.81, while smokers without visible air trapping had an AI of 0.91. A negative correlation between AI and neutrophils in BAL was observed. Smokers with regional air trapping had better lung function and less emphysema compared to smokers without.

We demonstrate inflammatory and structural changes in the lungs in smokers by means of HRCT and BAL. These changes are apparent even before clinical symptoms occur. The studies highlight the heterogeneity in smoking-related diseases which may be of importance in terms of disease progression and patient phenotypes.

LIST OF SCIENTIFIC PAPERS

I **Karimi R**, Tornling G, Grunewald J, Eklund A, Sköld CM. Cell recovery in bronchoalveolar lavage fluid in smokers is dependent on cumulative smoking history. PLoS One 2012; 7(3):e34232. Epub 2012 Mar 29.

II Reynisdottir G, **Karimi R**, Joshua V, Olsen H, Hensvold AH, Harju A, Engström M, Grunewald J, Nyrén S, Eklund A, Klareskog L, Sköld CM, Catrina AI. Structural lung changes and local anti-citrulline immunity are early features of anti citrullinated-proteins antibodies positive rheumatoid arthritis. Arthritis and Rheumatology 2014; 66, 31–39.

III **Karimi R,** Tornling G, Forsslund H, Mikko M, Wheelock ÅM, Nyrén S, Sköld CM. Lung density on high resolution computer tomography (HRCT) reflects degree of inflammation in smokers. Respir Research 2014, 15: 23.

IV **Karimi R**, Tornling G, Forsslund H, Mikko M, Wheelock ÅM, Nyren S, Sköld CM. Determination of air trapping in smokers by high resolution computer tomography (HRCT). In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-cyclic citrullinated peptide antibody
AI	Air trapping
BAL	Bronchoalveolar lavage
iBALT	Inducible bronchial associated lymphoid tissue
BMI	Body mass index
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
DLCO	Diffusion capacity for carbon monoxide
DPLD	Diffuse parenchymal lung diseases
DIP	Desquamative Interstitial Pneumonia
IPF	Idiopathic pulmonary fibrosis
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GOLD	Global initiative for chronic obstructive pulmonary disease
HU	Hounsfield units
HDS	Percentage of lung volume with higher density
HRCT	High resolution computed tomography
NK	Natural killer
PY	Pack years
PAD	Peptidylarginine deiminase
PLCH	Pulmonary Langerhans cell histiocytosis
RA	Rheumatoid arthritis
RB-ILD	Respiratory bronchiolitis associated interstitial lung diseases
RV	Residual volume
SPL	Secondary pulmonary lobule
TLC	Total lung capacity

1 INTRODUCTION

1.1 HISTORICAL BACKGROUND

Tobacco usage by Native Americans has a long history. In higher dosages as a hallucinogen tobacco was used only by "experienced" medicine men and shamans in ceremonies. It was not until the 18th century that tobacco emerged as a big commercial product and consequently an important financial resource for the United States government. Strong commercial interests in a free market and the opportunity for the tobacco industry to advertise easily via newspapers and films increased tobacco's popularity all over the word by the end of the 1930s. Health concerns regarding serious damage related to cigarette smoking became apparent through publications by Doll et al. in the British Medical Journal in the early 1950s, linking smoking to lung cancer [1, 2]. Increased mortality due to airway dysfunction and bronchitis symptoms and the association of airway mucus hypersecretion with emphysema were suggested in autopsy studies by Thurlbeck et al in 1963 [3].

1.2 SMOKING-RELATED DISEASES

During the past few decades a huge body of knowledge has been gathered, increasing our awareness of tobacco-related diseases. Among these, COPD is the most serious global health issue, accounting for almost 30% of all mortality attributed to tobacco smoking. This figure was estimated from nearly two million deaths in 2005 and COPD is expected to advance from the fifth to the fourth leading cause of death by 2030 [4, 5]. COPD is also a significant cause of disability, hospitalization and health-care costs worldwide [6]. Cigarette smoke affects almost every aspect of health, including the cardiovascular system with increased risk of heart failure and stroke. These together with cancer and respiratory diseases account for most tobacco-related deaths world-wide [7-10]. The prevalence of smoking-related malignancy is estimated to about 5%, mostly lung adenocarcinoma and other epithelial tumors [11, 12]. Smoking increases the prevalence of bladder cancer, mesothelioma and cervical cancer, and is a co-factor in several other malignant conditions [7, 8, 13]. Further, smoking has been suggested to initiate autoimmunity in genetically-susceptible individuals [14, 15]. Cigarette smoke has been associated directly to some diffuse parenchymal lung diseases (DPLD) [16], such as desquamative interstitial pneumonia (DIP) and respiratory-bronchiolitis-associated interstitial lung diseases (RB-ILD) and pulmonary Langerhans cell histiocytosis (PLCH). These conditions are treatable by smoking cessation and steroid therapy [17-20]. An association between cigarette smoke and increased risk of developing idiopathic pulmonary fibrosis (IPF) in former and current smokers has been shown in some studies [20-27] but not all [28].

1.3 EFFECT OF SMOKING ON THE IMMUNE SYSTEM

Cigarette smoke contains several thousand highly toxic components which affect the immune system in many different ways. Some agents in cigarette smoke are high potential carcinogens, several are pro-inflammatory and others such as nicotine and carbon monoxide have immune-suppressive effects [2, 29-34]. Cigarette smoke damages

respiratory epithelium directly with increased permeability, compromising the physical barrier against environmental pollutants and virus and bacteria [31,35, 36]. The normal process of repair and the apoptotic system is damaged by the oxidation of epithelial membrane lipids and the induction of extensive single-stranded DNA breaks [37]. Further, smoking activates alveolar macrophages, a key player in front-line defense against microbial agents. These increase in number and produce pro-inflammatory mediators that consequently recruit other inflammatory cells such as neutrophils and lymphocytes to the site of injury [29, 30, 38, 39]. Cigarette smoke compromises the ability of alveolar macrophages to phagocyte bacteria and clear apoptotic cells [40, 41]. This promotes a more sustained inflammatory process in the respiratory tract [42, 43]. The number and activity of natural killer cells (NK), an important element of the innate host defense against microorganisms, is reduced, thereby linking this effect to an increased risk of cancer and infections [44, 45]. A decreased level of immunoglobulin classes, except IgE, in smokers is reported in human and animal studies [33]. Skewing of several important elements in the immune system due to exposure to active or passive smoking together with individual susceptibility may explain the link between cigarette smoke and many diseases [43, 46].

1.4 SMOKING AND COPD

Chronic obstructive pulmonary disease is a complex disease where the most important environmental risk factor, tobacco smoke, induces an exaggerated inflammatory response in the lungs of genetically-susceptible individuals [5, 47-50]. The ongoing inflammation destroys the lung parenchyma (emphysema) and causes fibrosis and inflammation in small airways (remodeling), which subsequently contributes to increased airways resistance by narrowing and constricting the lumen [16, 51, 52]. Parenchymal destruction and increased alveolar size reduce elastic recoil and this in combination with remodeling contributes to not-fully-reversible airway obstruction [16, 53-58]. Smoking intensity is associated with decline in rate of FEV1 and increased frequency of exacerbation in COPD patients [59-62]. Overspill of inflammation from the lungs has been suggested as the main cause of systemic inflammation, increasing cardiovascular disease, osteoporosis, and diabetes [63, 64]. There are still limitations in our understanding of the underlying mechanisms behind COPD. Despite a chronic inflammation characterized mainly by neutrophils and macrophages in the small airways of the all smokers, for unknown reasons, some develop progressive emphysema and severe airway pathology while others manage to continue with airway inflammation with slower functional changes, which may stay subclinical [65, 66]. Neutrophils are found frequently in the small airways of smokers and correlate to the amount of smoking [30, 66, 67]. These cells may be attracted to the site by chemotactic factors released by activated macrophages in the respiratory bronchioles and alveolar sacs in epithelial lining fluid [67, 68].

The pathogenic role of neutrophils in smoking-related lung diseases is a matter of controversy. The number of neutrophils, the marginal pool, rises in the lung capillary in the presence of cigarette smoke [69] and correlates to the rate of decline in forced expiratory volume (FEV1) [69, 70]. However other investigators have found an inverse relation between

the number of neutrophils and the degree of tissue destruction in the lungs (i.e. emphysema), suggesting neutrophilic inflammation as a sign of better-preserved lung parenchyma [71, 72]. Nevertheless, macrophage and neutrophil infiltration seems to be orchestrated in an acute and non-specific defense against cigarette smoke injury, while lymphocyte infiltration with predominantly CD8+ cells in the lung may indicate a more advanced and specific humoral immune response with potential lung injury [66, 73-77]. The exact role of CD8+ cells in the pathogenesis of obstructive lung disease is not clear but an association between the number of CD8+ cells, and the severity of airway obstruction and the degree of alveolar destruction and apoptosis of alveolar epithelium, has been shown in smokers [70, 78]. A study on surgically resected lung tissue from smokers showed that several inflammatory cells including macrophages, neutrophils, lymphocytes and eosinophils correlated equally to the degree of tissue destruction with emphysema with no predominance of any single inflammatory cell [79].

1.5 SMOKING AND RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease characterized by multiple joints inflammation and destruction. Although the exact etiology of the disease is unknown, some data indicate a link to cigarette smoke [80-82]. Women are more susceptible than men and the prevalence among the general population is estimated to 0.5-1% [81]. Pulmonary involvement is a main extra-articular manifestation of RA with major impact on morbidity and premature mortality in this patient group [83-87]. Many structures in the lungs including airways, pulmonary vasculature, and pleura may be involved to differing extents but the most serious condition, with high mortality, is RA-associated intestinal lung disease (RA-ILD) [87, 88]. A high prevalence of pulmonary changes in newly-diagnosed RA, and indeed even prior to the onset of symptomatic inflammatory arthritis, has encouraged the hypothesis that the lungs may be a potential site of initiating autoimmunity [89-94]. Anti-cyclic citrullinated peptide antibody (ACPA) is a specific autoantibody which is highly predictive of disease severity and progression and is also a predictive marker of future disease development in the sub-group RA with (ACPA+) [95-97]. ACPA and lung changes together in newly-diagnosed RA, or prior to disease onset, provide further support for the hypothesis that in some susceptible individuals autoimmunity can be initiated at an extra-articular site, presumably the lungs, under the influence of smoking or other environmental agents[15, 98]. Elevated citrullinated peptides and corresponding catalyzing enzyme, peptidylarginine deiminase (PAD2) in bronchoalveolar lavage obtained from smokers' lungs may further strengthen this hypothesis [14]. A link between smoking and the initiation of an immune reaction with citrulline-modified protein with HLA-DR-shared epitope genes has been suggested [15].

2 ASSESSMENT OF INFLAMMATION AND STRUCTURAL CHANGES IN THE LUNG

2.1 QUANTIFICATION OF LUNG PARENCHYMA ON COMPUTED TOMOGRAPHY

Recent advances in computed tomography technique, along with our understanding of the various patterns of high-resolution computed tomography (HRCT) features in specific pathological conditions in the lung, have revolutionized the utility of this imaging modality. With excellent spatial resolution and high signal-to-noise ratio CT is indispensable for medical imaging of the lungs. It is used broadly in both the clinic and research [99, 100]. We are able nowadays to obtain detailed, thin section images of very high quality within just a few seconds of breath-holding, using multi-detector computed tomography (MDCT). Information from isotropic voxels can be added automatically to obtain both lung volume and attenuation reliably [101-103] (**Figure 1**). Further detailed morphological information can be extracted by reformatting sagittal and coronary images from axial planes. Attenuation values of the lungs are measured in Hounsfield units (HU). These are conventionally set to zero for pure water and -1000 HU for pure air and 40 HU for blood. Linear attenuation values from voxels average and provide information for quantitative assessment of the lung tissues. Density and structural measurements of the lung parenchyma and airways obtained from contiguous thin-slice images offer a unique opportunity to evaluate the distribution and presence of lung pathology in vivo [104-110].

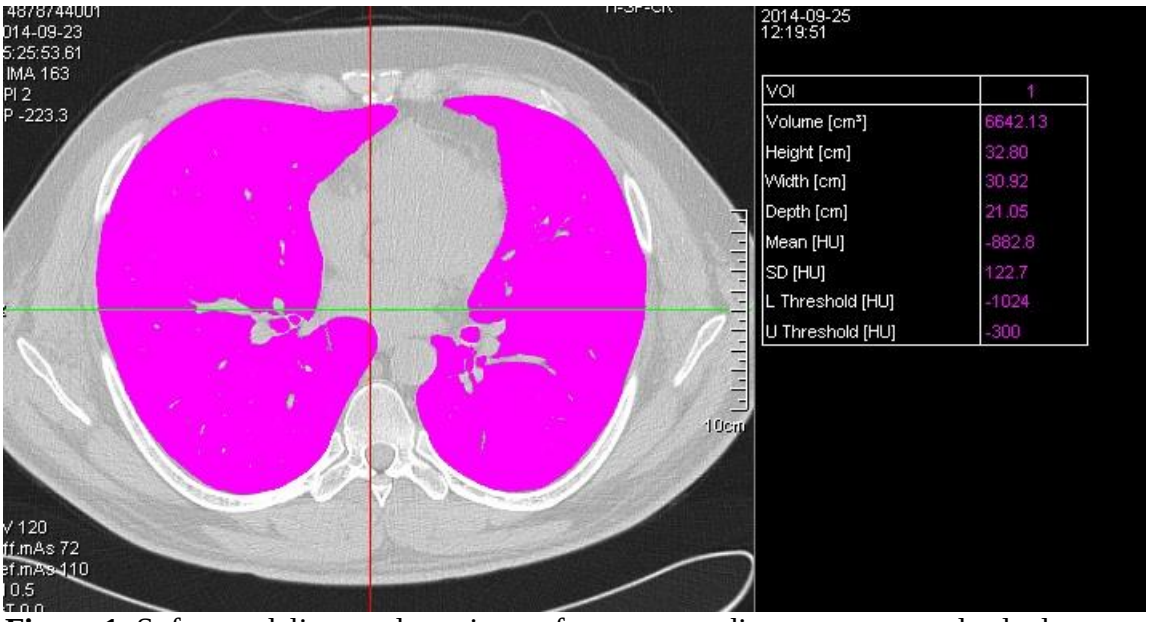

Figure 1: Software delineates lung tissues from surrounding structures and calculates values for lung volume and attenuation

Attenuation values are proportional to tissue density determined by the relative amounts of lung tissue, blood and air. In emphysema the number of voxels with lower attenuation values increases as a result of tissue loss. Attenuation values lower than -950 HU on slices

obtained on HRCT have been validated to represent microscopic and macroscopic emphysema [111-113]. The threshold of -910 HU was used originally on thicker slices obtained from conventional CT [114]. Another method which has shown correlation to emphysema in pathological specimens was originally to use attenuation values lower than the 5th percentile on a frequency-distribution curve as a measure of emphysematous tissue [115]. This approach has been modified in recent studies to the 15th percentile and has been implemented in clinical studies [108, 110, 116]. Both methods estimate the extent of disease reasonably and are recommended for cross-sectional studies, while the "15th" method is recommended in longitudinal studies [108, 116, 117]. CT densitometry is a valuable tool in assessing lung structure changes and provides very important data for understanding the pathogenesis of smoking-related lung diseases. Analogously with regions of lower attenuation as a surrogate for emphysema, lung parenchyma with increased attenuation can be used to indicate the intensity of inflammation. However, studies in this field are very limited [27]. CT densitometry has several limitations, including technical divergence between scanners, lack of a standard method for analyzing lung parenchyma and airways, degree of inspiration, and exposure of subjects to ionizing radiation, [118-120].

2.2 QUANTIFICATION OF AIRWAYS ON CT

Assessment of changes in the airways wall and lumen dimension due to inflammation and submucosal fibrosis –"remodeling" – has attracted much attention recently [121-123]. This method is technically more challenging than parenchymal lung densitometry measurements. This is because the diameters of small airways (i.e. <2mm), which are the epicenter of inflammation in smokers and COPD patients, is below the resolution of CT [121, 124, 125]. Despite the technical complexity of the method, Nakano et al. Showed that the wall thickness in small airways measured in histological specimens correlated to that in intermediate airways in the right apical bronchus (i.e. diameter nearly 7.5mm) measured with CT [123]. Hasegawa et al, refining the method using three-dimension reconstruction of the airways, measured wall thickness down to sixth-generation airways. They found a stronger correlation between wall thicknesses with FEV1 in smaller airways (sixth generation) than to intermediate (third generation). These studies indicate that small and large airways build an anatomical continuity, with coexisting morphological abnormality in both. Despite very important progress in this area the major obstacle, the limited resolution of current scanners for measuring small airways, is still unresolved. Consequently the method needs further refinement before it can be implemented clinically [126].

2.3 MORPHOLOGICAL FEATURES OF THE LUNG ON HRCT

HRCT findings play a central role in the detection and classification of small airways diseases and when correlated with clinical findings they can considerably improve diagnostic accuracy. The secondary pulmonary lobule (SPL) is the smallest functioning subunit of the lung separated by connective tissue septa. Each SPL is 1 -2.5 cm in diameter and contains a bunch of small airways radiating from central lobular bronchiole (1 mm in

diameter). SPL are very important anatomical landmarks which are not visible on normal HRCT. Bronchioles which are also invisible on normal HRCT become visible in the central part of the SPL with small airways disease due to infiltration of inflammatory cells and increased density in or around the bronchioles. These CT signs of small airways disease appear as faint ground-glass opacities [127]. Regional air trapping, which appears on end-expiratory thin-section CT, is characterized as regions with low attenuation area, due to under-ventilation. It follows the geographical border of SPL and may indicate small airways disease [128, 129]. Air trapping is a frequent finding on end-expiratory scans obtained from smokers with normal lung function and normal inspiratory scans, and it may be an indirect sign of small airways disease. Air trapping also appears sometimes within a few SPL in even apparently healthy never-smokers [130-132]. More serious conditions with widespread airways diseases such as infiltrative lung disease, occlusive pulmonary vascular disease and allergic alveolitis appear as extensive multi-lobular air trapping - the "mosaic pattern" – usually on both inspiratory and expiratory HRCT scans and mostly accompanied by serious respiratory symptoms [133]. Respiratory-bronchiolitis-associated interstitial lung disease (RB-ILD) overlaps pathologically with desquamating intestinal pneumonia (DIP). Both diseases are strongly related to smoking intensity and characterized histologically by sub-mucosal fibrosis and inflammation with accumulation of pigmented macrophages in bronchioles and alveolar ducts and spaces. The HRCT finding in these conditions appears as several stigmata including air trapping, micronoduli and patchy ground-glass opacity [134]. The two latter findings were shown to correspond, in pathological specimens, to accumulation of macrophages in respiratory bronchioles and in alveolar space, respectively [135].

2.4 VISUAL ASSESSMENT OF EMPHYSEMA AND AIRWAYS

The development of emphysema in the lungs of smokers is generally recognized and seldom causes interpretation difficulties on HRCT [104]. Centrilobular or (centriacinar) emphysema refers morphologically to the destruction of respiratory bronchioles located at the center of the secondary pulmonary lobules, while panlobular or (panacinar) indicates destruction of the whole acinus [136-138]. The former is strongly associated with cigarette smoking and associated with more small airways inflammation, and appears predominantly in the upper lobes while the latter is linked to α1-antitrypsin deficiency with lower-lobe predominance [136, 139]. It is likely that small airways disease spreads centrifugally and destroys the alveolar walls attached to the respiratory bronchioles, subsequently appearing on CT as centrilobular lesions [65, 136, 139]. These lesions are easily detectable on HRCT scans as small areas of low attenuation usually without walls, contrasting with the surrounding parenchyma with normal attenuation [140-142] (**Figure2**). With panlobular emphysema the entire secondary lobules are destroyed and the emphysema appears as a homogeneous pattern of low attenuation which can involve the entire lung [16, 143-145]. Several studies have documented the accuracy of CT in assessing the presence and extent of even mild emphysema with good correlation with pathological specimens [112, 142, 146]. Emphysema is detected in the lungs of a majority of smokers with no significant airways obstruction. Sometimes one-third of the lung can be destroyed by emphysema without any significant clinical symptom or reduced lung function [147, 148] [149]. In contrast to the accuracy of visual assessment of emphysema on HRCT, the judgment of

bronchial wall thickening and bronchial dilatation is more highly associated with inter-observer variation than any other morphological signs on HRCT [150, 151]. Nevertheless, with correct window settings, bronchial wall thickening is widely observed in smokers compared to never-smokers and it may be a typically widespread sign of HRCT in smokers with chronic bronchitis [127]. Bronchial wall thickening In COPD patients is partly associated with degree of airways obstruction and symptoms [152, 153]. Interpretation of bronchial dilatation, a cardinal sign of bronchiectasis, is based on approximate size equality between external diameters of bronchi and the adjacent artery. Although easy to remember, this rule is based on a study of healthy subjects with widely ranging bronchial sizes, with mean ratio of 0.98, range, 0.53-1.39 [154]; thus it should be used in the context of other clinical information.

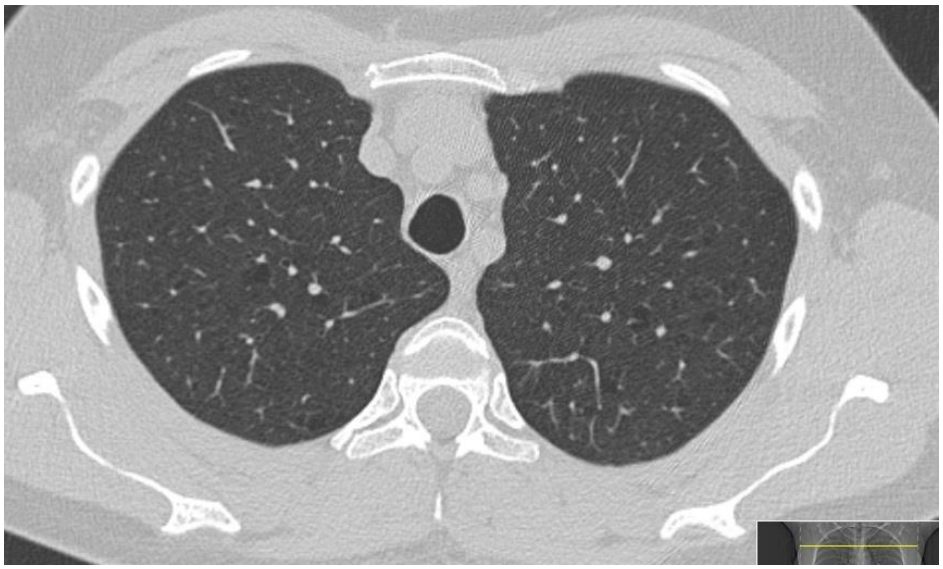

Figure 2: Centrilobular emphysema, easily distinguishable, in the central part of right and left over lobs of a 51 year-old female smoker

2.5 BRONCHOSCOPY AND BRONCHOALVEOLAR LAVAGE BAL

Considerable progress has been achieved since the introduction of rigid bronchoscopy by C. Jackson in 1904 and flexible fiber bronchoscopy by S. Ikeda in 1967. Flexible fiber bronchoscopes are available with different seizes and equipment to obtain biological material for both clinical and research purposes. Bronchoscopy with bronchoalveolar lavage (BAL) is a minimally-invasive procedure performed usually with outpatients. After application of local anesthesia the bronchoscope tip is wedged in a third- or fourth-generation-segmental bronchus (**Figure3**).The middle lobe or lingula is the usual site of lavage, because it is easily accessible with better fluid retrieval, when BAL is performed for research purposes and with diffuse or disseminated parenchymal abnormality.

BAL may be performed in other specific sites or in several when regional radiographic heterogeneity is present on HRCT [155, 156]. A number of 20-60 ml aliquots of body-warmed saline with a total volume of 100-300 are normally instilled and subsequently retrieved. Suction should be gentle to avoid airway collapse. Especially low suction should be applied with emphysema due to lack of airway support [157]. More than 40% of the instilled volume of the fluid should be recovered [156, 158, 159]. BAL has a low complication rate with minor discomfort such as coughing during the procedure or fever chills. The latter are associated to larger volumes, and may occur hours after BAL due to cytokine release [160, 161].

The gross visual appearance of BAL may be highly suggestive or virtually diagnostic of specific rare disease. One example is bloody BAL with diffuse alveolar hemorrhage (DAD), which has many different causes; or with pulmonary alveolar proteinosis [58] where the BAL fluid appears as cloudy, milky or light brown [162, 163].

BAL-cell profiles in smokers have been investigated in several studies confirming a four-to-six fold increase in cell concentration compared to never-smokers. This is mainly due to increase in the number of macrophages [14, 164-167]. Neutrophils and eosinophils are generally elevated in smokers [156, 168, 169]. An extreme increase in neutrophil percentages may indicate infection or diffuse lung injury while a percentage of eosinophils higher than 25% is likely caused by eosinophilic pneumonia or other eosinophilic lung disease [170-174]. BAL lymphocytes higher than 25% may indicate ILD related to granuloma, particularly when the CD4/CD8 ratio is more than 3.5 with sarcoidosis [167, 175]. Although BAL is a useful and well-tolerated clinical tool it cannot stand alone and should be interpreted in the context of clinical and radiographical information. BAL is particularly useful to rule out infection or hemorrhage as a cause of parenchymal infiltration on HRCT. As a research tool BAL has provided valuable insight into the pathogenesis of many rare lung diseases [176, 177].

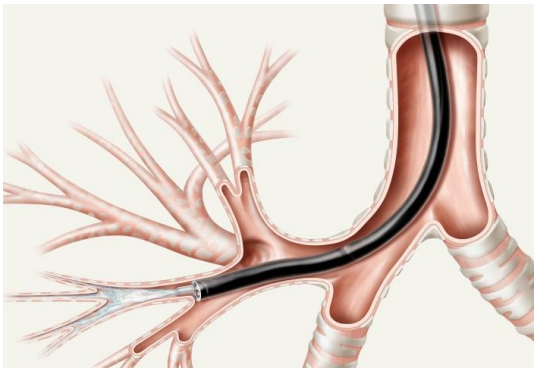

Figure 3: Simplified illustration of BAL

3 AIMS

The general aim of the work presented in this thesis was to assess inflammation and structural changes in the lungs of smokers and patients with COPD (GOLD I, II) and RA patients in the early stage of disease

The specific aims were:

-to study the effect of smoking on BAL fluid recovery and cell counts in smokers and ex-smokers with normal lung function, in order to establish reliable reference values for better interpretation of BAL cell counts in clinical and research settings;
-to assess the morphological features of CT scans in newly diagnosed RA and correlate these changes to the presence of ACPA and smoking status;
-to quantify inflammation in the lungs of smokers and COPD patients (GOLD I, II) current and ex-smokers by means of CT densitometry and BAL-cell differential counts, and
-to categorize morphological changes in the lungs of smokers, healthy controls and COPD patients (GOLD I, II) using CT scans obtained with expiration and inspiration with focus on air trapping and its correlation to BAL cells as a measure of small airways inflammation.

4 PAITENTS AND METHODOLOGY

A short description of the participants and the methods employed in the four studies underlying this thesis is presented here. Roman numerals (I-IV) refer to the corresponding papers.

Participants in study (I)

BAL cell counts and fluid recovery were studied retrospectively. The study encompassed 132 smokers with normal lung function (48/84, males/females, M/F) of mean age 39 years and smoking history of mean value 20.8 (PY), plus 44 ex-smokers (16/28, M/F), mean age 39 and smoking 5.3 (PY). A group of 295 (132/163, M/F), healthy never-smokers, an account of whom was published separately by Olsen et al [178], served as controls. All participants had been served as healthy controls in different investigations involving bronchoscopy and BAL between 1990 and 2009 in our department.

Participants in study II

Study II emerged as a result of collaboration between the Department of Rheumatology and the Department of Pulmonology at Karolinska University Hospital. A number of 105 patients with newly-diagnosed RA, (30/75, M/F), 70% ACPA+, 29% were current smokers, 74% ex-smokers and 26% never-smokers. A number of 43 age and gender matched, 67% current smokers with normal lung function and 33% never smokers, were included as controls. Control subjects were selected from participants in studies III and IV.

Participants in studies III, IV

Data analyzed in two studies (III, IV) were obtained from the same subjects. Forty smokers with normal lung function (20/20, M/F), mean age 54, mean smoking history 35 (PY), 40 healthy never-smokers, mean age 57, plus 40 COPD (GOLD, I-II), mean age 59, mean smoking 38 (PY), 31 current smokers and nine ex-smokers) were recruited, mainly by advertisement. None with any significant medical condition, infection, allergy or

respiratory disease except COPD were allowed to participate. Corticosteroid in any form was not allowed.

Pulmonary function test – studies I, II, III, IV

Participants in study I performed dynamic spirometry only if they were smokers and older than 40 years of age. Only subjects with FEV1/FVC>0.7 and FEV1>80% were included. Participants in studies II, III, and IV performed both dynamic spirometry and body plethysmography with measurement of DLCO. All measurements were performed in a standard way and were reported as percentages of predicted values according to international recommendations.

Bronchoscopy and BAL – studies I, II, III, IV

Bronchoscopy and BAL was performed on all participants in studies I, III, and IV, and 23 patients with RA, study II, according to a standard procedure in our department. The bronchoscopy procedure has been detailed earlier.

Preparation of BAL fluid and cells – studies I, II, III, IV

Fluid collected after BAL was kept on ice and analyzed immediately in the Lung Research Laboratory at our department. Debris was separated by filtration of BAL fluid through a Dacron net and then the volume was registered. A cell pellet was prepared after centrifugation and re-suspended in RPMI medium. The cells were counted in a Bűrker chamber. For differential counting, cytocentrifugation was employed prior to cell staining with May-Grűevald Giemsa.

II Immunohistochimical analysis (II)

Levels of IgG and IgA against ACPA in serum and BAL fluid were measured using a commercial kit. The total concentrations of IgG and IgA in serum and BAL were measured at the clinical immunology laboratory at Karolinska University Hospital.

Quantitative image analysis on HRCT – studies III, IV

Inspiratory and expiratory HRCT scans without contrast medium were obtained from all participants. A semi-automatic program was employed to differentiate lung parenchyma from surrounding structures by choosing predetermined attenuation values between -300 and -1024 HU, representing whole-lung parenchyma. Calculating the value of voxels generated mean lung attenuation and lung volume. In study III, the percentage of lung volume with attenuation between defined thresholds of -750 and -900 HU, labelled percentage of lung volume with higher-density HDS % (**Figure 6**), was calculated separately and was correlated to BAL cell differential cell counts. In study IV, the air trapping index (AI), was calculated as the ratio of mean lung attenuation at expiration and inspiration.

Qualitative image analysis on HRCT – study IV

All images were anonymized and then assessed in a random order independently by two reviewers blinded to all subject data. Morphological assessments including regional air trapping were judged on expiratory scans while emphysema, micronoduli and bronchial wall thickening and solid nodules were judged on inspiratory HRCT scans.

5 RESULTS AND DISCUSSION

Paper I

In Study I we found that both total cell number and cell concentration in BAL from smokers showed a four-fold increase with a wide inter-individual range. Macrophages accounted for almost 96% of the cells in smokers, while the corresponding percentages for never-smokers and smokers-and-ex-smokers were 90% and 88% respectively. Neutrophils and eosinophils were increased in smokers compared to never-smokers and ex-smokers while lymphocytes did not differ significantly. Cell concentration correlated positively with smoking history expressed as pack years (PY).The percentage of recovered BAL fluid decreased with increasing age and was significantly lower in male smokers than in female.

BAL as a diagnostic tool

In agreement with previous studies we found that smoking had a profound effect on BAL cell counts, especially macrophages [176, 177]. The increased number of BAL cells was dose-related and increased positively with increased pack years. This result agrees with that of Rennard et al who showed that reduction in cigarette smoking resulted in fewer macrophages and neutrophils [178]. The number of cells in BAL may represent the intensity of small airways inflammation in smokers, and this may differ widely depending on each subject's unique immune reaction to cigarette smoke. This inflammatory reaction seems mainly reversible at least in terms of cell number, according to our observation from ex-smokers. Subclinical inflammation in smokers is silent due to low resistance in small airways, and thus is undetectable on spirometry until a significant decline in lung function has occurred.

Many previous studies dealing with BAL in smokers are small and utilize different techniques particularly regarding instilled lavage volume. Despite technical inconsistency their results are consistent and in agreement with ours [176, 179, 180]. A wide range of cell numbers was obtained from smokers' BAL, and although this afforded very valuable information it cannot stand alone as diagnostic for smoking-related interstitial lung diseases. There is a risk of overlap between smokers with clinically significant symptoms and those with preclinical inflammation. Information from BAL in combination with radiographic signs and clinical symptoms may, however, provide specific diagnoses without the employment of invasive diagnostic procedures.

Paper II

The main focus of study II was to investigate structural lung changes on HRCT in a group of newly-diagnosed RA patients and, further, to correlate these structural changes with ACPA antibodies and smoking history. Structural changes were significantly higher in the lungs of all RA patients regardless of ACPA status than in controls. ACPA-positive RA patients had significantly higher morphological changes in the lung than ACPA-negative

ones, regardless of smoking status. Concentration of ACPA was significantly higher in BAL than in sera in ACPA-positive patients.

Is the lung the primary site for autoimmunization with ACPA-positive RA?

The majority of our RA patients had lung abnormalities on HRCT (54%) without any significant clinical symptoms. This indicates that HRCT is a powerful imaging tool to reveal early structural changes in the lung. Most of these changes were limited to a small portion of the lung, and barely affected lung function or caused clinical symptoms at this early stage (**Figure 4)**. These changes, however, in correlation to ACPA antibodies, strengthen the hypothesis that the lung can somehow be involved in autoimmunization. It is however possible that these ACPA patients had more intensive systemic inflammation and thereby more structural changes in the lung. In a study on subjects with positive ACPA prior to onset of inflammatory arthritis Demoruelle et al [87] found that pulmonary changes in76% of ACPA-positive subjects, of whom two developed inflammatory arthritis after a follow-up period of 13 months. The ability of lung immunity to react against inhaled pollutants such as cigarette smoke may initiate autoimmunity in genetically vulnerable subjects. Inducible bronchus-associated lymphatic tissue (iBALT) with abundant B lymphocytes in the submucosal airways of RA, and Sjögren's syndrome with pulmonary involvement and reaction between IgG and citrullinated fibrinogen, was identified in another study [181].

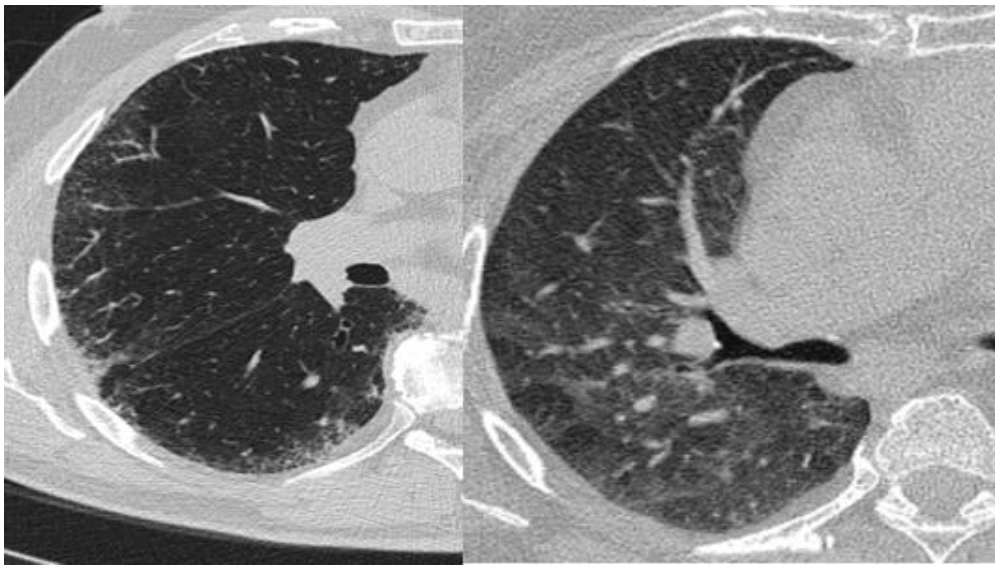

Figure 4: Examples of typical lung abnormalities in two patients with newly diagnosed RA, on the left scan mild area of fibrosis predominantly sub- pleural, on the right scan air trapping visible in the dependent and sub-pleural area of the lung

Paper III

In study III we demonstrated that lung density measured on HRCT, expressed as HDS%, as a surrogate for local inflammation, is correlated with total cell concentration obtained with BAL. Smokers had denser lungs than never-smokers and COPD patients. Lung density in females was higher regardless of smoking status but the difference diminished after correction for height.

Increased lung density and cell concentration in BAL mirrors inflammation

Lung density measurement algorithms have been developed according to the percentage of pixels with lower attenuation, called the density mask, for quantification of emphysema [112, 114]. However, there is only one study, by Lederer et al [25], where pixels with higher attenuation are investigated in relation to subclinical lung diseases. Lung density is a product of tissue volume, blood, cells and decrease of air. With inflammation in the lung the number of inflammatory cells increases and may indicate an overall increase in intensity of inflammation in lung parenchyma, and consequently increased lung density (**Figure 5)**. A uniform increase in lung density on HRCT in smokers without apparent lung disease is difficult to detect with the naked eye. Analogue with pixels with lower attenuation on the distribution curve as measurement for emphysema with shift on the frequency distribution curve to the left, with increased inflammation and consequently higher density the curve will shift to the right **(Figure 6)**. This method might be useful in smokers in an early preclinical stage of disease. The major challenge when measuring parenchymal inflammation in COPD is the net effect of increased density (inflammation) against decreased density (destruction). This became obvious in our study of COPD patients. In larger groups of COPD patients however this may be a useful method to categorize different sub-groups depending on lung density and smoking status.

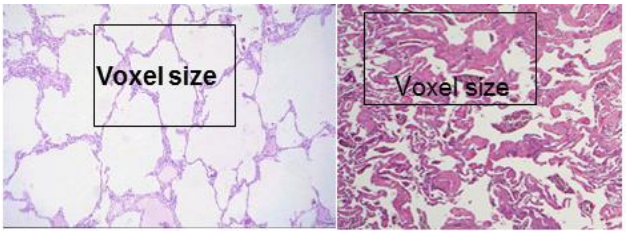

Figure 5: Left picture shows normal lung tissue compared to dens lung tissue due to inflammation on the right side

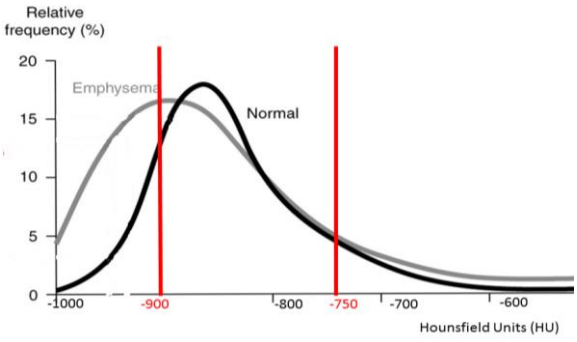

Figure 6: Attenuation values between -750 to -900 HU are highlighted on the frequency distribution curve

Paper IV

In study IV we investigated regional air trapping visible on expiratory HRCT in smokers with normal lung function as a measure of small airways disease. Further, we studied the relationship between regional air trapping and other morphological changes on HRCT with air trapping indices (AI). Regional air trapping was present frequently in smokers and in

never-smokers with normal lung function; but it was observed in just a few COPD patients. Smokers with normal lung function who had regional air trapping on expiratory HRCT had significantly lower AI than those without. This sub-group of smokers had better-preserved lung function and less emphysema. Lung attenuation on expiratory HRCT and lung function expressed as FEV_1 and FEV_1/FVC were positively correlated to neutrophil concentration in BAL. The AI correlated negatively to neutrophil concentration.

Sub-group of smokers in preclinical stage?

We demonstrated that HRCT is a powerful tool for assessing early pathological changes in smoker's lung with normal lung function and with no apparent clinical symptoms. Although quantitative methods are easily available and time-saving they lack the human eye's sensitivity towards early pathological changes in the lungs. Employing visual assessments and densitometry together, however, we were able to separate smokers with normal lung function in two distinct sub-groups. One was more susceptible to cigarette smoke with decline in lung function and emphysema while the other had preserved both function and parenchyma better. One interesting finding was the positive correlation of neutrophils to better lung function and preserved lung parenchyma. This finding may indicate that the role of neutrophils in pathogenies of COPD should be revised. Probably this sub-group of smokers is able to respond to the adverse effect of cigarette smoke better than the other. Another possibility can be that the other group was now in more advanced stage of preclinical disease.

6 Conclusions

-Cell counts and cell concentration showed a four- to fivefold increase among smokers compared to never-smokers and ex-smokers. The cell increase in smokers was due to macrophage dominance. The percentage of recovered BAL fluid declined with increased age. Female smokers had higher recovered fluid than male smokers.

-Morphological changes on HRCT were more frequent in all newly-diagnosed RA than among controls. RA patients with positive ACPA had more morphological changes than ACPA-negative RA patients. A higher concentration of ACPA in ACPA-positive RA patients was demonstrated in BAL than in serum.

- Lung density in smokers with normal lung function was higher than in never- smokers and COPD patients. Increased lung density in smokers with normal lung function was positively correlated to increased cell concentration in BAL. Lung density was higher in females than males regardless of smoking history.

- Visual assessment of morphological changes on expiratory and inspiratory HRCT combined with lung densitometry was a useful method for dividing smokers into two distinct sub-groups. Those with regional air trapping had better lung function and less emphysema. AI was higher in smokers without regional air trapping. Neutrophil concentration in BAL was positively correlated to AI in smokers.

7 FUTURE PLANS AND PERSPECTIVES

Participants in the cross-sectional studies reported in papers III and IV are well characterized at baseline. Data has been collected on lung function and inspiratory and expiratory HRC, blood samples, bronchial biopsies, BAL fluid etc. A follow-up study regarding changes in lung function, lung density and morphological changes shown on HRCT within a period of 7-10 years may contribute to the detection of some valuable predictive markers for disease progression and severity.

The participants in study II were newly-diagnosed RA patients examined at baseline with HRCT and spirometry. Blood samples were obtained from all and 23 cases underwent bronchoscopy and BAL. These base line data were from steroid- and methotrexate-naïve patients. A six-month follow-up study on these patients with HRCT and clinical evaluation has been already performed and data has been collected for analysis. The analysis may answer several important questions concerning the development of morphological changes on HRCT, whether they slow down or progress after methotrexate medication – a cornerstone of RA therapy – disease progress and other treatment responses in sub-groups of ACPA-positive and ACPA-negative RA patients related to pulmonary changes.

8 POPULÄRVENSKAPLIG SAMMANFATTNING

Bakgrund

Cigarettrökning skadar luftvägarna dels genom direkt påverkan på slemhinnan och dels genom initiering av en inflammatorisk process som mobiliserar kroppens immunceller till att utsöndra skadliga ämnen. Dessa ämnen i sin tur orsakar ytterligare skador både lokalt i lungor men även i andra organ, såsom hjärta, kärl och skelett. Den kroniska inflammationen i lungor leder till bindvävsomvandling med förträngning av små luftvägar och förlust av lungvävnad. Dessa förändringar kan manifestera sig i kroniskt obstruktiv sjukdom (KOL). Den inflammatoriska processen kan emellertid också fortskrida i många år utan påtagliga symptom eller påverkan på lungfunktionen. Den skadliga effekten av cigarettrök varierar starkt mellan olika individer och medför att det kan finnas olika manifestationer av tobaksrök hos olika individer, SK fenotyper. Spirometri används för att diagnostisera KOL och mäter enbart luftvägsobstruktionen men den ger ingen information om bakomliggande orsak till luftflödesbegränsningen. Dessutom ger både tidiga skador i små luftvägar och begynnande destruktion av lungvävnad(emfysem) inte utslag på spirometri och förblir ofta oupptäckta.

Rökning är också en känd riskfaktor för den vanligaste autoimmuna sjukdomen, ledgångsreumatism, reumatoid artrit (RA). Lungförändringarna på skiktröntgen är vanligt förekommande hos dessa patienter och specifika antikroppar mot kroppsegna ämnen, sannolikt från lungan, har påträffats hos personer som insjuknat i RA. Dessa antikroppar mot citrullinerad peptid (ACPA) kan ibland finnas redan före kliniska symtom.

Syftet med föreliggande avhandling är att på ett strukturerat sätt kartlägga aspekter avrökinducerad inflammation och strukturella förändringar i lungor och luftvägar i ett tidigt skede med fokus på två stora folksjukdomar, KOL och RA.

I delarbete I undersökte vi effekt av rökning på celler i lungsköljvätska, bronkoalveolärt lavage (BAL). Detta är en metod med vilken man genom ett smalt fiberoptiskt instrument nedförd i lungan, kan skölja upp celler från de djupa luftvägarna. Studien är en retrospektiv sammanställning av BAL resultat erhållna under åren 1990-2009 vid vår enhet. Etthundratrettiotvå rökare med normal lungfunktion och 44 exrökare inkluderades. Tvåhundranittiofem friska aldrigrökare tjänade som kontrollgrupp. Rökare hade cirka fyra gånger fler inflammatoriska celler i BAL än både aldrigrökare och exrökare. Den kumulativa cigarettkonsumtionen påverkade både totalantal celler och cellkoncentration i BAL. Returvolymen vätska minskade med stigande ålder och denna var också mindre hos rökande män än rökande kvinnor. Våra resultat kan användas vid tolkning av BAL i kliniska sammanhang och poängterer vikten av noggrann bedömning av rökstatus vid tolkning av resultat från lungsköljvätska.

Syftet med delarbete II var att undersöka strukturella och immunologiska förändringar i lunga vid nydebuterad reumatoid artrit (RA). Skiktröntgen genomfördes på 105

nydebuterade patienter med RA varav 24 även genomgick bronkoskopi inkluderande lungsköljning. . Antikroppar mot citullinerad peptid (ACPA+) påvisades hos 70 patienter. Fyrtiotre försökspersoner både rökare och aldrigrökare tjänade som kontrollmaterial. Lungskiktröntgen bedömdes av två experter och dessa förändringar relaterades till förekomst av ACPA+ och rökvanor. Patologiska förändringar i lungor upptäcktes i högre grad hos ACPA+ RA patienter (63 %) jämfört med ACPA- (37 %) kontroll (30 %). Lungförändringarna visade ingen korrelation till rökstatus. Nivåerna av ACPA var högre i lungsköljvätska jämfört med serum hos ACPA+ patienter. Studien visar att lungförändringar är vanliga hos RA patienter i tidigt sjukdomsskede och att dessa förändringar är korrelerade till förekomst av ACPA. Fynden förstärker hypotesen; att autoimmunitet kan initieras i lungor hos ACPA+ RA patienter.

Syftet med delarbete III var att studera lungtäthet mätt med skiktröntgen hos rökare med normal lungfunktion, friska aldrigrökare samt patienter med lindrig till måttlig KOL och att utröna om tätheten i rökarnas lungor speglar graden av inflammation i lungvävnad. Fyrtio rökare med normal lungfunktion, 40 friska aldrig rökare och 40 KOL patienter genomgick skiktröntgen av lunga samt lungsköljning. Lungtätheten var högre hos rökare jämfört med friska aldrigrökare och KOL patienter. Cellkoncentrationen i lungsköljvätska var positivt korrelerad till lungtäthet hos rökare. Kvinnor hade tätare lungor än män oavsett rökstatus. Studien visar att ökad lungtäthet hos rökare kan vara ett tecken på inflammation i lungor och att denna går att mäta med skiktröntgen.

Fyrtio rökare med normal lungfunktion, 40 friska aldrig rökare och 40 KOL genomförde skiktröntgen både i in- och utandning. Bilderna bedömdes både kvalitativt och kvantitativt av två oberoende experter."Airtrapping index", ett beräknat kvantitativt mått på engagemang av små luftvägar, var signifikant lägre hos rökare och aldrig rökare jämfört med KOL- patienter. Visuellt bedömd "airtrapping" fanns hos 63 % av rökarna, 45 % av aldrigrökarna och 8 % av KOL-patienterna. Emfysem detekterades hos 80 % av KOL-patienterna och 55 % av rökarna. Mikronoduli (små diffusa tunna förtätningar) fanns hos 58 % av rökarna och 38 % av KOL-patienterna. Bronkväggsförtjockning förekom hos 75 % av rökarna och 80 % av KOL-patienterna. Det saknades emfysem och mikronoduli hos aldrigrökarna men 12 % hade bronkväggsförtjockning. De rökare som hade tecken på visuell ” air trapping” hade bättre bevarad lungfunktion och mindre emfysem jämfört med rökarna utan. "Airtrapping index" visade negativ korrelation med neutrofila granulocyter, en viktig inflammatorisk cell, i lungsköljvätska.

Sammanfattningsvis har vi studerat inflammatoriska och strukturella förändringar i lungor hos rökare, KOL-patienter och RA patienter med hjälp av analys av skiktröntgen och lungsköljvätska. Dessa förändringar finns även i ett tidigt icke symtomgivande stadium hos rökare. Studierna visar att strukturella förändringar vid rökrelaterade sjukdomar är heterogena. Våra resultat ökar kunskaper om rökningens effekter på lungan som målorgan och påvisar att utfallet av rökning hos den enskilda patienten kan skilja sig åt.

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