Diplomarbeit

Increased levels of serum heat shock protein 27 as a possible marker for incipient obstructive pulmonary disease in a risk cohort

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1. ZUSAMMENFASSUNG

Hintergrund: Die chronisch obstruktive Lungenerkrankung (COPD) ist durch eine progressive Atemwegsobstruktion und eine abnormale entzündliche Immunantwort gekennzeichnet. Diese Erkrankung ist mit einer hohen Morbiditäts- und Mortalitätsrate weltweit vergesellschaftet; ein weiteres Ansteigen der Prävalenz wird prognostiziert. Trotz allem wird die Diagnose „COPD“ häufig erst in einem späteren Stadium der Erkrankung gestellt, da valide Biomarker zur Früherkennung bis dato noch nicht bekannt sind. Erhöhte Serumspiegel des Stressproteins heat shock protein (HSP) 27 in COPD Patienten und Rauchern – verglichen mit gesunden Nichtrauchern – wurden bereits in früheren Studien nachgewiesen. Basierend auf diesen Resultaten war es Ziel der jetzigen Studie einen möglichen Zusammenhang zwischen radiologischen Zeichen einer beginnenden COPD (Air Trapping [AT] und Emphysem [E]) und erhöhten HSP27 Serumwerten nachzuweisen.

Material und Methoden: 120 subjektiv gesunde Raucher wurden in diese Studie eingeschlossen. Serumproben wurden zum Zeitpunkt der Lungenfunktionsprüfung abgenommen. HSP27, pro-inflammatorische Zytokine, CXCR2 Chemokine, Matrix Metalloproteinasen, und Apoptose-Marker wurden anschließend mittels ELISA-Technik in den Serumproben bestimmt. Allen Studienteilnehmern wurde die Möglichkeit einer HR-CT Untersuchung der Lunge auf freiwilliger Basis angeboten.

Ergebnis: 94 der 120 Probanden willigten in eine HR-CT Untersuchung ein. AT oder AT+E konnte bei 57.5% der Probanden diagnostiziert werden. Probanden mit AT+E wiesen signifikant höhere HSP27 Serumkonzentrationen auf als Probanden ohne Lungenpathologie (NAD) (4618±1677 vs. 3282±1607 pg/ml; mean±SD, P=0.0081). HSP27 erwies sich in einem Regressionsmodell als guter diagnostischer Marker für AT+E (AUC 0.724; P=0.0033). Weiters wurde im Serum von Probanden mit AT+E signifikant höhere Interleukin-8 Serumspiegel gemessen als im Serum von Probanden ohne Lungenpathologien.

Diskussion: Erhöhte HSP27 Serumkonzentrationen zeigten unabhängig von klinischen Parametern einen eindeutigen Zusammenhang mit radiologischen Zeichen einer drohenden COPD. Die HSP27 Serumkonzentration würde sich somit als diagnostischer Marker zur Erkennung früher Anzeichen einer COPD anbieten.

2. ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a disease characterized by progressive airflow obstruction due to airway remodeling and an abnormal inflammatory response. This disease is amongst the leading causes of morbidity and mortality worldwide and its prevalence is expected to rise; yet, COPD still remains an underdiagnosed disease and currently no biomarkers for its early detection are known.
In former studies, we evidenced elevated levels of the stress protein heat shock protein (HSP) 27 in COPD patients and smokers as compared to healthy non-smokers. Based on these findings we hypothesized whether elevated HSP27 levels are associated with early radiological signs of COPD (air trapping and emphysema, AT and E respectively) detectable in HR-CT scan.

Materials and Methods: 120 apparently healthy smokers were included in this study. First, lung function testing with a portable testing device was performed and serum samples were obtained. HSP27 serum levels, inflammatory cytokines, CXCR2 chemokines, matrix metalloproteinases, and markers for apoptosis were determined with conventional available ELISA kits. Voluntary HR-CT scan was subsequently offered to all study subjects.

Results: 94 subjects underwent HR-CT examination: AT or AT+E was detected in 57.5%. Subjects with AT+E showed significantly higher HSP27 levels than those without any pathology (NAD) (4618±1677 vs. 3282±1607 pg/ml; mean±SD, P=0.0081). In a univariate logistic regression model including NAD and AT+E, the AUC of HSP27 in ROC curve was 0.724 (0.594-0.854 95% CI; P=0.0033), whereas HSP27 levels did not correlate with lung function. Further, proinflammatory interleukin-8 was also elevated in subjects who evidenced AT+E as compared to subjects without any pathology.

Conclusion: HSP27 serum levels positively correlated with early radiological signs of COPD, whereas lung function results neither correlated with HSP27 serum values nor with radiological findings. Elevated serum HSP27 level may serve as a potential biomarker to identify patients with early signs of COPD independent of lung function.

3. INTRODUCTION

3.1. Definition and Staging of COPD

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines, chronic obstructive lung disease (COPD) is

„…a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases.“[1]

COPD is not a single disease entity but a different group of conditions with the characteristic of obstructive airflow limitation. The main pathological entities are chronic bronchitis in combination with mucus hypersecretion and an obstructive ventilatory pattern characterized by permanent airway obstruction (forced expiratory volume in one second [FEV1]/forced vital capacity [FVC] ratio < 0.7). Additionally, other entities found in COPD are emphysema, which is defined as enlargement of distal airspaces caused by the destruction of airway walls, and small airway disease, which also plays a major role in the development of airway obstruction[2,3] (Figure 3.1-1).

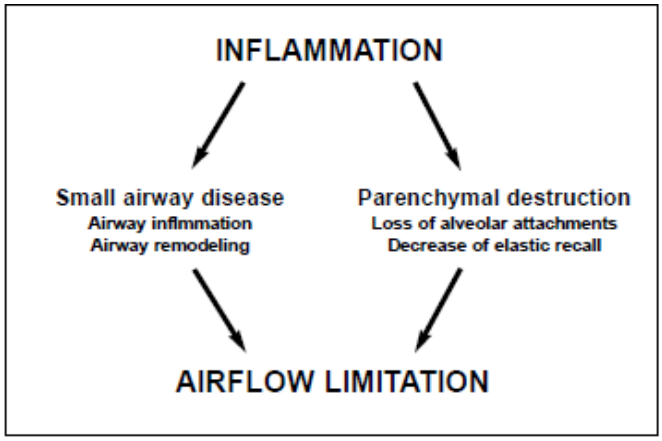


Figure 3.1-1: Mechanisms underlying airflow obstruction in COPD (adapted from the GOLD Guidelines 2007[1]).

The severity of COPD is classified into four stages by means of spirometry (Figure 3.1-2). The cut-off points have not been clinically validated and are usually not age-adjusted, but

rather used for purposes of convenience. Spirometric evaluation should be performed after the administration of a bronchodilator in order to minimize the variability of the test.

Stage I: Mild	$FEV_1/FVC < 0.70$ $FEV_1 \geq 80\%$ predicted
Stage II: Moderate	$FEV_1/FVC < 0.70$ $50\% \leq FEV_1 < 80\%$ predicted
Stage III: Severe	$FEV_1/FVC < 0.70$ $30\% \leq FEV_1 < 50\%$ predicted
Stage IV: Very Severe	$FEV_1/FVC < 0.70$ $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus chronic respiratory failure

Figure 3.1-2: Classification of COPD (adapted from the GOLD Guidelines 2007[1]).

In stage I, symptoms (such as cough and sputum production) might be present, but the abnormal lung function is usually not perceived by the individual. Medical advice is normally seeked in stage II when shortness of breath, cough and sputum production occurs, sometimes accompanied by an exacerbation of the disease. In stage III, lung function further declines resulting in a greater shortness of breath, a reduced exercise capacity and fatigue. Exacerbations occurring in this stage often affect quality of life rigorously. In stage IV, chronic respiratory failure (paO_2<60mmHg ± $paCO_2$>50mmHg) occurs. When clinical signs of right heart failure exist, patients are automatically staged COPD IV even if the FEV1 is above 30% predicted. Exacerbations at this stage are potentially life-threatening[1].

COPD GOLD stage 0 („At Risk“) is defined as the occurrence of symptoms (chronic cough and sputum production) despite a normal lung function[4]. This COPD stage appeared in the GOLD report in 2001, but has been removed from the GOLD-guidelines because of incomplete evidence that individuals meeting the definition of „Stage 0 – At Risk“ automatically progress on to GOLD stage I. However, it is accepted that the production of sputum and chronic cough are not normal processes and that underlying causes should be investigated[5]. Results of a 3-year follow-up study of more than 400 subjects presenting with symptoms of stage 0 revealed that about 40% of them remained stable, 1.4% progressed to GOLD stages I-II within only 3 years, and about 59% resolved to no symptoms. Persistent stage 0 was clearly associated with persistent smoking, depressive symptoms, the highest quartile of FEV1 decline per year, metabolic syndrome, and higher age. Due to this study,

excess decline of FEV1 may therefore identify a risk group that is likely to process to manifest COPD[6].

3.2. Epidemiology and Burden of COPD

Estimates for the prevalence of COPD are highly variable, ranging from about 5% to 22% for GOLD stage II or higher[7,8]. According to pooled prevalence estimates in a systematic review[9], 9.9% of subjects above 40 years suffer from COPD. In a large US survey on COPD[10] 10 million adults in the USA were found to suffer from COPD diagnosed by a physician, and about 24 million adults were found to evidence airflow limitation, indicating that COPD is a largely underdiagnosed disease. According to an Austrian study published in 2007[11], the overall prevalence of GOLD stage I or higher is 26.1% in individuals aged 40 years or older, increasing steeply with age. In 2005, an estimated one million of people suffered from COPD stage II-IV in Austria, and – until 2020 – an increase of more than 25% has been prognosticated[12].

Focusing on mortality, COPD was the fifth leading cause of death in high-income countries and the sixth leading cause of death in low- and middle-income countries in 2001, amounting to about 4% and 5% of total deaths, respectively[13]. Estimated mortality rates vary from 4.4/100,000 in Japan up to about 130/100,000 in China[14]. In Europe, respiratory diseases, and in particular COPD, are the third leading cause of death in the EU[15]. Mortality rates are generally higher in male subjects and increase with age and the severity of the disease. Due to the slow but steady progression of the disease, COPD is also one of the leading causes of disease burden worldwide based on disability-adjusted life years[13], appraising the number of healthy years lost owing to early death or disability[16]. In a multivariate hazard model adjusted for age, sex, race, and other parameters including participants of the first National Health and Nutrition Examination Survey (NHANES I) followed up for 22 years, the hazard ratio (HR) – defining the risk of death by reason of COPD or related conditions – for mild COPD was 1.2, for moderate COPD 1.5 and for severe COPD even 2.7 (Figure 3.2.)[17].

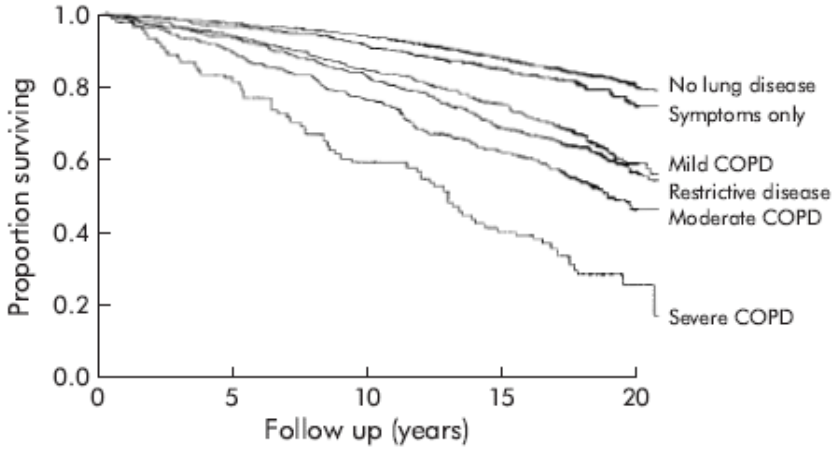


Figure 3.2.: Kaplan-Meier curve for death among 5542 participants stratified by the degree of lung function impairment (NHANES I) (adapted from Mannino et al 2003[17]).

Classifying patients by GOLD stages, 5-year mortality for COPD stage I has been reported to be 17% whereas in stage IV 73% of patients died within 5 years. During hospitalization by reason of exacerbation mortality rates rise and were estimated to be 2.5-10%[18]. The only factors having demonstrated a reduction in mortality in COPD are smoking cessation and oxygen treatment for patients with chronic respiratory failure[19,20].

Irrespective of the health aspects of COPD, enormous costs are caused by this disease year by year. According to the white book of the European Respiratory Society (ERS), the estimated annual cost amount to €38.7 billion. About 75% of the expenditures are related to the costs caused by the inability to work, 12% of the costs are related to ambulatory care, and each with 7.5% to hospitalization and medication[18]. Estimated additional costs for COPD patients range from $1,000 to $8,000 per year[14].

In addition to the enormous burden and the expenses caused by the disease itself, comorbidities related to systemic inflammation are known to be common in COPD patients: Recently, COPD has been associated with a fivefold increase in the odds of having had a cardiovascular disease, a threefold increase in the odds of having had a stroke and a twofold increase in the odds of having diabetes mellitus compared to healthy controls[21]. In the USA, COPD augmented both frequency and mortality of hospitalizations associated with comorbidities. From 1979 to 2001, percentages of hospital discharges with COPD increased from <5% to 12% mostly due to the increased proportion of those patients with COPD as secondary diagnosis[22].

3.3. Risk Factors for the Development of COPD

3.3.1. Tobacco Smoking

Corresponding to literature, there is no doubt that tobacco smoking is the major risk factor leading to the development of COPD[23]. In the 1950s the first studies investigating the relationship between active tobacco smoking and the risk of developing COPD were carried out[24]. Shortly afterwards, tobacco smoking has been established as not only a causative risk factor, but also as the most important risk factor in the development of COPD. Fletcher et al[25] designed the first prospective epidemiological study and investigated the early stages of the development of COPD. Within 8 years of follow-up, mucus hypersecretion, bronchial infections and airway obstruction were assessed every 6 months in almost 800 male study subjects. The main findings of this study were that loss of FEV1 is a continuous process that proceeds over years, and that non-smokers evidence a slower decline of FEV1 and hardly develop significant airflow obstruction as compared to smokers. Fletcher also reported that the susceptibility to smoke is a crucial issue: the more susceptible a subject is, the faster he or she will be affected by airway obstruction. Smoking individuals not susceptible to smoke are likely to undergo the same course of FEV1 decline than never-smokers (Figure 3.3.1-1).

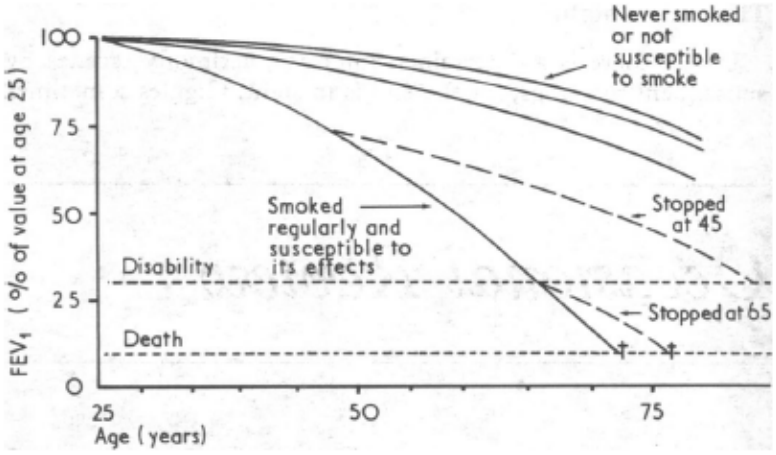


Figure 3.3.1-1: Effects of smoking, smoking cessation, and susceptibility to smoke on the decline of FEV1 (adapted from Fletcher et al 1977[25]).

About 15-20% of long-term smokers will develop COPD according to the International Agency for Research on Cancer[26]. In a large follow-up study[27] it has been concluded that 25-year incidence of moderate and severe COPD in continuous smokers is 20.7% and 3.6%, respectively, indicating an absolute risk of 25% for developing COPD. However, according to

Lundbaeck and colleagues[28], the prevalence rate of airway obstruction could be as high as 50% in people aged >70 years if they continue smoking. Mannino and coworkers[29] obtained similar results. They reported prevalence rates of 60% in smokers, 50% in ex-smokers, and up to 30% in non-smokers in the elderly population (75 – 84 years) of the US NHANES III population sample. Referring to the PLATINO study, which aimed at the description of the epidemiology of COPD in five major Latin American cities, and the Burden of Obstructive Lung Disease (BOLD) study, 3% to 15% – a percentage that varied due to different populations and the use of different methods – amongst never-smokers are prone to develop COPD[7,8].

Mortality in relation to smoking has been observed by Doll and colleagues[30] during 40 years of follow-up. The death rate ratios of continuing cigarette smokers aged 45 to 64 years were threefold and in those aged 65 to 84 twofold as compared to never-smokers. The age of starting smoking, the total pack-years (PYs) smoked and the current smoking status were found to be predictive of mortality due to COPD.

Smoking cessation is probably the most effective treatment in patients with COPD. By stopping smoking, lung function parameters in patients with early stages of COPD improve and the annual loss of FEV1 slows, whereas the use of bronchodilators has only a short-term effect in improving FEV1[31]. Especially before the age of 35 smoking cessation has a positive effect on long-term survival regarding COPD and other smoking-related diseases[30]. However, a more complex interaction between exogenous factors and individual susceptibility is proposed, as about half of COPD cases are due to non-smoking causes[17,28].

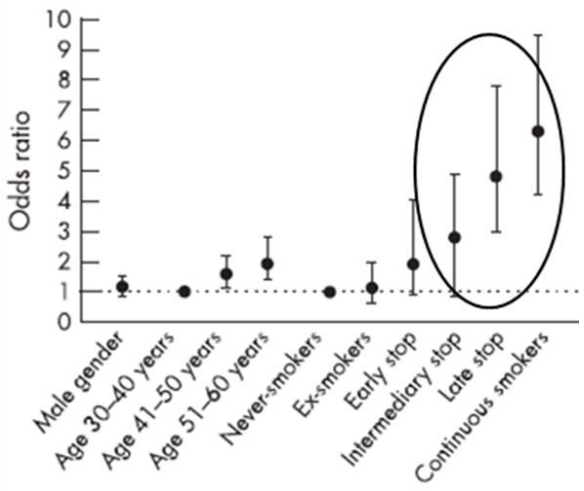


Figure 3.3.1-2: Significant predictors of COPD stage II or worse (odds ratios and 95% confidence intervals; adapted from Lokke et al 2006[27]).

3.3.2. Air Pollution and Occupational Exposures

The association between COPD exacerbations or aggravation of pre-existing COPD and high concentrations of outdoor air pollutants is supported by strong evidence[32]. Studies have mostly shown a relationship between higher outdoor-pollutant levels and lower lung function in children and young adults[33,34]. Increased prevalence rates of COPD diagnosis or symptoms[35] and elevated risk for COPD or respiratory hospitalization[36,37] were reported to occur in urban and more polluted areas.

Sunyer and coworkers[38] demonstrated the importance of the "urban factor", as prevalence and new onset of chronic phlegm were associated with traffic intensity (adjusted odds ratio [OR] 1.86) in this study cohort. Particles and gaseous pollutants were further found to be associated with reduced lung function and increased proportion of abnormal FEV1 in a follow-up from 10 to 18 years of age, representing risk factors for the development of COPD[39]. A beneficial effect of reducing air pollution has been demonstrated by the reduced proportional hazard mortality rate ratios for total, cardiovascular, and respiratory mortality with decreasing PM2.5 (particulate matter with a 50% cut-off aerodynamic diameter of 2.5µm) in the US six city study[40].

Regarding indoor air pollution, about 50% of all households and 90% of rural households worldwide use biomass fuel and coal as their main source for cooking and heating. The use of these fuels leads to high indoor concentrations of substances that are harmful to health, such as PM10, nitrogen dioxide, carbon monoxide, sulphur dioxide, formaldehyde, and polycyclic organic matter[41]. Many of these matters exceed the maximum allowable concentrations of international safety standards[42]. Indoor air pollution may increase the risk of acute and chronic respiratory disorders and lung function impairment: Recent estimates have revealed that 1.5 to 2 million deaths per year worldwide could be caused due to indoor air pollution, which is hereby amongst the 10 leading preventable risk factors that contribute to the global burden of disease[43]. In developing countries, about 50% of deaths from COPD are attributable to biomass smoke, of which 75% are women[13].

Further, indoor air pollution is a major risk factor for acute infections of the lower respiratory tract which are one of the most important causes of death for children living in developing countries and may predispose them to COPD[44].

The assessment of "occupational" COPD is difficult because of some reasons: The etiology of COPD is multifactorial; therefore, the differentiation between individuals with COPD due to occupational exposures and those with COPD due to other causes is hardly possible. Further, many workers with COPD are concurrently exposed to cigarette smoke and, moreover, exposed workers tend to have better baseline pulmonary function than the general population ("healthy worker effect")[45]. However, studies identified several areas of exposure related to high prevalence rates of COPD, namely the rural environment (farming), the plastic, textile, and rubber industry, and the mining, iron and steel, and wood industry[46,47]. Longitudinal studies found an association of COPD with occupational exposures in coal and hard-rock miners, tunnel workers and concrete manufacturers with a potentially greater effect of dust exposure than cigarette smoking in heavily exposed workers[48]. A significantly higher risk of death due to COPD in construction workers exposed to fumes and mineral dust as compared to unexposed workers was also reported[49]. Quantitative assessment of emphysema has shown a relationship between cumulative (coal) dust exposure and the degree of emphysema in several studies of coal and hard-rock miners[50].

The proportion of patients with COPD attributable to occupation has been estimated to be 19% overall and 31% in never-smokers[47]. A systematic epidemiological review conducted by the American Thoracic Society (ATS) showed that approximately 15% of COPD cases might be attributable to workplace exposure[51]. An estimate through workforce data and the CAREX database ascribed 318,000 deaths and approximately 3,7 million disability-adjusted life years to workplace-associated COPD in 2000[52]. Joint exposure to both occupational factors and smoking further increases the risk of developing COPD[53].

3.3.3. Respiratory Tract Infections

Fifty years ago Laurenzi and coworkers[54] revealed that pathogens are present in the lower respiratory tract of patients with COPD, in contrast to the sterile lung environments of healthy subjects. Typical bacteria such as non-typeable Haemophilus influenzae (NTHI) and Pseudomonas aeruginosa, atypical bacteria such as Chlamydia pneumoniae, adenoviruses, respiratory syncytial virus (RSV) and fungi have been implicated in chronic infection in COPD patients[55].

Due to epidemiological studies, frequent lower respiratory tract infections during childhood are an independent risk factor for the development of COPD later in life[44]. A study based on epidemiological surveys investigating the prevalence of respiratory symptoms in almost

10,000 subjects revealed that subjects with frequent respiratory infections during childhood suffered more frequently from chronic cough, chronic bronchitis and asthma than the control group[56]. Frequent lower respiratory tract infections during childhood further impair lung growth, which is later in life reflected in a lower FEV1[57].

Cell-free supernatants of NTHI, commonly causing severe pneumonia in children[58], Pseudomonas aeruginosa and Staphylococcus aureus were shown to impair ciliary function[59]. Bacterial products in the airways may be a potent stimulus for neutrophil migration into the airways, and as neutrophil elastase is also an inhibitor of ciliary activity, further damage of respiratory epithelium occurs[60,61]. An observation of subjects with present COPD revealed that patients with potentially pathogenic microorganisms in their sputum had higher neutrophil differential counts, higher levels of interleukin (IL)-8, leukotriene (LT)-B4, tumor necrosis factor (TNF) α and neutrophil elastase, and a more exaggerated neutrophil chemotactic response than subjects without colonization of the tracheobronchial tree[62]. If bacteria and bacterial products are able to cause neutrophil influx and degranulation, they could sustain chronic inflammation and contribute to parenchymal lung damage, small airway obstruction and exacerbations seen in COPD[63] (Figure 3.3.3.).

About 20 to 30% of acute lower respiratory tract infections in childhood are due to viral infections. Half of these are attributed to RSV, 14% to adenovirus, 7% to influenza and approximately 5% to parainfluenza[64].

RSV is a common pediatric pathogen and capable of evading the immune response by inducing skewed T helper type 2 (Th2) cell responses, antagonizing antiviral cytokines, mimicking chemokines and inhibiting apoptosis. RSV has been detected in more than 30% of patients with stable COPD and was associated with higher levels of IL-6 and IL-8 in sputum and a faster decline in FEV1 than in those patients without RSV colonization. These findings suggest that RSV may persist in COPD patients[65].

Adenoviruses often cause childhood respiratory disease and are able to remain in host cells in a latent form in which viral proteins are produced without replication of a complete virus[66]. It is assumable that latent adenoviral infections may amplify lung inflammation caused by cigarette smoke, as Adenoviral Early Region 1A (E1A) DNA persists in alveolar epithelial cells from COPD patients[66]. E1A transfected airway epithelial cells further produce higher amounts of inflammatory mediators such as IL-8, inter-cellular adhesion molecule (ICAM)-1

and growth factors such as connective tissue growth factor and transforming growth factor (TGF) β1[67,68].

Presumably, the impact of childhood lower respiratory tract infection on the prevalence of COPD is generally higher in developing countries where high incidence and inadequate treatment of these infections is usual[63].

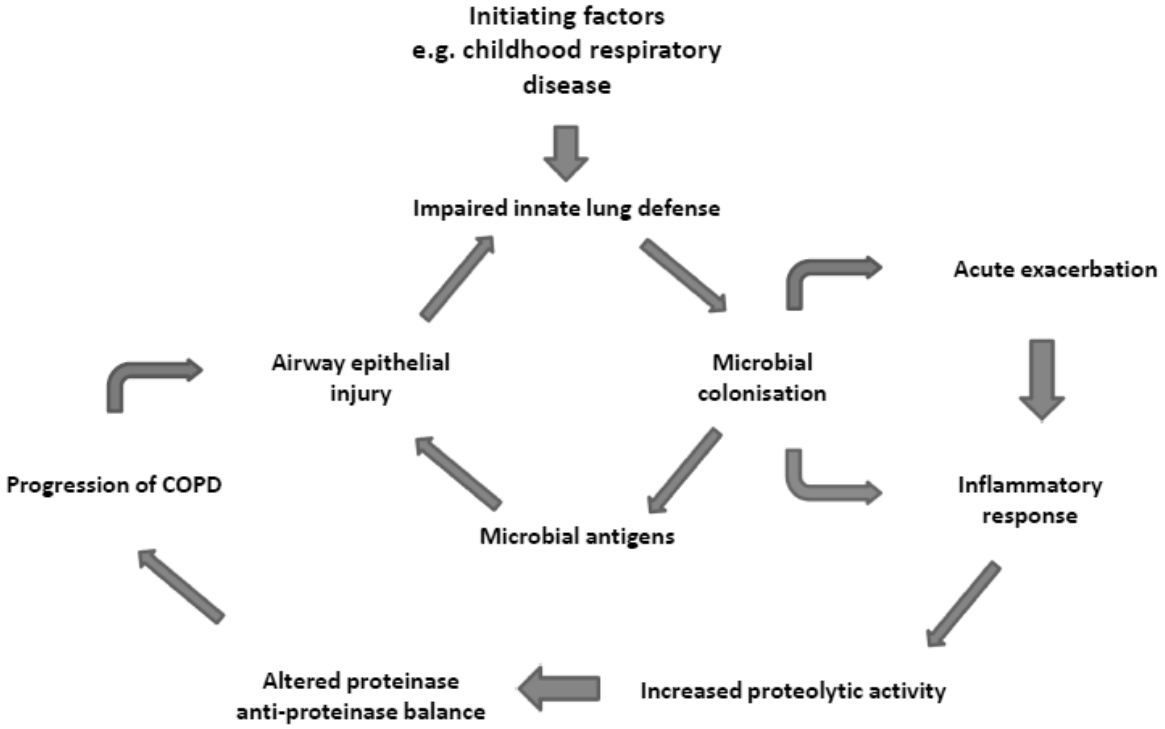


Figure 3.3.3.: The "vicious circle hypothesis" of infection and inflammation in COPD (adapted from Sethi et al 2000[63]). Initial insults to the airways are likely to cause impairment of the innate lung defense which facilitates bacterial colonization of the lower respiratory tract. Via the induction of inflammation, the innate lung defense is further disrupted and chronic inflammation is perpetuated.

3.3.4. Genetic Factors

Although tobacco smoking is accepted as the major risk factor for the development of COPD and various other exogenous risk factors are described, there is undeniable recognition that genetic factors also play a great role in the increased decline of lung function[69,70]. The finding that approximately only a fourth of smokers develops COPD[27] further corroborates the assumption that vulnerability to tobacco smoke varies depending on an individual´s genotype.

Family history seems to have an effect on the risk for developing COPD. Parental history of COPD and educational level have recently been demonstrated to be significant predictors of COPD, independent of parental history of smoking, personal lifetime smoking history, and

environmental tobacco exposure during childhood[71]. According to a Danish twin study the concordance rate for COPD amongst monozygotic twins was higher than in dizygotic twins. After adjusting for sex, smoking and age at first hospital admission for COPD, the risk of developing COPD in the co-twin of an affected twin was higher in monozygotic than in dizygotic twins, with HR 4.3[72].

A well-established genetic risk factor for the development of COPD is alpha1-antitrypsin (AAT) deficiency, an autosomal hereditary disorder clinically associated with emphysema and COPD in both smokers and non-smokers. AAT is a serine protease inhibitor encoded by the SERPINA1 gene located on chromosome 14. A missense mutation causes a variant, known as the Z allele (PI ZZ genotype), responsible for reduced serum levels of AAT[73]. The risk of developing COPD in subjects with heterozygous AAT deficiency (PI MZ) has been analyzed in several studies, however, with different results[74]. Silverman and coworkers[75] reported a significant genotype-environment interaction, comparing the relationship between FEV1 and PYs in PI ZZ, PI MZ, and PI MM subjects (Figure 3.3.4a and 3.3.4b). One of the latest studies comparing PI MZ individuals with PI MM individuals in two large populations demonstrated that PI MZ was associated with a lower FEV1/(F)VC ratio and with a slightly higher prevalence of emphysema than the control group[76].

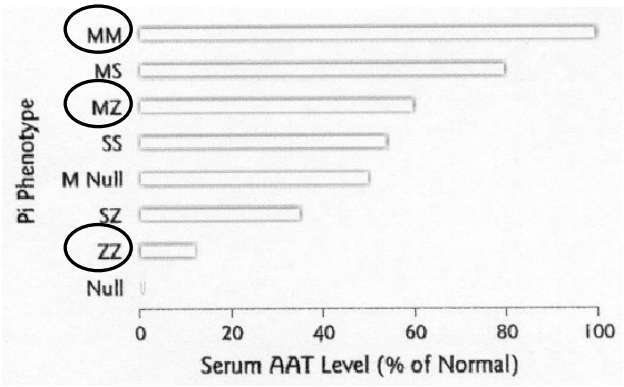


Fig. 3.3.4a

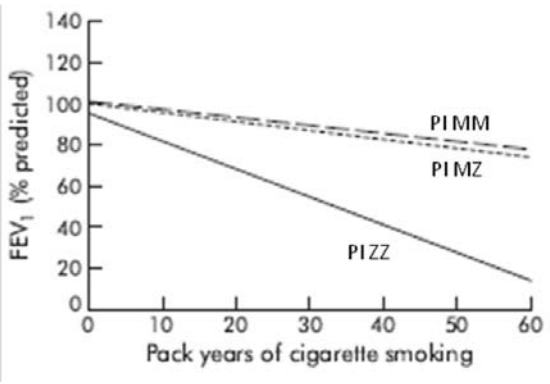


Fig. 3.3.4b

Figure 3.3.4a: PI phenotypes and corresponding serum AAT levels (adapted from Fregonese et al 2008[77]).

Figure 3.3.4b: PI ZZ subjects show a steeper FEV1 decline in response to PYs than PI MM and PI MZ subjects, indicating a genotype-environment interaction (adapted from Silverman et al 1992[75]).

There are a number of relatively rare genetic syndromes[45] that predispose to COPD and especially in the last years numerous studies have been carried out in order to assess genetic factors playing a possible role in the susceptibility to airway obstruction. Matrix proteins,

metalloproteinases, glutathione transferases and many other candidate genes have been discussed in the literature[78] (Table 3.3.4.).

Symbol	Name	Locus	Function
ELN	Elastin	7q11	Matrix
FBLN4	Fibulin 4	11q13	Matrix
FBLN5	Fibulin 5	14q32	Matrix
FBN1	Fibrillin	15q21	Matrix
ATP7A	Copper transporter	Xq13	Matrix
TGFB1	Transforming growth factor β1 (TGFβ1)	19q13	Matrix
LTBP4	Latent TGFβ binding protein 4	19q13	Matrix
SERPINA1	α_1-Antitrypsin	14q32	Anti-protease
SERPINE2	Serpin E2	2q33	Antiprotease
TIMP2	Tissue inhibitor of metalloproteinase 2	17q25	Antiprotease
MMP1	Metalloproteinase 1	11q22	Protease
MMP9	Metalloproteinase 9	20q11	Protease
MMP12	Metalloproteinase 12	11q22	Protease
EPHX1	Epoxide hydrolase 1	1q42	Detoxification
GST-P1	Glutathione S-transferase P1	11q13	Detoxification
GST-M1	Glutathione S-transferase M1	1p13	Detoxification
HMOX1	Heme oxygenase 1	22q13	Detoxification
SOD3	Superoxide dismutase 3	4p15	Detoxification
TNF	Tumour necrosis factor	6p21	Inflammation
GC	Group specific component	4q12	Inflammation

Table 3.3.4.: Candidate genes for COPD (adapted from Marciniak et al 2009[78]).

Lately, the role of Sox5, a member of the group D sex-determining region-related transcription factors, has been evaluated in COPD[79]. More than 20 single nucleotide polymorphisms were found to be related to COPD. Findings in a Sox5 –/– mouse model – abnormal lung development, delayed maturation and altered branching morphogenesis – further corroborated this hypothesis[79]. In another recent large case-control study more than 250 polymorphisms of genes with a possible relationship to COPD have been evaluated. Signal transducer and activator of transcription (STAT) 1, a key effectors of interferones, and NFKBIB/SIRT2 have been considered candidate genes[80]. Polymorphisms in the NFKBIB gene may alter the immune response to bacteria and therefore cause chronic airway inflammation. SIRT genes, however, are involved in the aging process, which agrees with the fact that emphysema resembles the aging lung in many ways[81].

3.4. Pathogenesis and Immunology of COPD

Multiple mechanisms contributing to the abnormal inflammatory response that characterizes COPD have been described in the literature. Early findings pointed out that neutrophil elastase, the target of alpha1-antitrypsin[82], and macrophage proteinases are primary effectors of lung destruction in COPD[83]. The interaction of innate and adaptive immune system, linked by dendritic cells (DCs) is known to be a root cause in the disease process (Figure 3.4.)[84].

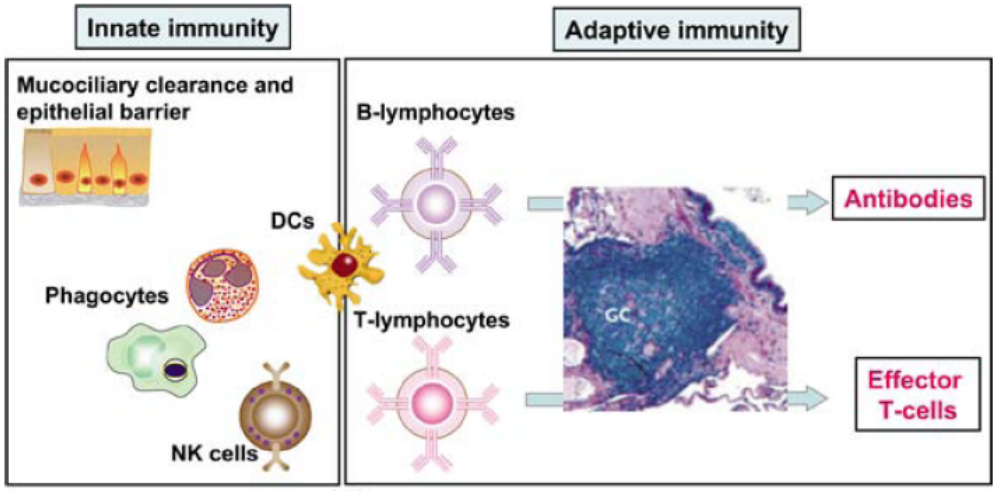


Figure 3.4.: Scheme of the effector cells of the innate and adaptive immune system. Abbreviations: NK cells: natural killer cells (adapted from Hogg et al 2008[85]).

Complementary mechanisms such as apoptosis of endothelial and epithelial cells, oxidative stress, age-related alterations, and viral infections are proposed to maintain inflammation, interfere with lung repair, and therefore further promote immune activation[66,86,87].

3.4.1. Initial Response to Airway Irritants – Players of the Innate Immune System

The initial response to airway pathogens and irritants, such as cigarette smoke, is believed to trigger an innate immune response. The innate defense system of the lung consists of the mucociliary clearance which acts in cooperation with monocytes and macrophages in order to remove particles[88]. Further, tight junctions connecting lung epithelial cells provide a physical barrier between tissue and airspace. Disruption of this barrier and tissue damage due to chronic cigarette smoke exposure initiate an acute inflammatory response[89].

Alveolar macrophages are believed to play a crucial role in the pathogenesis of COPD. They represent the first line of defense because of their special location in the interface between air

and lung tissue and are able to release reactive oxygen species (ROS), extracellular matrix proteins and lipid mediators such as prostaglandins, leukotrienes, cytokines and chemokines[90].

The number of macrophages in large and small airways, mainly in airway epithelium and subepithelium, of COPD patients was found to be significantly higher in COPD as compared to non-smokers (Figure 3.4.1-1a and 3.4.1-1b), and correlated with the degree of airflow limitation and PYs of smoking[91].

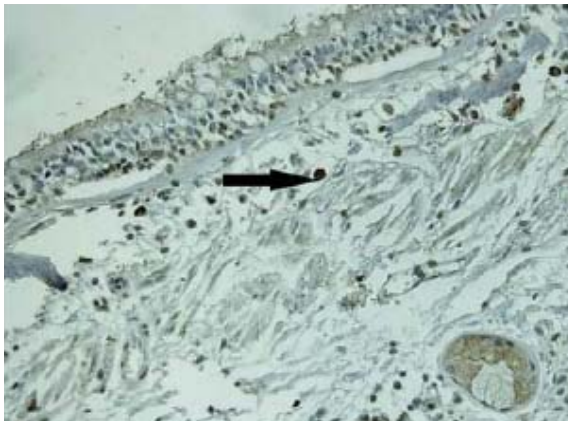


Fig. 3.4.1-1a

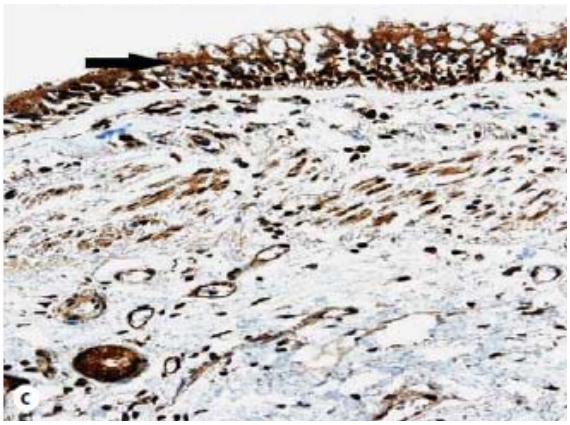


Fig. 3.4.1-1b

Figure 3.4.1-1.: NF-kBp65 positive cells, predominantly expressed in macrophages, stained immunohisto-chemically with NF-kBp65 antibody (brown) in non-smokers (**Fig.a**) and COPD patients (**Fig.b**) in large airways (adapted from Isajevs et al 2011[91]).

Cultured alveolar macrophages from smokers and COPD patients were found to release high amounts of matrix metalloproteinases (MMPs) (MMP-1, MMP-9) with increased immunoreactivity. The inhalation of cigarette smoke may induce the production of macrophage metalloelastase (MMP-12), which again induces chemotactic fragments that attract blood monocytes to the lung parenchyma[92]. In smokers with normal lung function, MMP-12, monocyte chemotactic protein (MCP)-1 and CCR5 – a receptor for macrophage inflammatory protein (MIP) 1α and 1β, regulated upon activation normal T cell expressed and secreted (RANTES) and MCP-2[93] – were shown to be increased, indicating the strong inflammatory potential of macrophages in individuals exposed to cigarette smoke[94]. The proinflammatory cytokines IL-1, IL-8 and TNFα were also found to be released in large amounts from alveolar macrophages of cigarette smokers and COPD patients[95]. The increased expression of ST2, which is a mediator released by Th2 cells and macrophages, in COPD

patients may be a possible negative feedback mechanism to control the inflammatory response and to prohibit further lung destruction[96].

Increased numbers of macrophages in COPD patients and smokers may be caused by the augmented expression of the anti-apoptotic long isoform of B cell leukaemia/lymphoma (Bcl)-X and the increased cytoplasmic expression of the cyclin-dependent kinase inhibitor $p21^{CIP/WAF-1}$, an inhibitory regulator of the cell cycle[97]. Additionally, reduced phagocytic potential of alveolar macrophages exposed to cigarette smoke has been reported[98] which, due to the defective clearance of apoptotic cells, may results in necrosis further causing tissue damage and exacerbating the inflammatory response[86].

There is much evidence in the literature that neutrophils are also primary effector cells in COPD[99]. The number of neutrophils was found to be increased in broncho-alveolar lavage fluid (BALF) of patients with COPD[100], and sputum neutrophilia was associated with greater airflow obstruction and accelerated decline in lung function in smokers[101]. Activated neutrophils can cause tissue damage through the release of various mediators. Elastases, which are capable of degrading elastin fibers and stimulating mucus secretion, MMPs, which can break down elastin and collagen, oxygen radicals, and defensis all contribute to tissue destruction[102]. The recruitment of neutrophils to the airways and to the parenchyma of the lung implies an interaction with adhesion molecules and is induced by chemotactic factors such as IL-8, and LT-B4. These factors may derive from alveolar macrophages and epithelial cells, but are also produced by neutrophils themselves[103]. Growth regulated oncogene alpha (GROα) and epithelial cell-derived neutrophil-activating peptide 78 (ENA78), other potential chemotactic factors for neutrophils, were also shown to be increased in smokers and COPD patients[104,105]. The tripeptides proline-glycine-proline (PGP) is another selective neutrophil chemoattractant enzymatically generated from extracellular matrix proteins, and was found to be increased in the sputum of COPD patients. Normally, PGP is inactivated by LT-A4 hydrolase (LT-A4H) through its peptidase activity[106]. In addition to its peptidase activity, LT-A4H generates the chemoattractant LT-B4 through its hydrolase activity. Interestingly, increased acetylation of PGP and inhibited peptidase but not hydrolase activity of LT-A4H by cigarette smoke extract was observed, leading to increased and prolonged neutrophil chemotaxis (Figure 3.4.1-2). This could be one of the explanations why neutrophilic inflammation is more persistent in the airways of patients with COPD as compared to healthy smokers[107].

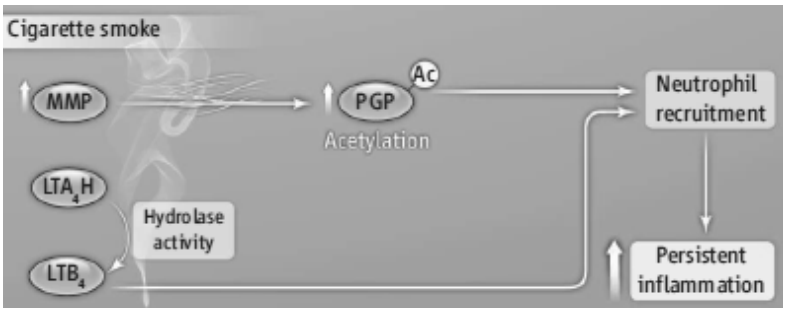


Figure 3.4.1-2: Inactivation of PGP by LT-A4H is inhibited by smoke; LT-A4H generates the chemoattractant LT-B4 through its hydrolase activity, which is not affected, and neutrophil recruitment is enhanced (adapted from Snelgrove et al 2010[107]).

Although this innate defense system responds quickly, it lacks specificity, memory, and has only limited diversity.

3.4.2. The Link between Innate and Adaptive Immune Response – Dendritic Cells

DCs act as "sentinel" cells of the innate immune system and alert the adaptive immune system to the presence of pathogens or to tissue injury.

Upon exposure to a pathogen via toll like receptors (TLRs), DCs process antigen and migrate to local lymph nodes. After undergoing maturation, DCs present antigens to T helper cells via major-histocompatibility-complex (MHC) class II proteins and the costimulatory molecules CD80/CD86 (B7.1/B7.2). The expression of IL-12 activates STAT4 inducing T cells to differentiate into Th1 cells which produce interferon (IFN) γ[108] (Figure 3.4.2.).

There is evidence in the literature that numbers of DCs are increased in lungs of COPD patients, although this topic is discussed controversially[109-111]. However, the DC-chemoattractant MIP-3α is highly expressed in COPD patients and numbers of CD4+ T cells expressing STAT4 are also increased. The expression of STAT4 and IFN-γ even correlated with the degree of airflow limitation in COPD patients[110,111].

T cells once activated by DCs can enter the lung parenchyma by means of the tissue-specific chemokine receptors[112]: CXCR3, CCR5, CXCR6 and the ligands IP-10 (interferon gamma-induced protein 10 kDa) and MIG (monokine induced by IFN-γ) were shown to be strongly expressed by structural cells in the airways of patients with COPD and correlated with disease severity[113].

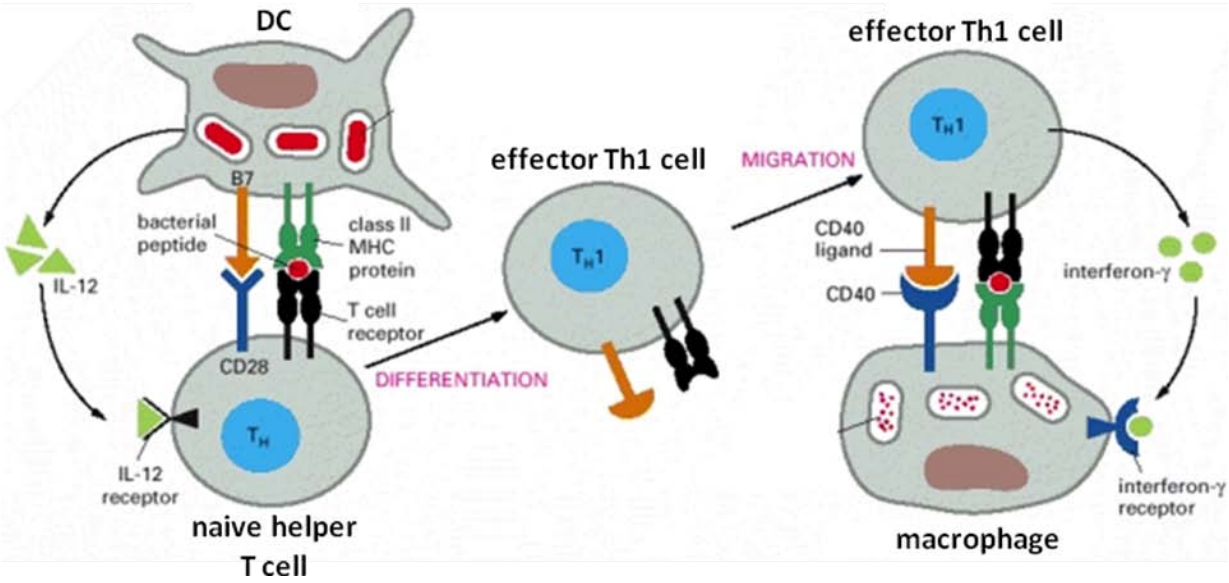


Figure 3.4.2.: A DC stimulates a naïve helper T cell to differentiate into an effector Th1 cell which in turn produces IFN-γ (adapted from[114]).

3.4.3. The Adaptive Immune Response

Results of histopathologic studies were the first to suggest a possible role of T lymphocytes in COPD. Finkelstein and coworkers[115] reported that the main cellular components of the inflammatory infiltrates within the airway walls of COPD patients are lymphocytes and macrophages.

Numbers of CD8+ lymphocytes were found to be increased in the small and large airways as well as in the lung parenchyma of COPD patients[116,117] and correlated with the degree of airflow limitation[117] (Figure 3.4.3-1). Chronic cigarette exposure of mice was proven to cause oligoclonal expansion of CD8+ T cells from lungs, which persists despite smoking cessation[118]. Knockout animal studies further showed that CD8 T cell deficient CD8-/- mice do not develop emphysema upon exposure to long-term cigarette smoke as compared to wildtype mice[119], corroborating the important role of CD8+ T cells in the development and progression of COPD.

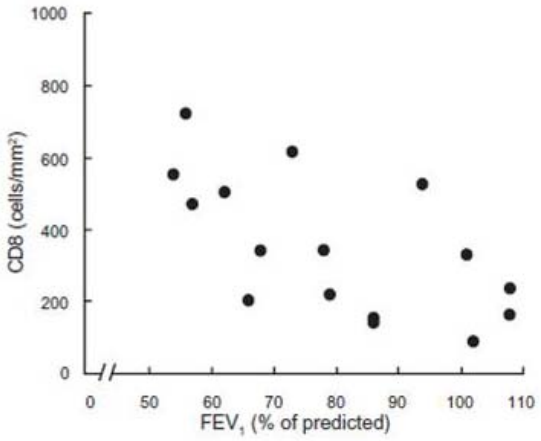


Figure 3.4.3-1: Inverse relationship between the presence of CD8+ lymphocytes in the airway wall and FEV1 in smokers with COPD (adapted from Saetta et al 1998[117]).

Lung parenchyma cells may be damaged directly by CD8+ T cells upon the release of cytolytic substances such as perforin and granzyme[120,121]. CD8+ T cells in patients with COPD exhibit increased cytotoxic activity: Higher concentrations of sputum perforin[121] and higher granzyme B levels and percentage of T cells expressing granzyme B and perforin in BALF of COPD patients and smokers – compared to healthy non-smokers – have been found[122]. The number of epithelial and endothelial cells undergoing apoptosis further increases with the extent of smoking and correlated with the number of CD8+ T cells found in the lungs of smoking COPD patients[123]. Hacker and colleagues[96] evidenced increased markers of apoptotic turnover in the systemic blood flow of COPD patients. They found elevated serum levels of cytokeratin 18 cleaved by caspase (ccCK-18) and histone-associated DNA fragments in COPD patients as compared to healthy controls.

As the contact to the extracellular matrix and integrin-mediated signals are important for cell survival, the loss of contact to the extracellular milieu due to the destruction of the extracellular matrix by proteases could be another signal for apoptosis and contribute to lung destruction in COPD[124]. The release of cytokines by CD8+ T cells such as IL-13 and IL-17 which are both able to up-regulate the expression of MMPs was also reported to play a role in sustaining tissue impairment in COPD[125,126].

Large numbers of CD4+ T cells were also found in the airways and parenchyma of smokers with COPD, predominantly near to bronchial associated lymphoid tissue (BALT)[127]. T cells isolated from lung tissues with severe emphysema showed oligoclonal expansion to conventional antigenic stimuli[128]. CD4+ lymphocytes in COPD patients and emphysema were

found to be predominately Th1 cells producing high amounts of IFN-γ, IP-10 and MIG. IP-10 and MIG bind to CXCR3, attract Th1 cells, and cause macrophages to secret MMP-12, which leads to further tissue destruction[129].

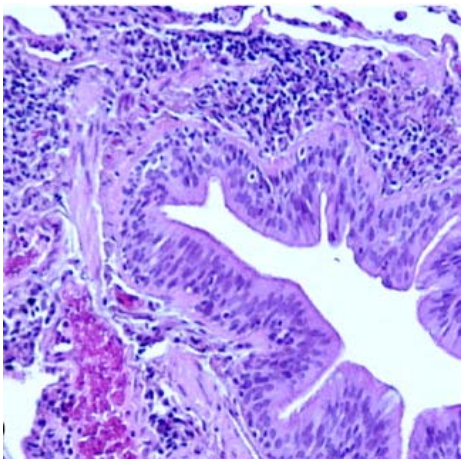


Figure 3.4.3-2: Inflammatory lymphocyte infiltrate in the adventitia of a bronchiole (adapted from Kim et al 2008[130]).

Regulatory T (T_{reg}) cells produce and induce anti-inflammatory cytokines in chronic inflammation; however, their role in COPD is discussed controversially. During acute exacerbations, CD4+ and CD8+ T_{reg} cells were found to be increased in the peripheral blood of COPD patients[131]. In cells obtained from BALF, increased expression of CD4, CD25 and forkhead box protein (FOX) P3 – an important transcription factor for the development of T_{reg} cells and the most commonly used marker to identify them[132] – was reported in smokers and COPD patients as compared to non-smokers[133]. In contrast, significantly fewer T_{reg} cells and less FOXP3 mRNA were found to be present in the lungs of subjects with emphysema compared with controls[134].

B cells organized in lymphoid follicles with an oligoclonal, antigen-specific reaction were present in the airways and parenchyma of COPD patients and mice exposed to cigarette smoke[135]. It appears that they play a role in local antigen specific immune responses although it is not certainly known which antigens may me involved: Microbial antigens, cigarette smoke derived antigens or autoantigens have been proposed[136].

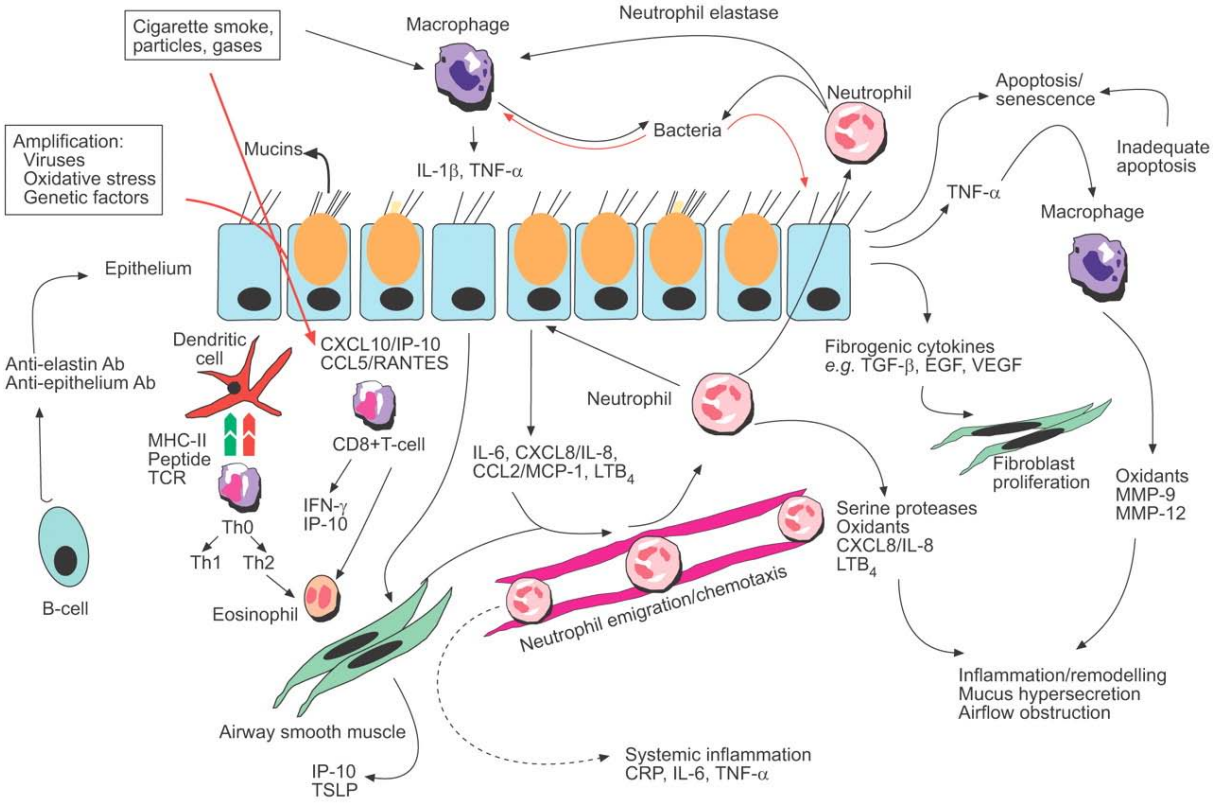


Figure 3.4.3-3: Summary of inflammatory and cellular interactions linking chronic cigarette exposure to the chronic inflammation in COPD. Activation of neutrophils, macrophages, epithelial cells, dendritic cells, T cells, B cells, fibroblasts and airway smooth muscle cells leads to the release of cytokines, chemokines and proteases. Abbreviations: Ab antibody; TCR T-cell receptor; CXCL CXC chemokine ligand; CCL CC chemokine ligand; TSLP thymic stromal lymphopoietin; CRP C-reactive protein; EGF epidermal growth factor; VEGF vascular endothelial growth factor; (adapted from Chung et al 2008[137]).

3.4.4. Evidence for Autoimmunity in COPD

As described previously, pulmonary inflammation in severe COPD includes large numbers of activated oligoclonal T cells, B cells, and other inflammatory cell types[138]. A self-perpetuating process seems to be obvious and indicates that the adaptive immune response in COPD and its persistence after smoking cessation might be due to a response to self-antigens[135,139]. Antigens may be associated with viral or bacterial infections often occuring in COPD patients[55], they might be derived from constituents of tobacco smoke, or may be self antigens altered by tobacco constituents. An association of antigens with persistent tissue destruction and apoptosis also seems to be possible[140].

The defective clearance of apoptotic cells supplies the self-antigens which are essential for autoantibody formation in autoimmune diseases such as systemic lupus erythematosus[141].

Alveolar macrophages in COPD have a reduced capacity to ingest apoptotic cells which leads to increased accumulation of apoptotic cells in COPD[98]. Because of the fact that apoptotic cell recognition typically induces an anti-inflammatory state in macrophages[142], a defective clearance might be one factor promoting lung inflammation[143].

Regarding persistent tissue destruction, evidence of increased elastin antibodies in subjects with emphysema as compared to controls was demonstrated by Lee and coworkers[134]. They suggested that cigarette smoke exposure induces cells of the innate immune system to secrete proteolytic enzymes that liberate lung elastin fragments, which could initiate B and T cell mediated immunity against elastin in susceptible individuals[134]. A link between pulmonary tissue damage and pathology of other elastin-rich organs and tissues – smokers are more susceptible to coronary artery disease and aortic aneurysms, for example – was proposed by the authors.

The potential importance of B cell response and autoantibody production in COPD and emphysema is corroborated by the presence of autoantibodies against pulmonary epithelial and endothelial cells in the serum of COPD patients. Intrapulmonary deposition of IgG complexes and C3, indicating an antibody mediated complement activation, was observed within alveolar septa and small airways of COPD patients but not in normal lung explants[144].

Another auto-antigen associated with COPD is the pulmonary ephithelial cytokeratin 18. Anti-CK-18 antibodies were found to be present in 76% of COPD patients and titers positively correlated with FEV1. CK18 neoepitopes may be released into the circulation following epithelial cell apoptosis (cleaved by caspase3)[145] and the resulting antibody-antigen interaction could promote COPD pathology. As described above, levels of caspase-cleaved CK-18 have already been found to be elevated in serum of COPD patients[96].

Anti-tissue antibodies and antinuclear antibodies (ANA), two common markers of autoimmunity, have been recently investigated in COPD patients. The prevalence of ANA and anti-tissue antibodies was 34% and 26% in COPD patients and 3% and 6% in controls, respectively. Anti-tissue antibody serum levels were further associated with severity of airflow limitation and gas transfer impairment[146].

Lambers and colleagues[147] found another evidence for autoimmune features in COPD. They described enhanced numbers of CD4+ cells lacking the costimulatory molecule CD28 and expressing NK-cell receptors in COPD patients. The authors concluded that chronic antigen exposure through contents of tobacco smoke leads to loss of CD28 and up-regulation of NK cell receptors expression on T cells in susceptible patients. Moreover, elevated numbers of

these circulating CD4+CD28null cells were shown to be present in patients with autoimmune disorders including rheumatoid arthritis[148] or ankylosing spondilytis[149].

In conclusion, the properties of autoantigens, T cells and antibodies in COPD support the idea that autoimmunity is an important issue in the pathogenesis of COPD[139].

3.5. Computed Tomography Assessment of Pathologic and Morphologic Changes in COPD

3.5.1. Multidetector High-Resolution Computed Tomography

High resolution computed tomography (HR-CT) is the gold standard for the evaluation of lung parenchyma and airways nowadays. This method is superior to chest radiography and conventional chest CT, notably in early stages of lung damage, as it is possible to obtain optimal spatial resolution and to characterize many disease processes that remain non-specific or occult with conventional radiography. Indications for HR-CT are, for example, pulmonary emphysema, diffuse lung disease, cystic lung disease, lung and pleural abnormalities, and small airway (bronchiolar) disease[150,151].

What characterizes HR-CT protocols are optimized technical parameters, such as a thin collimation of≤1.5mm and a high spatial frequency reconstruction algorithm to maximize fine lung details. Usually, volumetric acquisition technology is used, imaging the entire thorax helically. Generally, technical parameters include 100 – 120 kilovolt peak (kVp) at 40 – 100 milliampere-seconds (mAs), using higher mAs and kVp for larger patients and lower values for smaller patients and children. Thin collimation, as mentioned before, is important in order to reduce artifacts and to increase the spatial resolution which helps visualizing subtle findings. The 360° rotation time is about 350 – 500 milliseconds (ms) for current CT scanners. This is an important issue regarding the reduction of respiratory and cardiac motion artefacts[152,153]. In order to evaluate images, window levels between -500 and -600 Houndsfield units (HU) and window widths of 1500 – 2000 HU are used. For evaluating subtle differences in lung density such as emphysema, an increased contrast can be achieved using a lower window levels and a narrower window width[150,151].

Naturally, a reproducible technique with a radiation exposure that is as low as possible but still high enough to obtain images of sufficient diagnostic quality (ALARA principle of “as low as reasonably achievable”) should be used[154].

The secondary pulmonary lobule, containing the intralobular core structures (artery and airway) and the interlobular septa (containing veins that collect blood from the pulmonary acini and lymph vessels), is the smallest functional unit that can be pictured in HR-CT (Figure 3.5.1.)[155]. Generally, visualization of airways with a diameter of about 2mm, corresponding to sub-subsegmental bronchi, is possible with HR-CT scan. The pulmonary artery can be shown down to a diameter of 200µm in CT scan, providing recognition of a centrilobular region as an area around the tip of the visible artery[156].

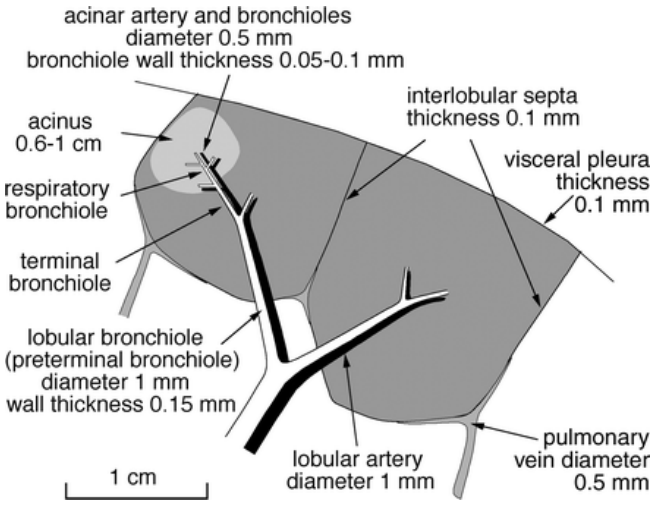


Figure 3.5.1.: Schematic illustration of two secondary pulmonary lobules with the approximate dimensions of their components (adapted from Webb 2006[155]).

3.5.2. Pulmonary Emphysema

Emphysema – per definition – is the abnormal, permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls157. Due to the loss of alveolar attachments to the airway wall, emphysema predisposes to expiratory collapse (Figure 3.5.2-1)[130].

In HR-CT scan, diagnosis of emphysema is based on the visual assessment of low attenuation areas surrounded by normal lung tissue. Visual detection schemes or more objective techniques – based upon lung density measurement –, each with its particular pros and cons, can be used to detect and quantify emphysema. Using visual interpretation schemes, correlations with histopathology and lung function were found. Experienced radiologists may even observe subtle patterns that are not yet accessible to objective quantification[158,159]. Thus, intra- and inter-observer variability commonly occurs using visual detection schemes[160].

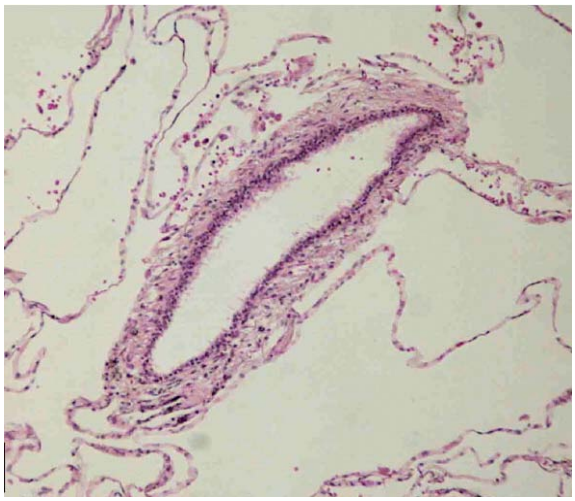


Figure 3.5.2-1: Emphysema causes loss of alveolar attachments to the airway wall (adapted from Kim et al 2008[130]).

Due to the distribution of low attenuation areas within secondary pulmonary lobules, emphysema can be characterized as centrilobular (-acinar), panlobular (-acinar), and paraseptal emphysema[155] (Figure 3.5.2-2). A brief overview of these three subtypes will be given below.

Figure 3.5.2-2: Subtypes of pulmonary emphysema; from left to right: centrilobular, panlobular, and paraseptal emphysema (adapted from Takahashi et al 2008[161]).

3.5.2.1. Centrilobular Emphysema

The centrilobular or centriacinar subtype of emphysema results from dilatation or destruction of the respiratory bronchioles, whereas distal alveolear ducts and sacs largely remain unaffected.

The lung parenchyma that surrounds the emphysematous lesions shows normal attenuation, and no border structure can be found between theses regions. In the perilobular region, the normal lung parenchyma is likely to be preserved. The 2nd and 3rd respiratory bronchioles are

mainly involved in this disease, and each lobule may be affected more or less severely. In mild to moderate centrilobular emphysema multiple areas of low attenuation have diameters of only several millimeters (Figure 3.5.2.1a). In contrast, the distinction between centrilobular and panlobular emphysema becomes more difficult when the destruction advances towards the periphery of the lobule in progressive disease (Figure 3.5.2.1b)[161-163].

Typically, centrilobular emphysema is found in the upper lung zone. Posterior and apical segments are predominantly affected in the upper lobe, and the superior segment is more involved in the lower lobe[162].

This subtype of emphysema is the one most commonly seen in cigarette smokers[164]. Anthracosis characteristically appears irregularly in the inner zone of the lung. A correspondence between the pigmented area and the centrilobular dilatation of the airspace is usually seen[161]. Further, it is associated with more severe small airway obstruction than the other emphysema subtypes[165].

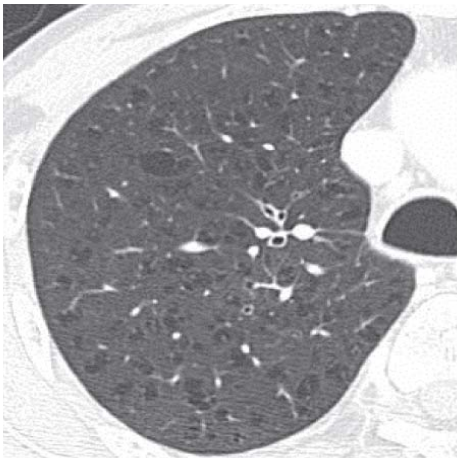


Fig. 3.5.2.1a

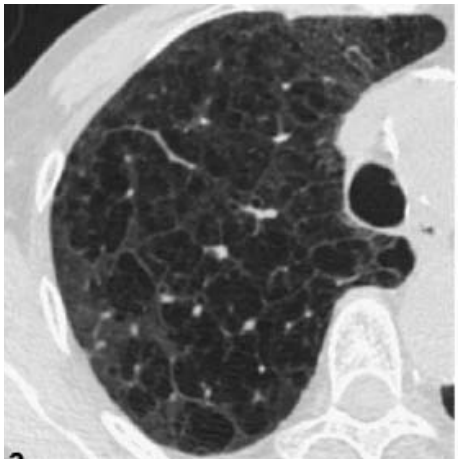


Fig. 3.5.2.1b

Figure 3.5.2.1.: Early stage centrilobular emphysema with tiny low attenuation fields (**Fig.a**) and severe centrilobular emphysema with focal areas of parenchymal destruction (**Fig.b**) (adapted from Takahashi et al 2008[161] and Litmanovich et al 2009[163]).

3.5.2.2. Panlobular Emphysema

The characteristic pattern of panlobular emphysema is the uniform destruction of the secondary pulmonary lobule, which appears as homogeneous areas of low attenuation in CT scan. In contrast to centrilobular emphysema, the lower lobe is predominantly affected, however, in severe cases the entire lung may be involved[166].

In mild panlobular emphysema the distinction between smaller alveoli from alveolar ducts and respiratory bronchioles becomes gradually lost. Alveoli lose their sharp angles, enlarge, and lose their contrast in size and shape with the alveolar ducts; the lung architecture appears simplified. In more severe disease, The abnormal enlargement becomes even more obvious when the disease deteriorates[162]. Panlobular emphysema may further occur in a localized form, where the distribution is multilobular, or in a diffuse form where the emphysematous lesions are not related to the zonal anatomy of the lung[167].

Differences between panlobular and centrilobular emphysema in HR-CT scan to keep in mind are the greater degree of lung inflation, the low contrast to the neighboring lung due to the involvement of the entire lobule, and the observation that bullous formation is less frequently in panlobular emphysema than in centrilobular emphysema[161,168].

AAT deficiency is considered a major cause of panlobular emphysema, although this pattern may also be seen in severe emphysema related to smoking[169]. Moreover, in subjects abusing methylphenidate (Ritalin) intravenously, lung CT findings were similar to those found in patients with ATT deficiency[170].

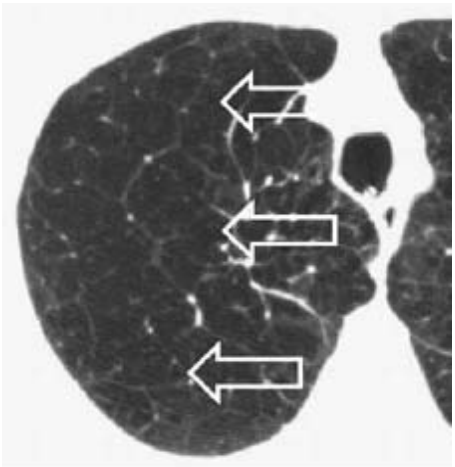


Fig. 3.5.2.2a

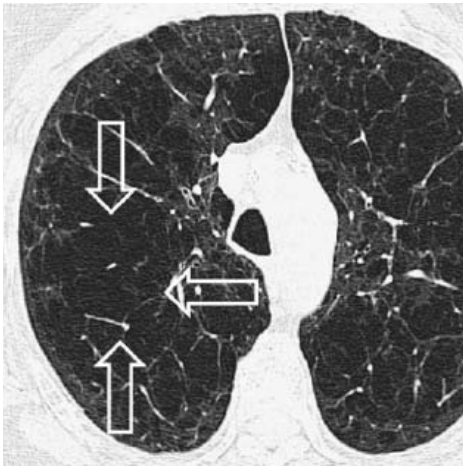


Fig. 3.5.2.2b

Figure 3.5.2.2.: Diffuse panlobular emphysema (**Fig.a**) and extensive panlobular emphysema with extensive, widespread destruction that almost efface the structure of the secondary lobule (**Fig.b**) (adapted from Litmanovich et al 2009[163]).

3.5.2.3. Paraseptal Emphysema

Paraseptal emphysema is characterized by single or multiple bullae with a diameter of 1cm or more, and a wall with a thickness of about 1mm or less (Figure 3.5.2.3.). In isolated forms of paraseptal emphysema, the lung parenchyma nearby is normal and does not present with

alterations of the airspace size. It is located in the periphery of the lung bordering the pleura or, in some cases it can also be located along interlobular septa. Most commonly, paraseptal emphysema occurs along the anterior and posterior side of the upper lobe[162,163,171].

Paraseptal emphysema occasionally occurs isolatedly, but it is found more often in association with centrilobular and panlobular emphysema, and also with fibrosis[161].

It is further one of the many causes for spontaneous pneumothoraces[172] although the exact pathogenesis is unclear. The relationship between paraseptal emphysema and thin, tall body habitus has led to the assumption that this subtype of emphysema appears due to the effects of gravitational pull on the lungs with a greater negative pleural pressure at the lung apices[162].

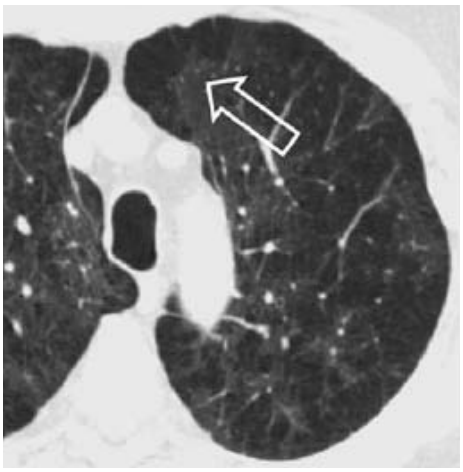


Fig. 3.5.2.3a

Fig. 3.5.2.3b

Figure 3.5.2.3.: CT section with destruction patterns predominating in the subpleural regions (arrow), corresponding to paraseptal emphysema (**Fig.a**). Inflated and fixed lung showing subpleural airspaces with smooth wall structures (**Fig.b**) (adapted from Litmanovich et al 2009[163] and Takahashi et al 2008[161]).

3.5.3. Small Airway Disease

The distal airways were first reported to be the main site of airflow limitation, and therefore an important factor in the pathophysiology in COPD, in the late 1960s[173]. In general, an inflammatory infiltrate and airway wall remodeling, which leads to thickened walls of the airways, can be found in small airway disease (Figure 3.5.3-1). These conditions contribute to a reduction of the airway diameter and an increasing resistance to flow[174].

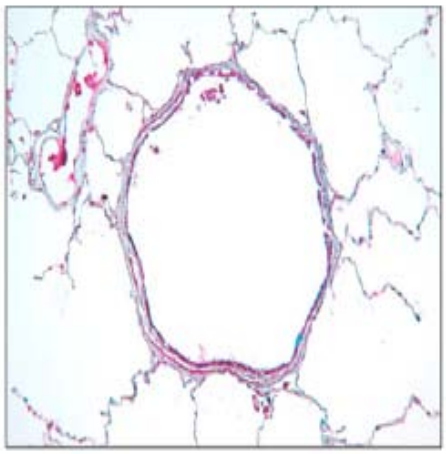


Fig. 3.5.3-1a

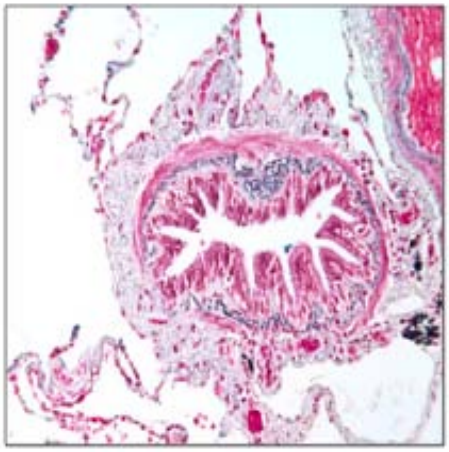


Fig. 3.5.3-1b

Figure 3.5.3-1: Sections of peripheral airways from smokers. **Fig.a** shows a nearly normal airway and **Fig.b** an airway with reduced lumen, airway wall remodeling and peribronchial connective tissue (adapted from Hogg et al 2004[175]).

Quantification of small airways disease with CT scan is less well developed than quantification of empyhsema. Direct visualization of small airway disease is not possible with current radiographic techniques; nevertheless, paired inspiratory and expiratory CT scans allow an indirect evaluation of small airway disease. These pathological changes may appear as so called air trapping on expiratory images[176,177].

"Air trapping" describes the retention of excess gas in the whole lung or parts of the lung at any stage of expiration. It is characterized as areas with decreased attenuation on expiratory CT scan as compared to the inspiratory images (Figure 3.5.3-2)[178].
To exclude any pre-existing heterogeneities, focal areas of air trapping, that are an early sign of airway disease, must respect findings on the inspiratory images, which can be caused by emphysema[179].

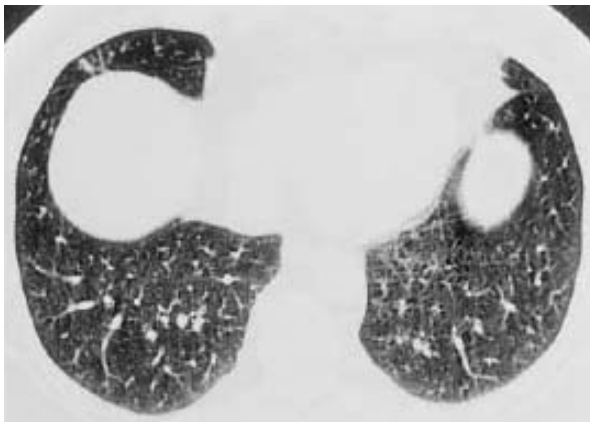


Fig. 3.5.3-2a

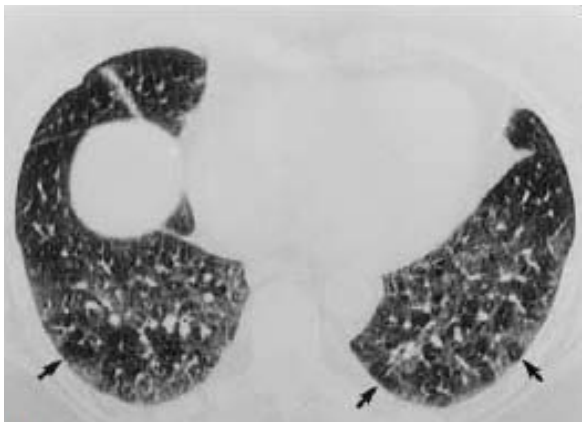


Fig. 3.5.3-2b

Figure 3.5.3-2: Female patient with normal lung function. Paired inspiratory (**Fig.a**) and expiratory (**Fig.b**) HR-CT shows physiological expiratory increase in lung density and also multiple areas of focal air trapping (arrows). The severity of air trapping was scored moderate and the extent was given as 26-50% (area occupied by air trapping with respect to the total lung area) (adapted from Kauczor et al 2006[180]).

Questions that are controversially discussed in the literature are whether the extent of air trapping correlates with pulmonary function tests, age or smoking[181-183].

(Focal) air trapping is a frequent finding not only in smokers and patients with pulmonary disease but also healthy young never-smokers, where percentages of about 55% were reported[184]. In a cohort of patients with known or suspected lung disease and a median age of 59 years, focal air trapping was observed in even 80%. In this study, no correlations of smoking history or blood gas analysis with air trapping were found[180].

In contrast to the above mentioned findings, about 75% of all small conducting airways need to be obstructed before significant airflow limitation is detectable with conventional pulmoary function testing. Clinical manifestations – until most of the airways are obstructed – are also limited. These findings may explain the slow development of early symptoms of COPD that are often neglected by the patients[174].

Indeed, a direct relationship between distal airway abnormalities and mortality was proven by Hogg and coworkers in 2007[185]: Histomorphometric measurements of small airways in patients with COPD GOLD III and IV undergoing lung volume reduction surgery showed that subjects with the least luminal occlusion lived twice as long as those with the greatest luminal occlusion after adjustment for FEV1, age and dyspnea.

3.6. The Search for COPD Biomarkers

The National Institute of Health (NIH) defines the term „biomarker“ as a *„characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention“*[186]. General characteristics of an ideal biomarker are listed in Table 3.6.

• Informs on the disease process and prognosis
• Reliable and reproducible in a routine clinical setting
• Inexpensive
• Measurable changes in response to intervention
• Little or no diurnal variation
• Sensitive, disease-specific, high positive and negative predictive values
• Sampling method acceptable to patients

Table 3.6.: Characteristics of an ideal biomarker (adapted from Patel et al 2010[187]).

Regarding COPD, the U.S. Center for Drug Evaluation and Research (CDER) indicates that *“with the exception of lung function tests, there are no well-validated biomarkers […] that can be used […] for COPD”*. They further concluded that *“the use of […] FEV1 as a marker of disease status has become validated as surrogate endpoint through years of clinical and regulatory experience and is commonly used and accepted as an endpoint to support efficacy”*. Sensitive radiological evaluation such as high resolution computed tomography (HR-CT), concentrations of gases in exhaled air or breath condensate, and inflammatory mediators in biological fluids are proposed as possible biomarkers that can be considered for use in studies[188].

Currently, several large clinical studies are in progress aiming at the identification of novel biomarkers in COPD.

In a study sponsored by the NIH (SPIROMICS: Subpopulations and intermediate outcome measures in COPD study) several thousand research subjects will be enrolled, phenotyped and followed at six clinical centers across the USA in order to identify subpopulations and surrogate markers of disease severity[189].

Another study is the 3-year longitudinal ECLIPSE study (Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints) more than 2,000 subjects with COPD are

investigated in order to find parameters that could characterize subgroups and predict disease progression[190].

About 10,000 study subjects are actually included in the COPDGene Study, in order to investigate underlying genetic factors in the development of COPD[191].

3.6.1. Blood-derived Biomarkers

Many circulating mediators, mainly markers of inflammation, have been studied in order to find potential biomarkers for COPD[192]. Only the most extensively studied proteins, namely C-reactive protein (CRP) and fibrinogen, will be mentioned here.

CRP is an acute phase protein released upon transcriptional control of IL-6; it activates the classical complement cascade and mediates phagocytosis[193]. CRP is not specific to COPD as it was found to be elevated in many other inflammatory and infective conditions[194]. Nevertheless, it has been widely studied in COPD patients: For example, a Danish group[195] showed that during 8 years of follow-up individuals with baseline serum CRP >3mg/L, as compared to those with levels≤3mg/L , had an increased risk of hospitalization and death because of COPD. Pinto-Plata and coworkers[196] found that – after exclusion of existing ischemic heart disease – CRP was increased in COPD patients as compared to controls, independent of the effect of cigarette smoking. However, patients using inhaled corticosteroids showed lower CRP levels. Moreover, modest associations of CRP and FEV1, BODE index (based on body mass index, obstructive ventilatory impairment, dyspnoea scale and exercise capacity) and inspiratory capacity/total lung capacity ratio could be demonstrated[197].

Mannino and coworkers[198] found that elevated levels of fibrinogen, which is also an acute phase reactant and regulated by IL-6[199], were related to the severity of COPD. Reduced lung function and increased cumulative incidence of COPD hospitalization in individuals with baseline plasma fibrinogen of >3.3g/L as compared to those with ≤2.7g/L was reported by Dahl and colleagues[200]. In a systematic review, a 0.47g/L difference in plasma fibrinogen between COPD patients and control subjects was estimated, confirming the former results[201].

There has been much interest in lung-specific markers because of their possible advantage to be specific to COPD. Clara-cell secretory protein 16 (CC-16) and surfactant protein D (SPD) were evaluated as part of the ECLIPSE study. CC-16 is an immunosuppressive protein secreted from cells in the terminal bronchioles.

Median serum CC-16 levels of current and former smokers with COPD were demonstrated to be significantly reduced as compared to healthy smokers and non-smokers. A weak correlation with FEV1 in former smokers could further be shown[202]. In COPD patients, SPD was elevated as compared to healthy controls but did not correlate with disease severity. Patients with serum SPD above the 95th percentile of non-smokers were at increased risk of exacerbations, whereas treatment with prednisolone resulted in decreasing serum SPD levels[203]. Surfactant protein A (SPA) has also been an issue of research interest. SPA is a lectin that contributes to innate host defense in the lung. SPA positive alveolar macrophages were found to be highly increased in COPD patients as compared to smokers without COPD[204]. Recently, Mazur and coworkers[205] have demonstrated that plasma SPA is associated with smoking history and lung function. Plasma levels were higher in current smokers than in quitters suggesting a possible involvement in the pathogenesis of cigarette smoking related lung diseases.

3.6.2. Airway-sampled Biomarkers

Many candidate markers, such as TNF-α, IL-6[206], MMPs[207], and high mobility group protein B1 (HMGB1)[208] have been investigated and found to be elevated in sputum of COPD patients. Yet, IL-8 is the most widely investigated marker and was found to be increased in the sputum of patients with stable COPD as compared to healthy smokers, non-smokers and asthma patients[209]. It further correlated with other inflammatory markers – e.g. myeloperoxidase as parameter of neutrophil activity and eosinophil cationic protein as parameter of eosinophil activity – in sputum of COPD patients. A negative correlation between IL-8 and FEV1/FVC was first described in the late 1990´s[210].

Generally, BAL is a good sampling method of peripheral lung cells. Percentages of macrophages and neutrophils[211] as well as various inflammatory mediators[212] were found to be significantly increased in BAL fluid of COPD patients as compared to healthy controls. However, BAL sampling is an invasive procedure and may even cause transient fever in patients. Therefore, it is presumably not suitable for the measurement of biomarkers in the general COPD population[213].

The research on biomarkers in exhaled air is an attractive approach, as the sampling of markers in exhaled air is a noninvasive and easily repeatable procedure[214]. However, to obtain

high reproducibility and sensitivity further investigation are required[215]. Nitric oxide (NO) as a biomarker in airway disease was proved not to be as useful in COPD patients as in asthma patients, since levels are normal or only slightly elevated during stable disease. Exhaled carbon monoxide (CO) is elevated in patients with COPD, but also in healthy smokers due to the high amounts of CO in cigarette smoke[216]. Moreover, passive smoking and environmental CO levels may further confound measurements.

Concentrations of ethane and pentane were found to be elevated in exhaled air of COPD patients. These volatile hydrocarbons are markers of lipid peroxidation as a result of oxidative stress and have been found to correlate with disease severity. Since concentrations are measured by gas chromatography, determination of these gases is quite a sophisticated approach[217].

Further, many mediators have been detected in exhaled breath condensate (EBC). Markers for oxidative stress, e.g. hydrogen peroxide, which is elevated in COPD patients and increases during exacerbations[218], and 8-isoprostane, a stable marker of oxidative stress that is also increased in COPD patients[219], were investigated. A multiplicity of inflammatory mediators, such as LT-B4, prostaglandine E2, IL-6 and MCP-1 were also reported to be increased in EBC of COPD patients[219,220]. Recently, results from the ECLIPSE study have been suggested a lower EBC pH in COPD patients as compared to healthy non-smokers, indicating tissue acidification associated with inflammation. However, there was no difference in pH between COPD patients and smokers without COPD[221]. Again, reproducibility and reliability of markers measured in EBC is a certain problem, as concentrations of mediators are often at the limit of detection[222].

3.7. Heat Shock Proteins

Heat shock proteins (HSPs) are highly conserved proteins induced by different kinds of stresses (Table 3.7.). These stress proteins have been found in virtually all eukaryotic and prokaryotic cells. Their classification is done according to their molecular weight, ranging from 7 to 110 kilo Dalton (kDa) [223].

TYPE OF STRESS	AGENT
ENVIRONMENTAL	Temperature
	Heavy metals
	Ethanol
	Oxygen radicals
METABOLIC	Hyperosmolality
	Glucose starvation
CLINICAL	Ischemia/Reperfusion
	Shock
	Anoxia
	Endotoxin

Table 3.7.: Types of stresses able to induce HSPs (adapted from Wheeler et al 2007[223]).

The main function of HSPs is believed to be that of a molecular chaperone assisting protein folding and sustaining protein homeostasis. In addition, several other functions have been described such as protection from cell death, immunomodulation, and regulation of cell development and evolution[224].

Cells respond to stress by increasing stress protein gene expression at a level that is proportional to the severity of the stressful stimuli[225]. Heat stress affects the tertiary and quaternary structure of intracellular proteins, which leads to their partial denaturation and unfolding. The intracellular accumulation of denatured or misfolded proteins is believed to be a signal resulting in the stress-induced gene expression of stress proteins[225,226].

A family of transcription factors, namely heat shock factors (HSFs), controls the regulation of stress protein gene expression. HSF1 appears to be the most important stress-inducible HSF; biochemical and genetic studies demonstrated the important role of HSF1 in the expression of stress proteins and the resistance to stress-induced apoptosis[227]. After heat shock or exposure to any other cellular stresses, HSF1 undergoes homotrimerization and rapidly translocates into the nucleus[228].

HSPs are amongst others direct inhibitors of cell death pathways. HSP70, for example, was shown to inhibit death induced by the stress kinase c-jun kinase[229]. Both HSP27 and HSP70

can inhibit caspase dependent apoptosis pathways via the direct interaction with intermediates in the death pathways[230].

Another potential mechanism by which the stress response may protect cells is the modulation of inflammatory responses. The stress response was shown to inhibit the expression of a number of genes related to inflammation, including e.g. TNF-α, IL-1β, IL-8 and RANTES[231,232,223]. The mechanisms by which the stress response inhibits the expression of proinflammatory genes involve inhibition of NF-kB: IkB kinase (IKK) has been identified as the most upstream target through which the stress response modulates NF-kB activity. IKK, as it phosphorylates the endogenous NF-kB inhibitor IkBα, is the limiting step in the activation of NF-kB. The inhibition of IKK inhibits phosphorylation and degradation of IkBα, and therefore NF-kB is kept in an inactive state[233,223].

3.7.1. Extracellular Heat Shock Proteins

Stress proteins have been originally considered to be intracellular proteins, however, they also exist and function outside the cell.

Mammalian cells express high levels of endogenous stress proteins after trauma or exposure to bacteria/bacterial proteins[234]. These stress proteins can be proinflammatory, lead to cytokine transcription and release[235], or act as stimulants of the adaptive immune response through their ability to bind antigenic peptides during antigen processing[236]. When such stress protein–peptide complexes are released from dead and dying cells, they bind directly to receptors on antigen-processing cells – cell surface glycoproteins called scavenger receptors are some of the most important receptors for HSPs[237] – and antigens can be delivered to MHC class I molecules on the surfaces of such cells through antigen cross-presentation[238] (Figure 3.7.1.).

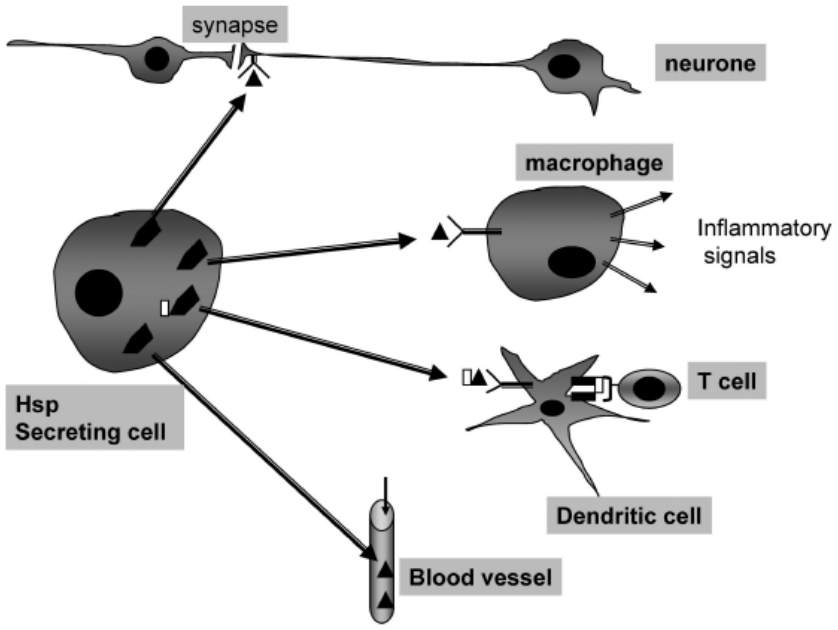


Figure 3.7.1.: Extracellular HSPs may interact with neuronal cells, monocytes or macrophages, or enter the circulation. HSPs may also be released conjugated to antigenic peptides and HSP-peptide-complexes are taken up by antigen presenting cells and further transferred to MHC class I molecules through antigen cross-presentation (adapted from Calderwood et al 2007[224]).

Stress proteins can also be anti-inflammatory. Such properties were noted specifically in inflammatory diseases such as rheumatoid arthritis. Therefore, HSPs can be both immunostimulatory or immunosuppressive, depending on the context[239] (Table 3.7.1.).

	Protein Function	
Stress Protein	Intracellular	Extracellular
Hsp27	Chaperone antideath	Anti-inflammatory
Hsp60	Chaperonin	Proinflammatory
Hsp70	Chaperone antideath	Immunoregulatory proinflammatory neuronal survival
Hsp90	Chaperone cell regulation	Proimmune prometastatic
Hsp110	Chaperone co-chaperone	Proimmune

Table 3.7.1.: Intracellular and extracellular properties of HSPs (adapted from Calderwood et al 2007[224]).

3.7.2. Heat Shock Proteins in COPD

HSPs are elevated in a wide spectrum of human diseases. For example, HSP27 and HSP70 mediate tumorigenesis in various human cancers through inhibition of programmed cell death, an important feature of cancer progression[240,241]. Increased levels of HSP60 have been associated with early presentation of cardiovascular disease[242], and elevated HSP70 levels have been further related to chronic heart failure[243] and myocardial infarction[244].

The role of extracellular HSPs in COPD has been investigated by Hacker and coworkers[245]. They found that levels of HSP27, HSP70 and HSP90α were significantly elevated in serum of COPD patients as compared to healthy controls. Especially serum HSP27 levels increased continuously with disease severity (Figure 3.7.2a) and showed a diagnostic potential to determine the occurrence of COPD in a logistic regression model (Figure 3.7.2b).

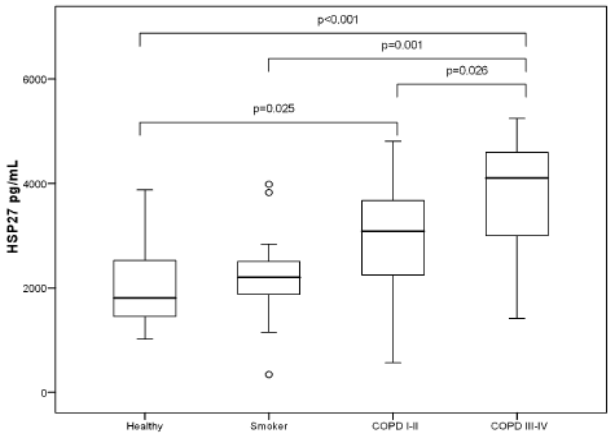


Fig. 3.7.2a

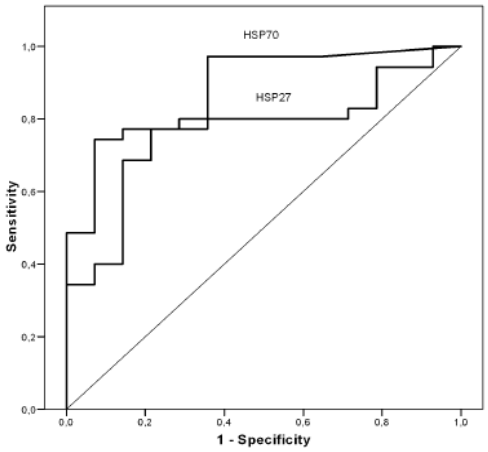


Fig. 3.7.2b

Figure 3.7.2.: Serum levels of HSP27 are significantly increased in COPD patients as compared to healthy smokers and non-smokers. Patients with COPD GOLD III-IV showed significantly higher levels of HSP27 than those with COPD GOLD I-II (**Fig.a**). HSP27 showed diagnostic potential to determine the occurrence of COPD in a logistic regression model (area under the curve 0.763) (**Fig.b**; adapted from Hacker et al 2009[245]).

3.8. Aims of the Study

Since HSP27 serum levels are known to be elevated in COPD patients as compared to healthy smoking and non-smoking controls[245], the aim of the present study was to evaluate serum levels of this stress protein in long-time smokers who still present with a normal lung function but already evidence signs of air trapping and/or emphysema in HR-CT scan.

Main criteria of our study were the assessment of air trapping and/or lung emphysema in a smoking study cohort and a possible correlation of HSP27 serum levels with these radiological signs of early airway obstruction and remodeling. We sought to investigate whether HSP27 serum values are altered before lung function parameters indicating an obstructive lung disease deteriorate in the disease process of COPD.

Furthermore, proinflammatory, pro-angiogenic, and chemotactic factors, markers for apoptosis and metalloproteinases – mediators known to play a role in the development of COPD – were evaluated using enzyme-linked immunosorbent assay (ELISA) technique.

4. MATERIALS AND METHODS

4.1. Study Population

The study protocol was approved by the Ethics Commission of the Medical University of Vienna. (No 091/2006). All clinical and laboratory tests were performed in accordance with the Declaration of Helsinki and the guidelines for Good Scientific Practice of the Medical University of Vienna. All study subjects gave informed and written consent.

A total number of 120 subjectively healthy smokers participated in this open cohort study. All study subjects were asked to answer a questionnaire regarding smoking habits and health-related behavior. Pulmonary function parameters (FVC[%], FEV1[%], FEV1/VC[%] ratio) were measured without the use of a bronchodilator, using a portable lung function testing device (PC Spirometry, SDS 104, Schiller AG, Linz, Austria). Results were evaluated by a pulmonologist. Predicted normal values were derived from the reference values of the Global Initiative for Obstructive Lung Disease. Blood samples were collected at the time of pulmonary evaluation, serum was obtained after centrifugation and aliquots were stored at -80°C until further evaluation. Exclusion criteria were known lung diseases (chronic obstructive pulmonary diseases, lung cancer, asthma, and alpha1-antitrypsin deficiency), relevant cardiopulmonary morbidities, autoimmune diseases and the use of immunomodulatory drugs (e.g. corticosteroids) within the past 14 days.

4.2. Enzyme-linked Immunosorbent Assay

4.2.1. Quantification of Serum HSP27

A commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to determine serum contents of HSP27 in our samples. 96-well microtitration plates were precoated with a capture antibody against human HSP27 and incubated overnight at room temperature. Plates were then washed and blocked with block buffer for 2 hours. Following another washing step, samples and standards with defined concentrations of antigen were incubated as described by the manufacturer. Plates were then washed and incubated with enzyme-linked polyclonal antibodies. After another washing step, horseradish-peroxidase-conjugate was applied for 20 minutes. Wells were washed, TMB (Sigma-Aldrich Corp, St. Louis, MO, USA) substrate solution was used for the detection of enzyme activity, and the

reaction was stopped using sulphuric acid (1N). Color development was monitored using a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). The optical density (OD) values obtained at 450 nm were compared to the standard curve calculated from OD values of standards with known concentrations of antigen. Specificity was demonstrated by the manufacturer by Western blot analysis of the protein bound by the capture antibody supplied in the kit. HSP70 cross reactivity was 0.23%, as stated by the manufacturer.

4.2.2. Quantification of Serum IL-1β, IL-6, TNFα, Soluble ST2, IL-8, ENA78, GROα, RANTES, MMP-1, MMP-7, MMP-9, HSP70, and Soluble RAGE

Commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to determine serum contents of the above mentioned proteins. 96-well microtitration plates were precoated with a capture antibody and incubated overnight. Plates were then washed and blocked with block buffer for 2 hours. Following another washing step, samples and standards with defined concentrations of antigen were incubated; plates were then washed again and incubated with enzyme-linked polyclonal antibodies. TMB (Sigma-Aldrich Corp, St. Louis, MO, USA) substrate solution was used for the detection of enzyme activity after addition of streptavidin conjugated horseradish-peroxidase. The reaction was stopped using sulphuric acid (1N). Plates were read at 450 nm on a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). The concentrations were calculated by comparing OD values of the samples with OD values of known concentrations of the standards.
According to the manufacturer, no significant cross-reactivity or interference could be observed in any of these assays.

4.2.3. Quantification of Serum HMGB1

A commercially available ELISA kit (IBL International, Hamburg, Germany) was used to determine serum contents of HMGB1. Diluent buffer, standards, positive controls and serum samples were pipetted into the respective wells of the 96-wells microtiter plate and incubated for 24 hours. Plates were then washed and incubated with enzyme conjugate for 2 hours at room temperature. Following another washing step, color solution was added to each well and plates were incubated for another 30 minutes at room temperature. The color reaction was stopped by adding stop solution provided by the manufacturer. Plates were read at 450 nm on a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). The concentrations

were calculated by comparing OD values of the samples with OD values of known concentrations of the standards.
According to the manufacturer, the coefficient of variation for inter-assay and intra-assay precision was 1.3 to 13.7%; HMGB2 cross-reactivity was < 2.0%.

4.2.4. Quantification of Serum ccCK-18

Levels of cytokeratin-18 neo-epitope M30 in serum samples were measured using the M30-Apoptosense® ELISA (Peviva, Bromma, Sweden). This ELISA uses an antibody recognizing a neo-epitope exposed after apoptosis-induced caspase cleavage of cytokeratin-18. The units which are measured by the M30 Apoptosense® ELISA are defined against a synthetic peptide containing the M30 and M5 epitopes (1 U/L=1.24 pM). In short, M30 HRP conjugate and serum samples or standards were pipetted into the respective wells of a microtiter plate and incubated for 4 hours. After 5 washing steps, TMB substrate provided by the manufacturer was added and after an incubation time of 20 minutes, stop solution (1.0 M sulphuric acid) was used to stop the color reaction. Serum concentrations were calculated by comparing OD values of the samples to OD values of the standard dilutions read at 450 nm on a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). Intra-assay and inter-assay precision was given as less than 10% for values > 100 U/L according to the manufacturer.

4.3. HR-CT Lung Imaging

4.3.1. Study Population

All study subjects were invited to undergo HR-CT scanning of the lung voluntarily. 94 out of 120 (78.3%) healthy smokers underwent a non contrast CT examination in inspiration and expiration after informed and written consent.

4.3.2. CT Scan

CT scanning was performed using a 16-detector MDCT scanner (Aquilion 16, Toshiba Medical Systems Europe, Zoetermeer, Netherlands). A non contrast spiral scan of the thorax was performed in inspiration and exspiration at a collimation of 16 x 0.75mm at 120kV/40mAs and a pitch of 1. Images were reconstructed at 1mm slice thickness and an increment of 0.8mm using a FC86 kernel and a lung window (-550/1600 window

level/window with). Images were then sent to an offline workstation (Syngo multi modality workplace, Siemens Medical Solutions, Erlangen, Germany) for further workup.
Images were analyzed by one reader blinded to clinical results, laboratory parameters and patient history. For each study subject the lung was assessed for the presence of air trapping and/or emphysema qualitatively. Air trapping was defined as regions of normal lung tissue in inspiration that did not exhibit an increase in attenuation and showed heterogeneous areas of low density beneath areas of high density in expiratory scans. Emphysema was defined as a permanent abnormal enlargement of airspaces distal to the terminal bronchiole, accompanied by the destruction of their walls.

4.4. Statistical Analysis

Statistical analysis was performed using SPSS Software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA). Data visualization was performed using GraphPad Prism5. Data are given as mean ± standard deviation (SD) or median and interquartile range (IQR; 1^{st} and 3^{rd} quartile) if data were not Gaussian distributed. To determine Gaussian distribution Shapiro-Wilk test was carried out. Either one-way ANOVA, for Gaussian distributed data, or Kruskal-Wallis test, were used to determine statistical significance between more than two study groups. Two-sided *T*-test, or, if data were not Gaussian distributed, Mann-Whitney-U test were performed for pair-wise comparisons between groups. Correlations were calculated using Pearson´s correlation coefficient. Categorical variables were compared using chi2 test. To assess the predictive capacity of HSP27 serum values receiver operating characteristic (ROC) curves with its area under the curve (AUC) were plotted. Bonferroni-Holm correction was used to adjust *P*-values for multiple testing. *P*-values <0.05 were considered statistically significant.

5. RESULTS

5.1. Initial Evaluation of Demographical Data, Lung Function Parameters and HSP27 Serum Levels

In Table 5.1., basic demographical data including lung function testing results and serum HSP27 values are shown

Comparing the initial study population with the split population that underwent HR-CT scan, none of the parameters were significantly altered.

	total study group	**HR-CT study group**	***P*-value**
	100%	**78.3%**	
n	120	94	-
M / F *n* **M / F *%***	53 / 67 44.2 / 55.8	42 / 52 44.7 / 55.3	*n.s.*
Age	43.1±9.7	43.4±9.5	*n.s.*
Pack Years	17.0 (10.5/31.0)	16.7 (10.8/33.9)	*n.s.*
HSP27 (pg/ml)	3623±1552	3761±1582	*n.s.*
Lung Function			
FVC%	90.2±11.5	90.6±11.7	*n.s.*
FEV1%	83.6±11.8	83.5±12.9	*n.s.*
FEV1/FVC	0.779±0.078	0.771±0.077	*n.s.*
FEV1/FVC≤0.7	15 (12.5%)	13 (13.8%)	*n.s.*

Table 5.1.: Characteristics of the initial study population and the split population that underwent HR-CT scan. Abbreviations: M: male; F: female. Data are given as mean±SD or as medians with IQR. Signs of airway obstruction were diagnosed by a specialist, according to lung function testing results.

5.2. HR-CT Scan revealed a High Prevalence of Air Trapping and Emphysema in our Study Population

Air trapping (AT) or both AT and emphysema (E) could be detected in 54 out of 94 study subjects (57.5%). In detail, 31 subjects (33.0%) presented with AT and 23 (24.5%) with both AT and E. E without AT was not detected in any of the study subjects. 40 (42.55%) study subjects did not evidence any airway pathology in HR-CT scan (nothing abnormal detected; NAD).

5.3. Study Subjects diagnosed with AT+E had the Longest Smoking History

Study subjects diagnosed with AT+E presented with significantly more pack years than study subjects without any pathology detected in HR-CT scan (NAD vs. AT+E: *P*=0.0372) and air trapping alone (AT vs. AT+E: *P*=0.0123) (Table 5.3.).

5.4. Lung Function Parameters did *not* differ significantly between Groups

There were no statistically significant differences between groups neither in lung function parameters (FVC[%], FEV1[%] and FEV1/FVC) nor in the percentage of subjects with FEV1/FVC≤0.7, indicating airflow obstruction (Table 5.3.).

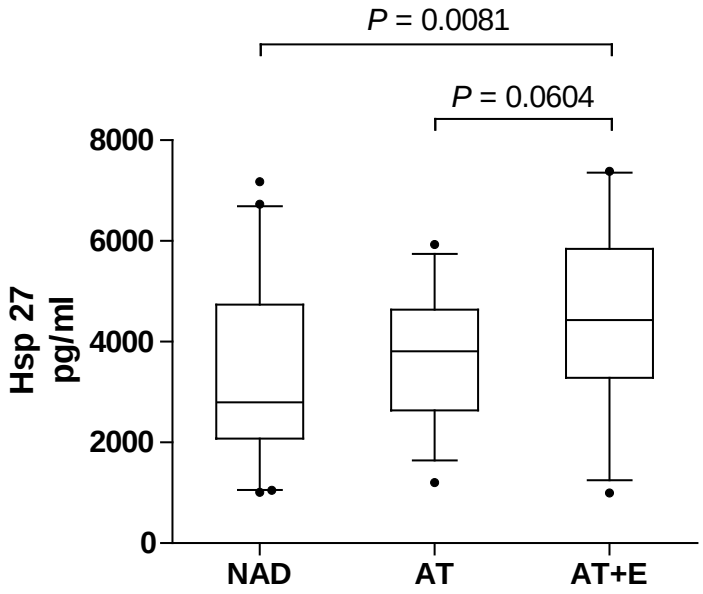
	nothing abnormal detected	**air trapping**	**air trapping and emphysema**	**total**	***P*-value**
n	40	31	23	94	-
M / F *n* M / F %	19 / 21 (47.5 / 52.5)	15 / 16 (48.4 / 51.6)	8 / 15 (34.8 / 65.2)	42 / 52 (44.7 / 55.3)	*n.s.*
Age	42.1±10.0	41.9±9.5	47.7±7.5	43.4± 9.5	*0.039*
Pack Years	15.6 (10.0/29.8)	14.8 (8.6/21.3)	33.0 (15.3/40.3)	16.7 (10.8/33.9)	*0.011*
Lung Function					
FVC%	92.4±10.5	90.4±12.5	87.7±12.5	90.6±11.7	*n.s.*
FEV1%	86.6±12.9	83.2±13.1	78.4±11.3	83.5±12.9	*n.s.*
FEV1/FVC	0.78±0.07	0.77±0.06	0.75±0.11	0.77±0.08	*n.s.*
FEV1/FVC≤0.7	5 (12.5%)	3 (9.7%)	5 (21.7%)	13 (13.8%)	*n.s.*

Table 5.3.: Characteristics of the study population that underwent HR-CT scan, divided into study subjects without any pathology (NAD) those with air trapping (AT), and those with both air trapping and emphysema (AT+E). Data are given as mean±SD or, if not Gaussian distributed, as medians with IQR.

5.5. Serum HSP27 was significantly elevated in Study Subjects with AT+E as compared to Subjects without any Pathology

Study subjects with AT showed slightly increased levels of serum HSP27 as compared to subjects without any pathology, but without statistically significant difference (*P*=0.187). In contrast, subjects diagnosed with AT+E showed significantly increased HSP27 serum levels as compared to NAD (*P*=0.0081) and also clearly higher amounts of serum HSP27 than

subjects with AT. The difference between AT and AT+E was not statistically significant (after Bonferroni-Holm correction: *P*=0.0604) (Figure 5.5.).



mean ± SD	**3282 ± 1607**	**3744 ± 1210**	**4618 ± 1677**

Figure 5.5.: HSP27 serum values. Subjects diagnosed with air trapping and emphysema showed the highest HSP27 serum levels as compared to the other groups.

5.6. HSP27 showed High Sensitivity and Specificity to determine the Presence of AT+E in our Study Population

In univariate logistic regression models including subjects without any pathology and subjects with AT+E, HSP27 had an AUC of 0.724 (0.594–0.854 95% CI; *P*=0.0033), indicating a high sensitivity and specificity of HSP27 as diagnostic marker in patients with both air trapping and emphysema (Figure 5.6a). ROC curve analysis for AT revealed an AUC of 0.607 (0.475–0.738 95% CI; *P*=0.126) (Figure 5.6b).

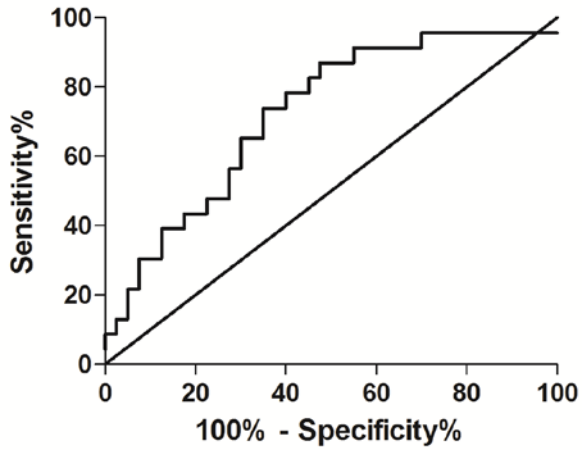


Fig. 5.6a

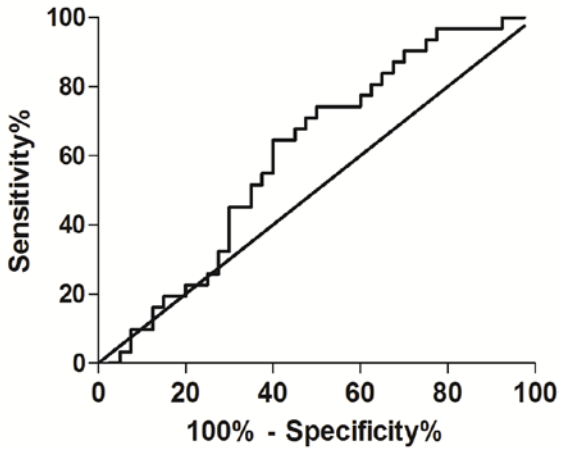


Fig. 5.6b

Figure 5.6.: ROC curve analysis in order to determine sensitivity and specificity of HSP27 to predict air trapping and emphysema (**Fig.a**) and air trapping without signs of emphysema (**Fig.b**).

5.7. Serum HSP27 Levels did *not* correlate with other Clinical Parameters

HSP27 serum levels were not associated with other clinical parameters such as age, smoking PYs and FEV1/FVC. Pearson´s correlation coefficients were as follows: age, r=-0.056; PYs, r=-0.028; FEV1/FVC, r=-0.059 (Figures 5.7a – 5.7c)

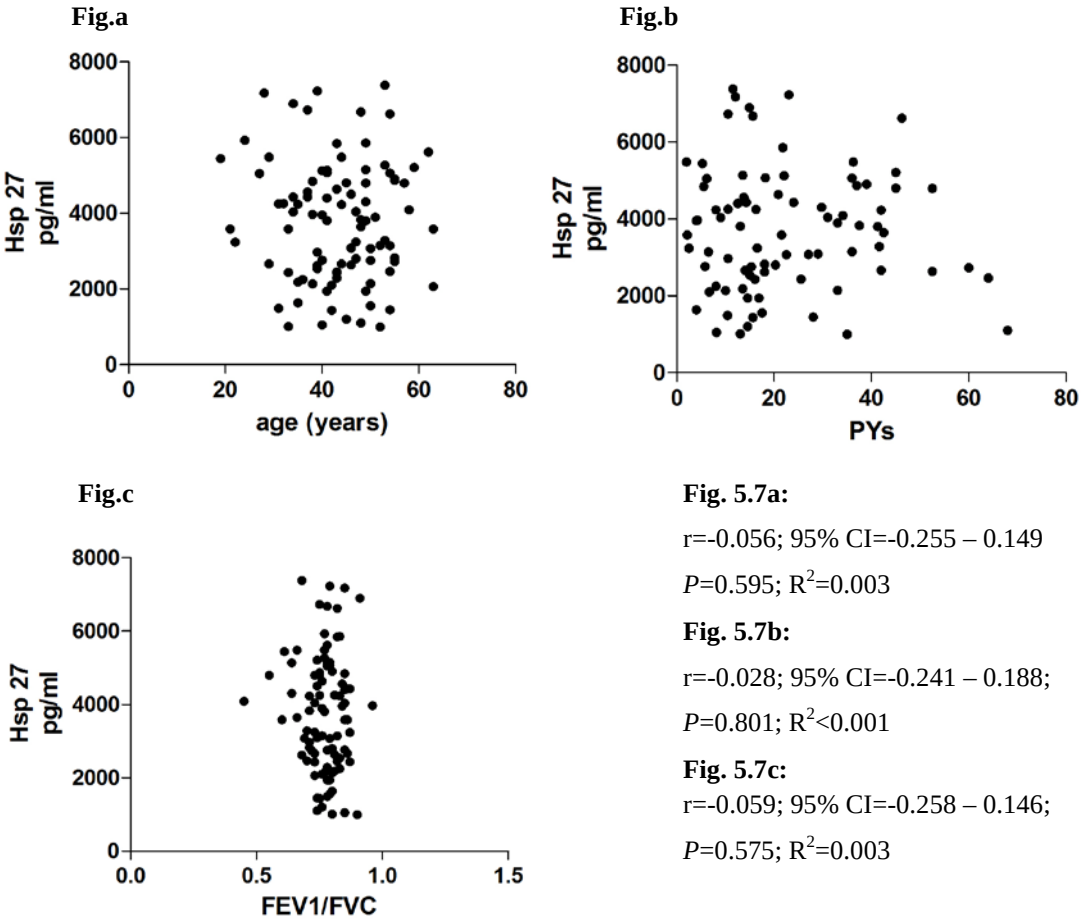


Fig. 5.7a:
r=-0.056; 95% CI=-0.255 – 0.149
P=0.595; R^2=0.003

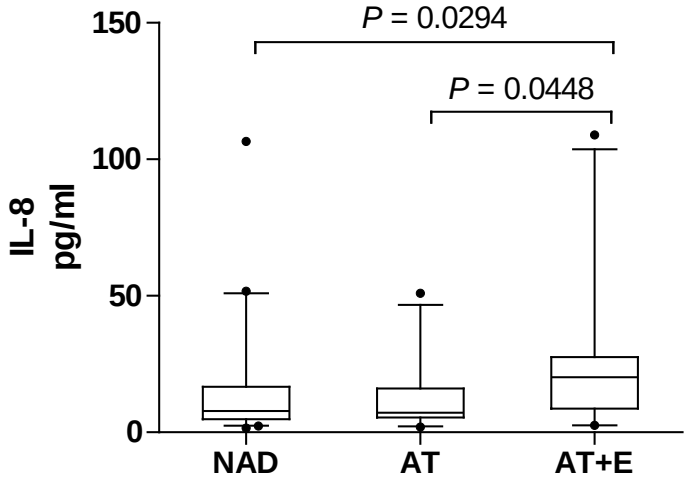
Fig. 5.7b:
r=-0.028; 95% CI=-0.241 – 0.188;
P=0.801; R^2<0.001

Fig. 5.7c:
r=-0.059; 95% CI=-0.258 – 0.146;
P=0.575; R^2=0.003

Figure 5.7.: Correlations of HSP27 with age, pack years, and FEV1/FVC ratio. Serum HSP27 levels did not correlate with any of these parameters.

5.8. IL-8 Serum Levels were significantly elevated in Study Subjects with AT+E as compared to the other Groups

We were able to show elevated serum levels of IL-8 in study subjects with AT+E as compared to IL-8 serum contents of subjects without any pathology detected in HR-CT scan (AT+E vs. NAD: *P*=0.0294) and subjects diagnosed with AT (AT+E vs. AT: *P*=0.0448). There was no significant difference between subjects without any pathology and subjects with AT (Figure 5.8.).



median (Q1/Q3)	**7.9 (4.8/16.7)**	**7.2 (5.4/16.1)**	**20.2 (8.7/27.6)**

Figure 5.8.: Subjects diagnosed with air trapping and emphysema showed a more than twofold increase in IL-8 serum levels as compared to the other groups.

5.9. Serum Levels of IL-1β, IL-6, TNFα, Soluble ST2, GROα, ccCK-18 (M30), HMGB1, RANTES, MMP-1, MMP-7, MMP-9, HSP70, and Soluble RAGE did not differ significantly between Groups

Serum levels of ENA78 differed significantly between groups (*P*=0.0313), but pair-wise comparisons did not show any significant result after Bonferroni-Holm correction. Serum concentrations of M30, MMP-1, MMP-7, MMP-9 and HSP70 were slightly increased (no statistical significance) in study subjects with AT+E as compared to subjects without any pathology and subjects with AT (Table 5.9.; Figures 5.9a – 5.9e).

HR-CT Findings	nothing abnormal detected		air trapping		air trapping + empyhsema		*P*-value
	Median (Q1/Q3)	Min–Max	Median (Q1/Q3)	Min–Max	Median (Q1/Q3)	Min–Max	
IL-1ß (pg/ml)	0.0 (0.0/2.67)	0.0–81.36	0.0 (0.0/0.08)	0.0–96.16	0.0 (0.0/1.95)	0.0–38.75	*0.916*
IL-6 (pg/ml)	0.0 (0.0/1.08)	0.0–76.26	0.0 (0.0/0.65)	0.0–23.15	0.19 (0.0/1.16)	0.0–46.25	*0.679*
TNF-α (pg/ml)	0.0 (0.0/0.0)	0.0–9.37	0.0 (0.0/0.0)	0.0–0.0	0.0 (0.0/0.0)	0.0–2.25	*0.132*
sST2 (pg/ml)	67.24 (41.19/137.1)	3.16–810.4	60.14 (32.36/125.7)	2.86–445.2	69.6 (9.93/206.1)	0.0–623.6	*0.573*
GRO-α (pg/ml)	24.79 (8.74/45.54)	0.0–116.9	27.39 (18.76/44.70)	0.0–112.7	25.42 (11.18/44.91)	0.0–107.3	*0.700*
M30 (U/L)	147.1 (108.6/223.8)	59.13–519.0	115.5 (96.55/164.3)	43.97–712.4	168.8 (130.1/264.1)	85.4–530.9	*0.062*
HMGB1 (ng/ml)	2.21 (1.53/3.52)	0.85–7.79	2.09 (1.36/2.87)	0.57–12.23	2.15 (1.53/4.0)	1.04–8.25	*0.618*
	Mean ± SD	**95% CI**	**Mean ± SD**	**95% CI**	**Mean ± SD**	**95% CI**	
ENA-78 (pg/ml)	2664 ± 1529	2175–3153	3575 ± 1694	2954–4196	2643 ± 1322	2024–3262	*0.031*
RANTES (ng/ml)	71.29 ± 26.17	62.81–79.78	74.18 ± 16.42	68.05–80.31	75.57 ± 19.37	67.20–83.95	*0.730*
MMP-1 (pg/ml)	2812 ± 1691	2264–3360	2467 ± 1433	1922–3012	3602 ± 2082	2701–4502	*0.062*
MMP-7 (ng/ml)	2.73 ± 0.71	2.49–2.97	2.62 ± 0.69	2.36–2.88	3.01 ± 0.80	2.66–3.36	*0.157*
MMP-9 (ng/ml)	1837 ± 857.9	1562–2111	1844 ± 647.8	1607–2082	2184 ± 1078	1718–2650	*0.252*
HSP70 (pg/ml)	282.3 ± 152.1	233.7–331.0	250.6 ± 149.6	194.7–306.4	311.6 ± 172.0	237.2–386.0	*0.371*
sRAGE (pg/ml)	363.5 ± 188.5	300.6–426.3	384.9 ± 188.0	314.7–455.1	283.0 ± 221.2	182.3–383.7	*0.175*

Table 5.9.: Results of serum evaluations.

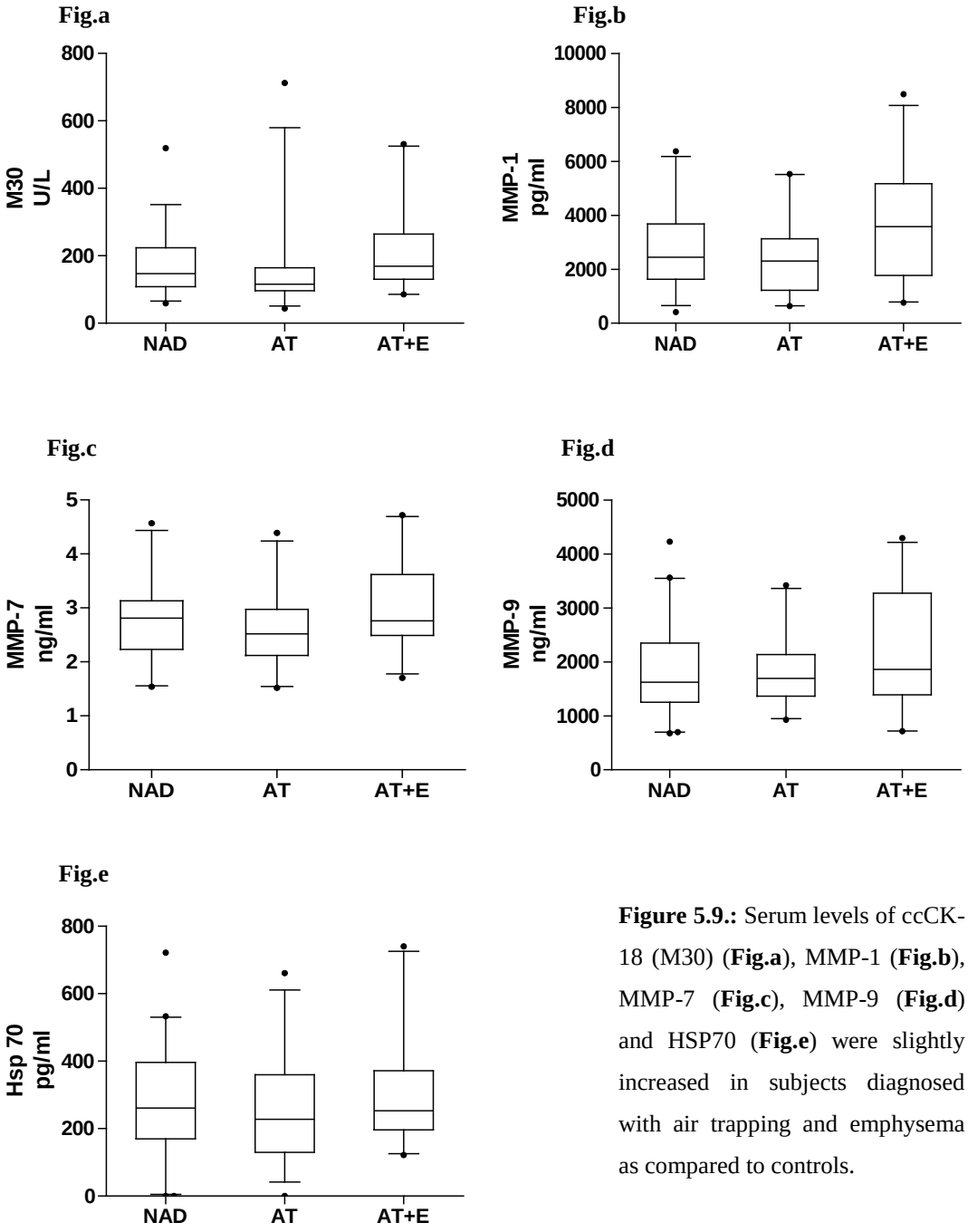


Figure 5.9.: Serum levels of ccCK-18 (M30) (**Fig.a**), MMP-1 (**Fig.b**), MMP-7 (**Fig.c**), MMP-9 (**Fig.d**) and HSP70 (**Fig.e**) were slightly increased in subjects diagnosed with air trapping and emphysema as compared to controls.

6. DISCUSSION

A triumvirate of symptoms (breathlessness, cough, sputum production), a history of risk factors such as smoking, and lung function parameters are currently the cornerstones of the diagnosis "COPD". Unfortunately, symptoms may overlap with other disorders requiring different treatment, risk factors are not always easy to quantify, and optimal ATS/ERS standards are not always used for disease assessment. Clinical judgment in interpretation of results is also an important issue[246], and valid results are often dependent on the patient´s effort and technique. Another controversially discussed topic are the cut-off values for spirometry: fixed value (FEV1/FVC<0.7) or lower limit of normal can be used[247,248]. For all these reasons, biomarkers for diagnosing COPD[187] and biomarkers for the early detection of COPD are required, as this disease continues to be severely under-diagnosed according to the BOLD study[8].

At least the following criteria should be considered before aiming at the detection of a disease in its preclinical stage: First, undetected disease would progress and cause relevant morbidity and mortality. Second, treatment of risk factors has a major effect on the further development of the disease, and third, an objective, simple, affordable and safe test is available to confirm the disease. All of these criteria are fulfilled by COPD[249].

Based on current knowledge of increased serum levels of HSP27 and HSP70 in patients with manifest COPD, we hypothesized that serum HSP27 may serve as a prognosticator for COPD development in smokers perceived healthy.

According to our primary hypothesis, we proved that elevated serum levels of HSP27 in smokers identified HR-CT verified lung pathology independently from spirometry analysis. To the best of our knowledge this is the first report that aims to correlate serum HSP27 levels with spirometry analysis and HR-CT in a study population "at risk".

The clinical relevance of our observation is twofold: We suggest that increased levels of serum HSP27 are an independent prognosticator of air trapping and emphysema in a study cohort of young smokers perceived subjectively healthy. ROC curve analysis revealed an AUC of 0.724 (P=0.003), indicating a high sensitivity and specificity of HSP27 as diagnostic marker for lung pathology. Since the only "deviation" of all our study subjects was inhalation of noxious substances, leakage of HSP27 into the circulation must mirror an immunological activation process.

HSP27 is a member of the so called small HSPs, a subfamily of the HSP group that share sequence homologies and biochemical properties such as phosphorylation and oligomerization. HSP27 is able to form oligomers up to 1000kDa, which is required for chaperone function[250,251].

Overexpression of HSP27 protects cells against apoptotic cell death which can be triggered by various stimuli such as hyperthermia, oxidative stress, ligation of the Fas/Apo-1/CD95 death receptor and cytotoxic drugs[252,253]. HSP27 is able to interfere with the activation of the cytochrome c/Apaf-1/dATP complex as it sequesters cytochrome c when released from the mitochondria into the cytosol and therefore inhibits the activation of pro-caspase-9[265]. A well documented function of HSP27 is the interaction with actin and intermediate filaments. The binding of HSP27 to F-actin prevents the disruption of the cytoskeleton resulting from cell stresses[254]. HSP27 was also shown to increase the antioxidant defense of cells by modulating reactive oxygen species[255] and to enhance the activation of the NFkB pathway which controls a variety of processes such as inflammatory responses[256].

HSP27 also affects one of the Fas-mediated apoptotic pathways. The phosphorylated form of HSP27 directly interacts with Daxx, which connects Fas signaling to the protein kinase Ask1 that mediates caspase-independent cell death[257]. Another function of this stress protein is the activation of the proteasome. It speeds up the degradation of irreversible denatured proteins and junk proteins by binding to ubiquitinated proteins and to the 26S proteasome[256].

Besides these intracellular functions, HSPs are also known to be released into the extracellular space following trauma or cell stress. This spillage of proteins serves as “danger signal” leading to cytokine transcription and release[235] and the induction of the adaptive immune system[236] as described above.

By interpreting accepted knowledge pertaining to HSP27, we conclude that toxins inhaled by cigarette smokers lead to an immune response that causes pulmonary changes detectable in HR-CT scan, and spillage of HSP27 into the pulmonary vascular network in subjects susceptible to COPD. Most intriguingly, this radiologically verified lung pathology was not associated with impaired lung function.

It is known that emphysema is sometimes found in people who maintain normal lung function[258]. This observation has become even more common since the introduction of the (HR-) CT scan, but the hypothesis that this early form of emphysema predicts a rapid decline in lung function has not been tested sufficiently yet. The assumption that COPD has a long subclinical course is corroborated by the findings that about 40% of heavy smokers develop

emphysema and only 15% develop airflow limitation[175], and that airflow limitation does not occur until 75% of small conducting airways are obstructed[174].
However, most probably these early morphological changes in our study cohort, accompanied by HSP27 release similar to that of patients with manifest COPD[245], will be followed by spirometric impairment and subsequently by the development of clinically manifest COPD in at least some of the studied individuals.

In line with above data we found elevated serum levels of ccCK-18, indicating apoptosis-dependent lung degeneration, in study subjects with AT+E as compared to controls. Of interest was also our finding that the CXCR2 cytokine IL-8 evidenced a more than two-fold increase in the AT+E group as compared to the NAD and the AT group. These observations are supported by recent literature that advocated IL-8 in the development of COPD[259]. A further insight was that RANTES, GRO-α and ENA-78 – the latter two are potential chemotactic factors for neutrophils and were found to be increased in COPD patients[104,105] – failed to demonstrate a clear correlation with HR-CT verified lung pathology in our study cohort. Inflammatory cytokines, matrix metalloproteinases, and soluble ST2 did not correlate with HR-CT results either. A further proinflammatory pathway, namely the sRAGE/sHMGB1 axis, did not show any significance in predicting lung pathology in the early course of COPD development although HMGB1 was found to be elevated in serum and sputum of patients with manifest COPD[208].

If we interpret our data correctly, a detailed picture is emerging. Chronic antigen exposure, e.g. through contents of tobacco smoke, leads to massive secretion of HSP27 into the vascular bed due to systemic immune activation. This immunological stress in “healthy smokers” was significantly associated with early COPD-associated radiological findings irrespective of lung function parameters. In conclusion, we suggest that heightened levels of serum HSP27 may serve as a marker for incipient COPD and may identify patients prone to develop COPD after further investigation and evaluation.

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8. LIST OF ABBREVIATIONS

AAT	alpha1-antitrypsin
ANA	antinuclear antibodies
ANOVA	analysis of variance
AT	air trapping
ATS	American Thoracic Society
AUC	area under the curve
BAL(F)	broncho-alveolar lavage (fluid)
BALT	bronchial associated lymphoid tissue
BOLD	Burden of Obstructive Lung Disease
CC-16	Clara-cell secretory protein 16
cc-CK18	caspase cleaved cytokeratin 18
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CDER	U.S. Center for Drug Evaluation and Research
CO	carbon monoxide
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	dendritic cell
E	emphysema
e.g.	for example
E1A	(adenoviral) early region 1A
EBC	exhaled breath condensate
ECLIPSE	Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ENA78	epithelial neutrophil-activating peptide 78
ERS	European Respiratory Society
F	female
FEV1	forced expiratory volume in one second
FOXP3	forkhead box protein P3

FVC	forced vital capacity
GOLD	the Global initiative for chronic Obstructive Lung Disease
GRO-α	growth regulated oncogene alpha
HMGB1	high mobility group box protein 1
HR	hazard ratio
HR-CT	high resolution computed tomography
HSF	heat shock factor
HSP	heat shock protein
HU	Houndsfield units
ICAM-1	inter-cellular adhesion molecule 1
IFN-γ	interferon gamma
IgG	immunoglobulin G
IKK	I kappa B kinase
IL	interleukin
IP-10	interferon gamma-induced protein 10 kDa
IQR	interquartile range
kDa	kiloDalton
KVp	kilovolt peak
LPS	lipopolysaccharide
LT-A4H	leukotriene A4 hydroxylase
LT-B4	leukotriene B4
M	male
MCP-1	monocyte chemotactic protein 1
MDCT	multidetector computed tomography
MHC	major histocompatibility complex
MIG	monokine induced by interferon gamma
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
NAD	nothing abnormal detected
NF-kB	nuclear factor kappaB
NHANES	National Health and Nutrition Examination Survey
NIH	National Institute of Health
NK-cell	natural killer cell
NO	nitric oxide

NTHI	non-typeable Haemophilus influenza
OD	optical density
OR	odds ratio
PGP	proline-glycine-proline
PM2.5, PM10	particulate matter with a 50% cut-off aerodynamic diameter of 2.5µm/10µm
PY	pack year
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
ROC	receiver operating characteristic
ROS	reactive oxygen species
RSV	respiratory syncytical virus
SD	standard deviation
SPA, SPD	surfactant protein A, surfactant protein D
SPIROMICS	Subpopulations and intermediate outcome measures in COPD study
STAT	signal transducer and activator of transcription
TGFβ	transforming growth factor beta
Th2	T helper type 2
TLR	toll like receptor
TNFα	tumor necrosis factor alpha
T_{reg} cells	regulatory T cells
VEGF	vascular endothelial growth factor

9. APPENDIX

9.1. Curriculum Vitae

PERSONAL BACKGROUND

Nationality: Austrian
Family Status: Single
Date of Birth: May 17th, 1987 in Vienna, Austria
Parents: Willibald and Christine Nickl

EDUCATION

2009/11 – Present	Scientific Co-Worker at the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration
2008/04 – Present	Student Research Fellow at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Austria
2005/10 – Present	Medical Student at the Medical University of Vienna, Austria
2005/06	Matura (high school graduation) with Distinction
2004/02 – 2004/08	Visiting Student at Escuela Normal Superior de Monteros, Argentina
1997 – 2005	Bundesgymnasium und Bundesrealgymnasium (High School) Frauengasse, Baden bei Wien, Austria
1993 – 1997	Primary School

CLINICAL TRAINING

2011/05	Clinical Clerkship at the Deparment of Neurology, Wilhelminenspital, Vienna, Austria (2 weeks).
2011/03	Clinical Clerkship at the Department of Dermatology, Wilhelminenspital, Vienna, Austria (2 weeks).
2011/01	Clinical Clerkship at the Department of Ophthalmology, General Hospital Vienna, Austria (2 weeks).
2010/12	Clinical Clerkship at the Department of Otorhinolaryngology, General Hospital Vienna, Austria (2 weeks).
2010/11	Clinical Clerkship at the Department of Obstetrics and Gynecology, General Hospital Vienna, Austria (5 weeks).
2010/10	Clinical Clerkship at St. Anna Children´s Hospital, Vienna, Austria (5 weeks).
2010/06	Clinical Clerkship at the Department of Surgery, General Hospital Vienna, Austria (2 weeks).
2010/05	Clinical Clerkship at the Department of Plastic Surgery, General Hospital of Vienna, Austria (1 week).

2010/05	Clinical Clerkship at the Department of Orthopaedics II, Orthopaedic Hospital Vienna-Speising, Austria (1 week).
2010/05	Clinical Clerkship at the Department of Surgery II, Krankenhaus Hietzing, Vienna, Austria (2 weeks).
2010/03	Clinical Clerkship at the Department of Neurosurgery (Anesthesia), General Hospital Vienna, Austria (3 weeks).
2009/12 – 2010/01	Clinical Clerkship at the Department of Internal Medicine II, General Hospital Vienna, Austria (5 weeks).
2008/09	Clinical Clerkship at the Department of Trauma Surgery, UKH Meidling, Vienna, Austria (4 weeks).
2008/07	Clinical Clerkship at the Department of Surgery, Krankenhaus Rudolfsstiftung, Vienna, Austria (4 weeks).
2008/02	Clinical Clerkship at the Department of Cardiology, Krankenhaus der Barmherzigen Schwestern, Vienna, Austria (2 weeks).
2007/11	Clinical Clerkship at the Department of Pathology, Thermenklinikum Mödling, Austria (2 weeks).
2007/09	Clinical Clerkship at the Department of Gynaecology and Obstetrics, Thermenklinikum Mödling, Austria (2 weeks).
2007/08	Clinical Clerkship at the Department of Cardiology, Krankenhaus der Barmherzigen Schwestern, Vienna, Austria (4 weeks).

CONTINUING EDUCATION

2009/03	Methodenseminar “Experimentelle biomedizinische Studien am Tier” – Methods Seminar “Experimental Biomedical Studies in Animals”, Univ.-Prof. Dr. Udo Losert, Vienna, Austria
2008/11	Methodenseminar “Statistik” – Methods Seminar “Statistics”, Mag. Michaela Schmöger, Vienna, Austria
2008/11	Biometrie II: Statistische Tests und Lebensdaueranalyse bei medizinischen Fragestellungen – Biometry II: Statistical Tests and Analysis of Survival in Medical Research, Vienna, Austria
2008/09	Biometrie I: Beschreibung und Visualisierung medizinischer Daten – Biometry I: Description and Visualization of Medical Data, Vienna, Austria

TEACHING ACTIVITY

2008/10 – 2009/10	Teaching Assistant at the Department of Cell Biology and Ultrastructure Research, Medical University of Vienna, O. Univ.-Prof. Dr. Margit Pavelka

CONGRESSES AND MEETINGS

2011/03	Update Cardiology 2011, Innsbruck, Austria

2010/12	2nd EACTS Meeting on Cardiac and Pulmonary Regeneration, Vienna, Austria
2010/10	Austrotransplant 2010 – 24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria
2010/10	Annual Meeting of the Austrian Society of Pneumology, Graz, Austria
2010/09	41st Annual Meeting of the Austrian Society of Internal Medicine, Salzburg, Austria
2010/06	51st Annual Meeting of the Austrian Society of Surgery, Linz, Austria
2010/03	ECR 2010 – European Congress of Radiology, Vienna, Austria
2009/10	Austrotransplant 2009 – 23rd Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Seefeld in Tirol, Austria
2009/09	40th Annual Meeting of the Austrian Society of Internal Medicine, Vienna, Austria
2009/09	Hanseatic Clinical Discussions No.4: Pneumology, Hamburg, Germany
2009/09	IV. Symposium "Cardiovascular Medicine – From Prevention to Intervention", Hamburg, Germany
2009/06	50th Annual Meeting of the Austrian Society for Surgery / 42th Annual Meeting of the Austrian Society of Gastroenterology and Hepatology (1st Austrian Surgical and Endoscopical Week), Vienna, Austria
2009/06	Annual Meeting of the Austrian Society of Cardiology, Salzburg, Austria
2009/02	53rd Annual Meeting of the Society of Thrombosis and Haemostasis Research, Vienna, Austria
2008/11	1st EACTS Meeting on Cardiac and Pulmonary Regeneration, Berne, Switzerland
2008/09	39th Annual Meeting of the Austrian Society of Internal Medicine, Graz, Austria

PUBLICATIONS

Articles:

Zimmermann M*, **Nickl S***, Roth GA, Hoetzenecker K, Mitterbauer M, Rozsas A, Torok S, Ostoros G, Laszlo V, Renyi-Vamos F, Steinlechner B, Klepetko W, Dome B, Ankersmit HJ.
Discrimination of clinical stages in non-small cell lung cancer patients by serum HSP27 and HSP70: A multi-institutional case-control study.
Lung Cancer-J IASLC; in submission (05/2011).
* contributed equally

Ankersmit HJ*, **Nickl S***, Hoeltl E, Toepker M, Lambers C, Mitterbauer A, Zimmermann M, Moser B, Bekos C, Steinlechner B, Klepetko W, Schenk P, Dome B.
Increased serum levels of HSP27 as a marker for incipient COPD in young smokers.
Eur J Clin Invest; under review (05/2011).
* contributed equally

Lichtenauer M, **Nickl S**, Hoetzenecker K, Mangold A, Mitterbauer A, Hacker S, Zimmermann M, Ankersmit HJ.
Effect of PBS Solutions on Chemokine Secretion of Human Peripheral Blood Mononuclear Cells.

American Laboratory 2011;43(1):30-33.

Mildner M, Storka A, Lichtenauer M, Mlitz V, Ghannadan M, Hoetzenecker K, **Nickl S**, Dome B, Tschachler E, Ankersmit HJ.
Primary sources and immunological prerequisites for sST2 secretion in humans.
Cardiovasc Res. 2010 Sep 1;87(4):769-77.

Hoetzenecker K, Adlbrecht C, Lichtenauer M, Hacker S, Hoetzenecker W, Mangold A, **Nickl S**, Mitterbauer A, Zimmermann M, Lang IM, Klepetko W, Ankersmit HJ.
Levels of sCD40L, TNF alpha, and TNF-RI in the Culprit Coronary Artery During Myocardial Infarction.
LabMedicine. 2009 Nov;40(11):660-664.

Mangold A, Szerafin T, Hoetzenecker K, Hacker S, Lichtenauer M, Niederpold T, **Nickl S**, Dworschak M, Blumer R, Auer J, Ankersmit HJ.
Alpha-Gal specific IgG immune response after implantation of bioprostheses.
Thorac Cardiovasc Surg. 2009 Jun;57(4):191-5.

Lichtenauer M, **Nickl S**, Hoetzenecker K, Mangold A, Moser B, Zimmermann M, Hacker S, Niederpold T, Mitterbauer A, Ankersmit HJ.
Phosphate buffered saline containing calcium and magnesium elicits increased secretion of Interleukin-1 Receptor Antagonist.
LabMedicine. 2009 May;40(5):290-293.

Abstracts:

Beer L, Werba G, **Nickl S**, Mitterbauer A, Zimmermann M, Wutzlhofer L, Ankerstmit HJ, Lichtenauer M.
Secretion of Cytokines and Chemokines by Peripheral Blood Mononuclear Cells is Triggered by Coagulation Products.
Annual Meeting of the Austrian Society of Cardiology, Salzburg, Austria, 2011/05.
[Epub ahead of print]

Lichtenauer M, Werba G, Mildner M, Hasun M, Baumgartner A, **Nickl S**, Mitterbauer A, Rauch M, Zimmermann M, Podesser BK, Klepetko W, Ankersmit HJ.
Administration of Anti-Thymocyte Globulin (ATG) Preserves Cardiac Function after Experimental Myocardial Infarction.
ISHLT 31st Annual Meeting and Scientific Sessions, San Diego, CA, USA. 2011/04.
[Epub ahead of print]

Lichtenauer M, Hoetzenecker K, Hasun M, Baumgartner A, Mildner M, **Nickl S**, Werba G, Zimmermann M, Mitterbauer A, Podesser B, Klepetko W, Ankersmit HJ.
Intramyocardial Injection of Irradiated Apoptotic Peripheral Blood Mononuclear Cells (PBMC) Preserves Ventricular Function after Myocardial Infarction.
ISHLT 31st Annual Meeting and Scientific Sessions, San Diego, CA, USA. 2011/04.
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Positive Korrelation zwischen erhöhten HSP27-Serumspiegel und Zeichen von Air trapping und Emphysem in der Thorax-Computertomographie
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10
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Nickl S, Lambers C, Kortuem B, Mitterbauer A, Zimmermann M, Hacker S, Weinhappel W, Ziesche R, Toepker M, Klepetko W, Ankersmit HJ.
Sekretionsprofil von Stress-Proteinen peripherer mononukleärer Blutzellen von COPD-Patienten und gesunden Kontrollen
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10.

Eur Surg 2010;42:Suppl 237:38-39.

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Evaluierung der Stress-Proteine HSP27, HSP70 und MMP9 im Serum von Patienten mit COPD und COPD at risk
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10.
Eur Surg 2010;42:Suppl 237:38.

Nickl S, Lambers C, Kortuem B, Mitterbauer A, Zimmermann M, Hacker S, Lichtenauer M, Hoetzenecker K, Klepetko W, Ankersmit HJ.
Screening einer gesunden Studienpopulation auf obstructive Lungenpathologien: Macht die hohe Inzidenz neu entdeckter Fälle von COPD die Erforschung von Serum-Markern zur Früherkennung dieser Erkrankung zur Notwendigkeit?
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Moser B, **Nickl S**, Lambers C, Kortuem B, Zimmermann M, Hacker S, Lichtenauer M, Ziesche R, Ankersmit HJ, Klepetko W.
RAGE-Rezeptor in PatientInnen mit COPD und Risiko für COPD: eine Subgruppenanalyse
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10.
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Werba G, Lichtenauer M, Mitterbauer A, Hacker S, Hoetzenecker K, **Nickl S**, Zimmermann M, Rauch M, Hasun M, Podesser BK, Ankersmit HJ.
Verbesserung der linksventrikulären Funktion nach Gabe von Anti-Thymozyten Globulin (ATG) im Modell des experimentellen Herzinfarkts
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10.
Eur Surg 2010;42:Suppl 237:42-43.

Mitterbauer A, Hoetzenecker K, Hasun M, Santner D, Mangold A, **Nickl S**, Zimmermann M, Podesser BK, Ankersmit HJ, Lichtenauer M.
Serum-freies Zellkulturmedium reduziert myokardialen Schaden nach Myokardinfarkt im Rattenmodell: Bedeutung für zelltherapeutische Methoden
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10.
Eur Surg 2010;42:Suppl 237:40-41.

Nickl S, Toepker M, Hoeltl E, Lambers C, Kortuem B, Hacker S, Mitterbauer A, Zimmermann M, Klepetko W, Ankersmit HJ.
Elevated Heat Shock Protein 27 serum levels positively correlate with the presence of air trapping and emphysema in lung CT scan.
Annual Meeting 2010 of the Austrian Society of Pneumology, Graz, Austria. 2010/10.
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Nickl S, Lambers C, Kortuem B, Mitterbauer A, Zimmermann M, Hacker S, Lichtenauer M, Hoetzenecker K, Klepetko W, Ankersmit HJ.
Lung function testing in a healthy study cohort reveals a high incidence of newly diagnosed lung pathologies: Potential role for serum markers?
Annual Meeting 2010 of the Austrian Society of Pneumology, Graz, Austria. 2010/10.
Wien Klin Wochenschr 2010;122/21–22:A54-55.

Moser B, **Nickl S**, Lambers C, Kortuem B, Zimmermann M, Hacker S, Lichtenauer M, Ziesche R, Ankersmit HJ, Klepetko W.
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Oral Poster Presentations:

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Positive Korrelation zwischen erhöhten HSP27-Serumspiegel und Zeichen von Air trapping und Emphysem in der Thorax-Computertomographie
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Irradiated cultured apoptotic peripheral blood mononuclear cells preserve ventricular function after myocardial infarction.
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Heightened extracellular levels of calcium and magnesium induce secretion of chemokines and anti-inflammatory cytokines.
40th Annual Meeting of the Austrian Society of Internal Medicine, Vienna, Austria. 2009/09.

Oral Presentations: "Increased HSP27 Serum Levels – A Novel Marker for COPD and Early Radiological Signs of COPD?"
(Oral Presentation)
Dept. of Thoracic Surgery, General Hospital Vienna/Medical University of Vienna, Austria. 23/03/2010.

"Discrimination of Clinical Stages in Lung Cancer Patients by Serum HSP 27"
(Oral Presentation)
Dept. of Thoracic Surgery, General Hospital Vienna/Medical University of Vienna, Austria. 23/03/2010.

„Screening einer gesunden Studienpopulation auf obstructive Lungenpathologien: Macht die hohe Inzidenz neu entdeckter Fälle von COPD die Erforschung von Serum-Markern zur Früherkennung dieser Erkrankung zur Notwendigkeit?"
(Oral Presentation)
24th Annual Meeting of the Austrian Society of Transplantation, Transfusion and Genetics, Villach, Austria. 2010/10.

"Elevated Heat Shock Protein 27 serum levels positively correlate with the presence of air trapping and emphysema in lung CT scan"
(Oral Presentation)
Annual Meeting 2010 of the Austrian Society of Pneumology, Graz, Austria. 2010/10.

"Investigation of T Lymphocytes, Cytokines and Markers for Apoptosis in Pulmonary Fibrosis Patients."
(Project Presentation)
Medical University of Vienna, Vienna, Austria. 05/2009.

AWARDS AND GRANTS

2011/02	Travel and Congress Grant (Austrian Society of Radiology & Scholarship Programme "Hellste Koepfe") for the 6th joint Meeting of the German and Austrian Society of Radiology / 92nd Meeting of the German Society of Radiology, Hamburg, Germany (June 2011).
2010/12	Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna.
2010/10	Poster Prize – Annual Meeting 2010 of the Austrian Society of Pneumology, Graz, Austria. (**"**Elevated Heat Shock Protein 27 serum levels positively correlate with the presence of air trapping and emphysema in lung CT scan")
2009/12	Leistungsstipendium – Scholarship for Outstanding Academic Achievement, Medical University of Vienna.
2008/12	Förderungsstipendium – Student Research Scholarship, Medical University of Vienna.

MEMBERSHIPS

2009/10	Austrian Society of Transplantation, Transfusion and Genetics

METHODS

Enzyme-linked immunosorbent essay (ELISA)
Cultivation of human and animal cell lines
Histology
Flow Cytometry

LANGUAGE SKILLS

Native German Speaker
Proficient in English
Proficient in Spanish

REFERENCES

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9.2. Scientific Posters

MEDIZINISCHE UNIVERSITÄT WIEN

Applied Immunology
Cardio - Thoracic Research in Surgery

Christian Doppler Laboratory
for the Diagnosis & Regeneration of Cardiac and Thoracic Diseases

Lung Function Testing in a Healthy Study Cohort Reveals a High Incidence of Newly Diagnosed Lung Pathologies: Potential Role for Serum Markers?

Nickl S[1,2], Lambers C[3], Kortuem B[1,2], Mitterbauer A[1,2], Zimmermann M[1,2], Hacker S[1,2], Lichtenauer M[1,2], Hoetzenecker K[1,2], Klepetko W[1], Ankersmit HJ[1,2]

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Introduction

Despite increased awareness in the past decades, chronic obstructive pulmonary disease (COPD) still remains an underdiagnosed disease. Although COPD is prevent- and treatable, its prevalence is still rising and it will be the third leading cause of death worldwide by 2020.

We therefore designed an open cohort study to investigate lung function results of subjectively healthy volunteers to determine the prevalence rate of chronic obstructive lung pathologies.

Material and Methods

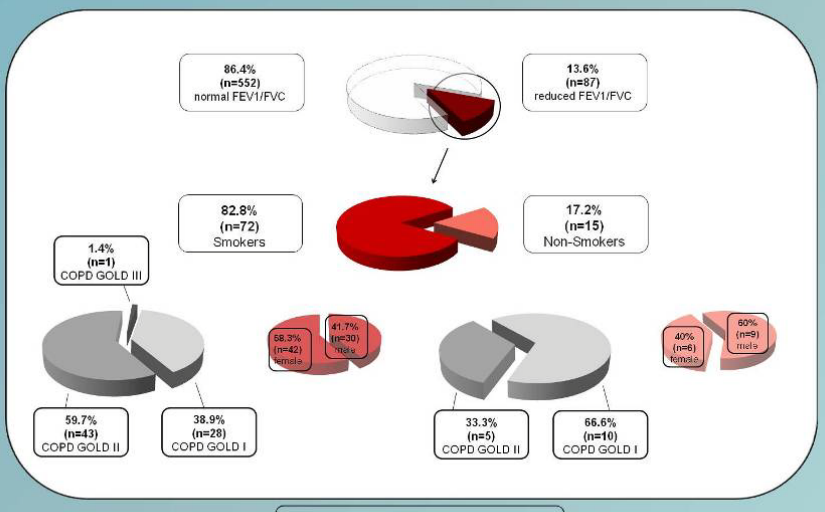
639 subjectively healthy subjects were included in this community based study, both smokers and non-smokers.

After informed and written consent, the study subjects were asked to answer a questionnaire regarding health related behaviour and smoking habits. Lung function testing with a portable pulmonary function testing device (PC Spirometry SDS 104 software Schiller AG, Baar, Switzerland) was performed without the use of a bronchodilator. Results where then evaluated by an experienced pulmonologist.

Results

In 87 out of 639 subjects (13.6%) we evidenced a reduced FEV1/FVC ratio < 0.7, indicating a present obstructive lung pathology. Of these 87 newly diagnosed COPD cases 72 subjects (82.8%) were smokers (S) (28 COPD GOLD I, 43 GOLD II and 1 GOLD III), amongst them 30 (41.7%) were male and 42 (58.3%) were female. 15 newly diagnosed COPD patients (17.2%) were non-smokers (N-S) (10 COPD GOLD I, 5 GOLD II), 9 (60%) male and 6 (40%) were female.

Between smokers and non-smokers diagnosed with COPD no significant differences regarding age (smokers [mean ± SD] 47.3 ± 11.5 vs. non-smokers 42.9 ± 8.7 years) or body mass index (S 24.4 ± 5.2 vs. N-S 24.7 ± 3.2 BMI) could be found. Smokers had an average smoking history of 27.1 ± 20.7 pack years.



Study results

Fig. 1 a

			Sex		
			m	f	Total
Non-Smoker	Obstruct.	1	6	4	10
	GOLD	2	3	2	5
	Total		9	6	15
(Ex-) Smoker	Obstruct.	1	12	16	28
	GOLD	2	17	26	43
		3	1	0	1
	Total		30	42	72

Fig. 1 b

		Mean ± SD	t-Test
BMI	Non-Smoker	24.70 ± 3.18	P = 0.822
	Smoker	24.39 ± 5.17	
Age (years)	Non-Smoker	42.93 ± 8.75	p = 0.169
	Smoker	47.31 ± 11.51	
Pack Years	Non-Smoker	0	\
	Smoker	27.15 ± 20.70	

Fig. 1 c

	Obstruction GOLD	Mean ± SD	t-Test
BMI	1	25.40 ± 3.90	p = 0.238
	2	23.63 ± 5.93	
Age (years)	1	46.86 ± 12.24	p = 0.913
	2	47.16 ± 10.91	
Pack Years	1	29.79 ± 23.05	p = 0.418
	2	25.45 ± 19.16	

Fig. 1a: Gender distribution (subjects with reduced FEV1/FVC)
Fig. 1b: No significant difference between mean BMI and mean age of non-smokers and smokers
Fig. 1c: No significant difference between mean BMI, mean age and mean pack years of study subjects diagnosed with GOLD stage I and GOLD stage II

Discussion

The study corroborates the fact that the majority of smokers are not aware of their pathologic lung function. Lung function testing revealed a high incidence of newly diagnosed lung pathologies. We believe that validated serum markers for the detection of early stage COPD/manifest COPD are needed for early identification of patients developing COPD.

Stress proteins HSP27, HSP70 and MMP9 in patients with COPD and COPD at risk

Nickl S[1,2], Lambers C[3], Kortuem B[1,2], Mitterbauer A[1,2], Zimmermann M[1,2], Hacker S[1,2], Weinhappel W[3], Ziesche R[3], Klepetko W[1], Ankersmit HJ[1,2].

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[2] Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Vienna, Austria
[3] Department of Pneumology, Medical University of Vienna, Austria

Introduction

Chronic obstructive pulmonary disease (COPD) is amongst the major causes of morbidity and mortality and its prevalence is still increasing.

In former studies we have demonstrated that patients with diagnosed COPD evidence a significant increment of serum levels of Heat Shock Protein (HSP) 27 and HSP70[1]. We therefore sought to investigate whether patients included in the AUVA-COPD follow up study (Dept. of Pneumology, Medical University of Vienna) evidence a similar serum protein pattern as previously published. Moreover, serum levels of Matrix Metalloproteinase (MMP) 9 were also investigated in this study cohort.

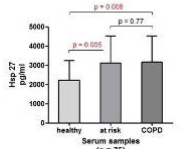
Material and Methods

The study cohort included a total of 75 subjects, termed "healthy" (H; n=17), "COPD at risk" (AR; n=32), and "COPD GOLD I-III" (COPD; n=26). Determination of disease severity was performed by an experienced pulmonologist. The included study subjects had previously undergone CT testing as well as spirometric and bronchoscopic evaluation. After informed and written consent, the study subjects were asked to answer a questionnaire regarding smoking- and health habits. Serum samples were drawn and stored at -80°C. Levels of HSP27, HSP70 and MMP9 were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

Results

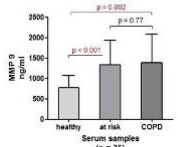
We showed that HSP27 serum levels were significantly elevated in COPD patients (mean ± SD: 3175 ± 1332 pg/ml) as compared to healthy controls (2206 ± 1044 pg/ml; H vs. COPD: p=0.008). Furthermore, patients with COPD at risk (3107 ± 1405 pg/ml) also showed an increment in serum HSP27 levels (H vs. AR: p=0.005). No differences in serum HSP70 levels between the three groups were determined in this study cohort. MMP9 levels were determined as follows: H (765.5 ± 303.7 pg/ml) vs. COPD (1379 ± 702.8 pg/ml): p=0.002; H vs. AR (1327 ± 603 pg/ml): p<0.001. Deductively, MMP9 serum levels were significantly higher in COPD and AR as compared to H.

Fig.1



	Mean ± SD
Healthy	2206 ± 1044 pg/ml
COPD at risk	3107 ± 1405 pg/ml
COPD GOLD I-III	3175 ± 1332 pg/ml

Fig.3

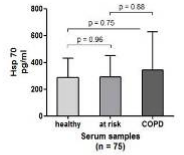


	Mean ± SD
Healthy	765.5 ± 303.7 ng/ml
COPD at risk	1327 ± 603 ng/ml
COPD GOLD I-III	1379 ± 702.8 ng/ml

Subject Category	Healthy (H)	At risk (AR)	COPD GOLD I-III (COPD)
N	17	32	26
Male / Female	9 / 8	27 / 5	23 / 3
Age	31.76 ± 13.3	45.97 ± 10.4	53.38 ± 7.8
Lung Function			
FVC (L)	4.70 ± 1.15	4.39 ± 0.89	4.15 ± 1.04
FEV1 (%)	92.18 ± 7.72	82.75 ± 12.99	57.92 ± 18.79
FEV1/VC (%)	81.47 ± 9.05	76.35 ± 5.38	52.12 ± 12.89
Smoking History			
Never-smoker	12	3	0
Ex-smoker			
(> 5a)	4	4	5
(<5a)	1	3	7
Current-smoker	0	22	14
Pack Years	1.8 ± 4.8	29.38 ± 27.3	50.73 ± 26.3
Body Weight (kg)	70.88 ± 16.2	83.1 ± 17.0	85.52 ± 14.0
Body Height (cm)	174.5 ± 8.5	175.6 ± 8.5	175.4 ± 7.8
BMI	23.29 ± 3.4	26.81 ± 5.6	27.91 ± 4.9

Table 1

Fig.2



	Mean ± SD
Healthy	285.6 ± 144.8 pg/ml
COPD at risk	291.3 ± 160.9 pg/ml
COPD GOLD I-III	341.9 ± 285.3 pg/ml

Figure 1:
Serum levels of HSP27 were significantly increased in patients with COPD and patients at risk for developing COPD as compared to healthy controls.

Figure 2:
Serum levels of HSP70 did not show significant differences between the three groups.

Figure 3:
Serum levels of MMP9 were significantly increased in patients with COPD and patients at risk for developing COPD as compared to healthy controls.

Table 1:
Demographics of the study subjects

Discussion

The present results corroborate our previous findings that patients suffering from COPD and patients at risk for developing COPD present with increased HSP27 serum levels. Moreover, serum MMP9 evidenced a similar significant increment as seen with HSP27. We suggest that HSP27 and MMP9 may function as diagnostic markers to identify patients at risk for developing COPD.

[1] Hacker S, Lambers C, Hoetzenecker K, et al.. Elevated HSP27, HSP70 and HSP90 alpha in chronic obstructive pulmonary disease - markers for immune activation and tissue destruction. Clin Lab. 2009;55(1-2):31-40.

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Applied Immunology
Cardio - Thoracic Research in Surgery

Christian Doppler Laboratory
for the Diagnosis & Regeneration of Cardiac and Thoracic Diseases

Stress protein secretion of peripheral blood mononuclear cells (PBMC) obtained from COPD patients and controls

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Introduction

We have previously demonstrated that patients with chronic obstructive pulmonary disease (COPD) and patients at risk for developing COPD evidence significantly higher levels of extracellular stress proteins Heat Shock Protein (HSP) 27, HSP70 and Matrix Metalloproteinase (MMP) 9.
HSP27, for example, is highly induced in PBMC from patients with systemic inflammatory response syndrome[1], however, the origin of these serum proteins in patients with COPD remains elusive. Therefore, we sought to investigate whether PBMC derived from patients with COPD, subjects at risk for developing COPD or healthy subjects secrete HSP27, HSP70 and MMP9, which plays an important role in tissue remodeling, under *in vitro* conditions.

Material and Methods

The study cohort included a total of 64 patients and controls. Subjects were termed "healthy" (H; n=15), "at risk" (AR; n=26), and "COPD GOLD I-III" (COPD; n=23). Determination of disease severity was performed by an experienced pulmonologist. The included study subjects had previously undergone CT testing as well as spirometric and bronchoscopic evaluation. Blood samples were drawn, PBMC were separated immediately by Ficoll density gradient centrifugation, and incubated (GIBCO CO2-independent Medium, Invitrogen, Carlsbad, CA, USA). PBMC supernatants (SN) were derived after 12 and 24 hours and analyzed for HSP27, HSP70 and MMP9 with commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA).

Results

MMP9 SN levels were significantly increased in "COPD" and "AR" as compared to "H", both after 12 and after 24 hours incubation period (after 12 hours: „H" vs. "AR" p=0.007; „H" vs. "COPD" p=0.006; after 24 hours: „H" vs. "AR" p=0.014; „H" vs. "COPD" p=0.006).
HSP27 SN levels were significantly increased in "AR" as compared to "H" after an incubation period of 12 hours: "H" vs. "AR" p=0.039. In the "COPD" group HSP27 SN levels were increased, but showed no significance ("H" vs. "COPD" p=0.07). HSP27 (24 hours incubation time) and HSP70 evaluation did not show any significant results.

Fig.1

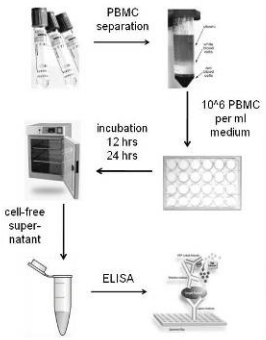
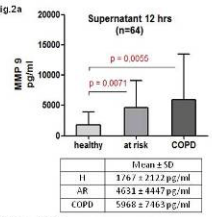


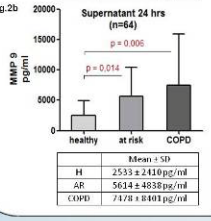
Fig.1
PBMC were separated using Ficoll density gradient centrifugation. Cells were then incubated at a concentration of 10^6 PBMC per milliter medium and incubated for either 12 or 24 hours under standard culture conditions. After centrifugation, cell-free supernatant was obtained and analyzed for HSP27, HSP70 and MMP9 using ELISA.

Fig.2a



	Mean ± SD
H	3767 ± 2122 pg/ml
AR	4631 ± 4447 pg/ml
COPD	5968 ± 7463 pg/ml

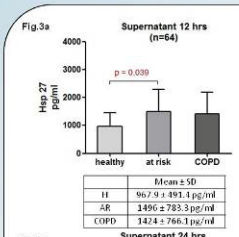
Fig.2b



	Mean ± SD
H	2533 ± 2410 pg/ml
AR	5614 ± 4838 pg/ml
COPD	7478 ± 8401 pg/ml

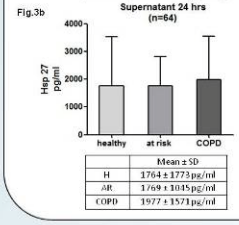
Fig.2
MMP9 levels of supernatants obtained from patients at risk for developing COPD and patients with COPD GOLD I-III were significantly higher as compared to healthy controls, both after 12 hours (Fig.2a) and after 24 hours (Fig.2b) incubation time.

Fig.3a



	Mean ± SD
H	967.9 ± 891.1 pg/ml
AR	1496 ± 783.3 pg/ml
COPD	1424 ± 766.1 pg/ml

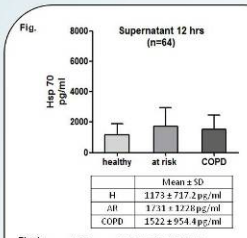
Fig.3b



	Mean ± SD
H	1764 ± 1773 pg/ml
AR	1769 ± 1045 pg/ml
COPD	1977 ± 1571 pg/ml

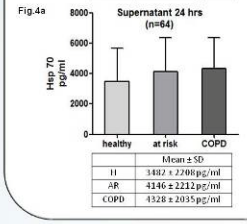
Fig.3
Supernatants obtained from patients at risk for developing COPD showed significantly higher amounts of HSP27 as compared to healthy controls (after 12 hours of incubation time - Fig.3a). After 24 hours of incubation time, no significant differences between the groups could be shown.

Fig.



	Mean ± SD
H	1173 ± 737.2 pg/ml
AR	1731 ± 1228 pg/ml
COPD	1522 ± 954.4 pg/ml

Fig.4a



	Mean ± SD
H	3482 ± 2208 pg/ml
AR	4146 ± 2212 pg/ml
COPD	4328 ± 2035 pg/ml

Fig.4
No significant differences regarding HSP70 supernatant-levels could be found between the groups, neither after 12 hours (Fig.4a) nor after 24 hours incubation time (Fig.4b). However, HSP70 levels were twice as high after 24 hours of incubation time than after 12 hours.

Discussion

We propose that PBMC obtained from COPD patients and subjects at risk for developing COPD are the source of increased levels of MMP9 and HSP27 *in vivo*.

1: Hashiguchi N, Ogura H, Tanaka H, et al. Enhanced expression of heat shock proteins in activated polymorphonuclear leukocytes in patients with sepsis. J Trauma 2001;51(6):1104-9.

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Elevated Heat Shock Protein 27 serum levels positively correlate with the presence of air trapping and emphysema in lung HR-CT scan

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Introduction

Despite increased awareness in the past decades, the prevalence of chronic obstructive pulmonary disease (COPD) is still rising and it is assumed that COPD will be the third leading cause of death in the near future.
In former studies we evidenced elevated levels of the stress protein Heat Shock Protein (HSP) 27 in COPD patients and smokers as compared to healthy controls[1]. Based on these findings we hypothesized whether elevated HSP27 levels correlate with signs of early airway obstruction related to COPD, such as air trapping (AT) and lung emphysema (E).

Material and Methods

120 apparently healthy smokers were included in this study. After informed and written consent, lung function testing with a portable testing device (PC Spirometry, SDS 104, Schiller AG, Linz) was performed and serum samples were obtained. HSP27 serum levels were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). High resolution computed tomography (HR-CT) scan (Aquilion 16, Toshiba Medical Systems, Neuss, Germany) of the lung was subsequently offered to all study subjects optionally.

Results

89 out of 120 subjects underwent CT examination voluntarily: AT and/or E was detected in 53 subjects (59.6%). 41 (77.4%) of them evidenced elevated HSP27 serum levels (mean ± SD: 4114 ± 1493 pg/ml). In 36 subjects no pathologies (NP) could be found in the CT scan (HSP27: 3322 ± 1503 pg/ml; AT and/or E vs. NP: p=0.016). Sensitivity and specificity of HSP27 values for the detection of AT and/or E were calculated by means of Receiver Operating Characteristic (ROC) curve (Area under the curve [AUC]=0.646).
Further analysis between subjects with AT, E, or both, and subjects without lung pathologies in the CT scan were carried out:
AT (with or without E) (HSP27 4201 ± 1490 pg/ml) vs. NP: p=0.009; AUC=0.66.
E (with or without AT) (HSP27 4618 ± 1677 pg/ml) vs. NP: p=0.003; AUC=0.71.
AT *and* E (HSP27 4914 ± 1594 pg/ml) vs. NP: p=0.0005 AUC= 0.76.
HSP27 levels did *not* correlate with pathologic lung function (correlation coefficient FEV1/FVC vs. HSP27 0.037; p=0.732).

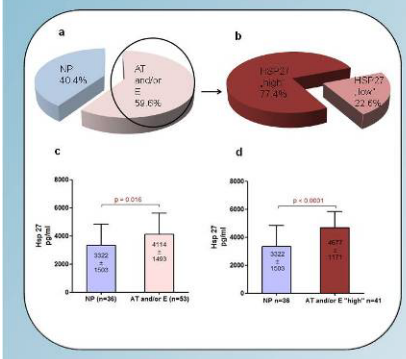

Figure 1:
a: Air trapping or emphysema was detected in 53 out of 89 study subjects (59.6%).
b: 77.4% of them had significantly elevated HSP27 serum values (>3000 pg/mL).
c: HSP27 serum levels were significantly higher in subjects that presented with AT and/or E than in subjects without lung pathologies (p=0.016).
d: Comparing "HSP27 high" subgroup with the healthy subroup: p<0.0001

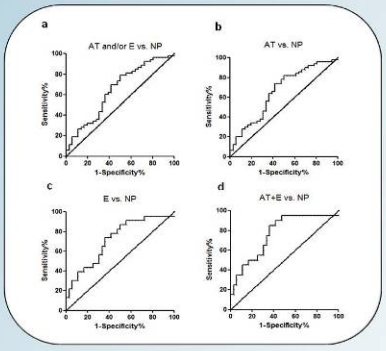

Figure 2:
Sensitivity and Specificity of HSP27 to detect air trapping and emphysema:
a: Subjects with air trapping and/or emphysema: AUC=0.646; p=0.0164.
b: Subjects with air trapping (independent of the presence of emphysema): AUC=0.66; p=0.009
c: Subjects with emphysema (independent of the presence of air trapping): AUC=0.71; p=0.003
d: subjects with both air trapping and emphysema: AUC=0.76; p=0.0005

a

	m		f		total	
Age (years)	45.08 ± 10.89		42.88 ± 8.4		43.84 ± 9.58	
	n	%	n	%	n	%
Non-Smoker (Ex-) Smoker	0 39	0 100	0 50	0 100	0 89	0 100
Air Trapping	22	56.4	29	58.0	51	57.3
Emphysem	8	20.5	15	30.0	23	25.8

b

	no pathologies (mean ± SD)	AT and/or E (mean ± SD)	P-value
FVC	4.25 ± 0.97	4.02 ± 0.67	n. s.
FVC%	93.39 ± 13.0	89.7 ± 12.3	n. s.
FEV1	3.27 ± 0.81	3.07 ± 0.58	n. s.
FEV1%	85.44 ± 12.34	81.49 ± 12.3	n. s.
FEV1/VC	74.61 ± 13.69	74.34 ± 13.93	n. s.
FEV1/VC%	88.17 ± 16.05	88.21 ± 16.41	n. s.

Figure 3:
a: Demographics of all study subjects
b: No significant differences between lung function results of study subjects with and study subjects without lung pathologies in the HR-CT.

Discussion

HSP27 serum levels positively correlated with early signs of COPD (air trapping and/or emphysema) in HR-CT scan analysis. In contrast, lung function results did *not* correlate with HSP27 serum values nor with lung pathologies detected in the CT scan. These data suggest that elevated levels of serum HSP27 may serve as a potential serum marker to identify patients with early signs of COPD independent of lung function.

[1] Hacker S, Lambers C, Hoetzenecker K, et al.: Elevated HSP27, HSP70 and HSP90 alpha in chronic obstructive pulmonary disease: markers for immune activation and tissue destruction. Clin Lab. 2009;55(1-2):31-40.